

University of Milano-Bicocca
Department of Biotechnology and Biosciences
Thesis for the degree of Doctor in Industrial Biotechnology
XXVIII Cycle



**BIOFUELS AND CHEMICALS PRODUCTION FROM RENEWABLE
RAW-MATERIALS**

**Exploiting yeasts diversity to bridge the gap between the proof-of-
concept and industrial success**

Coordinator: Prof. Marco Vanoni
Tutor: Prof. Paola Branduardi

Signori Lorenzo
N°701752

2015-2016

Thesis for the degree of Doctor in Industrial Biotechnology



BIOFUELS AND CHEMICALS PRODUCTION FROM RENEWABLE RAW-MATERIALS

Exploiting yeasts diversity to bridge the gap between the proof-of-concept and industrial success

SIGNORI LORENZO

Coordinator: Professor Marco Vanoni

Tutor: Professor Paola Branduardi

2015-2016

- It's always trivial the way we discover certain things.

And as these fall on our lives, growing from drop to flood.

The story of the horse that lost the nail and then lost the iron and the general lost his balance and the army lost the leadership and the King

finally lost the battle and lost the war.

A lost war because of a nail. -

To my family

RIASSUNTO

Il successo della bioraffineria deve passare per lo sviluppo di *cell factories* efficienti, robuste e versatili. Attualmente, microrganismi come lieviti, batteri e alghe, svolgono un ruolo chiave in numerosi processi industriali; tuttavia, molti dei ceppi industriali sono utilizzati per ragioni storiche, piuttosto che esser accuratamente selezionati a seconda della specifica applicazione. Inoltre, le richieste di maggiore produttività, produzione di composti non-convenzionali/non-naturali e della capacità di crescere su diversi substrati ha portato ad un grande interesse per un miglioramento degli attuali *workhorses* utilizzati a livello industriale e allo sviluppo di ceppi con nuove proprietà.

Il lievito modello *Saccharomyces cerevisiae* è attualmente il principale microorganismo utilizzato per la produzione di etanolo di prima generazione. Uno dei maggior ostacoli allo sviluppo di processi di seconda generazione è rappresentato dalla scarsa tolleranza di questo lievito ai composti inibitori rilasciati/prodotti durante il pretrattamento delle biomasse lignocellulosiche.

Nella prima parte di questo lavoro, sono stati descritti due diversi approcci per incrementare la tolleranza di *S. cerevisiae* ai composti derivanti dal pretrattamento delle biomasse.

Per quanto riguarda il primo approccio, l'effetto dell'over-espressione di un gene codificante un fattore trascrizionale (*YAP1*) e di un gene codificante per una NADH-citocromo b5 reduttasi mitocondriale (*MCR1*) è stato valutato in un ceppo industriale di *S. cerevisiae*. Durante coltivazioni batch, utilizzando idrolizzato di abete rosso, l'over-espressione di tali geni è

RIASSUNTO

risultata in un più rapido catabolismo degli esosi. Il secondo approccio ha mostrato che ingegnerizzando *S. cerevisiae* in modo che possa produrre endogenamente acido L-ascorbico è possibile incrementare la tolleranza all'acido acetico.

Nella seconda parte del lavoro, dato che i lieviti attualmente utilizzati a livello industriale sono solo la punta del proverbiale iceberg della diversità genetica presente in natura, lieviti non-*Saccharomyces* sono stati investigati per il loro potenziale a livello industriale.

I) *Kluyveromyces marxianus* (CBS 712) è stato scelto per alcune sue caratteristiche come la produzione di etanolo, la termotolleranza e la capacità di crescere su una vasta gamma di substrati. La sua capacità di crescere su xilosio e produrre etanolo è stata valutata mediante coltivazioni batch. Durante tali esperimenti, sia l'apporto di ossigeno che la temperatura si sono dimostrati parametri chiave che influenzano la capacità fermentativa del ceppo.

II) I lieviti oleaginosi *Rhodospiridium toruloides* (DSM 4444), *Lipomyces starkeyi* (DSM 70295) e *Cryptococcus curvatus* (DSM 70022) sono stati scelti come *cell factories* per la produzione di lipidi a partire da glicerolo grezzo. Per evitare la fase *lag* è stata sviluppata una strategia di feeding semplice ed efficiente, che ha permesso di ottenere con tutti e tre i lieviti un'elevata biomassa e produzione di lipidi. *L. starkeyi* è stato anche sottoposto ad un approccio di evoluzione diretta per incrementare la sua robustezza ai composti inibitori derivanti dal pretrattamento delle biomasse. I risultati preliminari sono promettenti ed esperimenti sono tutt'ora in corso.

RIASSUNTO

III) *Zygosacchomyces bailii* è stato scelto per la sua capacità di crescere a bassi pH e in presenza di considerevoli quantità di acidi organici deboli. Fermentazioni in bioreattore a basso pH ed in presenza di acido lattico sono state effettuate per valutare l'effetto fisiologico su questo lievito. Le analisi FTIR hanno rivelato che l'acido lattico induce cambiamenti macromolecolari a livello della struttura secondaria delle proteine intracellulari e alterazioni delle proprietà chimico-fisiche della membrana e della parete.

Infine, IV) *Candida lignohabitans* è stata identificata come nuova *cell factory* per la produzione di acidi organici a partire da materiali lignocellulosici e al fine di investigare in modo approfondito il suo metabolismo sono state effettuate coltivazioni in chemostato.

Complessivamente, il lavoro effettuato ha permesso di sviluppare ceppi industriali di *S. cerevisiae* maggiormente robusti che potrebbero essere utilizzati in processi fermentativi utilizzando idrolizzati lignocellulosici. Questo lavoro ha inoltre permesso di approfondire la conoscenza a livello metabolico e fisiologico di lieviti non-*Saccharomyces* con un grande potenziale a livello industriale.

RIASSUNTO

Parole chiave: Bioetanolo, Biodiesel, Acido lattico, Idrolizzati; Inibitori, Robustezza, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Rhodospiridium toruloides*, *Lipomyces starkeyi*, *Cryptococcus curvatus*, *Zygosacchromyces bailii*, *Candida lignohabitans*.

ABSTRACT

The success of the biorefinery concept will require efficient, robust and versatile cell factories. Currently, microorganisms, such as yeasts, bacteria and algae, are key players in numerous industrial processes. However, many industrial strains are currently used because of historical grounds, rather than being carefully selected for a specific application. Additionally, demands for increased productivity, wider substrate range utilization, and production of non-conventional/non-natural compounds, lead to a great interest in further improving the currently used industrial strains and the selection or development of strains with novel properties.

The model yeast *Saccharomyces cerevisiae* is currently employed as a *cell factory* for the production of several industrial products, and in particular for first generation ethanol. One of the major obstacles for the development of second generation ethanol production processes is the toxic effect of compounds released during the pre-treatment of lignocellulosic biomasses, which represent a more sustainable feedstock.

In the first part of this work, two different approaches to improve *S. cerevisiae* tolerance to compounds deriving from biomass pre-treatment are described.

In the former case, the effects of overexpressing genes encoding the transcription factor (*YAP1*) and the mitochondrial NADH-cytochrome b5 reductase (*MCR1*), either alone or in combination, was evaluated in an industrial xylose-consuming *S. cerevisiae* strain. During batch fermentation on undiluted and undetoxified spruce hydrolysate overexpression of either gene resulted in faster hexose catabolism.

ABSTRACT

The second approach was based on the comparison of a *S. cerevisiae* strain engineered to endogenously produce L-ascorbic acid (L-AA) with the parental strain. The vitamin C producing strain exhibited an increased tolerance compared to the parental strain when exposed to acetic acid at sublethal concentration.

In the second part of the work, since the currently used industrial yeasts represent only the tip of the proverbial iceberg of the genetic diversity present in nature, different non-*Saccharomyces* yeasts were investigated for their potential industrial applications.

I) Kluyveromyces marxianus (CBS 712) was chosen due to attractive specific traits such as production of ethanol, thermotolerance and ability to grow with a high specific growth rate on a wide range of substrates. Batch fermentations were performed to investigate its potential for xylose utilization and ethanol production. During these experiments, both oxygen supply and temperature were shown to be key parameters affecting its fermentation capability of sugars.

II) The oleaginous yeasts Rhodosporidium toruloides (DSM 4444), *Lipomyces starkeyi* (DSM 70295) and *Cryptococcus curvatus* (DSM 70022) were chosen as three of the most promising cell factories for lipid production using crude glycerol as sole carbon source. An efficient yet simple feeding strategy for avoiding the lag phase caused by growth on crude glycerol was developed, leading to high biomass and lipid production for all the tested yeasts. *L. starkeyi* was further subjected to an adaptive laboratory evolution approach to increase robustness against inhibitory substances that form

ABSTRACT

during the biomass pre-treatment. Preliminary results are promising and experiments are still ongoing.

III) *Zygosaccharomyces bailii* was chosen for its capability to grow at low pH and in the presence of considerable amounts of weak organic acids. The physiological effect of lactic acid was assessed by bioreactor fermentation at low pH in the presence of lactic acid. FTIR analyses revealed that lactic acid induced macromolecular changes in the overall intracellular protein secondary structures, and alterations in cell wall and membrane physico-chemical properties.

Finally, IV) *Candida lignohabitans* was investigated as a novel microbial host for organic acid production from lignocellulosic material. Chemostat cultivations were performed to deeply investigate its metabolism.

Overall, the work performed resulted in the development of industrial *S. cerevisiae* strains with improved traits that can match the requirements of lignocellulosic hydrolysate fermentation. The work also contributed to a better understanding of the metabolism and physiology of different non-*Saccharomyces* yeasts with a great industrial potential.

ABSTRACT

Keywords: Bioethanol, Biodiesel, Lactic acid, Hydrolysates; Inhibitors, Robustness, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Rhodospiridium toruloides*, *Lipomyces starkeyi*, *Cryptococcus curvatus*, *Zygosacchomyces bailii*, *Candida lignohabitans*.

LIST OF PUBLICATIONS

This thesis is based on the following research papers, which can be found at the end of the thesis.

- I. (Submitted) Signori L., Ami D., Posteri R., Giuzzi A., Mereghetti P., Porro D., Branduardi P. (2015) **Assessing an effective feeding strategy to optimize crude glycerol utilization as sustainable carbon source for lipid accumulation in *Rhodospiridium toruloides*, *Cryptococcus curvatus* and *Lipomyces starkeyi*.** Microbial Cell Factories.
- II. (Submitted) Kuanyshev N., Ami D., Signori L., Porro D., Morrissey J., and Branduardi P. (2015) **Assessing physio-macromolecular effects of lactic acid on *Zygosaccharomyces bailii* cells during microaerobic fermentation.** FEMS Yeast Research.
- III. Signori L., Passolunghi S., Ruohonen L., Porro D., Branduardi P. (2014) **Effect of oxygenation and temperature on glucose-xylose fermentation in *Kluyveromyces marxianus* CBS712 strain.** Microbial Cell Factories, 13:51.
- IV. Wallace-Salinas W., Signori L., Li Y., Ask M., Bettiga M., Porro D., Thevelein J., Branduardi P., Foulquié-Moreno M.R., Gorwa-

LIST OF PUBLICATIONS

- Grauslund M. (2014) **Re-assessment of *YAP1* and *MCR1* contributions to inhibitor tolerance in robust engineered *Saccharomyces cerevisiae* fermenting undetoxified lignocellulosic hydrolysate.** AMB Express.
- V. Martani F., Fossati T., Posterl R., Signori L., Porro D., Branduardi P. (2013) **Different response to acetic acid stress in *Saccharomyces cerevisiae* wild type and L-ascorbic acid producing strains.** Yeast, 30:365-78.

ABBREVIATIONS

ACL	Citrate lyase
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ALE	Adaptive laboratory evolution
ATMT	Agrobacterium-mediated transformation
ATP	Adenosine triphosphate
BG	Billion gallons
BL	Billion liters
C5	Pentose sugar
C/N	Carbon to nitrogen ratio
CBP	Consolidated Bioprocessing
CTR	Carbon dioxide transfer rate
EU	European union
FAS	Fatty acid synthetase
FDA	Food and Drug Administration
GAP	Glyceraldehyde 3-phosphate dehydrogenase
GEMs	Genome-scale metabolic models
GHG	Greenhouse gases
GMO	Genetically modified organism
GRAS	Generally regarded as safe
H ₂ SO ₄	Sulfuric acid
HCl	Chloridric acid
HMF	5-Hydroxymethyl furfural

ABBREVIATIONS

KBBE	Knowledge based bio-economy
KOH	Potassium hydroxide
LA	Lactic acid
LB	Lipid bodies
LCA	Life Cycle Assessment
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOCH ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PDH	Pyruvate dehydrogenase
PLA	Polylactic acid
PPP	Pentose phosphate pathway
RQ	Respiratory quotient
SCOs	Single-cell oils
SE	Steryl esters
SHCF	Separate Hydrolysis and Co-Fermentation
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
TAG	Triacylglycerol
TCA	Tricarboxylic acid cycle

ABBREVIATIONS

XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XK	Xylulokinase
XR	Xylose reductase

CONTENTS

1. INTRODUCTION

1.1. MOVING TOWARD A BIOECONOMY	01
1.1.1. The Biorefinery concept	04
1.2. RENEWABLE RAW MATERIALS	07
1.2.1. Composition of lignocellulosic biomasses	08
1.2.1.1. Pretreatment of lignocellulosic materials	12
1.2.1.2. Hydrolysis	15
1.2.2. Crude glycerol from biodiesel production	18
1.3. YEAST CELL FACTORY	20
1.3.1. <i>Saccharomyces cerevisiae</i>	23
1.3.2. <i>Kluyveromyces marxianus</i>	25
1.3.3. <i>Rhodospiridium toruloides</i>	27
1.3.4. <i>Cryptococcus curvatus</i>	28
1.3.5. <i>Lipomyces starkeyi</i>	28
1.3.6. <i>Zygosaccharomyces bailii</i>	29
1.3.7. <i>Candida lignohabitans</i>	31
1.4. MICROBIAL CHALLENGES IN LIGNOCELLULOSIC BIOMASS UTILIZATION	33
1.4.1. Xylose utilization in yeast	33
1.4.2. Lignocellulose derived inhibitors and their impact on cells	38

CONTENTS

1.5.	IMPROVING YEAST STRAINS	45
1.5.1.	Rational metabolic engineering	46
1.5.2.	Non-targeted approaches: Evolutionary engineering	48
1.6.	ETHANOL AS TRANSPORTATION FUEL	52
1.6.1.	From raw materials to ethanol: a schematic overview	55
1.6.2.	Fermentation	56
1.6.3.	Process configuration for lignocellulosic bioethanol production	59
1.7.	BIODIESEL	63
1.7.1.	Chemistry of biodiesel	64
1.7.2.	Lipids from yeast	67
1.7.3.	Steps involved in microbial biodiesel recovery	71
1.8.	MICROBIAL PRODUCTION OF ORGANIC ACIDS	74
1.8.1.	Lactic acid	76
1.8.2.	Itaconic acid	79
2.	CONCLUSIONS AND FUTURE PERSPECTIVES	83
3.	ACKNOWLEDGMENTS	87
4.	REFERENCES	89

1.1. MOVING TOWARD A BIOECONOMY

Our strong dependence on fossil fuels is a consequence of the intensive use and consumption of petroleum derivatives which, combined with diminishing petroleum resources, causes environmental and political concerns.

Nowadays, for the transportation sector the world's primary source of energy is oil. World demand is approximately 96 million barrels a day and is expected to increase to about 116 million barrels a day by 2030 [1]. In order to simultaneously reduce the dependence on oil and mitigate climate change, alternative production chains for transport and chemical sectors are necessary.

In this context, the term 'bio-economy' has emerged in different contexts, but until now, a comprehensive and common definition still needs to be developed.

The term bio-economy is composed of the words 'bio' and 'economy', which implies the '*opportunity to reconcile economic growth with environmentally responsible action*' [2]. The existing synonyms, i.e., bio-based economy or knowledge-based bio-economy (KBBE), are often used interchangeably.

As reported in Golembiewski and coworkers [3], the concept of a knowledge-based bio-economy (KBBE) has been introduced by the European Commission in 2004 but its importance has already been highlighted before e.g., by Hardy declaring that '*The bio-based economy can*

INTRODUCTION

and should be to the 21st century what the fossil-based economy was to the 20th century' [4].

Currently, European research framework programs like 'HORIZON 2020' are aiming at promoting innovations within research areas of the evolving bio-economy [5].

It is widely recognized that there is not a single solution to the previously described problems and that combined actions are needed. Interdisciplinary research not only faces engineering and natural sciences-related challenges but also socio-economic challenges such as societal expectations affecting the adoption of new bio-economic products and processes. Hence, an integration of concepts and platforms from different disciplines is required to explore the prerequisites for implementing the bio-based economy.

Currently, we still lack a standard measure to capture the current size of the bio-economy as well as to monitor its evolutionary progress, i.e., the transition from a fossil to a bio-based economy [6] (**Figure 1**).

In Golembiewski and coworkers [3] three main challenges were identified for the implementation of the bioeconomy: *I) the complex knowledge base challenge*, at the beginning of the bio-economy new knowledge and capabilities will have to be created and acquired; *II) the converging technologies challenge*, new technologies across disciplines and, even more complex, across value chains will have to be developed; and *III) the commercialization and market diffusion challenge*, the development of the

INTRODUCTION

bio-economy will have to be accompanied by the market adoption and diffusion of the developed technologies.

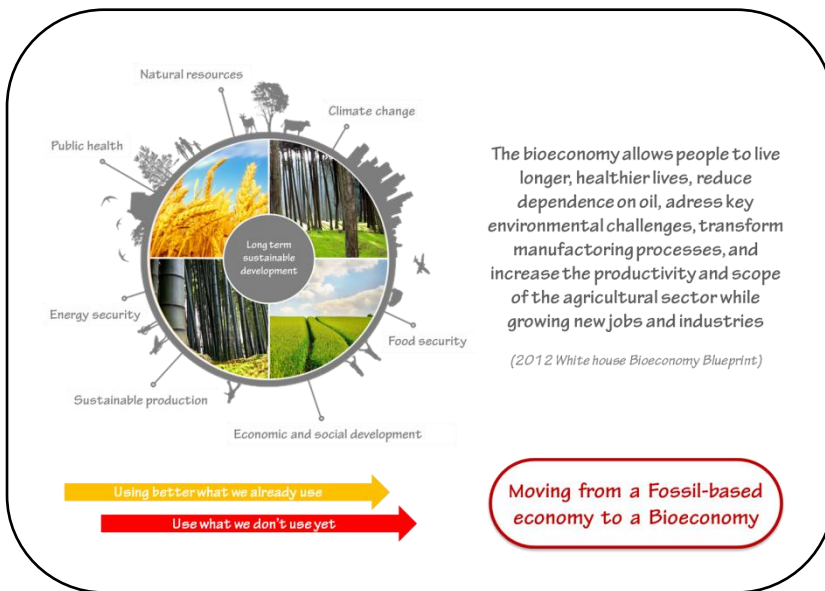


Figure 1. Definition of Bioeconomy [7].

Still there is no common understanding of the concept on bio-economy, focus should be set on how to best manage collaborations between different disciplines to obtain radical innovations and on how to establish standardized measures allowing to track the implementation of the bio-economy.

1.1.1. The Biorefinery concept

In this global context of fossil energy dependence linked to oil and gas prices, it is essential to promote and increase the fraction of biobased products. Production and development of these new products are based on the biorefinery concept.

A biorefinery is the integral upstream, midstream, and downstream processing of biomass into a range of products [8] (**Figure 2**).

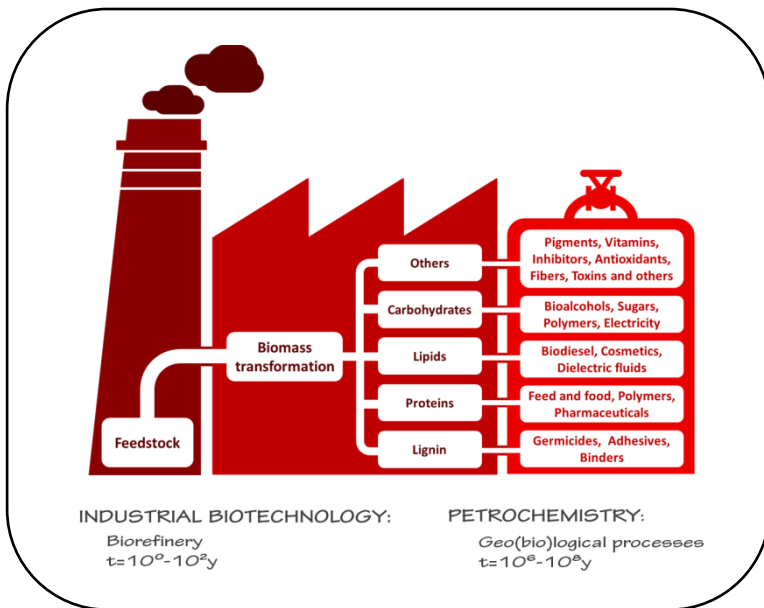


Figure 2. Schematic representation of the biorefinery concept.

INTRODUCTION

It has been developed as a concept to use all kinds of biomass from forestry, agriculture, aquaculture, and residues from industry and households including wood, agricultural crops, organic residues (both plant and animal derived), forest residues, and aquatic biomass (algae and seaweeds).

A biorefinery should produce a spectrum of marketable products and energy. The products can be both intermediates and final products, and include food, feed, materials, and chemicals; whereas energy includes fuels, power, and/or heat [9].

Biorefineries are expected to contribute to an increased competitiveness and wealth of the countries by supplying products and energy in an economically, socially, and environmentally sustainable manner. New competences, new job opportunities, and new markets are also expected to elaborate. Furthermore, the development of biorefineries is expected to contribute to the implementation of several European, North American, and global policies and initiatives.

The production of biobased products could generate 10–15 US billion\$ of revenue for the global chemical industry [9]. The potential for chemical and polymer production from biomass has been comprehensively assessed in several reports and papers [10, 11].

As clearly shown in **Table 1**, starting materials, processes, and also products are currently quite different between traditional refineries and biorefineries. However, it might be still very attractive to integrate biomass

INTRODUCTION

processing in traditional refineries as a way to upgrade conventional refineries.

Table 1. Overview of the major similarities and dissimilarities of petrochemical refineries and biorefineries [9].

	REFINERY	BIOREFINERY
Feedstock	Feedstock relatively homogeneous	Feedstock heterogeneous regarding bulk components e.g., carbohydrates, lignin, proteins, oils, extractives, and/or ash Most of the starting material present in polymeric form (cellulose, starch, proteins, lignin)
	Low in oxygen content. The weight of the product (mole/mole) generally increases with processing	High in oxygen content. The weight of the product (mole/mole) generally decreases with processing It is important to perceive the functionality in the starting material
	Some sulfur present. Sometimes high in sulfur	Low sulfur content. Sometimes high in inorganics, especially silica
Building block composition	Main building blocks: Ethylene, propylene, methane, benzene, toluene, xylene isomers.	Main building blocks: Glucose, xylose, fatty acids (e.g., oleic, stearic, sebacic)
(Bio)chemical processes	Almost exclusively chemical processes	Combination of chemical and biotechnological processes
	Introduction of heteroatoms (O, N, S)	Removal of oxygen
	Relative homogeneous processes to arrive to building blocks: Steam cracking, catalytic reforming	Relative heterogeneous processes to arrive to building blocks
	Wide range of conversion chemistries	Smaller range of conversion chemistries: Dehydration, hydrogenation, fermentation Smaller range of conversion chemistries: Dehydration, hydrogenation, fermentation
Chemical intermediates produced at commercial scale	Many (e.g. Polyesters, Polypropylene, PET, Nylon 6.6, Epoxy resins, Solvents, Polyesters fibers)	Few but increasing (e.g., ethanol, furfural, biodiesel, mono-ethanolglycol, lactic acid, succinic acid, ...)

INTRODUCTION

Biorefineries can create a process chain that considers biomass as a resource alternative to coal, crude oil, or natural gas in order to create C2-, C3-, or C4-based chemical platforms.

One of the key prerequisites for a successful biorefinery is to invite key stakeholders from separate backgrounds (agriculture/forestry, transportation fuels, chemicals, energy, etc.) to discuss common processing topics and stimulate deployment of developed technologies in multi-disciplinary partnerships.

1.2. RENEWABLE RAW MATERIALS

The industrial production of biofuels and biochemicals still remains economically unsustainable if expensive and edible substrates are considered [12].

To be economical feasible, many industrial fermentation processes require renewable raw materials having zero or even negative costs. However, low value nutrient sources are often challenging to utilize due to their heterogeneous nature and the presence of contaminant often resulting as inhibitory compounds.

As attempts to reduce the cost of microbial culture media, researchers have investigated the use of various agro-industrial residues including whey permeate, sewage sludge, lignocellulosic biomass, crude glycerol, animal fat, flour-rich waste streams, sugar cane molasses, monosodium glutamate wastewater [13-19] and others.

INTRODUCTION

Currently, the most attractive and studied renewable raw material is by far lignocellulosic biomass, due to its large amount and universal availability: lignocellulosic materials are the most abundant organic compounds in the biosphere, participating in approximately 50 % of the terrestrial biomass.

Also crude glycerol, that is the main byproduct (about 10 % w/w) of the conversion of oils into biodiesel, is recently attracting increasing interests due to its low cost and increasing availability.

In the next paragraphs (1.2.1. and 1.2.2.) lignocellulosic biomass and crude glycerol will be discussed in detail.

1.2.1. Composition of lignocellulosic biomasses

Worldwide annual production of lignocellulose biomass has been estimated to approach 10 - 50 billion dry tons [20].

The major constituents of lignocellulose materials are: *I*) cellulose, *II*) hemicellulose, and *III*) lignin and their relative concentrations largely depend on the plant material used [21].

- I. Cellulose*, the major structural component of plant cell wall, is a high molecular weight linear condensation polysaccharide consisting of several repeated cellobiose (an oligomer of two anhydrous glucose units joined together with β (1 \rightarrow 4) glycosidic bond) units. Despite the fact that it is a polar molecule with several hydroxyl groups, cellulose is insoluble in water [22]. Cellulose exists in different crystalline structures with some more amorphous

INTRODUCTION

regions, and its properties vary in relation to the degree of polymerization [23].

- II. *Hemicellulose* (a collective term of polysaccharides) is the second major carbohydrate constituent of lignocelluloses. In contrast to cellulose, it is highly branched and it has a lower degree of polymerization. Hemicelluloses is made up of a heterogeneous group of polysaccharides and contains both hexose (D-galactose, D-glucose and D-mannose) and pentose (L-arabinose and D-xylose) sugars [20].

The most common type of sugar polymer of hemicellulose family is xylan. Further, hemicelluloses may also contain small amounts of other sugars such as α -L-rhamnose and α -L-fucose, organic acids such as acetic, 4-O-methyl glucuronic, galacturonic, and ferulic acid and the acetyl groups can be partially substituted for the hydroxyl groups of sugars [24].

Hemicellulose is linked to cellulose and lignin by hydrogen bonds and covalent bonds, respectively [25].

- III. *Lignin* is another major component of lignocellulose biomass and it is, by far, nature's dominant source of aromatic polymer [26]. Lignin is an amorphous and highly branched irregular complex polymer, predominantly constituting of three phenylpropane units as the major building blocks: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol [27]. Lignin has been described as the cellular glue that holds cellulose and hemicellulose fibers together; its high

INTRODUCTION

molecular weight, structural complexity, and insolubility in water make its degradation (as well as its valorization) very challenging [28]. Lignocellulose is a natural barrier that not only gives rigidity and strength to the plant cell, but also protects it from attack by microorganisms and insects. As a substrate for bio-products production, however, depolymerization of the lignocellulosic structure is a technological challenge.

Apart from these three major components, plant cell walls also contain a minor amounts of other substances such as pectins, proteins, extractives, several inorganic compounds, and ashes, but they do not have significant impact in forming the lignocellulose structure.

Lignocellulosic biomass is highly abundant and diverse, and include: agricultural, industrial, and municipal wastes, and residues derived from there.

In general, three major types of lignocellulose biomass can be used as renewable feedstock: softwoods (e.g. spruce and pine), hardwoods (e.g. willow, aspen and oak) and grasses (e.g. wheat, rice and barley). Softwoods and hardwoods differ in structure and composition of the hemicellulose: softwood hemicellulose has a higher proportion of mannose and glucose units than hardwood hemicellulose, which usually contains a higher proportion of xylose units [29].

INTRODUCTION

Table 2. Composition of different types of lignocellulosic biomass (% of dry matter).

Biomass	Glucan	Xylan	Galactan	Arabinan	Mannan	Lignin
Agricultural waste						
Corn stover ¹	36.4	18.0	1.0	3.0	0.6	16.6
Wheat straw ¹	38.2	21.2	0.7	2.5	0.3	23.4
Switchgrass ¹	31.0	20.4	0.9	2.8	0.3	17.6
Bagasse ¹	40.2	21.1	0.5	1.9	0.3	25.2
Rapeseed straw ³	34.7	19.4	-	8.3	-	28.1
Rice straw ¹	34.2	24.5	-	-	-	11.9
Softwoods						
Spruce ⁶	43.4	4.9	-	1.1	12.0	28.1
Pine ¹	46.4	8.8	-	2.4	11.7	29.4
Hardwoods						
Sycamore ¹	53.1	17.1	-	1.7	2.7	23.2
Willow ⁵	43.0	24.9	-	1.2	3.2	24.2
Paper waste						
From MSW ¹	56.0	8.3	-	-	5.6	30.1
From newsprint ²	64.4	4.6	-	0.5	16.6	21.0
From chemical pulps ⁴	60-70			20-10		05-10

MSW, municipal solid waste;

References: ¹[30], ²[31], ³[32], ⁴[33], ⁵[34] and ⁶[35].

However, different species of plants have significant differences in the proportions of the main components [36] (**Table 2**). Also, the ratios between these components vary in the same plant species depending on age, stage of growth, and other conditions [37].

Therefore, while such abundance suggest that there is almost an unlimited supply of lignocellulosic biomass, the heterogeneous chemical composition imposes different technical challenges.

1.2.1.1. Pretreatment of lignocellulosic materials

The recalcitrant nature of lignocellulosic materials makes a pretreatment step necessary. The purpose of the pretreatment is mainly to delignify the biomass and decrease the cellulose crystallinity. This leads to an increased accessible surface area and increased porosity and thus enables easier hydrolysis of the carbohydrate polymers.

Typical goals of pretreatment include: production of highly digestible solids that enhances sugar yields during enzyme hydrolysis, avoiding the degradation of sugars (mainly pentoses), including those derived from hemicellulose, minimizing the formation of inhibitors for subsequent fermentation steps, recovery of lignin for conversion into valuable coproducts and to be cost effective by operating in reactors of moderate size and by minimizing heat and power requirements.

The pretreatment can be performed in numerous different ways, usually divided into *I*) physical, *II*) biological, *III*) chemical and *IV*) physico-chemical methods [38].

- I. Physical* pretreatment involves breakdown of biomass size and crystallinity by milling or grinding. Improved hydrolysis results due to the reduction in crystallinity and improved mass transfer

INTRODUCTION

characteristics from reduction in particle size. This method is expensive and likely will not be used in a full-scale process.

- II.* *Biological* pretreatment, as normally defined, involves the use of microorganisms (mainly fungi) to degrade lignin and hemicellulose but leave the cellulose intact. Lignin degradation occurs through the action of lignin degrading enzymes secreted by the fungi. Even though biological pretreatments involve mild conditions and are low cost, the disadvantages are the low rates of hydrolysis and long pretreatment times required compared to other technologies [39].
- III.* Various *chemical* pretreatments were studied aiming at the removal of the hemicellulosic fraction, the cleavage of the bindings between the lignin and the polysaccharides and the reduction of the cellulose crystallinity degree before the enzymatic hydrolysis of cellulose. In particular, some chemicals such as acids, alkali, organic solvents, and ionic liquids have been reported to have significant effect on the native structure of lignocellulosic biomass [40]. Even though a lot of these processes reach high efficiency, disadvantages comprise the requirement of plants constructed with materials which have great resistance for drastic reaction conditions, especially concerning the aspect of environment corrosiveness.
- IV.* The *physico-chemical* category includes the vast majority of pretreatment technologies such as Steam pretreatment (or

INTRODUCTION

Steam Explosion), Liquid Hot Water pretreatment, Wet Oxidation pretreatment, Ammonia Fibre/ Freeze Explosion, Ammonia Recycle Percolation, Aqueous Ammonia pretreatment and Organosolv pretreatment.

Steam explosion is the most commonly used method for the pretreatment of lignocellulosic materials [41]. In this method, biomass is treated with high-pressure saturated steam and then the pressure is suddenly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160 - 260 °C (corresponding pressure, 0.69 - 4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The steam mixture is held for a period of time to promote hemicellulose hydrolysis, and the process is terminated by an explosive decompression. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis.

Steam explosion can be effectively enhanced by addition of H₂SO₄, CO₂ or SO₂ as a catalyst. The use of acid catalyst increases the recovery of hemicellulose sugars, decreases the production of inhibitory compounds and improve the enzymatic hydrolysis on the solid residue [39, 42].

INTRODUCTION

Steam explosion is an attractive process because it makes limited use of chemicals, it does not result in excessive dilution of the resulting sugars; and it requires low energy input with no recycling or environmental cost. However, also this pretreatment has some disadvantage linked to an incomplete destruction of lignin-carbohydrate matrix, resulting in the risk of condensation and precipitation of soluble lignin components making the biomass less digestible, destruction of a portion of the xylan in hemicellulose and possible generation of fermentation inhibitors at higher temperatures.

Each methods have inherent advantages and disadvantages, and effectiveness is strongly substrate-dependent [43].

Ideally, the pretreatment should be applicable to a range of raw materials with low energy demand at low capital and operating costs. The most common practice is that different pretreatment methods are combined in a cost effective way.

1.2.1.2. Hydrolysis

After the raw materials has been pretreated, the polymeric and oligomeric sugars have to be further degraded to monomers by a hydrolysis step. There are mainly two ways to do it: *I)* chemically, by acid catalysis and *II)* biologically, enzymatic treatment.

- I. Acid hydrolysis* can be carried out with either concentrated or diluted acid, with differences between the methods.

INTRODUCTION

The concentrated acid process operates at low temperatures (around 30 - 40 °C), usually utilising H₂SO₄ because of its low cost. The hydrolysis yields are good, with up to 90 % of the theoretical hexoses and pentoses released. In addition to this, the degradation of sugars into inhibitory compounds is low [44]. However, drawbacks of the method include the requirement of an efficient acid recovery process as well as the corrosiveness of the acid used [45]. It is, therefore, difficult to make the process economically feasible and there is nowadays little focus on this process.

The diluted acid process operates with significantly lower concentrations of acid, usually less than 2 %. For this reason the hydrolysis has to be operated at a higher temperature (180 - 230 °C) than those illustrated for the treatment with concentrated acid, in order to achieve high reaction rates [45]. The high temperature and low pH causes degradation of the sugar monomers into toxic compounds. The sugar yields from this process are around 50 - 60 % and up to 90 % of the theoretical, for the cellulosic and the hemicellulosic fraction, respectively.

- II. *Enzymatic hydrolysis* is performed usually using enzyme cocktails whose composition depends on the type of solid fraction [46].

INTRODUCTION

These enzymes are produced naturally by a wide range of organisms such as fungi that grow on rotting wood or microorganisms present in the rumen of cows, and of various insects [47]. The enzymes produced by these organisms are referred to as cellulolytic and hemicellulolytic enzymes, respectively.

The most famous producer of cellulolytic enzymes is the fungus *Hypocrina jecorina*, formerly known as *Trichoderma reesei*. Three major classes of cellulases are known to be necessary for complete hydrolysis of cellulose into glucose monomers: endo-1,4- β -D-glucanases, exo-1,4- β -D-glucanases and 1,4- β -D-glucosidases [48]. Endo-glucanases cleave β -1,4-glycosidic bonds randomly inside the cellulose polymer. Thereby, two chains with twice as many ends are created. This is utilised by the exo-glucanases, or cellobiohydrolases as they are also called, which cleave off cellobiose units from both ends of cellulose fibres. The cellobiose units are in turn hydrolysed into two glucose molecules by the 1,4- β -glucosidases.

Even if pretreatment is often designed to degrade the hemicellulose into monomeric sugars, the supplementation of hemicellulolytic enzymes during hydrolysis can be advantageous. As example, enzymatic activities such as endo-xylanase, exo-xylanase and β -xylanase can be required for the degradation of xylan into monomers. Moreover, synergistic

INTRODUCTION

effects have been observed when cellulolytic and hemicellulolytic enzymes are used together [49].

1.2.2. Crude glycerol from biodiesel production

Crude glycerol can be considered as a waste product deriving from the biodiesel production process. In fact, the triglyceride trans-esterification, necessary to produce methyl- or ethyl-esters, releases glycerol at the end of the process. It is estimated that for every 100 kg of biodiesel produced 10 kg of crude glycerol are generated [50].

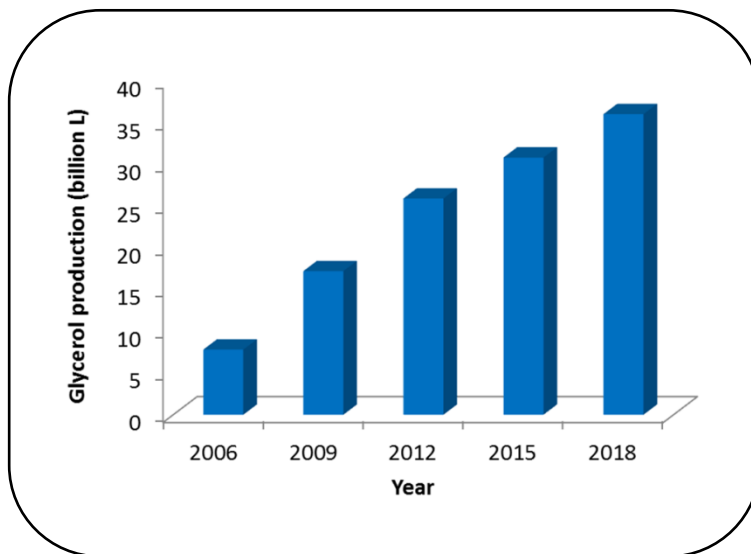


Figure 3. World's scenario of crude glycerol [51].

INTRODUCTION

Glycerol is considered one of the most interesting carbon sources, due to its low cost and its increasing availability (**Figure 3**). However, an efficient valorization of crude glycerol is difficult to achieve, despite this would help significantly in reducing the cost of the overall biodiesel production. This is because of the several impurities present in the crude glycerol such as residual methanol, NaOH, carry-over fat/oil, some esters, and low amounts of sulfur compounds, proteins, and minerals [52, 53].

Therefore, crude glycerol contains too many contaminants for a useful application in chemistry or pharmacy, and the purification process is too costly and energy-intensive [54].

Nowadays, in some countries, crude glycerol is treated as industrial wastewater or simply incinerated, making biodiesel a “grey” rather than a green fuel [52]. In addition, since glycerol can be used as a substrate from different microorganisms, it cannot be released in the environment [55, 56].

For these reasons, the microbial conversion of crude glycerol into value added compounds attracts increasing interest and represent an alternative and “environmentally-friendly” strategy. Several papers underline how economic and environmental benefits could be gained if an industrially relevant process of adding value to crude glycerol will be developed [57].

1.3. YEAST CELL FACTORY

The development of economically feasible and sustainable biotechnological processes as alternatives to oil based chemistry will require efficient, robust and versatile cell factories [58].

Nowadays, microorganisms, such as yeasts, bacteria, and algae, are key players in numerous industrial processes, ranging from the production of traditional fermented foods and beverages to recombinant proteins and other high-value molecules [59].

In particular, yeasts have a large market: the global market reached 5.9 billion US\$ in 2013 and is projected to reach 9.2 billion US\$ by 2019 [60], with a projected 5-year (compound) annual growth rate of 7.9 %. Many of these industrial processes rely on the model yeast *Saccharomyces cerevisiae* (that will be presented more in detail in the paragraph 1.3.1). However, as reported in literature [61], sometimes this yeast is not the optimal host, as example for large-scale production of heterologous proteins. Moreover, in the emerging era of bio-economy microbial cell factories will have to efficiently utilize more sustainable, cheaper and generally available carbon sources, especially lignocellulose and, in this context, *S. cerevisiae* cannot directly utilize xylose (the second most abundant sugar in lignocellulosic biomass after glucose) and arabinose [62, 63].

Disadvantages such as these have promoted a search for alternative hosts. Biotechnologists have summarized all non-*S. cerevisiae* yeasts as “non-conventional” yeasts. What unifies them is a short history of genetic and

INTRODUCTION

biological characterization [64]. Notwithstanding the fact that *S. cerevisiae* remains by far the most widely used industrial yeast species to date, other, so-called non-conventional yeasts, such as *Pichia pastoris* (syn. *Komagataella pastoris*), *Hansenula polymorpha* (syn. *Ogataea parapolyomorpha*), *Yarrowia lipolytica*, *Pichia stipitis* (syn. *Scheffersomyces stipitis*) and *Kluyveromyces lactis* are emerged as valuable contributors to industrial fermentation processes.

Despite of this, there is still significant need of improvement. This is because many industrial strains are currently used because of historical grounds, rather than being carefully selected for a specific application, and are therefore often suboptimal for their purposes. Additionally, demands for increased productivity, wider substrate range utilization, and production of non-conventional compounds in industry, as well as changing consumer preferences, lead to a great interest in further improving the currently used industrial strains and the selection or development of strains with novel properties.

Recent (meta)genomics studies indicate that the natural fungal biodiversity is enormous and largely unexplored, with the current industrial strains only representing a small fraction of the natural biodiversity [65]. This implies that nature possibly provide a wide variety of species and strains that may prove to be superior for certain industrial fermentations.

Despite the immense wealth of natural yeast diversity, the extremely selective and specific conditions of industrial fermentations sometimes require a combination of phenotypic traits that might not be commonly

INTRODUCTION

encountered in nature. Several techniques have therefore been developed to artificially increase the existing yeast diversity and generate variants that may perform better in industrial settings. Genetic modifications as well as non-GMO approaches such as hybridization, mutagenesis, and directed evolution approaches have contributed to the expansion of strains (potentially) useful for industrial purposes [59]. However, each of these techniques has its shortcomings and limitations. In short, non-GMO techniques are often limited to phenotypes that allow efficient selection, and they often involve a risk of improving one property at the expense of others. GMO techniques, on the other hand, require a deep knowledge of the underlying genetics and are in many cases not well received by consumers. The ever-growing knowledge of yeast physiology and the continuously expanding biotechnological toolbox now allow more adventurous techniques. Interestingly, several of these novel techniques combine aspects of both classic non-GMO techniques and genetic modification. These techniques includes: transposon mutagenesis, global transcription machinery engineering (gTME), coupling of zinc fingers with transcription factors, targeting glycosylases to embedded arrays for mutagenesis (TaGTEAM) and others.

Perhaps the greatest promise lies in the combination of carefully selecting the best strains from the immense natural biodiversity, followed by a combination of technique(s) to further improve these natural yeasts in order to generate superior variants for industrial use [59].

INTRODUCTION

Overall, the new knowledge and technologies summarized above clearly show that there are enormous opportunities to obtain superior industrial yeasts and the gains are promising on the route to a true bio-economy.

1.3.1. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae belongs to the phylum Ascomycetous, subphylum Saccharomycotina. *S. cerevisiae*, commonly known as bakers' yeast, is probably the most studied and characterized yeast. It is known for its ability to ferment under fully aerobic conditions glucose to ethanol and carbon dioxide, thus been classified as a Crabtree-positive yeast. Because of this property, it has been used since ancient times for the production of alcoholic beverages and food [66].

In the past 50 years, *S. cerevisiae* has become one of the main cell factories for the production of chemicals from biomass in industrial biotechnology. The reasons underlying the choice of *S. cerevisiae* are several: *I*) is recognized by the American Food and Drug Administration (FDA) as an organism generally regarded as safe (GRAS); *II*) it can tolerate relatively low pH, thus allowing the recovery of the acid in its carboxylated form, decreasing the associated downstream costs; *III*) it does not produce toxins harmful for humans; *IV*) It is very osmo-tolerant *V*) it has a very high glycolytic flux thus through appropriate engineering strategies it is possible to obtain high productivities.

S. cerevisiae has also become an important eukaryotic cell model, since its biochemical and signal transduction pathways are similar to those of higher

INTRODUCTION

eukaryotic organisms, while remaining much simpler to culture. The study of *S. cerevisiae* has contributed to the development of an impressive variety of recombinant DNA technologies. Functional analysis of the genes present in its genome, the first complete eukaryotic genome published [67], has helped to better understand a variety of cellular processes. Moreover, the development of genome-scale metabolic models (GEMs), contributed to the identification of new, non-intuitive targets for metabolic engineering applications [68, 69].

Several works aimed at the engineering of *S. cerevisiae* as cell factory for the production of different classes of compound were published in the last decades, from bioethanol [70, 71] to organic acids, such as succinic [72, 73] and lactic acid [74], and recombinant therapeutic proteins such as insulin [75] and the antimalarial precursor artemisinic acid through the engineering of the isoprenoid pathway [76]. Several studies have also shown that it is possible to expand the substrate range of *S. cerevisiae* for utilization of naturally abundant pentose sugars like xylose and arabinose [62, 77].

The development of advanced molecular biology techniques and the establishment of robust fermentation technologies together with its GRAS status, makes *S. cerevisiae* still one of the best platforms to produce chemicals and biofuels [78].

1.3.2. *Kluyveromyces marxianus*

Kluyveromyces marxianus belongs to Hemiascomycetous phylum, subphylum Saccharomycotina. It is phylogenetically related to *S. cerevisiae* and is a sister species to the better-known *Kluyveromyces lactis* [79].

K. marxianus is classified as facultative fermentative and Crabtree-negative [80]. It is important to note that it cannot grow under strictly anaerobic conditions and that the occurrence of ethanol formation is almost exclusively linked to oxygen limitation [80, 81].

Several *K. marxianus* strains have obtained the “generally regarded as safe” (GRAS) status, similarly to *S. cerevisiae* and *K. lactis* [82] and this is important from an industrial point of view in terms of process approval by regulatory agencies.

This yeast is of particular interest because of traits that render it especially suitable for industrial application: *I*) the ability to grow at temperatures up to 45 °C [83, 84], *II*) the high capacity of converting substrate into biomass [85] and *III*) the highest specific growth rate among eukaryotes [86]. Moreover, this yeast possesses the natural ability to excrete enzymes: this is a desired property for cost-efficient downstream processing of low- and medium-value enzymes [87].

Several different biotechnological applications have been investigated with this yeast: production of enzymes (β -galactosidase, β -glucosidase, inulinase, and polygalacturonases, among others) [88], of single-cell protein, of flavour and fragrance (F&F) compounds [89], of ethanol (including high-temperature and simultaneous saccharification-fermentation processes),

INTRODUCTION

reduction of lactose content in food products, production of bioingredients from cheese-whey, bioremediation, as an anticholesterolemic agent and as a host for heterologous protein production [88].

One important aspect on the physiology of *K. marxianus* is the fact that significant differences in growth parameters, such as μ_{\max} and $Y_{x/s}$, have been reported not only for different strains within the species but also for the same strain when investigated in different laboratories [85]. This makes difficult to gain fundamental knowledge on the metabolism and physiology of this yeast. As suggested in Fonseca and coworkers [88], it would be necessary that researchers started using one or two strains with characteristics that give to *K. marxianus* a clear advantage over other yeasts and this would allow the development of efficient molecular genetic tools.

Currently, introduction of DNA into *K. marxianus* can be achieved by electroporation or by Lithium Acetate transformation using protocols adapted from *S. cerevisiae* and *K. lactis* [90-92].

Until one year ago there was no publicly available a full genome sequence for this species and only 20 % of the CBS 712 strain genome was randomly sequenced [79]. However in 2015 the complete genome sequence and transcriptome analysis of *K. marxianus* DMKU 3-1042 have been published [93]. These data together with new genomic and molecular tools will facilitate in the next future the development of novel applications with this yeast.

1.3.3. *Rhodosporidium toruloides*

The oleaginous yeast *Rhodosporidium toruloides* (formerly known as *Rhodotorula glutinis* or *Rhodotorula gracilis*) belongs to the phylum Basidiomycetes, subphylum Pucciniomycotina [94].

R. toruloides, is a non-pathogenic fungus that can accumulate lipids to more than 70 % of its dry cell weight [95, 96]. In addition, this specie, often called “pink yeast”, has also been reported as a source of carotenoids [97, 98] and biotechnologically important enzymes, such as cephalosporin esterase [99] and epoxide hydrolase [100].

R. toruloides has already been proved to be capable to grow on a broad range of substrates including but not limited to: *I*) sugars (e.g. glucose and xylose), *II*) glycerol, *III*) lignocellulosic hydrolysate, and *IV*) sludge hydrolysate [95, 101].

Moreover, the robustness and tolerance to inhibitory compounds of this yeast has been well demonstrated [102, 103].

The genome of the yeast *R. toruloides* MTCC457 was sequenced in 2012 [104]. In the same year, Zhu and coworkers [105] published a multi-omic map of *R. toruloides* providing valuable resource for efforts to rationally engineer lipid-production pathways.

Transformation of *R. toruloides* by protoplast has been described in 1985 [106], but this method suffered from low efficiency. However, in 2014 an *Agrobacterium*-mediated transformation (ATMT) protocol for effective gene integration into the *R. toruloides* genome was published [107].

1.3.4. *Cryptococcus curvatus*

The oleaginous yeast *Cryptococcus curvatus* belongs to the phylum Basidiomycetes, subphylum Agaricomycotina. *C. curvatus* is a unicellular, anamorphic yeast with an ovoid shape and has a polysaccharide capsule and multilamellate cell wall containing xylose, mannose, and mannitol. This yeast form soft mucoid colonies that can become yellow and wrinkled after one month.

The organism is stable, very easy to grow with minimal nutritional requirements [16] and when grown with high C/N ratio can accumulate up to 60 % of its cellular dry weight as lipids [16, 108]. This accumulated oil is mainly triglyceride (80 - 90 %) [109], with a fatty acid profile rich in 16 and 18 carbon acids showing Δ^9 , Δ^{12} and Δ^{15} desaturation, comparable with plant seed oil [110].

C. curvatus can grow on a broad range of substrates including, but not limited to: *I*) sugars (e.g. glucose, xylose, galactose, mannose, fructose, ribose, maltose, cellobiose, sucrose, and lactose), *II*) glycerol and *III*) whey concentrate or permeate [111, 112].

The genome sequence of *C. curvatus* is available (assembly ASM102816v1), but at the moment no transformation protocol has been published for this yeast.

1.3.5. *Lipomyces starkeyi*

The oleaginous yeast *Lipomyces starkeyi* belongs to the phylum Ascomycetous, subphylum Saccharomycotina. *L. starkeyi* is a unicellular

INTRODUCTION

yeast with an ovoid/round shape that form mucoid colonies with a white/cream colour.

Several biochemical and physiological studies [113] have shown that only a few trace elements influence the growth and lipid content of this yeast [114-116] and that temperature affects lipid composition, with the highest lipid production rate reported at 28 °C [115, 117].

L. starkeyi has the ability to accumulate over 70 % of its cell biomass as lipid under defined culture conditions [118]. This yeast has also others important biotechnological capabilities such as the production of dextranases [119, 120] and delta 12-desaturases [121].

L. starkeyi can grow on a broad range of substrates including, but not limited to: I) sugars (e.g. glucose, xylose, galactose, arabinose), II) whey permeate [122], III) ethanol [111], IV) glycerol and V) sewage sludge [118]. Lipid production is maximal when glucose and xylose are supplied in a ratio of approximately 2:1 [116].

An efficient transformation system for *L. starkeyi* was reported in Calvey and coworkers [123] and in 2011 a proteomic analysis of *L. starkeyi* cells has been published [124].

Currently, no genome sequence of *L. starkeyi* strains is available in literature.

1.3.6. *Zygosaccharomyces bailii*

The yeast *Zygosaccharomyces bailii* belongs to Hemiascomycetous phylum, subphylum Saccharomycotina.

INTRODUCTION

Colony morphology has been described as smooth, convex, and white to cream in color. However, great phenotypic diversity has been observed among *Z. bailii* strains [125].

Z. bailii is osmotolerant and grows preferentially on fructose [126]. This species is similar in some respects to the brewing yeast *S. cerevisiae*, fermenting in aerobic conditions and in anaerobic conditions with suitable nutritional supplementation.

Z. bailii represent the most significant spoilage yeast within the genus, especially in acidic food products [127]. Its success as a spoilage yeast results from a number of physiological traits of the species, in particular, its remarkable resilience against weak acids. *Z. bailii* is reported to be highly resistant to sorbic, benzoic, acetic and propionic acids and to sulphite and hydroxycinnamic acids. It is also able to tolerate high concentrations of ethanol and other sanitizers and to grow in a wide range of pH (2.0–7.0) and water activities (0.80–0.99) [128].

These characteristics make *Z. bailii* an attractive candidate to allow fermentation processes to be performed under otherwise restrictive conditions, or to be used in heterologous protein and metabolite production due to its high resilience to a number of environmental stresses, high specific growth rate and high biomass yield [127]. The use of *Z. bailii* was already found to be successful for the production of lactic acid, L-ascorbic acid (vitamin C) and vitamin B12 [129, 130]. More recently, the potential of *Z. bailii* for the production of bioethanol has also been reported [131].

INTRODUCTION

A quantitative proteomic analysis was recently performed to elucidate the mechanisms underlying the adaptive response and intrinsic high tolerance of *Z. bailii* cells to sub-lethal concentrations of acetic acid [132]. Proteome analysis revealed that the response to acetic acid involves an increased activity of different carbohydrate metabolic processes together with a requirement for increased ATP production, in order to assure acetate catabolism and cell detoxification.

The development of molecular biology tools for *Z. bailii*, such as the isolation of stable auxotrophic mutants and release of a set of vectors allowing ectopic gene expression, is also relatively recent [133].

Until few years ago there was no available genome sequences for species of this genus. The genome sequence of the *Z. bailii* type strain CLIB213^T (=ATCC58445), was released only in 2013 [134]. More recently, the sequencing and annotation of the genome of the *Z. bailii* yeast strain ISA1307 was also described [127].

1.3.7. *Candida lignohabitans*

The yeast *Candida lignohabitans* (also described as *Sugiyamaella lignohabitans*; [135]) belongs to the phylum Ascomycetous, subphylum Saccharomycotina. *C. lignohabitans* strains were isolated from tenebrionid beetles inhabiting rotten logs [136].

Currently, very few information are available in literature regarding its biochemistry, metabolism, or physiology. Actually, the first paper

INTRODUCTION

investigating this yeast and highlighting its potential as a novel microbial cell factory for biorefinery applications was published in 2015 [137].

C. lignohabitans can grow on a broad range of substrates including several hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose). Moreover, in rich medium it is able to consume ethanol, lactate, glycerol and acetate [137].

This yeast has also the remarkable capability to convert both hexoses and pentoses into ethanol, that is a property that very few yeast strains possess [138].

As described by Bellasio and coworkers [137] *C. lignohabitans* is able to grow on rich medium supplemented with hydrolyzed lignocellulosic material and has a remarkable pH tolerance. Therefore, this yeast seems a promising reliable microbial host for production of organic acids from lignocellulosic material.

C. lignohabitans can be efficiently transformed by an electroporation method. Unlike the majority of species belonging to the *Candida* genus, *C. lignohabitans* is not part of the CTG clade therefore metabolic engineering can be performed without any substitution of CTG codons [137].

GAP (glyceraldehyde 3-phosphate dehydrogenase) promoter and GAP terminator demonstrated to be appropriate regulation sequences for the expression of genes in this yeast.

Currently, no genome sequence of *C. lignohabitans* strains is available in literature.

1.4. MICROBIAL CHALLENGES IN LIGNOCELLULOSIC BIOMASS UTILIZATION

Several challenges are associated with the production of biofuels and/or chemicals from lignocellulosic biomasses by yeast.

One challenge is associated with osmotic stress during fermentations at very high sugar/solids content (so-called “very high gravity fermentations”) [139]. Another challenge is related to the fact that often large-scale facilities (e.g. ethanol plants) are not run under sterile conditions and contamination by both bacterial species and yeast species may affect the production of interest [140, 141]. Contaminants may reduce the product yield and/or produce compounds that can inhibit the yeast growth.

In addition to these, two major challenges associated with lignocellulosic material utilization are: the fermentation of pentose sugars and the tolerance to hydrolysate-derived inhibitors and to the final product (e.g. high ethanol concentrations). These will be discussed in detail in the next paragraphs (1.4.1. and 1.4.2.).

1.4.1. Xylose utilization in yeast

As previously described, pentose (C5) accounts for a considerable fraction of the total sugar in several lignocellulosic materials. Consequently, a microorganism capable of utilizing pentose and, in particular xylose, is needed for economically viable utilization of lignocellulosic feedstocks.

INTRODUCTION

Two different xylose pathways have been successfully introduced into *S. cerevisiae* by heterologous expression: I) the XR/XDH pathway, commonly found in aerobic yeasts and fungi, and II) the XI pathway, commonly found in bacteria.

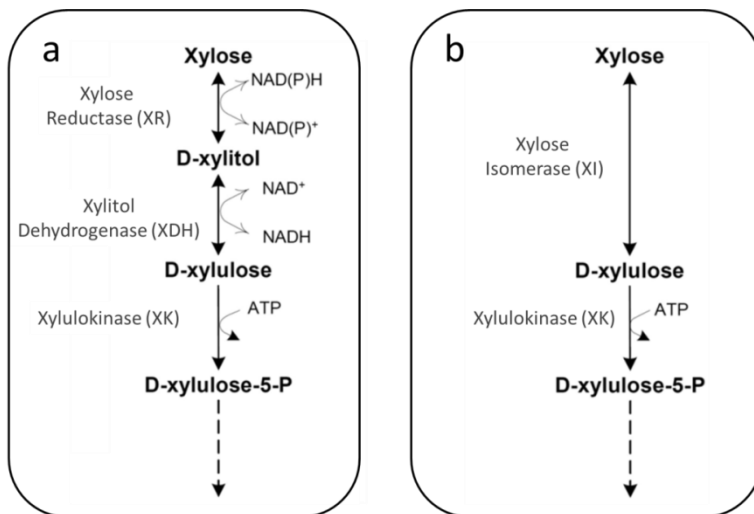


Figure 4. Bacterial (a) and fungal (b) pentose utilization pathways.

In this paragraph both pathways will be described underlying advantages and disadvantages associated with their introduction in *S. cerevisiae*.

- I. Most of the yeast and fungi that can utilize xylose have an oxido-reductive pathway consisting of a xylose reductase (XR), a xylitol dehydrogenase (XDH) and a xylulokinase (XK) (**Figure 4a**). The most studied xylose utilizing eukaryote is the yeast *Schefferomyces (Pichia) stipitis* [142]. The introduction of *S.*

INTRODUCTION

stipitis XIL1 and *XIL2* genes enabling *S. cerevisiae* to utilize xylose was reported for the first time in 1993 [143, 144]. In the XR/XDH pathway, D-xylose is first reduced to xylitol, which is then oxidized to D-xylulose. The XR catalyzes the conversion of xylose to xylitol using electrons stored in reduced cofactors. While some species use only NADPH as cofactor, others have a dual cofactor specificity and can use both NADPH and NADH, but still with an higher specificity for NADPH [145]. The XDH enzyme catalyzes the conversion of xylitol to xylulose and uses exclusively NAD^+ as cofactor.

Since XR prefers NADPH, while XDH utilizes NAD^+ , NADH generated during XDH conversion cannot be re-oxidized in the XR step [146]. This results in a cofactor imbalance and XR/XDH recombinant *S. cerevisiae* strains secrete xylitol during anaerobic xylose utilization. Different strategies, which include cofactor engineering and enzyme engineering, have been applied to reduce cofactor imbalance in xylose engineered *S. cerevisiae* strains. The most successful strategy so far was to generate variants of XR with altered cofactor specificity. Strains expressing these engineered variants produce ethanol with higher yield due to a significant reduced production of xylitol and are also able to grow anaerobically on xylose as sole carbon source [147]. However, the growth on xylose during batch fermentation in a glucose/xylose mixture remains low [148].

INTRODUCTION

It must be pointed out that *S. cerevisiae* does possess the genes for an endogenous oxido-reductive pathway. There are several genes encoding enzymes for xylose reduction (*YJR096W*, *GCY1*, *GRE3* and *YPR1*), xylitol oxidation (*XYL2*, *SOR1*, *SOR2* and *XDH1*) and only one encoding a XK (*XKS1*) [149]. The expressions of these genes are however very low, but the simultaneous over-expression of *GRE3* and *XYL2* has been shown to confer a xylose-positive phenotype [150, 151].

- ii. The bacterial pathway has also been identified in the fungal species *Pyromyces sp.* [152]. It consist of two enzyme (**Figure 4b**): a xylose isomerase (XI) and a xylulokinase (XK). The XI catalyzes the conversion of xylose directly to xylulose, without the use of any cofactor. Due to problems in balancing the redox co-factors in the XR/XDH pathway, efforts have been made to develop recombinant strains expressing a gene encoding XI. However, most genes encoding XI are of bacterial origin and are not translated into fully functional enzymes when expressed in yeast [153, 154]. The recently reported site-directed mutagenesis of the *XylA* gene from *Pyromyces* has led to significant and rapid progress in the development of XI-based xylose fermenting *S. cerevisiae* strains [155]. However, strains with the XI pathway require extensive metabolic engineering combined with long adaptation in xylose medium before their performance can match that of less evolved XR/XDH strains

INTRODUCTION

[156, 157]. The main advantage is still the potential of obtaining high ethanol yields due to very low xylitol production.

As shown in **Figure 4** the XK gene is common for both pathways and is important for achieving a high flux through any of the pathways. XI-strains with endogenous XK activity consume only a fraction of the available xylose and excrete the xylulose formed intracellularly [158, 159]. Similarly, in XK/XDH strains low XK activity leads to xylitol excretion and low ethanol production [160]. The majority of current developed strains only have a single extra copy of the XK gene integrated in the genome to enable xylose utilization. This because in all the tested cases [161, 162] a high XK activity lead to reduced xylitol yield, and increased ethanol yield consequently, but also lead to a reduced xylose consumption rate as well as lower aerobic growth rate.

Many recombinant xylose fermenting strains of *S. cerevisiae* currently carry additional copies of the genes encoding the enzymes active in the non-oxidative part of the PPP [163]. These modifications, together with the deletion of *GRE3* gene, proved to be highly beneficial for xylose fermentation using *S. cerevisiae*.

Another bottlenecks for an efficient xylose consumption in *S. cerevisiae* regards sugar transporters. *S. cerevisiae* has no specific transporter for xylose but the Hxt transporters (glucose transporters) as well as Gal2p transporter are able to facilitate the uptake of xylose [164, 165].

Despite the several publications, a *S. cerevisiae* strain that can efficiently co-ferment glucose and xylose is still missing. Probably, this will not be

INTRODUCTION

possible until a specific xylose transporter will be identified with an affinity for xylose that is at least equal that for glucose.

1.4.2. Lignocellulose derived inhibitors and their effects on the cells

During the pretreatment and non-enzymatic hydrolysis the lignocellulosic materials are not only broken down into the desired sugar monomers but degradation products with inhibitory effects to the subsequent fermentation are also formed. Furthermore, the degradation into the inhibitory compounds leads to a loss of fermentable sugars.

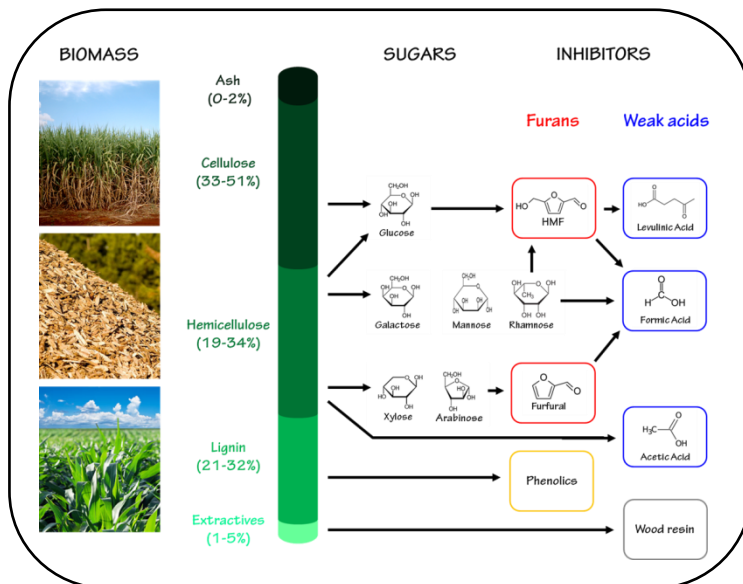


Figure 5. Schematic representation of the lignocellulosic biomass composition and of the main products deriving from its pretreatment and hydrolysis [modified from 166].

INTRODUCTION

The concentration as well the type of such inhibitory compounds can vary greatly depending on the starting raw materials and the adopted pretreatment method.

Commonly, the lignocellulose derived inhibitors are divided into three classes: I) carboxylic acids, II) furan aldehydes and III) phenolic compounds [167].

- I. *Origin and nature:* Carboxylic acids such as formic acid, levulinic acid and acetic acid are the degradation products formed at highest concentration (0.5 - 5 g L⁻¹). Acetic acid is formed by the initial hydrolysis of acetyl groups in hemicellulose, but is also the end product from many oxidation reactions. Formic acid is formed when furfural and HMF are broken down while levulinic acid is formed by HMF degradation [168].

Effects and mechanisms: Undissociated weak acids are liposoluble and can diffuse across the plasma membrane. The growth-inhibiting effect on microorganisms has been proposed to be due to the inflow of undissociated acid into the cytosol [169]. In the cytosol, dissociation of the acid occurs due to the neutral intracellular pH, thus decreasing the cytosolic pH. The concentration of undissociated acids in lignocellulosic hydrolysates is very dependent on pH: it is a function of pH and pKa, and increases with decreasing pH (Henderson-Hasselbach equation). The values of pKa at zero ionic strength for acetic, formic, and

INTRODUCTION

levulinic acid are 4.75 (25°C), 3.75 (20°C) and 4.66 (25°C), respectively.

The inhibitory effect of weak acids has been ascribed to energy uncoupling and intracellular anion accumulation [166]. According to the uncoupling theory, the drop in intracellular pH resulting from inflow of weak acids is neutralized by the action of the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis [169]. Additional ATP must be generated in order to maintain the intracellular pH and under anaerobic conditions this is achieved by increased ethanol production at the expense of biomass formation. At high acid concentrations, the proton pumping capacity of the cell is exhausted, resulting in depletion of the ATP content, dissipation of the proton motive force, and acidification of the cytoplasm. According to the intracellular anion accumulation theory, the anionic form of the acid is captured inside the cell and the undissociated acid will diffuse into the cell until equilibrium is reached. This is supported by the fact that formic acid is more toxic than levulinic acid which, in turn, is more detrimental than acetic acid.

Despite their toxicity, and according to what said before about the flux to the ATP production, low concentrations of weak acids (<100 mmol L⁻¹) have been shown to exert a stimulating effect on the ethanol production at pH 5.5 by *S. cerevisiae* [170].

INTRODUCTION

II. Origin and nature: 5-hydroxymethyl furfural (HMF) and 2-furaldehyde (furfural), are formed as dehydration products of hexoses and pentoses sugars, respectively. The level of furans varies according to the type of raw material and the pretreatment procedure. Furfural is usually found at lower levels than HMF. However, it is often enough (around 1 g L^{-1}) to be inhibitory [166].

Effects and mechanisms: These compounds have been shown to inhibit cell growth, decrease ethanol productivity, induce DNA damage and inhibit several enzymes in glycolysis, thus posing a serious challenge for the feasibility of lignocellulosic ethanol production [171].

Several mechanisms may explain the inhibition effects of ethanol fermentation by furans. In vitro measurements showed that furfural and HMF directly inhibited alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALDH).

S. cerevisiae has been shown to be capable of converting HMF and furfural to less inhibitory compounds, as long as the concentrations are below lethal levels. Under anaerobic conditions, HMF and furfural are mainly converted to their corresponding alcohols, furan dimethanol and furfuryl alcohol, respectively, while during fully respiratory metabolism, furfural is converted to furoic acid [172]. HMF has been reported to be converted at a lower rate than furfural, which might be due to lower membrane permeability, and

INTRODUCTION

cause a longer lag-phase in growth. The proposed mechanism for the intracellular conversion of HMF and furfural has been hypothesized to be NAD(P)H-dependent reduction and NAD(P)⁺-dependent oxidation by oxidoreductases under anaerobic conditions and fully respiratory metabolism, respectively [173].

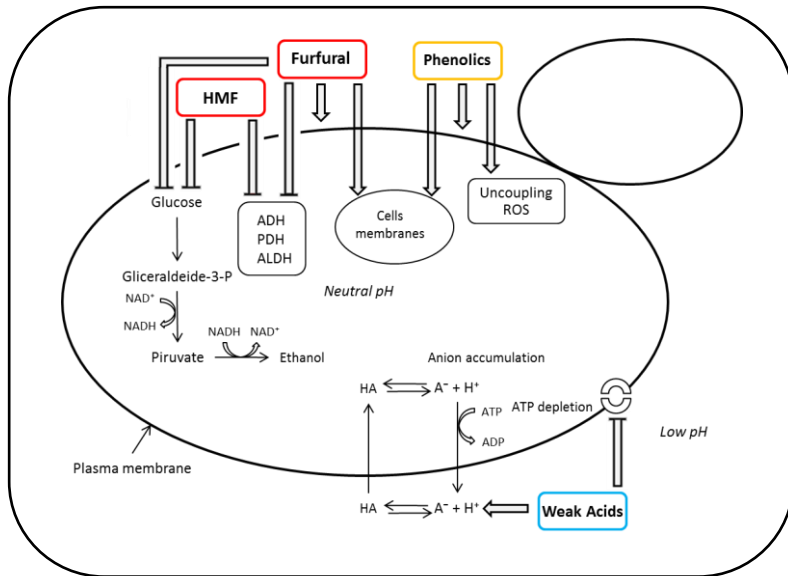


Figure 6. Schematic view of known inhibition mechanisms of furans, weak acids and phenolic compounds in *S. cerevisiae*. HMF: inhibition of ADH (alcohol dehydrogenase), (PDH) pyruvate dehydrogenase and ALDH (aldehyde dehydrogenase), inhibition of glycolysis (either enzyme and/or cofactors). Furfural: same as HMF, plus cell membrane damages. Weak acids: ATP depletion, toxic anion accumulation and inhibition of aromatic amino acids uptake. Phenolic compounds: uncoupling, generation of reactive oxygen species and membrane damage [166].

III. Origin and nature: A wide range of phenolic compounds are generated due to lignin breakdown and also carbohydrate degradation during acid hydrolysis. The amount and type of phenolic compounds depend on the biomass source, since lignin in different raw materials has different degrees of methoxylation, internal bonding and association with hemicellulose and cellulose in the plant cell wall.

Effects and mechanisms: Phenolic compounds have been suggested to exert a considerable inhibitory effect in the fermentation of lignocellulosic hydrolysates and low molecular-weight phenolic compounds are more inhibitory to *S. cerevisiae* than the equivalent high molecular-weight [167]. However, the mechanism of inhibition has not been elucidated, largely due to a lack of accurate qualitative and quantitative analyses. Phenolic compounds may act on biological membranes, causing loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices. Weakly acidic phenolic compounds may destroy the electrochemical gradient by transporting the protons back across the mitochondrial membranes.

The understanding of the inhibitory mechanisms of individual compounds and their interaction effects, as well as the influence of environmental parameters such as pH, have increased considerably in recent years and, hopefully, they will allow the development of efficient lignocellulose-based production processes.

INTRODUCTION

Table 3. Common inhibitory compounds present in lignocellulosic hydrolysates from spruce, willow, wheat straw, sugar cane bagasse and cornstover [166].

GROUP OF COMPOUNDS	CONCENTRATIONS (g L ⁻¹)				
	Spruce ^{a,b}	Willow ^c	Wheat ^d	Sugar cane ^e	Corn stover ^f
Furan derivatives					
5-hydroxymethyl-2-furaldehyde (HMF)	5.9 ^a - 2.0 ^b	n.q.	n.i.	0.6	0.06
2-Furaldehyde	1.0 - 0.5	n.q.	n.i.	1.9	11
2-Furoic acid	n.i.	n.q.	0.007		
Aliphatic acids					
Acetic acid	2.4	n.q.	1.6	4.4	1.6
Formic acid	3.1 - 1.6	n.q.	1.4	1.4	
Levulinic acid	0.9 - 2.6	n.q.	n.i.		
Phenolic compounds					
R1 = R2 = H 4-hydroxybenzaldehyde	n.i.	0.010	0.021		
R1 = H, R2 = OCH3 Vanillin	0.12	0.430	0.032		
R1 = R2 = OCH3 Syringaldehyde	0.107		0.024		
R1 = R2 = H 4-hydroxyacetophenone	n.i.	n.i.	0.004		
R1 = H, R2 = OCH3 Acetovanillone	n.i.	n.i.	0.008		
R1 = R2 = OCH3 Acetosyringone	n.i.	n.i.	0.039		
R1 = R2 = H 4-hydroxybenzoic acid	0.005 - 0.039	n.q.	0.010		
R1 = H, R2 = OCH3 Vanillic acid	0.034 - 0.017	n.q.	0.067		
R1 = R2 = OCH3 Syringic acid	n.i.	n.q.	0.022		
R1 = R2 = H Phenol	n.i.	0.035			
R1 = H R2 = OH Cathecol	0.009 - 0.002	0.440			
R1 = OH R2 = H Hydroquinone	0.017	n.i.			
R1 = R2 = CH R3 = CHO Coniferyl aldehyde	0.035 - 0.054	n.i.			
R1 = CO R2 = CH3 R3 = Acetoguaiacone	0.007	n.i.			
R1 = CO or COH2 or CH2	0.146	n.i.			
R2 = COH2 or CO					
R3 = CH3 or COH2 Hibbert's ketones					
R1 = COOH Cinnamic acid n.i.	n.i - 0.001	n.i.			

Biomass source and pretreatment employed:

^a upper values; two-step dilute acid spruce (*Picea abies*)

^b lower values, one-step dilute acid spruce

^c dilute acid willow (*Salix caprea*)

^d wet oxidation wheat straw (*Triticum aestivum* L.)

^e steam pretreatment sugar cane bagasse

^f steam pretreatment corn stover

n.q. not quantified; n.i. not identified.

1.5. IMPROVING YEAST STRAINS

Both traditional and novel yeast-based industries can benefit from using superior yeasts that yield increased production efficiency and/or product quality.

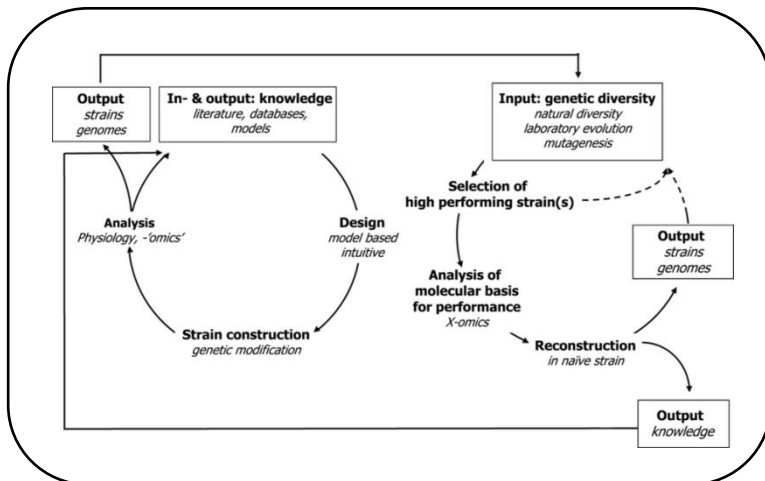


Figure 7. The ‘forward’ (rational) metabolic engineering and ‘reverse’ (inverse) metabolic engineering cycles and their interaction [174].

As previously discussed, demands for increased productivity, wider substrate range utilization and production of non-conventional compounds in industry, lead to a great interest in further improving the currently used industrial strains and the development of strains with novel properties.

INTRODUCTION

Two main strategies (**Figure 7**) are commonly used for improving yeasts: *I*) rational metabolic engineering approaches and *II*) non-targeted approaches.

In the first case, also known as “forward metabolic engineering” (as opposite to “inverse metabolic engineering”), a deep understanding of the pathways and mechanisms behind the phenotype of interest is required [174].

The non-targeted strategies, on the other hand, have usually been used when the background information about the biochemistry or genetics of the relevant phenotype has been limited, or when the desired phenotype involves interactions between multiple metabolic pathways and regulatory elements [175].

In the next paragraphs (1.5.1. and 1.5.2.) these two strategies will be briefly presented. In particular, concerning non-targeted approaches the attention will be focused on evolutionary engineering.

1.5.1. Rational metabolic engineering

Metabolic engineering is the technological discipline that allows the introduction of specific modification to metabolic pathways to improve cellular properties through the manipulation of enzymatic, transport, and regulatory functions of the cell by using recombinant DNA technology [176, 177].

Genomic sequencing efforts have expanded the initial focus from local metabolic pathways to global gene targets, such as competing pathways,

INTRODUCTION

redox balancing and regulatory elements that might not be obvious to be considered. By making changes at these higher regulatory levels, the overall cellular setting can be reprogrammed to give the desired phenotype.

Strains can also be metabolically engineered to produce not-native products through the heterologous expression of biosynthetic pathways deriving from a different organism. Metabolic engineering has been successfully employed for the production of natural and unnatural products in several organisms.

In modern industrial biotechnology, yeast metabolic engineering played a key role in large-scale processes for production of several compounds: second generation ethanol (BP/Verenium, DSM/Poet, Dupont/Danisco, Mascoma and others), succinic acid (DSM/Roquette and Bio-Amber/Cargill), butanols (Butamax and Gevo), and isoprenoid-derived chemicals (Amyris, Firmenich and others) [178], among others.

However, as reported in Cakar and coworkers [175], application of rational metabolic engineering present different problems: *I*) requirement of extensive biochemical and genetic information on the metabolism or metabolic pathway(s) of interest, regulatory factors, enzymes involved and their kinetics, flux-limiting steps, etc.; *II*) complexity of cellular physiological responses; and *III*) difficulties of cloning in industrial strains.

Thus, the engineering of strains by metabolic engineering approach is usually confined to well-characterized hosts, such as *E. coli*, *S. cerevisiae* and *Bacillus subtilis* [179].

INTRODUCTION

Despite these negative aspects, metabolic engineering stands to gain significantly from advances in complementary biological fields. Omics technologies and computational systems biology will provide large amounts of data about a cellular state, whereas protein engineering and synthetic biology will provide toolsets for new ways to manipulate a cell to improve the cellular properties [178].

1.5.2. Non-targeted approaches: Evolutionary engineering

To overcome limitations of 'rational' metabolic engineering, a 'bottom-up' approach called 'inverse metabolic engineering' was defined by Bailey and coworkers [180] as an alternative strategy. In particular, continuous evolution procedures based on the application of a selection procedure to obtain a desired phenotype are generally called 'evolutionary engineering' [181].

Evolutionary engineering, also referred to as direct evolution, or adaptive laboratory evolution (ALE) is performed by applying a selection pressure for the desired phenotype.

Mutagenesis is often performed at the start of the evolutionary process to increase the genomic diversity of the population. Moreover, rational metabolic engineering has also been performed before the evolutionary engineering approach, as example to broaden the substrate range of *S. cerevisiae* to include xylose and arabinose [157, 182].

Evolutionary engineering involves systematic approaches: 1) repeated batch cultivations can be performed in the presence of a selective pressure, or

INTRODUCTION

alternatively, //) prolonged chemostat cultivations can be performed under selective conditions.

- I. In repeated batch cultivations cells are allowed to pass through lag and exponential growth phases before a small fraction of the culture is transferred into fresh medium. After which the procedure is repeated until the desired phenotype is obtained. Over time the selection pressure (e.g. concentration of inhibitors) is increased to select the desired phenotype. Repetitive progress through the growth stages has been reported to result in a selection favoring certain characteristics such as a shorter lag phase and an increased specific growth rate at non-limiting substrate concentrations [181]. Evolutionary engineering by repetitive batch cultivation has successfully been performed to improve the robustness of *S. cerevisiae* to hydrolysates as well as specific inhibitors such as furfural and HMF [183-185].
- II. In chemostat cultivations cells are cultivated in the presence of (for example) inhibitory compounds for extended periods. The advantages of chemostat cultivations are constant growth rates and population densities. Furthermore, it is possible to control nutrient supply and environmental conditions such as pH and oxygenation. A major drawback is instead the costs of operation. In continuous cultures, the growth rate is kept constant (or in certain experimental setups continuously

INTRODUCTION

increasing) by the limitation of a major growth nutrient, such as glucose, nitrogen or phosphate. The nutrient limitation results in a selection pressure for phenotypes with an increased affinity for the limited substrate and consequently a higher specific growth rate at suboptimal concentrations of the limiting nutrient [186, 187]. Evolutionary engineering in chemostat cultivations has been performed to improve the robustness of *S. cerevisiae* to both pretreated sugar cane bagasse and spruce hydrolysate [188].

Typical ALE experiments are performed for somewhat between 100 and 2000 generations and usually take a few weeks up to a few months. In Dragosits and coworkers [189] was estimated that within 100 to 500 generations (corresponding to up to 2 months of selection for a typical *E. coli* or *S. cerevisiae* culture), a fitness increase of up to 50 - 100 % can be achieved.

During ALE, several phenotypes will occur at first and compete for 'dominance' in the total population. Stable phenotypes will accumulate rapidly, although clonal interference [190], bet hedging [191, 192], genetic hitchhiking [193] and fluctuating growth environments can lead to significant population heterogeneity [189, 194]. Thus, it cannot be assumed that a homogenous population is present during any point of a laboratory evolution experiment. It should also be noted that selection for improved fitness in a specialized environment often leads to significant tradeoffs in other stressful or selective conditions [175].

INTRODUCTION

The stability of the evolved strains should be properly addressed by sub-cultivating the improved clones in non-inhibitory medium and thereafter reassessing the superiority of the phenotypes. Once the superior phenotype has been confirmed the next step is the elucidation at molecular level of the factor(s) responsible for the improved phenotype.

Successful evolutionary engineering examples exist with a variety of industrially important microorganisms, such as *Escherichia coli* [195], *Acetobacter aceti* [196], *Bacillus subtilis* [197], *Actinobacillus succinogenes* [198] and *Mannheimia succiniciproducens* [199]. However, it is important to note that improving industrial yeast strains by evolutionary engineering may be more difficult than improving laboratory strains. The main reason is that industrial yeast strains are usually polyploid and are consequently less likely to accumulate relevant recessive mutations by laboratory evolution.

Evolutionary engineering simply mimics the nature by random mutation of the microorganisms own genes. Thus, *S. cerevisiae* and other microorganisms to be used in food and beverage industry will not be considered as GMOs, when improved by evolutionary engineering, and will most likely have higher public acceptance as also mentioned previously [200, 201].

To conclude, evolutionary engineering is a powerful approach with widespread use in improving industrially important and genetically complex properties of *S. cerevisiae* and other microorganisms. It is expected that emerging high-throughput technologies will change evolutionary engineering experiments by decreasing their costs and increasing their

precision and allowing high-throughput continuous and batch processing of multiple samples.

1.6. ETHANOL AS TRANSPORTATION FUEL

There are a number of different biofuels on the market and under development, e.g. biogas, biodiesel, biohydrogen and biobutanol [202]. However, the predominant one is without doubt ethanol.

Already back in 18th century ethanol was used as a fuel in combustion engines. In 1908, Ford Model T (known as T-Ford) was demonstrated to run on either gasoline or pure alcohol. However, by then, interest in using ethanol as a fuel was declined due to the available large quantities of low cost gasoline.

Nowadays, in many countries ethanol is blended with gasoline in various amounts for the use in flexible fuel vehicles [203].

Countries in different parts of the world have set high goals for utilization of biofuels. For example, the European Union (EU) has established that by 2020, 10 % of the fuel used for transportation should be biofuels. The US Department of Energy Office also has a scenario that by 2030, 30 % of the gasoline demand should be replaced by biofuels [204].

Most of the world's ethanol (>90 %) is currently being produced from sugary or starchy feedstocks, sources often called as the "first generation" bioethanol raw materials (**Figure 8**) [205].

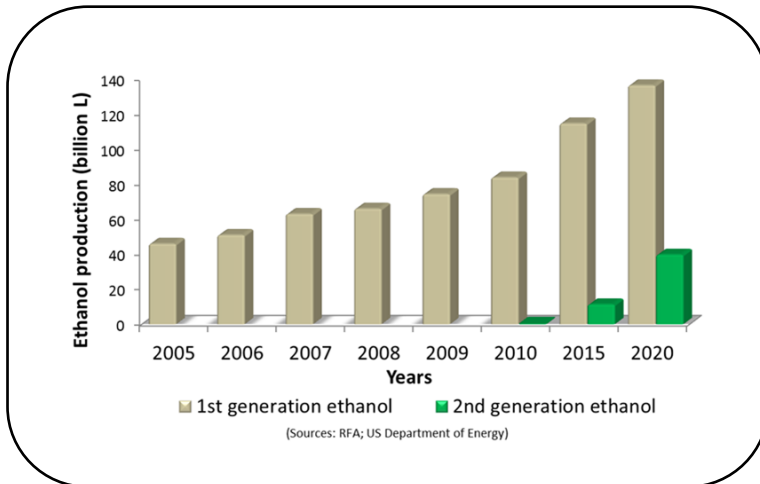


Figure 8. Trend in ethanol production [206].

In 2013, world's top fuel ethanol producers were United States with 13.3 billion gallons (bg) and Brazil with 6.3 bg, together accounting for 84 % of world production [207]. Major feedstocks used in US and in Brazil are corn and sugar cane, respectively. The advantages of first generation bioethanol is that the feedstocks has a high sugar content, conversion processes are fairly simple, and the technology is well established. Most of the Life Cycle Assessment (LCA) studies have shown a net reduction in GHG emissions when bioethanol is used to replace gasoline [208-210].

However, the potential of first generation bioethanol as fossil fuels substitute is limited by different factors: 1) competition for land and water

INTRODUCTION

used with food and fiber production [211, 212]; *II*) high production and processing costs that often require government subsidies in order to compete with petroleum products [213] and *III*) widely varying assessments of the net greenhouse gas (GHG) reductions once land-use change is taken into account [214].

Even though crops grown for biomass feedstocks take up less than 2 % of world's arable land, many authorities agree that selected first generation biofuels have contributed to past increases in world commodity prices for food and animal feeds, at least in part [215].

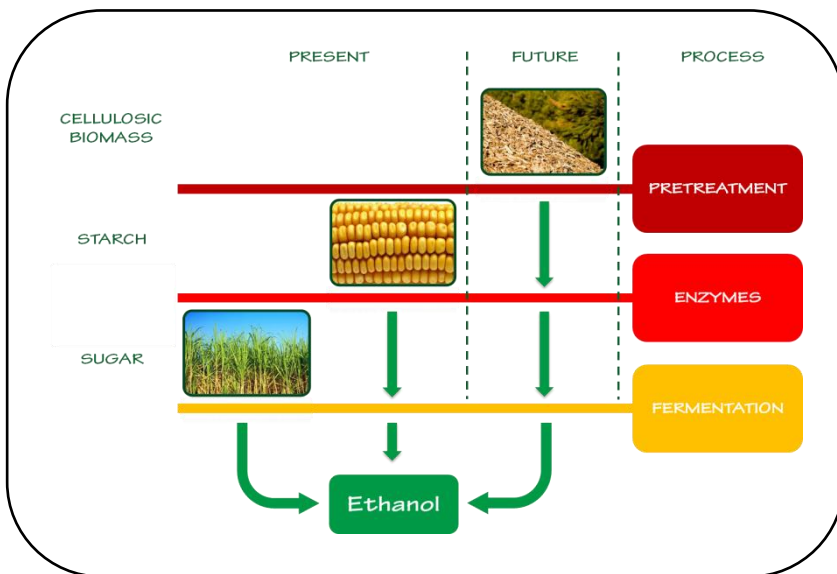


Figure 9. Schematic representation of the first- vs second-ethanol production process.

The cumulative impacts of these various concerns have stimulated the interest in developing “second generation” bioethanol produced from non-edible and possibly waste biomasses (**Figure 9**). When commercialized, the cost of second generation bioethanol has the potential to be more comparable with standard fossil fuels and would be a cost effective route to renewable, low carbon energy for road transport [216].

At present, the production of such fuel is still not cost effective because there are a number of technical barriers that need to be overcome before their potential can be realized. In particular, bioconversion of lignocellulose to bio-ethanol is difficult due to: *I*) the resistant nature of biomass to breakdown, *II*) the variety of sugars which are released when the hemicellulose and cellulose polymers are broken and the need to find or genetically engineer organisms to efficiently co-ferment these sugars, and *III*) costs for harvest and storage of low density lignocellulosic feedstocks.

1.6.1. From raw materials to ethanol: a schematic overview

By definition, second-generation bioethanol refers to ethanol produced by biological fermentation of residual biomass [217, 218]. Although the nature of lignocellulosic raw materials vary considerably, certain process steps are common regardless of the type and source of feedstock:

- Collection: harvesting and storage of the lignocellulosic feedstocks;
- Milling/Chipping: these procedures can be used to reduce the particle size. Common lignocellulosic material can be reduced

to 10-30 mm by chipping and 0.2-2 mm by grinding or milling methods;

- Pre-treatment: process where the structural carbohydrates that compose the biomass are made more accessible for the subsequent steps (for details see paragraph 1.2.1.1.);
- Hydrolysis: breakdown of the polymeric carbohydrates into simple sugars that can be fermented by the microorganisms into ethanol (for details see paragraph 1.2.1.2.);
- Fermentation: conversion of the carbohydrates into ethanol by the selected microorganism or culture (for details see paragraph 1.6.2.);
- Downstream processing: recovery of the ethanol from the fermentation broth (typically by distillation) and management of the remaining streams.

All these steps contribute to the cost of the final product, making important to reduce the energy input in each step [219].

1.6.2. Fermentation

Fermentation is the key step in bioethanol production, in which the sugars obtained from the raw material are converted to ethanol by microorganisms. There are a number of different microorganisms able to produce ethanol as a by-product when they utilize sugars for growth. Examples can be found among both bacteria and fungi, like *Escherichia coli*, *Zymomonas mobilis*, *Kluyveromyces marxianus* and *Pichia stipitis*, among

INTRODUCTION

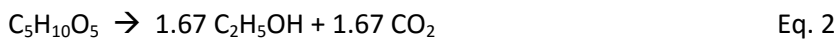
others [220]. However, there are issues with these microorganisms, such as low ethanol productivity or low ethanol tolerance. To date, *S. cerevisiae* is preferred due to its long history of utilization for both ethanol production and baking, and the fact that it has GRAS status (for details see paragraph 1.3.1.). This yeast is a facultative aerobe which means that it can sustain growth in both the presence and absence of oxygen, although ergosterol and unsaturated fatty acids are needed to maintain growth under strict anaerobic conditions [221]. *S. cerevisiae* is known as a Crabtree-positive yeast, which means that it is also able to produce ethanol in the presence of oxygen through respire-fermentative metabolism. This phenomenon occurs when the glucose concentration is above a critical level (>1 mM) and at high glucose consumption rates it produces ethanol even under fully aerobic conditions [222].

Although glucose is the preferred carbon and energy source, other hexoses present in lignocellulosic materials, such as mannose and galactose, can also be catabolized by *S. cerevisiae*. It also possesses many of the traits important for production of second generation bioethanol, such as high ethanol yields and productivity and relatively high inhibitor tolerance.

However, *S. cerevisiae* lacks an active intrinsic pathway for utilization of pentose sugar and in particular for xylose, that is the second most abundant sugar in lignocellulosic materials. Moreover, despite its natural tolerance to inhibitory compounds, concentrations reached during second generation ethanol production process are often too severe to ensure adequate growth of the yeast.

INTRODUCTION

In yeast, during glycolysis 1 mol of glucose is converted to 2 mol of pyruvate with the concomitant production of 2 mol of ATP and 2 mol of NADH. If molecular oxygen is present as terminal electro acceptor (i.e. under aerobic conditions) the pyruvate and NADH formed in glycolysis can be further oxidized to CO₂ and H₂O in mitochondria in the TCA cycle and respiration chain, with the concomitant production of ATP by oxidative phosphorylation. Instead, under anaerobic conditions yeast ferment pyruvate to ethanol via acetaldehyde in a two-step process catalyzed by pyruvate decarboxylase and alcohol dehydrogenase, respectively. Ethanol production regenerate NAD⁺ from NADH, which makes the entire pathway from glucose to ethanol redox neutral. This process lead to the production of 2 mol of ATP per mol of glucose (i.e. eight times less than respiration). Theoretically, 2 moles of ethanol can be obtained by fermenting 1 mole of hexose (Eq. 1). The yield from 1 mole of pentose is lower (Eq. 2).



However, part of the sugars is used for growth and biomass production, and therefore the experimentally obtained yields can never correspond to the theoretical ones. Furthermore, depending on the metabolic pathway that the microorganism uses, other metabolites can be produced alongside ethanol, therefore decreasing the final yield.

1.6.3. Process configuration for lignocellulosic bioethanol production

The majority of the work reported in literature relies on straightforward sequential application of the production process involving pre-treatment of the biomass, hydrolysis, fermentation and product recovery. Simultaneous occurrences or a combination of these steps could hugely impact on the economics of bioethanol production from yeast.

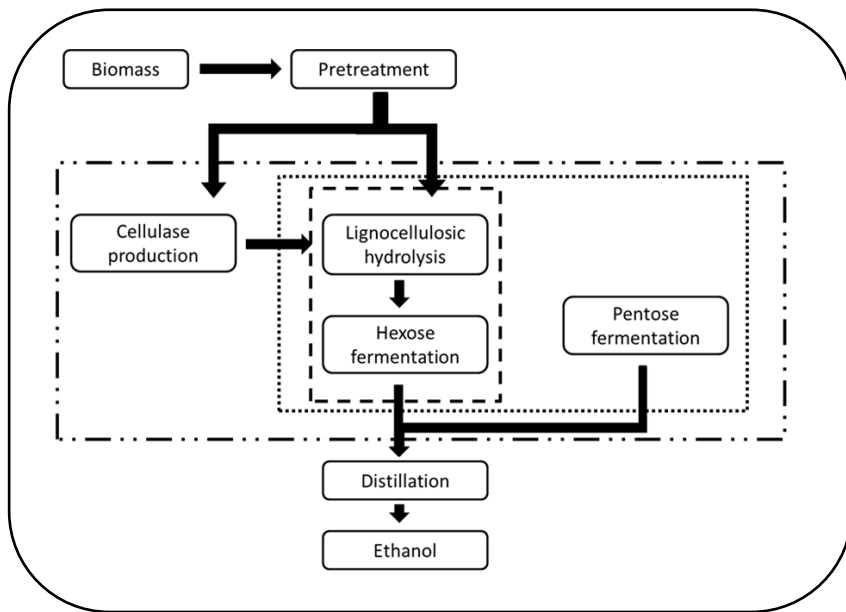


Figure 10. Schematic representation of an ethanol production process starting from lignocellulosic biomass. The different possible strategies are here reported using different dotted boxes.

INTRODUCTION

Different process approaches can play an important role in the reduction of ethanol production costs (**Figure 10**), those so far identified includes: *I*) Simultaneous Saccharification and Fermentation (SSF) *II*) Separate Hydrolysis and Fermentation (SHF), *III*) Simultaneous Saccharification and Co-Fermentation (SSCF), *IV*) Separate Hydrolysis and Co-Fermentation (SHCF) and *V*) Consolidated Bioprocessing (CBP). Every process configuration has its advantages and drawbacks (**Table 4**).

- I.* Over the past decade SSF has become the preferred process, since end-product inhibition of the enzymes can be avoided by performing fermentation in the same vessel at the same time as hydrolysis [223]. Therefore, compared to SHF, the requirement for enzyme is lower and the bioethanol yield is higher in SSF. Furthermore, the higher bioethanol concentration in SSF production also reduces foreign contamination [224]. The capital investment cost of the plant is also reduced as fewer tanks are required. However, a disadvantage of SSF is that the operating temperature must be a compromise between the optimal temperatures for hydrolysis and fermentation.
- II.* In SHF, the enzymatic hydrolysis is performed separately from the fermentation step. Since hydrolysis and fermentation occur in separate vessels, each step can be performed at optimum conditions [225]. More specifically, it enables enzymes to operate at optimum activities to produce more substrates for

INTRODUCTION

yeast fermentation. Furthermore, the yeast produced during the SHF process can be recycled after fermentation of the hydrolysate, which is not possible in SSF [226]. However, the accumulation of hydrolysis products leads to one of the drawbacks of SHF: glucose and cellobiose inhibit the activities of the cellulases so the rate of hydrolysis is progressively reduced [227]. Moreover, the operating costs are higher due to the need to use separate vessels.

- III.* SSCF can be considered as an improvement of SSF. In fact, microorganisms usually applied for bioethanol production cannot utilize all the sugar sources derived from hydrolysis. For example, the wild-type strain of *S. cerevisiae* is unable to use pentose, and this represents a waste of biomass and reduces the bioethanol yield. To overcome this problem, recombinant yeast or cellulosic enzyme cocktails are introduced during fermentation to convert a wide range of both hexoses and pentoses. The hydrolysis and fermentation steps are combined in one vessel for SSCF; hence it has the same characteristics as SSF, such as low cost, short process time, reduced contamination risk and less inhibitory effects [228].
- IV.* SHCF combines the advantages of SHF and SSCF. The hydrolysis and fermentation processes in SHCF take place in separate vessels so that each step can be performed at its optimal conditions. Besides, since in SHCF the microbes utilize both

INTRODUCTION

pentoses and hexoses effectively in the co-fermentation process, the bioethanol yield is higher than SHF. However, there is, to date, only few literatures on SHCF operations.

Table 4. Comparison of the different fermentation process configurations.

PROCESS	ADVANTAGES	DISADVANTAGES
SHF	Hydrolysis and fermentation take place at optimum conditions	Inhibitory effects Increased contamination
SSF	Low quantity of enzyme input High ethanol yield Reduced foreign contamination Less inhibitory effects Lower cost	Either hydrolysis or fermentation can be performed under optimal conditions Difficulty in process control
SHCF	High bioethanol yield Hydrolysis and fermentation take place at optimum conditions	High enzyme load Increased contamination risk Inhibitory effects
SSCF	Shorter process time High bioethanol yield Less contamination risk	High enzyme load Either hydrolysis or fermentation can be perform under optimal conditions
CBP	Cost effective Energy efficient	Lack of suitable organisms Difficulty in process control

- V. CBP simultaneously combines biomass hydrolysis, utilization of liberated sugars and fermentation in one bioreactor [229]. Theoretically, CBP is energy efficient because of the reduction of process steps and is overall more cost effective than SSCF [230]. However, the crucial problem is to develop an organism that singularly combine all the features during the process. Among all the CBP potential microbes, thermophilic bacteria,

such as *Clostridium thermocellum*, are believed feasible as they possess cellulolytic and ethanologenic characteristics under high temperature conditions [231].

1.7. BIODIESEL

Biodiesel is a biodegradable, non-toxic, almost sulfurless and non-aromatic environmentally friendly alternative to diesel fuel [232]. It is defined as the fatty acid alkyl monoesters derived from renewable feedstocks, such as vegetable oils and animal fats.

Biodiesel has a high cetane number, good lubricity properties, an energy content comparable to conventional mineral diesel fuels, and is easily mixed with its conventional counterpart. The molecular weights of the methyl esters are similar to diesel fuels, making their transport properties and melting points superior to the fats and oils from which they were derived. Technically, biodiesel can be considered a good quality component for mixing into diesel fuel, usually at concentrations up to 20 %, provided it is produced to adequate quality specifications.

The global biodiesel industry has grown significantly over the past decade. The EU has arguably been the global leader in biodiesel production [233] and the global biodiesel market is estimated to reach 37 billion gallons by 2016.

INTRODUCTION

Currently, the end cost of the biodiesel mainly depends on the price of feedstock. The high cost of the food-grade oils causes to increase the cost of biodiesel and prevents its usage.

To become an economically viable alternative fuel and to survive in the market, biodiesel must compete economically with diesel fuel.

1.7.1. Chemistry of Biodiesel

There are four chemical processes used to solve the high viscosity problem of triglycerides: dilution, microemulsification, pyrolysis (thermal cracking), and transesterification [234]. Among these four, the commonly preferred process is transesterification.

In the transesterification reaction, a catalyst is used to enhance the reaction rate and improve the fuel's features. Among the catalysts used for transesterification, alkaline catalysts (NaOH, KOH, NaOCH₃, etc.) are most commonly preferred when compared to acid (H₂SO₄, HCl, etc.) and lipase (biologic) catalysts since they are faster, and lower catalyst amount is sufficient to carry out the reaction [232]. The alcohol used for transesterification is usually methanol.

The general scheme of the transesterification reaction is presented in **Figure 11**.

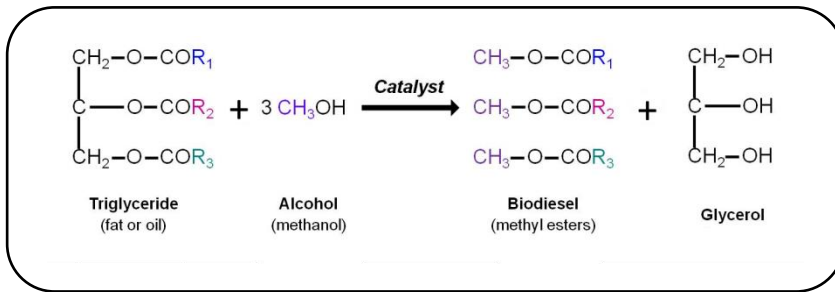


Figure 11. Transesterification reaction.

In principle, transesterification is a reversible reaction, although in the production of biodiesel the back reaction does not occur or is negligible because the glycerol formed is not miscible with the product, leading to a two-phase system. Nevertheless, an excess of alcohol is usually employed to force the reaction towards the products side. The stoichiometry of reaction is a 3:1 molar ratio of alcohol to oil, to produce 3 mol of biodiesel and 1 mol of glycerol. Though, in practice it is usually increased from 6:1 to 1000:1 to favor the formation of products and increase its performance.

The main constituents of oils and fats are triglycerides, representing about 90 – 98 % of total mass. Triglycerides are composed of three fatty acids (R-COOH) and one glycerol [C₃H₅(OH)₃].

As shown in **Table 5** fatty acids vary in their carbon chain length and in the number of double bonds (unsaturation level).

INTRODUCTION

Table 5. The chemical structure of commons fatty acids.

Fatty Acid	Chemical Structure
Myristic (14:0)	$\text{CH}_3(\text{CH}_2)_{12}\text{COOH}$
Palmitic (16:0)	$\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$
Palimtoleic (16:1)	$\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Stearic (18:0)	$\text{CH}_3(\text{CH}_2)_{16}\text{COOH}$
Oleic (18:1)	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Linoleic (18:2)	$\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Linolenic (18:3)	$\text{CH}_3\text{CH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Arachidic (20:0)	$\text{CH}_3(\text{CH}_2)_{18}\text{COOH}$
Arachidonich (20:4)	$\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_3\text{COOH}$

The physical and chemical fuel properties of biodiesel basically depend on the fatty acids distribution of the triglyceride used in the production.

The quality of biodiesel can be influenced by a number of factors: the quality of the feedstock, the fatty acid composition of the vegetable oil or animal fat, the production process and the other materials used in this process, the post-production parameters and the handling and storage.

The European standard EN 14214, adopted by all 31 member states of the European Committee for Standardization (CEN), regulate biodiesel proprieties in terms of: ester content, density, viscosity, flash point, sulfur content, carbon residue, cetane number, sulfated ash, water content, total contamination, copper strip corrosion, oxidation stability, etc.

Quality standards for biodiesel are continuously updated, due to the evolution of compression ignition engines, ever stricter emission standards

and re-evaluation of the eligibility of feedstocks used for the production of biodiesel.

1.7.2. Lipids from yeast

An emerging potential alternative for biodiesel production is represented by microbial lipids, also referred as single-cell oils (SCOs), which could lead to a green and sustainable biodiesel production process, with no competition with the food supply chain [235].

Microbial oil show similar chemical composition and energy value to plant and animal oils [236]. A number of microorganisms belonging to the genera of algae, bacteria, yeast and fungi have the ability to accumulate lipids under specific cultivation conditions [237]. Among them, the utilization of oleaginous yeast is advantageous due to fast growth rate and high oil content compared to algae [233].

Oleaginous yeasts are typically found, but not exclusively, in genera such as *Candida*, *Cryptococcus*, *Rhodotorula*, *Rhizopus*, *Trichosporon*, *Lipomyces*, and *Yarrowia* [238].

Under conditions of nutrient limitation, these yeasts may accumulate lipids to levels exceeding 70 % of their biomass. They consist of 80 - 90 % triacylglycerols (TAG) and a small fraction of steryl esters (SE), collectively called neutral lipids. These storage lipids accumulate in a specialized compartment of the cell known as the lipid body (LB) that consists of a lipid core encased in a phospholipid monolayer within which many proteins with diverse functions are embedded [239].

INTRODUCTION

De novo fatty acid synthesis in yeast is carried out by the fatty acid synthetase (FAS) enzymatic complex in the cytosol and requires the constant supply of acetyl-CoA and malonyl-CoA. The former is the initial biosynthetic unit and the latter the elongation unit. The origin of acetyl-CoA, however, differs in oleaginous and non-oleaginous species. Non-oleaginous yeasts produce acetyl-CoA from glycolysis of fermentable sugars by breaking down pyruvate in the mitochondrion and by the cytosolic pyruvate-acetaldehyde-acetate pathway. In oleaginous microorganisms, an additional source of acetyl-CoA exists: its precursor is the excess of citrate exported from the Krebs cycle out of the mitochondrion, via the malate/citrate shuttle. Citrate is then cleaved within the cytosol by ATP citrate lyase (ACL), an enzyme present in all oleaginous microorganisms studied so far [240].

The fatty acids produced by the de novo lipid synthesis or incorporated from the medium are then esterified either in the glycerol backbone or in a sterol molecule to form TAGs and SE, respectively. The whole process is known as the storage lipid pathway, and its end products form the neutral lipid fraction of the cell, packed inside the LB.

It is now well established that cultivation conditions, nitrogen availability, temperature, pH, metal traces, and mineral concentrations all influence lipid accumulation in oleaginous yeasts [96].

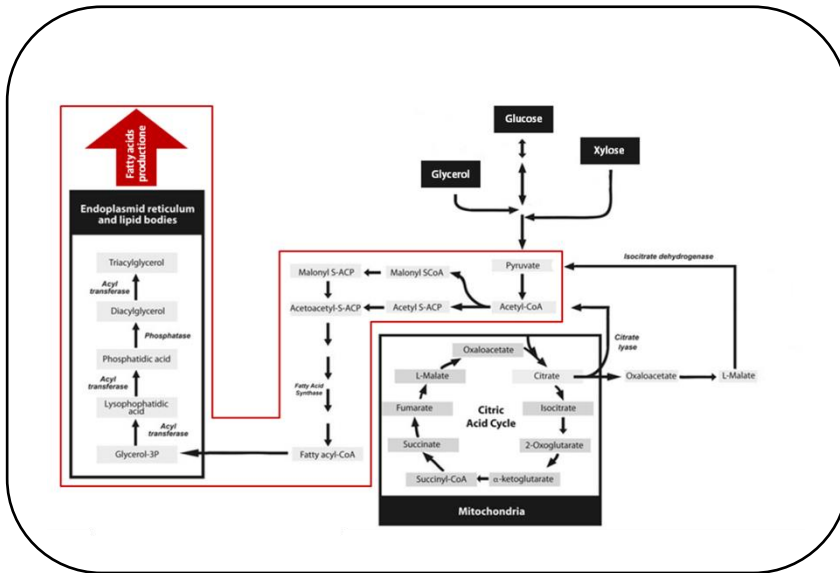


Figure 12. Microbial metabolism of glucose and glycerol to produce single-cell oil [241].

Cells begin to accumulate lipids when an essential element for cell proliferation becomes limiting in the medium and the carbon source is present in excess. Once cell proliferation becomes limiting, carbon is directly channeled into lipid synthesis with the resulting buildup of neutral lipids. Then, in the presence of an excess of carbon source, depletion of any essential nutrient can be used for setting on lipid accumulation, like nitrogen, magnesium, zinc, iron, or phosphorus. However, nitrogen limitation is generally used (often referred to as a high C/N ratio). This is the easiest condition to control and it results in the most efficient accumulation

INTRODUCTION

induction factors. The reason of this efficiency lies in an additional regulation affecting the TCA cycle with the excessive production of the main precursor of fatty acid synthesis.

A typical profile for the accumulation of lipid in an oleaginous microorganism is shown in **Figure 13**.

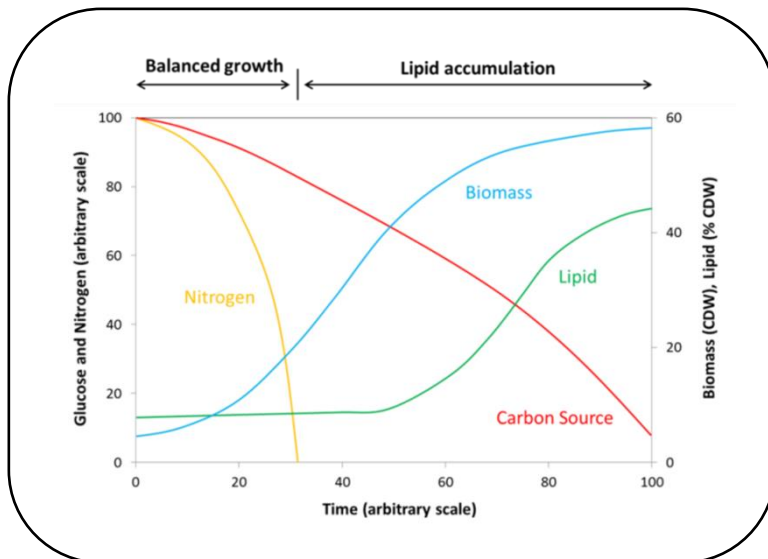


Figure 13. Idealized representation of the process of lipid accumulation in an oleaginous microorganism. The composition of the culture medium is formulated so that the supply of nitrogen, which is usually an ammonium salt is growth limiting. After its exhaustion, cells do not multiply any further, but they continue to assimilate glucose (the usual carbon feedstock). This is then channeled into the synthesis of storage lipid (triacylglycerol) within the cells. The extent of lipid accumulation is dependent upon the individual microorganism-lipid contents may vary between 20 and 70 % of the biomass [242].

INTRODUCTION

In order to fine tune the C/N ratio, there are different culture options such as batch, fed-batch, and continuous fermentation modes. Briefly, in batch mode all minerals and carbon substrates are initially mixed in the reactor, with a high initial C/N ratio to boost lipid accumulation. No further monitoring of the C/N ratio is possible in this mode of culture. In the continuous mode, the C/N ratio stays constant throughout culture and regulation of substrate concentration leads to the fine tuning of growth rate: lower growth rates promote more extensive lipid accumulation. Finally, fed-batch mode allows a precise control of nutrient and substrate flow rates. This ensures efficient and reproducible lipid production.

Currently, the production of microbial oil is more expensive than that of vegetable oils [12]. The cost of feedstock or carbon source required for the production of microbial lipids accounts for 60 to 75 % of the total costs of the biodiesel. Therefore, it is clear that the production of microbial oil using cheap substrates, such as waste streams (e.g. whey, crude glycerol, lignocellulosic hydrolysates), is a key parameter in order to develop an economically and environmentally viable biodiesel production process [108].

1.7.3. Steps involved in microbial biodiesel recovery

The separation of individual components from microorganisms offers a wider range of opportunities for byproduct utilization [243]. High-value metabolites and lipids for biofuels can be extracted to be sequentially or independently processed depending on the transformation technology.

INTRODUCTION

Different steps are involved in processing microbial extracts: *I*) harvesting, *II*) drying, *III*) cell disruption, *IV*) lipid extraction, and *V*) transesterification of the extracted lipid.

- I.* The first step in the production of biofuels from microbial extracts requires the transformation of biomass from diluted cultures to concentrated pastes which are much more suitable for downstream processing. Microbial harvesting can be achieved through filtration, flocculation, flotation, centrifugation, and sedimentation.
- II.* Drying is commonly used to extend shelf life of the final product or, depending on the final application, as a pre-processing step. Depending on the selected microorganism, different technologies can be applied for biomass dehydration such as sun drying, low-pressure drying, spray drying, drum drying, fluidized bed drying, or freeze drying.
- III.* Cell disruption is often required for recovering intracellular products from microorganisms, such as microalgae and yeast, which are protected by extremely tough cell walls. Typical laboratory extraction protocols involve unit operations such as freeze drying, pulverization, cell homogenization, sonication, autoclaving, osmotic shock, or microwaving, but none are good candidates for use at industrial scale. Extraction of intracellular lipids from yeast also requires breakage of the thick yeast cell

INTRODUCTION

wall. This process is commonly achieved by using acids, bases, enzymes, or by applying physical and mechanical pressure.

- IV. After cell disruption, the most common way of extracting different metabolites is by using solvents. The solvent extraction should meet several requirements such as being inexpensive, volatile, pure, water-immiscible, and poor extractors of unwanted components. Hexane, ethanol, chloroform, and diethyl ether are some of the most common solvents used.

For yeast, several specific lipid extraction technologies have been proposed in recent years. These involve the use of solvents at near-critical or supercritical conditions, or the use of pressurized liquids of different solvent mixtures [244, 245]. Direct extraction from fermentation broths has also been studied by using homogenizers under high pressure, microwaves and subsequent enzymatic treatments, and solvents [246]. However, only some of these approaches have been scaled-up, and their feasibility and cost-effectiveness are justified only when value-added specialty lipids or metabolites are produced.

- V. The transesterification reaction is a key step in biodiesel production and has been extensively described in the previous paragraph (1.7.2.).

1.8. MICROBIAL PRODUCTION OF ORGANIC ACIDS

Organic acids are broadly distributed in nature, and humans have used them since early ages. Organic acids find their wide application in the food, pharmaceutical and chemical industries [247, 248].

Nowadays, organic acids constitute a significant portion of the fermentation market in the world. Microbial production is an important economic alternative to the chemical synthesis for many of them, since chemical synthesis often requires very harsh conditions and involves many steps.

Several organic acids can be produced through fermentations: citric acid, lactic acid, fumaric acid, propionic acid, malic acid, α -ketoglutaric acid, 5-ketoglutaric acid, 2-ketoglutaric acid, gluconic acid, acetic acid, kojic acid, itaconic acid, etc [247, 249]. For some organic acids like citric, lactic, gluconic, and itaconic advances in the fermentation technology have helped to manufacture organic acid at industrial scale. However, others are not currently being produced commercially by fermentation, but have significant potential for future manufacture by this route (**Table 6**). It is noteworthy that for many organic acids the actual market is small, but an economical production process will create new markets by providing new opportunities for the chemical industry [247].

Table 6. Organic acids as high volume products^a [247].

Number of carbon atoms	Organic acid	Annual production (t)	Annual production by microbial process (t) ^b	Projected market volume (t)	Use (examples)
C ₂	Acetic acid	7 000 000	190 000		Vinylacetate for polymers, ethylacetate as "green" solvent
C ₂	Oxalic acid	124 000	-		Synthetic intermediate, complexing agent
C ₃	Acrylic acid	4 200 000	-		Polymer production
C ₃	3-hydroxypropionic acid	n.a.	-	Up to 3 600 000	Potential substitute for acrylic acid and production of biodegradable polymers
C ₃	Lactic acid	150 000	150 000		Food and beverages, biodegradable polymer production
C ₃	Propionic acid	130 000	n.a.		Food and feed
C ₄	Butyric acid	50 000	n.a.		Therapeutics, aroma, fragrance
C ₄	Fumaric acid	12 000	-	> 200 000	Food and feed, polyesters resins
C ₄	Malic acid	10 000	-	> 200 000	Potential to replace maleic anhydride
C ₄	Succinic acid	16 000	-	> 270 000	Potential to replace maleic anhydride, manufacture of tetrahydrofuran, polymers
C ₅	Itaconic acid	15 000	15 000		Speciality monomer
C ₅	Levulinic acid	450	-	High	Possible precursor for bulk chemicals
C ₆	Adipic acid	2 500 000	-		Production of nylon 6,6 esters used as plasticizers and lubricants
C ₆	Ascorbic acid	80 000	-		Food additive
C ₆	Citric acid	1 600 000	1 600 000		Food additive
C ₆	Glucaric acid	n.a.	-	High	Production of new nylons, new building-block
C ₆	Gluconic acid	87 000	87 000		Food additive, metal chelator

n.a., no data are available

^aThese numbers are intended to give the reader an impression of the order of magnitude in which relevant acids are on the market. One should be aware that reliable market data are not often found in the public domain.

^bThe "-" indicates that these acids are not microbially produced on an industrial level to our knowledge.

The quickest way to establish an industrial process for microbial organic acid production is the exploitation of natural producers with thorough bio-process engineering. The choice of the cell factory influences by-product formation and therefore influences costs. Strain robustness inevitably has to be considered for a process to be viable at large scale.

To improve the established processes for organic acid production, the access to cheap substrates is crucial. However, the choice of the carbon

INTRODUCTION

source cannot be made without thinking about the purification: purification costs are higher with less purified substrates and by-products can also constitute an important problem.

As reported in [247] an integrated picture of the production processes is currently missing in the academic world and the obstacles for a large-scale commercialization should be studied without ignoring the greater context of industrial manufacturing.

To conclude, a large number of organic acids by microbial fermentations are already economically viable and with the increase of the market will increase also the need for the development of highly efficient biotechnological processes. In this context, in the next paragraphs lactic acid and itaconic acid will be presented in detail.

1.8.1. Lactic acid

Lactic acid (2-hydroxy prop ionic acid) is the most widely occurring carboxylic acid in nature and was discovered in 1780 by Carl Wilhelm Scheele, a Swedish chemist that isolated it in the form of dense brown liquid from milk.

Lactic acid is a three carbon organic acid: one terminal carbon atom is part of an acid or carboxyl group, the other terminal carbon atom is part of a methyl or hydrocarbon group and a central carbon atom having an alcohol carbon group. Lactic acid exists in two optically active isomeric forms.

It can be manufactured by chemical synthesis or carbohydrate fermentation. Though chemical synthesis produces a racemic mixture,

INTRODUCTION

stereo specific acid can be made by carbohydrate fermentation depending on the strain being used.

Lactic acid has a long history in the food industry for its application as an acidulant, flavoring agent, pH buffering or preservative [250, 251]. It is also used as an additive in animal feed, in detergents and in pharmaceutical preparations. In contrast to other food acids it has a mild acidic taste. It is non-volatile odorless and is classified as GRAS by FDA in the US. In the cosmetic field, the lactic acid can be used as a moisturizing agent, anti-acne, anti-tartar, and to brighten and rejuvenate the skin [251]. Lactic acid is being used in many small scale applications like pH adjustment hardening baths for cellophanes used in food packaging, terminating agent for phenol formaldehyde resins, alkyd resin modifier, solder flux, lithographic and textile printing developers, adhesive formulations, electroplating and electro-polishing baths, detergent builders [252]. Furthermore, the presence of both carboxylic and hydroxyl groups in the lactic acid molecule enables its conversion into different technologically useful chemicals such as pyruvic acid, acrylic acid, 1,2-propanediol and lactate ester via chemical and biotechnological routes [253-255], making it a primary chemical platform.

However, the most interesting application from an environmental point of view, is the production and the application of its derivative polylactic acid (PLA), since such biopolymer could replace traditional petrochemical-based plastic. PLA is a biodegradable aliphatic polyester industrially obtained from renewable resources, such as corn or sugar beets. The monomer, lactic acid

INTRODUCTION

(LA), is mainly produced by a bacterial fermentation batch process. In the last decades, increasing efforts have been registered both from academia and industry towards understanding and deepening the large-scale production processes of PLA. Accordingly, a large fraction of the degradable polymer market from renewable resources is nowadays covered by this type of material. Moreover, due to its biodegradability and biocompatibility, PLA has been approved by the regulatory agencies of many countries for medical applications such as suture threads, implantable scaffolds, bone fixation devices, and micro- and nano-capsules [256].

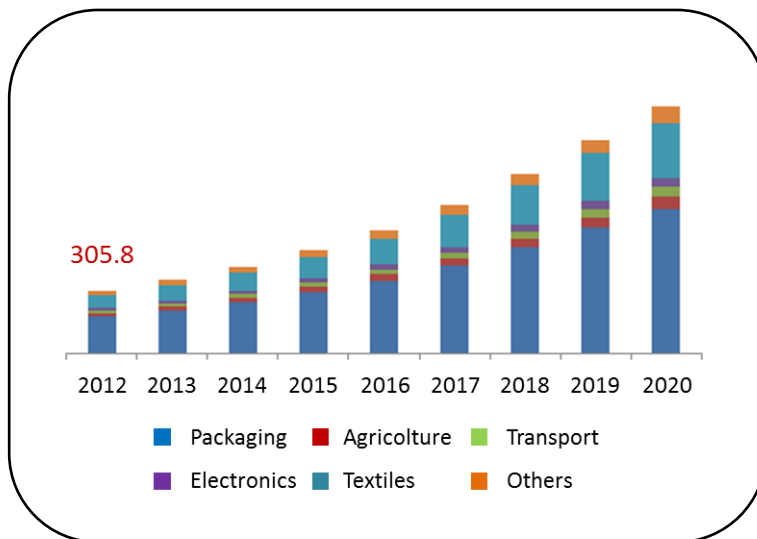


Figure 14. Global PLA market by application, 2012 - 2020, (Kilo Tons) [257].

INTRODUCTION

Currently, It is possible to produce various lactate-containing polyesters by one-step fermentation of recombinant microorganisms equipped with the engineered PLA biosynthesis system [258]. The new challenge is to maximize the production of lactate-containing polymers in a cost-effective manner.

1.8.2. Itaconic acid

Itaconic acid is a naturally occurring unsaturated 5-C dicarboxylic acid which is also known as methylenesuccinic acid or methylenebutanedioic acid.

Since the 1960s the production of itaconic acid is achieved by the fermentation with *Aspergillus terreus* on sugar containing media [259]. Besides *A. terreus*, itaconic acid is known to be produced also by other fungi like *Ustilago zaeae*, *Ustilago maydis*, *Candida* sp., and *Rhodotorula* sp. [259-261].

Currently, *A. terreus* is still the dominant production host, because so far only bred strains of this species can reach levels of up to 80 - 86 g L⁻¹ [262, 263]. Although this is already a substantial amount, it cannot be compared with the production of citric acid where titers over 200 g L⁻¹ are steadily obtained in industrial processes. Transferred to the itaconic acid production a maximal theoretical titer of about 240 g L⁻¹ should be achievable [264].

Several attempts have been made to find microorganisms with improved characteristics including greater yields, higher tolerance to shear stress, and/or tolerance to higher end product concentration.

INTRODUCTION

The pathway for the biosynthesis of itaconic acid was firstly proposed in 1957 [265] and subsequently confirmed by tracer experiments with ^{14}C and ^{13}C labeled substrates. Starting from a sugar substrate like glucose, the carbon molecules are processed via glycolysis to pyruvate. Then the pathway is split and part of the carbon is metabolized to acetyl-CoA, releasing a carbon dioxide molecule. The other part is converted to oxaloacetate so that the previously released carbon dioxide molecule is again incorporated. In the first steps of the citric acid cycle, citrate and *cis*-aconitate are formed. In the last step, the only itaconic acid pathway dedicated step, *cis*-aconitate decarboxylase (CadA) forms itaconic acid releasing carbon dioxide.

The formation of carboxylic acids, like citric and itaconic acid, involves the shuttling of intermediate metabolites between different intracellular compartments and utilizes the different enzymatic capabilities of the respective compartment.

The key enzyme of the pathway, CadA, is not located in the mitochondria but in the cytosol, whereas the enzymes preceding in the pathway, namely citrate synthase and aconitase, are found in the mitochondria. The proposed mechanism is that *cis*-aconitate is transported via the malate-citrate antiporter into the cytosol [266]. However, so far it was not shown whether *cis*-aconitate makes use of the mitochondrial malate-citrate antiporter or uses another mitochondrial carrier protein to be translocated to the cytosol [267]. Itaconic acid is valuable as a monomer because of its unique chemical properties, which derive primarily from its methylene

INTRODUCTION

group and its possession of two carboxylic acid groups. Itaconic acid is able to take part in addition polymerization, giving polymers with many free carboxyl groups that confer advantageous properties on the polymer. It can either be self-polymerised or can act as a co-monomer with other monomers to form heteropolymers.

In the future it can function as a substitute for acrylic and methacrylic acid used for the production of plastics [262]. However, the current market price of itaconic acid, estimated of \$ 2 per kg, is still too high for these applications. The identification of novel host able to grow on renewable waste raw materials (e.g. whey, crude glycerol, lignocellulosic biomass), having zero or even negative costs, could make itaconic acid production economically competitive.

Recently, *Candida lignohabitans*, was proposed as a novel microbial cell factory for itaconic acid production [137] and experiments are currently ongoing to increase itaconic acid production and productivity.

- In a paved road it's comfortable to walk, but no flowers grow -

CONCLUSIONS AND FUTURE PERSPECTIVES

Biorefineries can provide a significant contribution to sustainable development, generating added value from renewable biomasses use and producing a range of biobased products, biofuels and energy.

Despite the great relevance of biorefineries, there are still numerous technical challenges that need to be overcome before any large-scale commercialization can succeed.

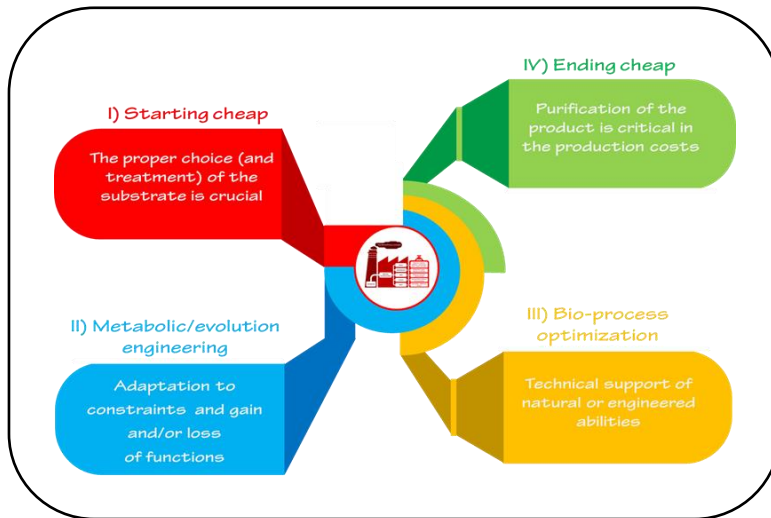


Figure 15. The biorefinery road map.

For approaching to the ideal biorefinery road map (**Figure 15**), it is possible to identify the main bottlenecks and relevant aspects for the implementation of processes: *I)* valorisation of cheap raw materials, *II)* engineering the cell factories to achieve high production and productivity of

CONCLUSIONS AND FUTURE PERSPECTIVES

the compound of interest as well as to improve stress resistance and *III/IV*) improvement of the production process and of the downstream phase(s) to maximize yields and minimize costs.

In respect to the first point (*I*), different renewable raw material were discussed in this thesis (**Chapter 1, 3, 4 and 6**). In particular, in **Chapter 3** crude glycerol was used as sole carbon and energy source for lipids accumulation from oleaginous yeasts. Instead, in **Chapter 1** an industrial and engineered *S. cerevisiae* strain was used for bioethanol production from 100 % undetoxified lignocellulosic hydrolysate. All these represent cheap substrates whose valorisation would contribute to the development of economically feasible production process.

The developments of strains with higher productivities and/or more tolerance to inhibitory compounds was another major goal (*II*). In this study, both rational metabolic engineering approaches and non-targeted approaches were applied. In particular, in **Chapter 1** overexpression of the transcription factor *YAP1* and the mitochondrial reductase *MCR1* in the already robust strain GSE16 resulted in an even faster hexose catabolism in the presence of spruce hydrolysate-derived inhibitors. Instead, in **Chapter 4** an adaptive laboratory evolution approach resulted in a *L. starkeyi* strain with improved tolerance towards lignocellulose-derived inhibitors.

In **Chapter 2 and 5** the promising cell factories *Z. bailii* and *K. marxianus* were investigated for some of the traits that render them particularly attractive for industrial applications. From these studies a deep

CONCLUSIONS AND FUTURE PERSPECTIVES

understanding of the pathways and mechanisms behind the phenotype of interest was gained.

Together with the development of improved cell factories the development of efficient and robust production process is a primary requirement (*III*). In **Chapter 3** an efficient and robust feeding strategy for avoiding the lag phase caused by growth on crude glycerol was developed. Moreover, in **Chapter 6** *C. lignohabitans* chemostat cultivations were used as source of data to write a program script for the control of the oxygen supply during batch fermentations.

The downstream step (*IV*) was not directly addressed during this thesis. However, it was always taken into consideration, given its relevance in determining the economic feasibility of the process.

Overall, the present study investigated the main aspects of a biorefinery and offered some examples of how these technical challenges could be overcome.

However, we have to remember that the overcome of technical challenges will not be sufficient to ensure a bright future to biorefineries. Various measures have to be undertaken to induce a significant impact of bio-based production, such as: *I*) creation of new markets for businesses to support bio-based products and encourage competition; *II*) creation of public-private partnerships, *III*) public engagement and communication initiatives to explain how bio-based products could become a realistic supplement to fossil-based products.

CONCLUSIONS AND FUTURE PERSPECTIVES

It is probable that economy will continue to rely of fossil-based production for the time being, but biorefineries may (and should) have a major role to play in supplementing our growing demand for sustainability.

ACKNOWLEDGEMENTS

Many inspiring and supportive persons deserve my deepest gratitude. Especially I would like to express my gratitude to the following persons:

First of all I would like to thank my supervisor, Professor Paola Branduardi for giving me this opportunity and for her guidance during my PhD studies. I admire her leadership and effort in creating a well-functioning laboratory group. Thank her for believing and trusting in me.

My deepest thanks go to my mentor Simone Passolunghi, first of all to share all his knowledge and passion for bioreactor fermentations with me.

Thanks to professor Danilo Porro because he forced me to become more critical toward my own work.

I wish to thank Professor Michael Sauer and its great group (in particular Martina Bellasio) for the beautiful experience during my stay in Vienna.

Valeria Wallace and Ying-Ying for the fruitful collaboration during my stay in Lund.

I would like to greatly thank Erba Gabriele and Giovanna Tengattini, as well as two excellent traveling companions I found in them two good friends.

ACKNOWLEDGEMENTS

I would like to thank Paolo Frati, several times he could send me to hell instead he brought me considerable happiness during my last year of PhD.

All the former and present members of the Brandulab are acknowledged for all the support over the years. Special thanks to Francesca Martani, Stefano Bertacchi, Marco Brambilla, Riccardo Posteri, Tiziana Fossati, Nurzhan Kuanyshhev and Stefano Bertagnoli (If I forgot someone apologizes) for sharing their time with me and for all the times I interrupted their work with my questions.

A special thanks to Francesca Marano and Nadia Berterame, it is commendable how, all along my PhD, they helped me to overcome difficult moments with equally technical/moral support and alcohol.

To conclude, Marta and my family deserve the deepest thanks, no words can express my gratitude for their never-ending support and endurance.

REFERENCES

1. International Energy Agency: **Oil market report**. www.iea.org/oilmarketreport/; 2015.
2. Bioeconomy Council: **En route to the biobased economy**. 2013.
3. Golembiewski B, Sick N, Broering S: **The emerging research landscape on bioeconomy: What has been done so far and what is essential from a technology and innovation management perspective?** *Innovative Food Science & Emerging Technologies* 2015, **29**:308-317.
4. Hardy RWF: **The Bio-based Economy**. J. Janick and A. Whipkey; 2002.
5. European Commission: **Innovating for sustainable growth: A bioeconomy for Europe**. 2012.
6. McCormick K, Kautto N: **The Bioeconomy in Europe: An Overview**. *Sustainability* 2013, **5**:2589-2608.
7. US Department of State: **Bioeconomy Blueprint**. 2012.
8. de Jong E, Jungmeier, G: **Biorefinery concepts in comparison to petrochemical refineries**. *Industrial Biorefineries and White Biotechnology*; 2015.
9. de Jong E, Higson A, Walsh P, Wellisch M: **Product developments in the bio-based chemicals arena**. *Biofuels Bioproducts & Biorefining-Biofpr* 2012, **6**:606-624.
10. Bozell JJ, Petersen GR: **Technology development for the production of biobased products from biorefinery carbohydrates-the US Department of Energy's "Top 10" revisited**. *Green Chemistry* 2010, **12**:539-554.
11. Chojnacka K: **Innovative bio-products for agriculture**. *Open Chemistry* 2015, **13**:932-937.
12. Koutinas AA, Chatzifragkou A, Kopsahelis N, Papanikolaou S, Kookos IK: **Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production**. *Fuel* 2014, **116**:566-577.
13. Gabardo S, Rech R, Rosa CA, Zachia Ayub MA: **Dynamics of ethanol production from whey and whey permeate by immobilized strains of *Kluyveromyces marxianus* in batch and continuous bioreactors**. *Renewable Energy* 2014, **69**:89-96.
14. Jiang L, Pei H, Hu W, Ji Y, Han L, Ma G: **The feasibility of using complex wastewater from a monosodium glutamate factory to cultivate *Spirulina subsalsa* and accumulate biochemical composition**. *Bioresource Technology* 2015, **180**:304-310.
15. Yan D, Lu Y, Chen Y-F, Wu Q: **Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production**. *Bioresource Technology* 2011, **102**:6487-6493.
16. Liang Y, Cui Y, Trushenski J, Blackburn JW: **Converting crude glycerol derived from yellow grease to lipids through yeast fermentation**. *Bioresource Technology* 2010, **101**:7581-7586.
17. Dionisi D, Anderson JA, Aulenta F, McCue A, Paton G: **The potential of microbial processes for lignocellulosic biomass conversion to ethanol: a review**. *Journal of Chemical Technology and Biotechnology* 2015, **90**:366-383.

REFERENCES

18. Papanikolaou S, Chevalot I, Komaitis M, Marc I, Aggelis G: **Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures.** *Applied Microbiology and Biotechnology* 2002, **58**:308-312.
19. Tsakona S, Kopsahelis N, Chatzifragkou A, Papanikolaou S, Kookos IK, Koutinas AA: **Formulation of fermentation media from flour-rich waste streams for microbial lipid production by *Lipomyces starkeyi*.** *Journal of Biotechnology* 2014, **189**:36-45.
20. Zhao X, Zhang L, Liu D: **Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose.** *Biofuels Bioproducts & Biorefining-Biofpr* 2012, **6**:465-482.
21. Mood SH, Golfeshan AH, Tabatabaei M, Jouzani GS, Najafi G, Gholami M, Ardjmand M: **Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment.** *Renewable & Sustainable Energy Reviews* 2013, **27**:77-93.
22. Lindman B, Karlstrom G, Stigsson L: **On the mechanism of dissolution of cellulose.** *Journal of Molecular Liquids* 2010, **156**:76-81.
23. Perez J, Munoz-Dorado J, de la Rubia T, Martinez J: **Biodegradation and biological treatments of cellulose, hemicellulose and lignin: An overview.** *International Microbiology* 2002, **5**:53-63.
24. Mussatto SI, Roberto IC: **Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review.** *Bioresource Technology* 2004, **93**:1-10.
25. Jin Z, Katsumata KS, Lam TBT, Iiyama K: **Covalent linkages between cellulose and lignin in cell walls of coniferous and nonconiferous woods.** *Biopolymers* 2006, **83**:103-110.
26. Ragauskas AJ, Beckham GT, Biddy MJ, Chandra R, Chen F, Davis MF, Davison BH, Dixon RA, Gilna P, Keller M, et al: **Lignin Valorization: Improving Lignin Processing in the Biorefinery.** *Science* 2014, **344**:709.
27. Frei M: **Lignin: characterization of a multifaceted crop component.** *The Scientific World Journal* 2013, **2013**:436517-436517.
28. Rubin EM: **Genomics of cellulosic biofuels.** *Nature* 2008, **454**:841-845.
29. Palmqvist E, Hahn-Hagerdal B: **Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification.** *Bioresource Technology* 2000, **74**:17-24.
30. Wiseloge A, Tyson S, Johnson D: **Biomass feedstock resources and composition.** pp. 105–118. Handbook on Bioethanol: Production and Utilization: C.E. Wyman; 1996: 105–118.
31. Caparros S, Garrote G, Ariza J, Jesus Diaz M, Lopez F: **Xylooligosaccharides production from *Arundo donax*.** *Journal of Agricultural and Food Chemistry* 2007, **55**:5536-5543.
32. Wiseloge AE, Agblevor FA, Johnson DK, Deutch S, Fennell JA, Sanderson MA: **Compositional changes during storage of large round switchgrass bales.** *Bioresource Technology* 1996, **56**:103-109.
33. Tengborg C, Stenberg K, Galbe M, Zacchi G, Larsson S, Palmqvist E, Hahn-Hagerdal B: **Comparison of SO₂ and H₂SO₄ impregnation of softwood prior to steam**

REFERENCES

- pretreatment on ethanol production. *Applied Biochemistry and Biotechnology* 1998, **70-2**:3-15.
34. Sassner P, Galbe M, Zacchi G: **Bioethanol production based on simultaneous saccharification and fermentation of steam-pretreated *Salix* at high dry-matter content.** *Enzyme and Microbial Technology* 2006, **39**:756-762.
 35. Sannigrahi P, Ragauskas AJ, Tuskan GA: **Poplar as a feedstock for biofuels: A review of compositional characteristics.** *Biofuels Bioproducts & Biorefining-Biofpr* 2010, **4**:209-226.
 36. Menon V, Rao M: **Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept.** *Progress in Energy and Combustion Science* 2012, **38**:522-550.
 37. Pauly M, Keegstra K: **Plant cell wall polymers as precursors for biofuels.** *Current Opinion in Plant Biology* 2010, **13**:305-312.
 38. Taherzadeh MJ, Karimi K: **Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review.** *International Journal of Molecular Sciences* 2008, **9**:1621-1651.
 39. Sun Y, Cheng JY: **Hydrolysis of lignocellulosic materials for ethanol production: a review.** *Bioresource Technology* 2002, **83**:1-11.
 40. Swatloski RP, Spear SK, Holbrey JD, Rogers RD: **Dissolution of cellulose with ionic liquids.** *Journal of the American Chemical Society* 2002, **124**:4974-4975.
 41. Agbor VB, Cicek N, Sparling R, Berlin A, Levin DB: **Biomass pretreatment: Fundamentals toward application.** *Biotechnology Advances* 2011, **29**:675-685.
 42. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M: **Features of promising technologies for pretreatment of lignocellulosic biomass.** *Bioresource Technology* 2005, **96**:673-686.
 43. Chandra RP, Bura R, Mabee WE, Berlin A, Pan X, Saddler JN: **Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics?** *Biofuels* 2007, **108**:67-93.
 44. Galbe M, Zacchi G: **A review of the production of ethanol from softwood.** *Applied Microbiology and Biotechnology* 2002, **59**:618-628.
 45. Taherzadeh MJ, Karimi K: **Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review.** *Bioresources* 2007, **2**:472-499.
 46. Merino ST, Cherry J: **Progress and challenges in enzyme development for Biomass utilization.** *Biofuels* 2007, **108**:95-120.
 47. Wilson DB: **Microbial diversity of cellulose hydrolysis.** *Current Opinion in Microbiology* 2011, **14**:259-263.
 48. Wood TM: **Properties of cellulolytic enzyme-systems.** *Biochemical Society Transactions* 1985, **13**:407-410.
 49. Zhang J, Tuomainen P, Siika-aho M, Viikari L: **Comparison of the synergistic action of two thermostable xylanases from GH families 10 and 11 with thermostable cellulases in lignocellulose hydrolysis.** *Bioresource Technology* 2011, **102**:9090-9095.
 50. Kolesarova N, Hutnan M, Bodik I, Spalkova V: **Utilization of Biodiesel By-Products for Biogas Production.** *Journal of Biomedicine and Biotechnology* 2011.

REFERENCES

51. Nanda M, Yuan Z, Qin W, Poirier MA, Chunbao X: **Purification of Crude Glycerol using Acidification: Effects of Acid Types and Product Characterization.** vol. Volume 1 Issue 1. Austin Journal of Chemical Engineering 2014.
52. Anand P, Saxena RK: **A comparative study of solvent-assisted pretreatment of biodiesel derived crude glycerol on growth and 1,3-propanediol production from *Citrobacter freundii*.** *New Biotechnology* 2012, **29**:199-205.
53. Chatzifragkou A, Papanikolaou S: **Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes.** *Applied Microbiology and Biotechnology* 2012, **95**:13-27.
54. Fonseca Amaral PF, Ferreira TF, Fontes GC, Zarur Coelho MA: **Glycerol valorization: New biotechnological routes.** *Food and Bioproducts Processing* 2009, **87**:179-186.
55. Johnson DT, Taconi KA: **The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production.** *Environmental Progress* 2007, **26**:338-348.
56. Saenge C, Cheirsilp B, Suksaroge TT, Bourtoom T: **Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids.** *Process Biochemistry* 2011, **46**:210-218.
57. Nicol RW, Marchand K, Lubitz WD: **Bioconversion of crude glycerol by fungi.** *Applied Microbiology and Biotechnology* 2012, **93**:1865-1875.
58. Kavsek M, Strazar M, Curk T, Natter K, Petrovic U: **Yeast as a cell factory: current state and perspectives.** *Microbial Cell Factories* 2015, **14**.
59. Steensels J, Snoek T, Meersman E, Nicolino MP, Voordeckers K, Verstrepen KJ: **Improving industrial yeast strains: exploiting natural and artificial diversity.** *Fems Microbiology Reviews* 2014, **38**:947-995.
60. Kellershohn J, Russell I: **Yeast biotechnology.** Book: Advances in Food Biotechnology; 2015.
61. Porro D, Branduardi P: **Yeast cell factory: fishing for the best one or engineering it?** *Microbial Cell Factories* 2009, **8**.
62. Cai Z, Zhang B, Li Y: **Engineering *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: Reflections and perspectives.** *Biotechnology Journal* 2012, **7**:34-46.
63. Zhou H, Cheng J-s, Wang BL, Fink GR, Stephanopoulos G: **Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*.** *Metabolic Engineering* 2012, **14**:611-622.
64. Mattanovich D, Sauer M, Gasser B: **Yeast biotechnology: teaching the old dog new tricks.** *Microbial Cell Factories* 2014, **13**.
65. Liti G, Carter DM, Moses AM, Warringer J, Parts L, James SA, Davey RP, Roberts IN, Burt A, Koufopanou V, et al: **Population genomics of domestic and wild yeasts.** *Nature* 2009, **458**:337-341.
66. Piskur J, Rozpedowska E, Polakova S, Merico A, Compagno C: **How did *Saccharomyces* evolve to become a good brewer?** *Trends in Genetics* 2006, **22**:183-186.

REFERENCES

67. Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, et al: **Life with 6000 genes.** *Science* 1996, **274**:546-&.
68. Bro C, Regenbreg B, Forster J, Nielsen J: **In silico aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production.** *Metabolic Engineering* 2006, **8**:102-111.
69. Grotkjaer T, Papp B, Oliver SG, Villadsen J, Nielsen J: **A systematic study of the transcriptional regulation on the metabolic fluxes in *Saccharomyces cerevisiae*.** *Journal of Biotechnology* 2005, **118**:S4-S4.
70. Lu Y, Cheng Y-F, He X-P, Guo X-N, Zhang B-R: **Improvement of robustness and ethanol production of ethanologenic *Saccharomyces cerevisiae* under co-stress of heat and inhibitors.** *Journal of Industrial Microbiology & Biotechnology* 2012, **39**:73-80.
71. Wallace-Salinas V, Signori L, Li Y-Y, Ask M, Bettiga M, Porro D, Thevelein JM, Branduardi P, Foulquie-Moreno MR, Gorwa-Grauslund M: **Re-assessment of *YAP1* and *MCR1* contributions to inhibitor tolerance in robust engineered *Saccharomyces cerevisiae* fermenting undetoxified lignocellulosic hydrolysate.** *AMB Express* 2014, **4**:56-56.
72. Ito Y, Hirasawa T, Shimizu H: **Metabolic engineering of *Saccharomyces cerevisiae* to improve succinic acid production based on metabolic profiling.** *Bioscience Biotechnology and Biochemistry* 2014, **78**:151-159.
73. Raab AM, Gebhardt G, Bolotina N, Weuster-Botz D, Lang C: **Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid.** *Metabolic Engineering* 2010, **12**:518-525.
74. Porro D, Brambilla L, Ranzi BM, Martegani E, Alberghina L: **Development of metabolically engineered *Saccharomyces-cerevisiae* cells for the production of lactic-acid.** *Biotechnology Progress* 1995, **11**:294-298.
75. Kjeldsen T: **Yeast secretory expression of insulin precursors.** *Applied Microbiology and Biotechnology* 2000, **54**:277-286.
76. Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, et al: **Production of the antimalarial drug precursor artemisinic acid in engineered yeast.** *Nature* 2006, **440**:940-943.
77. Matsushika A, Inoue H, Kodaki T, Sawayama S: **Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives.** *Applied Microbiology and Biotechnology* 2009, **84**:37-53.
78. Hong K-K, Nielsen J: **Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries.** *Cellular and Molecular Life Sciences* 2012, **69**:2671-2690.
79. Llorente B, Malpertuy A, Blandin G, Artiguenave F, Wincker P, Dujon B: **Genomic exploration of the hemiascomycetous yeasts: 12. *Kluyveromyces marxianus* var. *marxianus*.** *Febs Letters* 2000, **487**:71-75.
80. Vandijken JP, Weusthuis RA, Pronk JT: **Kinetics of growth and sugar consumption in yeasts.** *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 1993, **63**:343-352.

REFERENCES

81. Bellaver LH, de Carvalho NMB, Abrahao-Neto J, Gombert AK: **Ethanol formation and enzyme activities around glucose-6-phosphate in *Kluyveromyces marxianus* CBS 6556 exposed to glucose or lactose excess.** *Fems Yeast Research* 2004, **4**:691-698.
82. Hensing MCM, Rouwenhorst RJ, Heijnen JJ, Vandijken JP, Pronk JT: **Physiological and technological aspects of large-scale heterologous-protein production with yeasts.** *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 1995, **67**:261-279.
83. Limtong S, Sringiew C, Yongmanitchai W: **Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*.** *Bioresource Technology* 2007, **98**:3367-3374.
84. Anderson PJ, McNeil K, Watson K: **High-efficiency carbohydrate fermentation to ethanol at temperatures above 40-degrees-c by *Kluyveromyces-marxianus* var *marxianus* isolated from sugar mills.** *Applied and Environmental Microbiology* 1986, **51**:1314-1320.
85. Fonseca GG, Gombert AK, Heinzle E, Wittmann C: **Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source.** *Fems Yeast Research* 2007, **7**:422-435.
86. Groeneveld P, Stouthamer AH, Westerhoff HV: **Super life - how and why 'cell selection' leads to the fastest-growing eukaryote.** *Febs Journal* 2009, **276**:254-270.
87. Hensing M, Vrouwenvelder H, Hellinga C, Baartmans R, Vandijken H: **Production of extracellular inulinase in high-cell-density fed-batch cultures of *Kluyveromyces-marxianus*.** *Applied Microbiology and Biotechnology* 1994, **42**:516-521.
88. Fonseca GG, Heinzle E, Wittmann C, Gombert AK: **The yeast *Kluyveromyces marxianus* and its biotechnological potential.** *Applied Microbiology and Biotechnology* 2008, **79**:339-354.
89. Morrissey JP, Etschmann MMW, Schrader J, de Billerbeck GM: **Cell factory applications of the yeast *Kluyveromyces marxianus* for the biotechnological production of natural flavour and fragrance molecules.** *Yeast* 2015, **32**:3-16.
90. Nonklang S, Abdel-Banat BMA, Cha-aim K, Moonjai N, Hoshida H, Limtong S, Yamada M, Akada R: **High-Temperature Ethanol Fermentation and Transformation with Linear DNA in the Thermotolerant Yeast *Kluyveromyces marxianus* DMKU3-1042.** *Applied and Environmental Microbiology* 2008, **74**:7514-7521.
91. Antunes DF, de Souza CG, de Moraes MA: **A simple and rapid method for lithium acetate-mediated transformation of *Kluyveromyces marxianus* cells.** *World Journal of Microbiology & Biotechnology* 2000, **16**:653-654.
92. Iborra F: **High-efficiency transformation of *Kluyveromyces-marxianus* by a replicative plasmid.** *Current Genetics* 1993, **24**:181-183.
93. Lertwattanasakul N, Kosaka T, Hosoyama A, Suzuki Y, Rodrussamee N, Matsutani M, Murata M, Fujimoto N, Suprayogi, Tsuchikane K, et al: **Genetic basis of the highly efficient yeast *Kluyveromyces marxianus*: complete genome sequence and transcriptome analyses.** *Biotechnology for Biofuels* 2015, **8**.

REFERENCES

94. Sampaio JP, Golubev WI, Fell JW, Gadanho M, Golubev NW: ***Curvibasidium cygneicollum* gen. nov., sp nov and *Curvibasidium pallidicorallinum* sp nov., novel taxa in the Microbotryomycetidae (Urediniomycetes), and their relationship with *Rhodotorula fujisanensis* and *Rhodotorula nothofagi*.** *International Journal of Systematic and Evolutionary Microbiology* 2004, **54**:1401-1407.
95. Li Y, Zhao Z, Bai F: **High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture.** *Enzyme and Microbial Technology* 2007, **41**:312-317.
96. Ratledge C, Wynn JP: **The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms.** *Advances in Applied Microbiology, Vol 51* 2002, **51**:1-51.
97. Freitas C, Nobre B, Gouveia L, Roseiro J, Reis A, da Silva TL: **New at-line flow cytometric protocols for determining carotenoid content and cell viability during *Rhodosporidium toruloides* NCYC 921 batch growth.** *Process Biochemistry* 2014, **49**:554-562.
98. Buzzini P, Innocenti M, Turchetti B, Libkind D, van Broock M, Mulinacci N: **Carotenoid profiles of yeasts belonging to the genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces*, and *Sporidiobolus*.** *Canadian Journal of Microbiology* 2007, **53**:1024-1031.
99. Politino M, Tonzi SM, Burnett WV, Romancik G, Usher JJ: **Purification and characterization of a cephalosporin esterase from *Rhodosporidium toruloides*.** *Applied and Environmental Microbiology* 1997, **63**:4807-4811.
100. Botes AL, Litthauer D, van Tonder A, van Dyk MS: **Physico-chemical properties of the epoxide hydrolase from *Rhodosporidium toruloides*.** *Biotechnology Letters* 1999, **21**:1137-1144.
101. Xu J, Zhao X, Wang W, Du W, Liu D: **Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast *Rhodosporidium toruloides* and the individual effect of some impurities on lipid production.** *Biochemical Engineering Journal* 2012, **65**:30-36.
102. Hu C, Zhao X, Zhao J, Wu S, Zhao ZK: **Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*.** *Bioresource Technology* 2009, **100**:4843-4847.
103. Huang C, Chen X-f, Xiong L, Chen X-d, Ma L-l, Chen Y: **Single cell oil production from low-cost substrates: The possibility and potential of its industrialization.** *Biotechnology Advances* 2013, **31**:129-139.
104. Kumar S, Kushwaha H, Bachhawat AK, Raghava GPS, Ganesan K: **Genome Sequence of the Oleaginous Red Yeast *Rhodosporidium toruloides* MTCC 457.** *Eukaryotic Cell* 2012, **11**:1083-1084.
105. Zhu Z, Zhang S, Liu H, Shen H, Lin X, Yang F, Zhou YJ, Jin G, Ye M, Zou H, Zhao ZK: **A multi-omic map of the lipid-producing yeast *Rhodosporidium toruloides*.** *Nature Communications* 2013, **4**.
106. Tully M, Gilbert HJ: **Transformation of *Rhodosporidium toruloides*.** *Gene* 1985, **36**:235-240.

REFERENCES

107. Lin X, Wang Y, Zhang S, Zhu Z, Zhou YJ, Yang F, Sun W, Wang X, Zhao ZK: **Functional integration of multiple genes into the genome of the oleaginous yeast *Rhodospiridium toruloides***. *Fems Yeast Research* 2014, **14**:547-555.
108. Thiru M, Sankh S, Rangaswamy V: **Process for biodiesel production from *Cryptococcus curvatus***. *Bioresource Technology* 2011, **102**:10436-10440.
109. Ratledge CC, Z: **Microbial and algal oils: do they have a future for biodiesel or as commodity oils**. vol. Vol. 20, No. 7. *Lipid Technology* 2008.
110. Hassan M, Blanc PJ, Granger LM, Pareilleux A, Goma G: **Influence of nitrogen and iron limitations on lipid production by *Cryptococcus curvatus* grown in batch and fed-batch culture**. *Process Biochemistry* 1996, **31**:355-361.
111. Ageitos JM, Vallejo JA, Veiga-Crespo P, Villa TG: **Oily yeasts as oleaginous cell factories**. *Applied Microbiology and Biotechnology* 2011, **90**:1219-1227.
112. Daniel HJ, Otto RT, Binder M, Reuss M, Syldatk C: **Production of sophorolipids from whey: development of a two-stage process with *Cryptococcus curvatus* ATCC 20509 and *Candida bombicola* ATCC 22214 using deproteinized whey concentrates as substrates**. *Applied Microbiology and Biotechnology* 1999, **51**:40-45.
113. Oguri E, Masaki K, Naganuma T, Iefuji H: **Phylogenetic and biochemical characterization of the oil-producing yeast *Lipomyces starkeyi***. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 2012, **101**:359-368.
114. Naganuma T, Uzuka Y, Tanaka K: **Using inorganic elements to control cell-growth and lipid-accumulation in *Lipomyces starkeyi***. *Journal of General and Applied Microbiology* 1986, **32**:417-424.
115. Suutari M, Priha P, Laakso S: **Temperature shifts in regulation of lipids accumulated by *Lipomyces-starkeyi***. *Journal of the American Oil Chemists Society* 1993, **70**:891-894.
116. Zhao X, Kong X, Hua Y, Feng B, Zhao Z: **Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi***. *European Journal of Lipid Science and Technology* 2008, **110**:405-412.
117. Suutari M, Rintamaki A, Laakso S: **The effect of temperature on lipid classes and their fatty acid profiles in *Lipomyces starkeyi***. *Journal of the American Oil Chemists Society* 1996, **73**:1071-1073.
118. Angerbauer C, Siebenhofer M, Mittelbach M, Guebitz GM: **Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production**. *Bioresource Technology* 2008, **99**:3051-3056.
119. Kang H-K, Park J-Y, Ahn J-S, Kim S-H, Kim D: **Cloning of a Gene Encoding Dextranase from *Lipomyces starkeyi* and its Expression in *Pichia pastoris***. *Journal of Microbiology and Biotechnology* 2009, **19**:172-177.
120. Millson SH, Evans IH: **Multiple dextranases from the yeast *Lipomyces starkeyi***. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 2007, **92**:399-404.

REFERENCES

121. Lomascolo A, Dubreucq E, Galzy P: **Study of the Delta 12-desaturase system of *Lipomyces starkeyi***. *Lipids* 1996, **31**:253-259.
122. Akhtar P, Gray JI, Asghar A: **Synthesis of lipids by certain yeast strains grown on whey permeate**. *Journal of Food Lipids* 1998, **5**:283-297.
123. Calvey CH, Willis LB, Jeffries TW: **An optimized transformation protocol for *Lipomyces starkeyi***. *Current Genetics* 2014, **60**:223-230.
124. Liu H, Zhao X, Wang F, Jiang X, Zhang S, Ye M, Zhao ZK, Zou H: **The proteome analysis of oleaginous yeast *Lipomyces starkeyi***. *Fems Yeast Research* 2011, **11**:42-51.
125. Domizio P, Romani C, Comitini F, Gobbi M, Lencioni L, Mannazzu I, Ciani M: **Potential spoilage non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae***. *Annals of Microbiology* 2011, **61**:137-144.
126. Emmerich W, Radler F: **The anaerobic metabolism of glucose and fructose by *Saccharomyces bailii***. *Journal of General Microbiology* 1983, **129**:3311-3318.
127. Mira NP, Muensterkoetter M, Dias-Valada F, Santos J, Palma M, Roque FC, Guerreiro JF, Rodrigues F, Sousa MJ, Leao C, et al: **The Genome Sequence of the Highly Acetic Acid-Tolerant *Zygosaccharomyces bailii*-Derived Interspecies Hybrid Strain ISA1307, Isolated From a Sparkling Wine Plant**. *DNA Research* 2014, **21**:299-313.
128. Martorell P, Stratford M, Steels H, Fernandez-Espinar MT, Querol A: **Physiological characterization of spoilage strains of *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* isolated from high sugar environments**. *International Journal of Food Microbiology* 2007, **114**:234-242.
129. Branduardi P, Valli M, Brambilla L, Sauer M, Alberghina L, Porro D: **The yeast *Zygosaccharomyces bailii*: a new host for heterologous protein production, secretion and for metabolic engineering applications**. *Fems Yeast Research* 2004, **4**:493-504.
130. Sauer M, Branduardi P, Valli M, Porro D: **Production of L-ascorbic acid by metabolically engineered *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii***. *Applied and Environmental Microbiology* 2004, **70**:6086-6091.
131. Paixao SM, Teixeira PD, Silva TP, Teixeira AV, Alves L: **Screening of novel yeast inulinases and further application to bioprocesses**. *New Biotechnology* 2013, **30**:598-606.
132. Guerreiro JF, Mira NP, Sa-Correia I: **Adaptive response to acetic acid in the highly resistant yeast species *Zygosaccharomyces bailii* revealed by quantitative proteomics**. *Proteomics* 2012, **12**:2303-2318.
133. Dato L, Branduardi P, Passolunghi S, Cattaneo D, Riboldi L, Frascotti G, Valli M, Porro D: **Advances in molecular tools for the use of *Zygosaccharomyces bailii* as host for biotechnological productions and construction of the first auxotrophic mutant**. *Fems Yeast Research* 2010, **10**:894-908.
134. Galeote V, Bigey F, Devillers H, Neuveglise C, Dequin S: **Genome Sequence of the Food Spoilage Yeast *Zygosaccharomyces bailii* CLIB 213T**. *Genome announcements* 2013, **1**.

REFERENCES

135. Urbina H, Frank R, Blackwell M: ***Scheffersomyces cryptocercus*: a new xylose-fermenting yeast associated with the gut of wood roaches and new combinations in the Sugiyamaella yeast clade.** *Mycologia* 2013, **105**:650-660.
136. Houseknecht JL, Hart EL, Suh S-O, Zhou JJ: **Yeasts in the Sugiyamaella clade associated with wood-ingesting beetles and the proposal of *Candida bullrunensis* sp nov.** *International Journal of Systematic and Evolutionary Microbiology* 2011, **61**:1751-1756.
137. Bellasio M, Mattanovich D, Sauer M, Marx H: **Organic acids from lignocellulose: *Candida lignohabitans* as a new microbial cell factory.** *Journal of Industrial Microbiology & Biotechnology* 2015, **42**:681-691.
138. Hahn-Hagerdal B, Karhumaa K, Fonseca C, Spencer-Martins I, Gorwa-Grauslund MF: **Towards industrial pentose-fermenting yeast strains.** *Applied Microbiology and Biotechnology* 2007, **74**:937-953.
139. Pereira FB, Guimaraes PMR, Gomes DG, Mira NP, Teixeira MC, Sa-Correia I, Domingues L: **Identification of candidate genes for yeast engineering to improve bioethanol production in very high gravity and lignocellulosic biomass industrial fermentations.** *Biotechnology for Biofuels* 2011, **4**.
140. Skinner KA, Leathers TD: **Bacterial contaminants of fuel ethanol production.** *Journal of Industrial Microbiology & Biotechnology* 2004, **31**:401-408.
141. de Souza Liberal AT, da Silva Filho EA, de Moraes JOF, Simoes DA, de Moraes MA, Jr.: **Contaminant yeast detection in industrial ethanol fermentation must by rDNA-PCR.** *Letters in applied microbiology* 2005, **40**:19-23.
142. Kurtzman CP, Suzuki M: **Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces*.** *Mycoscience* 2010, **51**:2-14.
143. Kotter P, Ciriacy M: **Xylose fermentation by *Saccharomyces-cerevisiae*.** *Applied Microbiology and Biotechnology* 1993, **38**:776-783.
144. Tantirungkij M, Nakashima N, Seki T, Yoshida T: **Construction of xylose-assimilating *Saccharomyces cerevisiae*.** *Journal of Fermentation and Bioengineering* 1993, **75**:83-88.
145. Yablochkova EN, Bolotnikova OI, Mikhailova NP, Nemova NN, Ginak AI: **The activity of xylose reductase and xylitol dehydrogenase in yeasts.** *Microbiology* 2003, **72**:414-417.
146. Bruinenberg PM, Debot PHM, Vandijken JP, Scheffers WA: **NADH-linked aldose reductase - the key to anaerobic alcoholic fermentation of xylose by yeasts.** *Applied Microbiology and Biotechnology* 1984, **19**:256-260.
147. Runquist D, Hahn-Hagerdal B, Bettiga M: **Increased Ethanol Productivity in Xylose-Utilizing *Saccharomyces cerevisiae* via a Randomly Mutagenized Xylose Reductase.** *Applied and Environmental Microbiology* 2010, **76**:7796-7802.
148. Bengtsson O, Hahn-Hagerdal B, Gorwa-Grauslund MF: **Xylose reductase from *Pichia stipitis* with altered coenzyme preference improves ethanolic xylose fermentation by recombinant *Saccharomyces cerevisiae*.** *Biotechnology for Biofuels* 2009, **2**.

REFERENCES

149. Wenger JW, Schwartz K, Sherlock G: **Bulk Segregant Analysis by High-Throughput Sequencing Reveals a Novel Xylose Utilization Gene from *Saccharomyces cerevisiae***. *Plos Genetics* 2010, **6**.
150. Traff KL, Jonsson LJ, Hahn-Hagerdal B: **Putative xylose and arabinose reductases in *Saccharomyces cerevisiae***. *Yeast* 2002, **19**:1233-1241.
151. Toivari MH, Salusjarvi L, Ruohonen L, Penttila M: **Endogenous xylose pathway in *Saccharomyces cerevisiae***. *Applied and Environmental Microbiology* 2004, **70**:3681-3686.
152. Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MSM, de Laat W, den Ridder JJJ, Op den Camp HJM, van Dijken JP, Pronk JT: **High-level functional expression of a fungal xylose isomerase: the key to efficient ethanol fermentation of xylose by *Saccharomyces cerevisiae*?** *Fems Yeast Research* 2003, **4**:69-78.
153. Amore R, Wilhelm M, Hollenberg CP: **The fermentation of xylose - an analysis of the expression of *Bacillus* and *Actinoplanes* xylose isomerase genes in yeast**. *Applied Microbiology and Biotechnology* 1989, **30**:351-357.
154. Gardonyi M, Hahn-Hagerdal B: **The *Streptomyces rubiginosus* xylose isomerase is misfolded when expressed in *Saccharomyces cerevisiae***. *Enzyme and Microbial Technology* 2003, **32**:252-259.
155. Lee S-M, Jellison T, Alper HS: **Directed Evolution of Xylose Isomerase for Improved Xylose Catabolism and Fermentation in the Yeast *Saccharomyces cerevisiae***. *Applied and Environmental Microbiology* 2012, **78**:5708-5716.
156. Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT: **Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain**. *Fems Yeast Research* 2005, **5**:925-934.
157. Wisselink HW, Toirkens MJ, Wu Q, Pronk JT, van Maris AJA: **Novel Evolutionary Engineering Approach for Accelerated Utilization of Glucose, Xylose, and Arabinose Mixtures by Engineered *Saccharomyces cerevisiae* Strains**. *Applied and Environmental Microbiology* 2009, **75**:907-914.
158. van Maris AJA, Winkler AA, Kuyper M, de Laat WTAM, van Dijken JP, Pronk JT: **Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: Xylose Isomerase as a key component**. *Biofuels* 2007, **108**:179-204.
159. Madhavan A, Tamalampudi S, Srivastava A, Fukuda H, Bisaria VS, Kondo A: **Alcoholic fermentation of xylose and mixed sugars using recombinant *Saccharomyces cerevisiae* engineered for xylose utilization**. *Applied Microbiology and Biotechnology* 2009, **82**:1037-1047.
160. Ho NWY, Chen ZD, Brainard AP: **Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose**. *Applied and Environmental Microbiology* 1998, **64**:1852-1859.
161. Lee TH, Kim MD, Park YC, Bae SM, Ryu YW, Seo JH: **Effects of xylulokinase activity on ethanol production from D-xylulose by recombinant *Saccharomyces cerevisiae***. *Journal of Applied Microbiology* 2003, **95**:847-852.
162. Van Vleet JH, Jeffries TW, Olsson L: **Deleting the para-nitrophenyl phosphatase (pNPPase), PHO13, in recombinant *Saccharomyces cerevisiae* improves growth and ethanol production on D-xylose**. *Metabolic Engineering* 2008, **10**:360-369.

REFERENCES

163. Chu BCH, Lee H: **Genetic improvement of *Saccharomyces cerevisiae* for xylose fermentation.** *Biotechnology Advances* 2007, **25**:425-441.
164. Saloheimo A, Rauta J, Stasyk OV, Sibirny AA, Penttila M, Ruohonen L: **Xylose transport studies with xylose-utilizing *Saccharomyces cerevisiae* strains expressing heterologous and homologous permeases.** *Applied Microbiology and Biotechnology* 2007, **74**:1041-1052.
165. Sedlak M, Ho NWY: **Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant *Saccharomyces* yeast.** *Yeast* 2004, **21**:671-684.
166. Almeida JRM, Modig T, Petersson A, Hahn-Hagerdal B, Liden G, Gorwa-Grauslund MF: **Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*.** *Journal of Chemical Technology and Biotechnology* 2007, **82**:340-349.
167. Klinka HB, Thomsen AB, Ahring BK: **Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass.** *Applied Microbiology and Biotechnology* 2004, **66**:10-26.
168. Ulbricht RJ, Northup SJ, Thomas JA: **A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions.** *Fundamental and Applied Toxicology* 1984, **4**:843-853.
169. Verduyn C, Postma E, Scheffers WA, Vandijken JP: **Effect of benzoic-acid on metabolic fluxes in yeasts - a continuous-culture study on the regulation of respiration and alcoholic fermentation.** *Yeast* 1992, **8**:501-517.
170. Pampulha ME, Loureirodias MC: **Combined effect of acetic-acid, pH and ethanol on intracellular pH of fermenting yeast.** *Applied Microbiology and Biotechnology* 1989, **31**:547-550.
171. Ask M, Bettiga M, Mapelli V, Olsson L: **The influence of HMF and furfural on redox-balance and energy-state of xylose-utilizing *Saccharomyces cerevisiae*.** *Biotechnology for Biofuels* 2013, **6**.
172. Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW: **Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran.** *Journal of Industrial Microbiology & Biotechnology* 2004, **31**:345-352.
173. Liu ZL, Moon J, Andersh BJ, Slininger PJ, Weber S: **Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*.** *Applied Microbiology and Biotechnology* 2008, **81**:743-753.
174. Oud B, van Maris AJA, Daran J-M, Pronk JT: **Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast.** *Fems Yeast Research* 2012, **12**:183-196.
175. Cakar ZP, Turanli-Yildiz B, Alkim C, Yilmaz U: **Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties.** *Fems Yeast Research* 2012, **12**:171-182.
176. Koffas M, Roberge C, Lee K, Stephanopoulos G: **Metabolic engineering.** *Annu Rev Biomed Eng* 1999, **1**:535-557.

REFERENCES

177. Bailey JE: **Toward a science of metabolic engineering.** *Science* 1991, **252**:1668-1675.
178. Nielsen J, Larsson C, van Maris A, Pronk J: **Metabolic engineering of yeast for production of fuels and chemicals.** *Current Opinion in Biotechnology* 2013, **24**:398-404.
179. Gong J, Zheng H, Wu Z, Chen T, Zhao X: **Genome shuffling: Progress and applications for phenotype improvement.** *Biotechnol Adv* 2009, **27**:996-1005.
180. Bailey JE, Shurlati A, Hatzimanikatis V, Lee K, Renner WA, Tsai PS: **Inverse metabolic engineering: A strategy for directed genetic engineering of useful phenotypes.** *Biotechnology and Bioengineering* 1996, **52**:109-121.
181. Sauer U: **Evolutionary engineering of industrially important microbial phenotypes.** *Advances in biochemical engineering/biotechnology* 2001, **73**:129-169.
182. Wahlbom CF, van Zyl WH, Jonsson LJ, Hahn-Hagerdal B, Otero RRC: **Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054.** *Fems Yeast Research* 2003, **3**:319-326.
183. Heer D, Sauer U: **Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain.** *Microbial Biotechnology* 2008, **1**:497-506.
184. Almario MP, Reyes LH, Kao KC: **Evolutionary Engineering of *Saccharomyces cerevisiae* for Enhanced Tolerance to Hydrolysates of Lignocellulosic Biomass.** *Biotechnology and Bioengineering* 2013, **110**:2616-2623.
185. Liu ZL, Slininger PJ, Gorsich SW: **Enhanced biotransformation of furfural and hydroxymethylfurfural by newly developed ethanologenic yeast strains.** *Applied Biochemistry and Biotechnology* 2005, **121**:451-460.
186. Jansen MLA, Diderich JA, Mashego M, Hassane A, de Winde JH, Daran-Lapujade P, Pronk JT: **Prolonged selection in aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* causes a partial loss of glycolytic capacity.** *Microbiology-Sgm* 2005, **151**:1657-1669.
187. Wright J, Bellissimi E, de Hulster E, Wagner A, Pronk JT, van Maris AJA: **Batch and continuous culture-based selection strategies for acetic acid tolerance in xylose-fermenting *Saccharomyces cerevisiae*.** *Fems Yeast Research* 2011, **11**:299-306.
188. Koppram R, Albers E, Olsson L: **Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass.** *Biotechnology for Biofuels* 2012, **5**.
189. Dragosits M, Mattanovich D: **Adaptive laboratory evolution - principles and applications for biotechnology.** *Microbial Cell Factories* 2013, **12**.
190. Kao KC, Sherlock G: **Molecular characterization of clonal interference during adaptive evolution in asexual populations of *Saccharomyces cerevisiae*.** *Nature Genetics* 2008, **40**:1499-1504.
191. Beaumont HJE, Gallie J, Kost C, Ferguson GC, Rainey PB: **Experimental evolution of bet hedging.** *Nature* 2009, **462**:90-U97.

REFERENCES

192. Rainey PB, Beaumont HJE, Ferguson GC, Gallie J, Kost C, Libby E, Zhang X-X: **The evolutionary emergence of stochastic phenotype switching in bacteria.** *Microbial Cell Factories* 2011, **10**.
193. Barton NH: **Genetic hitchhiking.** *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 2000, **355**:1553-1562.
194. Cooper TF, Lenski RE: **Experimental evolution with *E. coli* in diverse resource environments. I. Fluctuating environments promote divergence of replicate populations.** *Bmc Evolutionary Biology* 2010, **10**.
195. Weikert C, Sauer U, Bailey JE: **Use of a glycerol-limited, long-term chemostat for isolation of *Escherichia coli* mutants with improved physiological properties.** *Microbiology-Uk* 1997, **143**:1567-1574.
196. Steiner P, Sauer U: **Long-term continuous evolution of acetate resistant *Acetobacter aceti*.** *Biotechnology and Bioengineering* 2003, **84**:40-44.
197. Fleming G, Dawson MT, Patching JW: **The isolation of strains of *Bacillus-subtilis* showing improved plasmid stability characteristics by means of selective chemostat culture.** *Journal of General Microbiology* 1988, **134**:2095-2101.
198. Roca C, Albuquerque G, Reis M: **Evolutionary engineering of *Actinobacillus succinogenes* for improved succinic acid production on glycerol.** *Journal of Biotechnology* 2010, **150**:S373-S373.
199. Lee JW, Lee SY: **Proteome-based physiological analysis of the metabolically engineered succinic acid producer *Mannheimia succiniciproducens* LPK7.** *Bioprocess and Biosystems Engineering* 2010, **33**:97-107.
200. Cakar ZP, Seker UOS, Tamerler C, Sonderegger M, Sauer U: **Evolutionary engineering of multiple-stress resistant *Saccharomyces cerevisiae*.** *Fems Yeast Research* 2005, **5**:569-578.
201. Kutyna DR, Varela C, Stanley GA, Borneman AR, Henschke PA, Chambers PJ: **Adaptive evolution of *Saccharomyces cerevisiae* to generate strains with enhanced glycerol production.** *Applied Microbiology and Biotechnology* 2012, **93**:1175-1184.
202. Naik SN, Goud VV, Rout PK, Dalai AK: **Production of first and second generation biofuels: A comprehensive review.** *Renewable & Sustainable Energy Reviews* 2010, **14**:578-597.
203. Goldemberg J: **Ethanol for a sustainable energy future.** *Science* 2007, **315**:808-810.
204. Viikari L, Vehmaanpera J, Koivula A: **Lignocellulosic ethanol: From science to industry.** *Biomass & Bioenergy* 2012, **46**:13-24.
205. Mohr A, Raman S: **Lessons from first generation biofuels and implications for the sustainability appraisal of second generation biofuels.** *Energy Policy* 2013, **63**:114-122.
206. US Department of Energy: **Trend in ethanol production.** 2013.
207. Renewable Fuels Association: **World ethanol production.** <http://www.ethanolrfa.org/>; 2014.
208. Dias De Oliveira ME, Vaughan BE, Rykiel EJ, Jr.: **Ethanol as fuels: Energy, carbon dioxide balances, and ecological footprint.** *Bioscience* 2005, **55**:593-602.

REFERENCES

209. von Blottnitz H, Curran MA: **A review of assessments conducted on bio-ethanol as a transportation fuel from a net energy, greenhouse gas, and environmental life cycle perspective.** *Journal of Cleaner Production* 2007, **15**:607-619.
210. Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM: **Ethanol can contribute to energy and environmental goals.** *Science* 2006, **311**:506-508.
211. Fargione J, Hill J, Tilman D, Polasky S, Hawthorne P: **Land clearing and the biofuel carbon debt.** *Science* 2008, **319**:1235-1238.
212. Searchinger T, Heimlich R, Houghton RA, Dong F, Elobeid A, Fabiosa J, Tokgoz S, Hayes D, Yu T-H: **Use of US croplands for biofuels increases greenhouse gases through emissions from land-use change.** *Science* 2008, **319**:1238-1240.
213. Doornbusch R, Steenblik R: **Biofuels: is the cure worse than the disease?** : Organisation for Economic Co-operation and Development (OECD); 2007.
214. Organisation for Economic co-operation and development: **Annual report.** 2008.
215. Sims REH, Mabee W, Saddler JN, Taylor M: **An overview of second generation biofuel technologies.** *Bioresource Technology* 2010, **101**:1570-1580.
216. Shell: **Sustainability.** www.shell.com; 2015.
217. Galbe M, Zacchi G: **Pretreatment of lignocellulosic materials for efficient bioethanol production.** *Biofuels* 2007, **108**:41-65.
218. Taylor MP, Eley KL, Martin S, Tuffin MI, Burton SG, Cowan DA: **Thermophilic ethanologenesis: future prospects for second-generation bioethanol production.** *Trends in Biotechnology* 2009, **27**:398-405.
219. Cardona CA, Sanchez OJ: **Fuel ethanol production: Process design trends and integration opportunities.** *Bioresource Technology* 2007, **98**:2415-2457.
220. Geddes CC, Nieves IU, Ingram LO: **Advances in ethanol production.** *Current Opinion in Biotechnology* 2011, **22**:312-319.
221. Andreasen AA, Stier TJ: **Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium.** *Journal of cellular physiology* 1954, **43**:271-281.
222. Verduyn C, Zomerdijk TPL, Vandijken JP, Scheffers WA: **Continuous measurement of ethanol-production by aerobic yeast suspensions with an enzyme electrode.** *Applied Microbiology and Biotechnology* 1984, **19**:181-185.
223. Erdei B, Franko B, Galbe M, Zacchi G: **Separate hydrolysis and co-fermentation for improved xylose utilization in integrated ethanol production from wheat meal and wheat straw.** *Biotechnology for Biofuels* 2012, **5**.
224. Chen M, Wang F: **Optimization of a fed-batch simultaneous saccharification and co-fermentation process from lignocellulose to ethanol.** vol. Vol. 49,. pp. pp. 5775-5585: Industrial & Engineering Chemistry Research; 2010: pp. 5775-5585.
225. Tomas-Pejo E, Oliva JM, Ballesteros M: **Realistic approach for full-scale bioethanol production from lignocellulose: a review.** *Journal of Scientific & Industrial Research* 2008, **67**:874-884.
226. Olofsson K, Bertilsson M, Liden G: **A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks.** *Biotechnology for Biofuels* 2008, **1**.

REFERENCES

227. Balat M, Balat H, Oz C: **Progress in bioethanol processing.** *Progress in Energy and Combustion Science* 2008, **34**:551-573.
228. Chandel AK, Chan ES, Rudravaram R, Narasu ML, L.V. R, Ravindra P: **Economics and environmental impact of bioethanol production technologies: an appraisal.**, vol. 2(1). pp. 14-32: *Biotechnology and Molecular Biology Review*; 2007: 14-32.
229. Jouzani GS, Taherzadeh MJ: **Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: a comprehensive review.** *Biofuel Research Journal-Brj* 2015, **2**:152-195.
230. Lynd LR, van Zyl WH, McBride JE, Laser M: **Consolidated bioprocessing of cellulosic biomass: an update.** *Current Opinion in Biotechnology* 2005, **16**:577-583.
231. Xu Q, Singh A, Himmel ME: **Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose.** *Current Opinion in Biotechnology* 2009, **20**:364-371.
232. Canakci M, Sanli H: **Biodiesel production from various feedstocks and their effects on the fuel properties.** *Journal of Industrial Microbiology & Biotechnology* 2008, **35**:431-441.
233. Li Q, Du W, Liu D: **Perspectives of microbial oils for biodiesel production.** *Applied Microbiology and Biotechnology* 2008, **80**:749-756.
234. Ramadhas AS, Jayaraj S, Muraleedharan C: **Use of vegetable oils as IC engine fuels - A review.** *Renewable Energy* 2004, **29**:727-742.
235. Leiva-Candia DE, Pinzi S, Redel-Macias MD, Koutinas A, Webb C, Dorado MP: **The potential for agro-industrial waste utilization using oleaginous yeast for the production of biodiesel.** *Fuel* 2014, **123**:33-42.
236. Beopoulos A, Nicaud J-M, Gaillardin C: **An overview of lipid metabolism in yeasts and its impact on biotechnological processes.** *Applied Microbiology and Biotechnology* 2011, **90**:1193-1206.
237. Subramaniam R, Dufreche S, Zappi M, Bajpai R: **Microbial lipids from renewable resources: production and characterization.** *Journal of Industrial Microbiology & Biotechnology* 2010, **37**:1271-1287.
238. Thevenieau F, Nicaud J-M: **Microorganisms as sources of oils.** vol. 20(6): EDP Sciences; 2013.
239. Thiam AR, Farese RV, Jr., Walther TC: **The biophysics and cell biology of lipid droplets.** *Nature Reviews Molecular Cell Biology* 2013, **14**:775-786.
240. Ratledge C: **Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production.** *Biochimie* 2004, **86**:807-815.
241. Armenta RE, Valentine MC: **Single-Cell Oils as a Source of Omega-3 Fatty Acids: An Overview of Recent Advances.** *Journal of the American Oil Chemists Society* 2013, **90**:167-182.
242. Cohen Z, C R: **Single cell oil.** AOCS Press; 2005.
243. Chisti Y: **Biodiesel from microalgae.** *Biotechnology Advances* 2007, **25**:294-306.
244. Hegel PE, Camy S, Destrac P, Condoret JS: **Influence of pretreatments for extraction of lipids from yeast by using supercritical carbon dioxide and ethanol as cosolvent.** *Journal of Supercritical Fluids* 2011, **58**:68-78.

REFERENCES

245. Milanesio J, Hegel P, Medina-Gonzalez Y, Camy S, Condoret J-S: **Extraction of lipids from *Yarrowia Lipolytica***. *Journal of Chemical Technology and Biotechnology* 2013, **88**:378-387.
246. Jin G, Yang F, Hu C, Shen H, Zhao ZK: **Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast *Rhodospiridium toruloides***. *Bioresource Technology* 2012, **111**:378-382.
247. Sauer M, Porro D, Mattanovich D, Branduardi P: **Microbial production of organic acids: expanding the markets**. *Trends in Biotechnology* 2008, **26**:100-108.
248. Yin X, Li J, Shin H-d, Du G, Liu L, Chen J: **Metabolic engineering in the biotechnological production of organic acids in the tricarboxylic acid cycle of microorganisms: Advances and prospects**. *Biotechnology Advances* 2015, **33**:830-841.
249. Sanchez-Riera F: **Production of organic acids**. vol. V. *Biotechnology: Encyclopedia of Life Support Systems (EOLSS)*.
250. Abdel-Rahman MA, Tashiro Y, Sonomoto K: **Recent advances in lactic acid production by microbial fermentation processes**. *Biotechnology Advances* 2013, **31**:877-902.
251. Valli M, Sauer M, Branduardi P, Borth N, Porro D, Mattanovich D: **Improvement of lactic acid production in *Saccharomyces cerevisiae* by cell sorting for high intracellular pH**. *Applied and Environmental Microbiology* 2006, **72**:5492-5499.
252. Narayanan N, Roychoudhury PK, Srivastava A: **L (+)lactic acid fermentation and its product polymerization**. *Electronic Journal of Biotechnology* 2004, **7**:167-U162.
253. Lira CT, McCrackin PJ: **Conversion of lactic acid to acrylic acid in near-critical water**. *Industrial & Engineering Chemistry Research* 1993, **32**:2608-2613.
254. Elferink S, Krooneman J, Gottschal JC, Spoelstra SF, Faber F, Driehuis F: **Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri***. *Applied and Environmental Microbiology* 2001, **67**:125-132.
255. Rossi G, Sauer M, Porro D, Branduardi P: **Effect of HXT1 and HXT7 hexose transporter overexpression on wild-type and lactic acid producing *Saccharomyces cerevisiae* cells**. *Microbial Cell Factories* 2010, **9**.
256. Graupner N, Herrmann AS, Muessig J: **Natural and man-made cellulose fibre-reinforced poly(lactic acid) (PLA) composites: An overview about mechanical characteristics and application areas**. *Composites Part a-Applied Science and Manufacturing* 2009, **40**:810-821.
257. Grand View Research: **Lactic acid and Poly Lactic Acid (PLA) market analysis by application (Packaging, Agriculture, Transport, Electronics, Textiles) and segment forecasts to 2020**. <http://www.grandviewresearch.com/>; 2014.
258. Yang JE, Choi SY, Shin JH, Park SJ, Lee SY: **Microbial production of lactate-containing polyesters**. *Microbial Biotechnology* 2013, **6**:621-636.
259. Willke T, Vorlop KD: **Biotechnological production of itaconic acid**. *Applied Microbiology and Biotechnology* 2001, **56**:289-295.
260. Haskins RH, Thorn JA, Boothroyd B: **Biochemistry of the Ustilaginales. XI. Metabolic products of *Ustilago zaeae* in submerged culture**. *Canadian journal of microbiology* 1955, **1**:749-756.

REFERENCES

261. Tabuchi T, Sugisawa T, Ishidori T, Nakahara T, Sugiyama J: **Itaconic acid fermentation by yeast belonging to the genus *Candida***. *Agricultural and Biological Chemistry* 1981, **45**:475-479.
262. Okabe M, Lies D, Kanamasa S, Park EY: **Biotechnological production of itaconic acid and its biosynthesis in *Aspergillus terreus***. *Applied Microbiology and Biotechnology* 2009, **84**:597-606.
263. Kuenz A, Gallenmueller Y, Willke T, Vorlop K-D: **Microbial production of itaconic acid: developing a stable platform for high product concentrations**. *Applied Microbiology and Biotechnology* 2012, **96**:1209-1216.
264. Li A, van Luijk N, ter Beek M, Caspers M, Punt P, van der Werf M: **A clone-based transcriptomics approach for the identification of genes relevant for itaconic acid production in *Aspergillus***. *Fungal Genetics and Biology* 2011, **48**:602-611.
265. Bentley R, Thiessen CP: **Biosynthesis of itaconic acid in *Aspergillus terreus*. I. Tracer studies with C14-labeled substrates**. *The Journal of biological chemistry* 1957, **226**:673-687.
266. Jaklitsch WM, Kubicek CP, Scrutton MC: **The subcellular organization of itaconate biosynthesis in *Aspergillus terreus***. *Journal of General Microbiology* 1991, **137**:533-539.
267. Blumhoff ML, Steiger MG, Mattanovich D, Sauer M: **Targeting enzymes to the right compartment: Metabolic engineering for itaconic acid production by *Aspergillus niger***. *Metabolic Engineering* 2013, **19**:26-32.

Chapter 1.

ORIGINAL ARTICLE

Open Access

Re-assessment of *YAP1* and *MCR1* contributions to inhibitor tolerance in robust engineered *Saccharomyces cerevisiae* fermenting undetoxified lignocellulosic hydrolysate

Valeria Wallace-Salinas^{1†}, Lorenzo Signori^{2†}, Ying-Ying Li^{3,4}, Magnus Ask⁵, Maurizio Bettiga⁵, Danilo Porro², Johan M Thevelein^{3,4}, Paola Branduardi², María R Foulquié-Moreno^{3,4} and Marie Gorwa-Grauslund^{1*}

Abstract

Development of robust yeast strains that can efficiently ferment lignocellulose-based feedstocks is one of the requirements for achieving economically feasible bioethanol production processes. With this goal, several genes have been identified as promising candidates to confer improved tolerance to *S. cerevisiae*. In most of the cases, however, the evaluation of the genetic modification was performed only in laboratory strains, that is, in strains that are known to be quite sensitive to various types of stresses. In the present study, we evaluated the effects of overexpressing genes encoding the transcription factor (*YAP1*) and the mitochondrial NADH-cytochrome b5 reductase (*MCR1*), either alone or in combination, in an already robust and xylose-consuming industrial strain of *S. cerevisiae* and evaluated the effect during the fermentation of undiluted and undetoxified spruce hydrolysate. Overexpression of either gene resulted in faster hexose catabolism, but no cumulative effect was observed with the simultaneous overexpression. The improved phenotype of *MCR1* overexpression appeared to be related, at least in part, to a faster furfuraldehyde reduction capacity, indicating that this reductase may have a wider substrate range than previously reported. Unexpectedly a decreased xylose fermentation rate was also observed in *YAP1* overexpressing strains and possible reasons behind this phenotype are discussed.

Keywords: *Saccharomyces cerevisiae*; Hydrolysate; Inhibitors; *YAP1*; *MCR1*; Ethanol

Introduction

Production of second-generation bioethanol from lignocellulosic biomass requires robust *Saccharomyces cerevisiae* strains with improved capacity to cope with the toxic compounds formed during the biomass pre-treatment, among which are 5-hydroxymethylfurfural (HMF), furfural, weak organic acids and phenolic compounds (Parawira and Tekere 2011). This has led to extensive studies to decipher mechanisms behind the compounds toxicity and the yeast natural tolerance responses to them and, among others, genes involved in detoxification and yeast tolerance to individual inhibitors have been identified, such as *ADH6*,

HAA1 or *PMA1* (Haitani et al. 2012; Mira et al. 2010; Petersson et al. 2006); for a more exhaustive review, see (Almeida et al. 2009a; Liu 2011). *YAP1* is another interesting candidate for industrial strain engineering because it encodes a transcription factor (Yap1p) that simultaneously controls a wide range of stress-related targets (Toone and Jones 1999). Notably, its overexpression has a beneficial role in the response of laboratory *S. cerevisiae* towards HMF, furfural, and different concentrations of hydrolysate (Alriksson et al. 2010; Kim and Hahn 2013; Ma and Liu 2010; Sundström et al. 2010). Another interesting and complementary candidate for gene overexpression is *MCR1* that encodes the mitochondrial NADH-cytochrome b5 reductase (Hahne et al. 1994; Meineke et al. 2008). Previous experiments performed in our group revealed that overexpression of *MCR1* in *S. cerevisiae* resulted in a reduced lag

* Correspondence: Marie-Francoise.Gorwa@tmb.lth.se

[†]Equal contributors

¹Applied Microbiology, Department of Chemistry, Lund University, P.O. Box 124, SE-22100 Lund, Sweden

Full list of author information is available at the end of the article

phase and faster growth rate when the yeast was grown with high concentrations of acetic acid (Signori et al., personal communication). This weak acid is one of the inhibitors directly affecting xylose metabolism in *S. cerevisiae* (Almeida et al. 2011; Bellissimi et al. 2009; Casey et al. 2010; Helle et al. 2003). Still, considering that many of the studies about strain improvement towards hydrolysate-derived inhibitors concern laboratory strains, it is difficult to predict the real effect of these changes in an industrial, and more robust, strain background.

The objective of the present study was to evaluate the effect of overexpressing *YAP1* and *MCR1*, either alone or in combination, in process-like conditions, that is using a robust industrial *S. cerevisiae* strain and undetoxified lignocellulosic hydrolysate. For this, the strain GSE16 was chosen as background strain for engineering since it combines a robust industrial background with the ability to ferment xylose, using the xylose isomerase pathway (Demeke et al. 2013b). This robust strain was developed by a combination of different strategies including rational metabolic engineering, mutagenesis, evolutionary engineering, genome shuffling and meiotic recombination (Demeke et al. 2013a,b). Moreover, and as part of the genetic engineering strategy used in the current study, the overexpression of *YAP1* was combined with the deletion of the chaperone-encoding gene *APJ1* since deletion of this gene has been previously reported to enable growth on rich medium at inhibitory ethanol concentrations for the parental strain (Swinnen et al. 2012). The relevance of overexpressing *YAP1* and *MCR1* in an industrial background was confirmed for undetoxified spruce hydrolysate fermentation. We also uncovered unexpected interactions between *YAP1* overexpression and xylose metabolism.

Materials and methods

Strains

S. cerevisiae strains utilized in this study are presented in Table 1. *Escherichia coli* DH5 α and *E. coli* NEB 5- α were used for sub-cloning and were grown on Luria-

Bertani (LB) medium supplemented with 100 mg.L⁻¹ ampicillin, when required. Plasmids utilized in the study are described in Table 2.

Molecular biology methods

Standard molecular biology methods were used for all cloning procedures. (Sambrook and Russel 2001). ThermoScientific GeneJET plasmid miniprep kit (ThermoScientific, Lithuania) was used for plasmid extraction. E.Z.N.A Cycle-Pure Kit (Omega Biotek, USA) was used for purification of polymerase chain reaction (PCR) products. Qiagen Qiaquick gel extraction kit (Qiagen GmbH, Germany) was used to extract DNA from agarose gels. All DNA-modifying enzymes were purchased from ThermoScientific. Primers for PCR and sequencing of DNA constructs were ordered from and performed by MWG (MWG-Biotek AG, Germany). All primers are shown in Additional file 1: Table S1. Transformation of *E. coli* was performed using the Inoue Method (Sambrook and Russel 2001). Either the lithium acetate (LiAc) method (Gietz et al. 1995), a modified version of it that uses dimethyl sulfoxide (DMSO) (Hill et al. 1991) or electroporation (Benatuil et al. 2010), were used as transformation methods of *S. cerevisiae*.

Construction of plasmids pJET1,2-attB-KanMX-attP, pJET1,2-attB-hph-attP and pBEVY-Nat-PhiC31

The antibiotic markers KanMX and hph expressed under the TEF promoter and terminator were amplified with the primers Fw-A1-attP-tefpr and Rv-A2-attB-teft and cloned into pJET1,2 (Thermo Scientific, Belgium) following the protocol of the kit. The resulting plasmids were called pJET1,2-attB-KanMX-attP and pJET1,2-attB-hph-attP respectively. The PhiC31 integrase was amplified from pCMVInt (Addgene, Cambridge, Massachusetts, USA) using the primers Fw-PstI-PhiC31 and Rv-BamHI-PhiC31 and cloned into the *PstI* and *BamHI* sites of pBEVY-Nat giving the pBEVY-Nat-PhiC31.

Table 1 *Saccharomyces cerevisiae* strains used in the current work

<i>S. cerevisiae</i> strains	Genotype	Plasmid	Source
CEN.PK 102-5b	<i>Mat a, ura 3-52, his2Δ1, leu 2-3/112</i>		(van Dijken et al. 2000)
CEN.PKc	CEN.PK 102-5b	[pYX012; pYX022; pYX042]	This work
TMB3400	<i>S. cerevisiae</i> industrial strain		(Almeida et al. 2009b)
GSE16	GS1.11-26 + backcrossing with a segregant of Ethanol Red that is tolerant towards acetic acid; <i>MATa/a</i>		(Demeke et al. 2013b)
GSE16 - YAP1	GSE16- <i>APJ1-1::TDH3p-YAP1-CYC1t</i>		This work
GSE16 - MCR1	GSE16- <i>YLR446W-1::TPIp-MCR1</i>		This work
GSE16 - MCR1-YAP1	GSE16- <i>YLR446W-1::TPIp-MCR1, APJ1-1::TDH3p-YAP1-CYC1t</i>		This work
GSE16 - $\Delta\Delta$ APJ1	GSE16- <i>APJ1/APJ1::attL/attL</i>		This work

Table 2 Plasmids used in the current work

Plasmids	Relevant features	Origin
p426GPD	Multicopy <i>URA3</i> 2 μ m <i>TDH3p-CYC1t</i>	(Mumberg et al. 1995)
p426GPD -YAP1	<i>TDH3p-YAP1-CYC1t</i>	This work
pUG6	<i>kanMX</i> flanked by <i>loxP</i> sites	(Güldener et al. 1996) (EUROSCARF; accession number P30114)
YE-plac 112 KanR	Multicopy, KanMX	(Jeppsson et al. 2003)
YE-plac 112 KanR-YAP1	<i>TDH3p-YAP1-CYC1t</i>	This work
pJET1,2	Multicopy	Thermo Scientific, Belgium
pJET1,2-attB-KanMX-attP	Multicopy, KanMX under TEFp	This work
p-intYAP1	<i>TDH3p-YAP1-CYC1t</i> - KanMX attB/attP system; integrative	This work
pSTBlue-1	multi-purpose cloning vector with dual kanamycin/vampicillin resistance.	Novagen (EMD Millipore)
pSTBlue-YLR446W	pSTBlue-1 with the <i>YLR446W</i> gene cloned into the multiple cloning region	This work
pYX012	Integrative; <i>URA3</i> ; TP11p	R&D System, Inc., Wiesbaden, D
pYX012-LoxPkanMXLoxP	pYX012, with the KanMX cassette flanked by <i>loxP</i> sites, deriving from pUG6	This work
pYX012-LoxPkanMXLoxP-MCR1	pYX012-LoxPkanMXLoxP with the <i>MCR1</i> gene inserted in the MCS under the control of the TP11 promoter	This work
pSTBlue-YLR446WA-LoxPkanMXLoxP-MCR1	pSTBlue-YLR446W with the LoxPkanMXLoxP-MCR1 cassette, deriving from pYX012 LoxPkanMXLoxP-MCR1, inserted into the <i>YLR446W</i> sequence	This work
pSH65	Centromeric plasmid, GAL1p-cre, ble ^r	(Güldener et al. 2002) (EUROSCARF; accession number P30122)
pJET1,2-attB-hph-attP	Multicopy, hph under TEFp	This work
pBEVY-Nat-phiC31	Multicopy, phiC31 integrase	This work

Construction of *S. cerevisiae* strains *S. cerevisiae* GSE16-YAP1

The open reading frame of the *YAP1* gene was amplified from *S. cerevisiae* strain TMB3500 (Almeida et al. 2009b), using primers YAP1-F and YAP1-R. The amplicon was ligated into p426GPD (Mumberg et al. 1995) resulting in p426GPD-YAP1 used for transformation of *E. coli* DH5 α cells and followed by sequence verification. The cassette from p426GPD-YAP1 was amplified using primers GPD-YAP1-F and CYC1t-YAP1-R. The amplified cassette was ligated into pUG6 after restriction with *AatII* and *PvuII*, resulting in pUG6-YAP1. Amplification of the two homologous regions (HR) used for integration of the *YAP1* cassette into the *APJ1* locus were performed from *S. cerevisiae* GSE16 (Demeke et al. 2013b) with primers HR1-F/HR1-R and HR2-F/HR2-R. Amplicon for HR2 was first ligated into pUG6-YAP1 and transformed into *E. coli* DH5 α cells resulting in pYAP1-HR2. Amplicon for HR1 was ligated into pYAP1-HR2 and transformed into *E. coli* DH5 α cells resulting in pYAP1-HR2-HR1. The selection cassette (KanMX) flanked by the attB and attP sites was amplified from pJET1,2-attB-KanMX-attP using attBP-F and attBP-R. The amplicon was ligated into pYAP1-HR2-HR1 and transformed into *E. coli* NEB 5-alpha resulting in p-intYAP1. The nucleotide sequence of each amplicon

was verified after every subsequent cloning step. *S. cerevisiae* strain GSE16 was used for expression of the *YAP1* construct. In this strain, the expression cassette containing the transcription factor *YAP1* was integrated by linearization of the integrative cassette using *AatII* and *NotI*, followed by transformation using a DMSO-modified version of the LiAc method (Hill et al. 1991). The selection of colonies was done on YNB plates with 150 μ g.mL⁻¹ geneticin G418 (Sigma). Verification of the correct insertion (*APJ1* locus) was done by sequencing using primers Ver.ins1-F/Ver.ins1-R and Ver.ins2-F/Ver.ins2-R. The resulting strain was named GSE16-YAP1.

S. cerevisiae GSE16-MCR1

The open reading frame of *MCR1* was amplified from CEN.PK 102-5b using primers MCR1-F and MCR1-R. The amplified DNA fragment (1544 bp) was cloned into pSTBlue (*EcoRV* site) and used to transform *E. coli* DH5 α cells. A 1.5 Kb *EcoRI* fragment containing *MCR1* was isolated from pSTBlue-MCR1 and cloned into the MCS of the yeast integrative plasmid pYX012, resulting in pYX012-MCR1. In parallel, the ORF YLR446W was amplified from *S. cerevisiae* CEN.PK 102-5b using primers YLR446W-F and YLR446W-R and cloned into pSTBlue (*EcoRV* site) resulting in pSTBlue-YLR446W. The selection

cassette (KanMX) flanked by two LoxP sites was amplified from pUG6 using primers YLR446W Lox-F and Lox-R, and cloned into pYX012-MCR1 plasmid (*KpnI* site). The expression cassette (LoxPKanMXLoxP + promoter + *MCR1* ORF + terminator) was amplified using primers YLR446W Lox-F and YLR446W TER-R and cloned into pSTBlue-YLR446W after restriction with *BtgI* and *BsrGI* (this double digestion allowed the removal of the inner part of the *YLR446W* gene (~601 bp)). Each amplicon was verified by sequencing analysis after every subsequent cloning step. *S. cerevisiae* strain GSE16 was used for expression of the *MCR1* construct. The expression cassette containing *MCR1* was integrated into GSE16 after PCR amplification using primers YLR446W-F and YLR446W-R. Transformation was carried out using a DMSO-modified version of the LiAc method (Hill et al. 1991). Correct insertion in the *YLR446W* locus was verified by PCR. The resulting strain was named GSE16-MCR1. The removal of the dominant marker (KanMX) was obtained by transforming GSE16-MCR1 with pSH65.

***S. cerevisiae* GSE16-MCR1-YAP1**

The strain was constructed from GSE16-MCR1 by integration of p-intYAP1 previously digested with *AatII* and *NotI* (see *S. cerevisiae* strain overexpressing *YAP1*). The correct integration in the *APJ1* locus was verified by PCR.

***S. cerevisiae* GSE16- $\Delta\Delta APJ1$**

The selection cassettes (KanMX and hph) flanked by the attB and attP sites were amplified by PCR from pJET1,2-attB-KanMX-attP and pJET1,2-attB-hph-attP using primers Fw-APJ1-A1 and Rv-APJ1-A2 with 50 bp homologues regions. Deletion of the two *APJ1* alleles was carried out by integrating both selection cassettes into the *APJ1* loci of GSE16 (Demeke et al. 2013b) using an adapted electroporation method (Benatuil et al. 2010). *APJ1* double deletion colonies were selected from YPD plates with hygromycin (300 $\mu\text{g}\cdot\text{mL}^{-1}$) and geneticin (G418) (200 $\mu\text{g}\cdot\text{mL}^{-1}$), and checked by PCR using the primers Fw-APJ1-check and Rv-APJ1-check. To loop out the markers, the colonies were transformed with pBevy-Nat-phi31 and selected in YPD plates with 100 $\mu\text{g}\cdot\text{mL}^{-1}$ of nourseotricin. These colonies were also checked on YPD hygromycin and YPD geneticin plates. The plasmids containing the integrase were lost by growing the colonies in YPD liquid medium overnight and transferred twice. The resulting strain was named GSE16- $\Delta\Delta APJ1$.

Spruce hydrolysate Spruce hydrolysate was obtained from SEKAB E-Technology AB (Örnsköldsvik, Sweden), and consisted of the non-detoxified liquid fraction of spruce after a pretreatment by SO_2 catalyzed steam explosion. It is referred in this work as spruce hydrolysate and had the following sugar composition: 11 $\text{g}\cdot\text{L}^{-1}$ glucose,

17 $\text{g}\cdot\text{L}^{-1}$ mannose, 4 $\text{g}\cdot\text{L}^{-1}$ galactose, and 10 $\text{g}\cdot\text{L}^{-1}$ xylose. All the batch fermentations were carried out with the same batch of spruce hydrolysate which was kept at 4°C.

Anaerobic batch fermentations of spruce hydrolysate

Inoculum was prepared by growing the cells overnight in 1 L shake flasks containing 100 mL defined mineral medium with vitamins (Verduyn et al. 1992) with glucose as carbon source (20 $\text{g}\cdot\text{L}^{-1}$) buffered with phthalate buffer (50 mM, pH 5.0). After centrifugation and a washing step with deionized water, the pellet was resuspended with 20 mL of fermentation medium, and immediately used for inoculation of the fermenter. The fermentation medium consisted of 100% (v/v) spruce hydrolysate supplemented with 10 $\text{g}\cdot\text{L}^{-1}$ of xylose, 1 $\text{g}\cdot\text{L}^{-1}$ yeast extract, 0.5 $\text{g}\cdot\text{L}^{-1}$ $(\text{NH}_4)_2\text{HPO}_4$ and 0.025 $\text{g}\cdot\text{L}^{-1}$ $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The pH of the hydrolysate was adjusted to 5.0 with 8 M KOH prior to supplementation. The fermentations were carried out in 1.2 L MultiFors fermenters with 0.5 L working volume. Temperature was maintained at 30°C and the pH was kept at 5.0 by addition of 3 M KOH. Oxygen free conditions were maintained by sparging N_2 at 0.2 $\text{L}\cdot\text{min}^{-1}$, and the agitation was set to 600 rpm. Reactors were inoculated to a biomass concentration of 1 $\text{g}\cdot\text{L}^{-1}$ (cdw). Cultivations were performed in biological duplicates for each investigated strain. Specific conversion rates of furfural and HMF were calculated assuming a pseudo-steady state during the exponential growth on glucose.

Anaerobic batch fermentations in mineral medium

Inoculum was prepared by growing the cells overnight in 100 mL shake flasks containing 20 mL defined mineral medium with vitamins (Verduyn et al. 1992) with glucose as carbon source (20 $\text{g}\cdot\text{L}^{-1}$) buffered with phthalate buffer (50 mM, pH 5.0). Fermentations were carried out in 100 mL glass bottles with 40 mL working volume of the same mineral medium and vitamins supplemented with 5 $\text{g}\cdot\text{L}^{-1}$ of glucose and 20 $\text{g}\cdot\text{L}^{-1}$ of xylose as carbon and energy sources. Temperature was maintained at 30°C and stirring was set at ca. 160 rpm. Oxygen-limited conditions were obtained by sealing the bottles and sparging N_2 at 0.2 $\text{L}\cdot\text{min}^{-1}$ for at least 5 minutes before inoculation. A cotton-filled syringe was inserted through the rubber stopper using a needle to avoid accumulation of gas inside the bottles. Bottles were inoculated to a biomass concentration of ca. 1 $\text{g}\cdot\text{L}^{-1}$ (cdw). Cultivations were performed in biological duplicates for each investigated strain.

Analysis of substrates and products Controlled volumes of samples were taken regularly for analysis. Biomass was followed by OD620 measurements during the length of the fermentations and determination of cell dry weight measurements were also done at time zero (just after inoculation) and at times 42 h and 92 h. For biomass

determination, the cell pellet from 5 mL culture was washed with distilled water and dried on Gelman filters (ϕ 47 mm Supor-450, 0.45 μ m) in a microwave oven (350 W) for 8 minutes. Ethanol, glycerol, acetic acid, HMF and furfural were analysed by high performance liquid chromatography (HPLC; Waters Corporation, MA, USA) using an Aminex HPX-87H column (Bio-Rad, CA, USA) at 65°C. The mobile phase was 5 mM sulphuric acid with a flow rate of 0.6 mL.min⁻¹. Analysis of glucose, mannose, xylose, galactose and xylitol was performed on a Shodex™ SP-0810 sugar column (Showa Denko K.K, Japan) at 85°C with water as mobile phase and 0.6 mL.min⁻¹ flow rate. All compounds were detected with a refractive index detector (Shimadzu, Tokyo, Japan, and Waters 2414, MA, USA respectively). Yields were calculated based on HPLC measurements.

Results

Genetic engineering of the xylose consuming industrial strain GSE16

It has previously been reported that, whereas overexpression of the transcription factor *YAP1* resulted in a strain with a faster sugar consumption rate when fermenting 60% (v/v) of spruce hydrolysate, the same strain was severely inhibited at higher concentrations of the substrate (Alriksson et al. 2010). However the study was performed using laboratory strains with a sensitive genetic background towards hydrolysate-derived inhibitors (Martin and Jönsson 2003). From these results and further studies carried out in our group (data not shown), the importance of the genetic background and the initial inhibitors concentrations on the effect of *YAP1* overexpression was further highlighted, and stressed the necessity of assessing the effect of gene modification under more industrial relevant conditions, that is using up-to-date engineered industrial strains and process-like fermentation conditions.

In this study, overexpression cassettes containing the genes that encode the transcription factor *YAP1* and the mitochondrial NADH-cytochrome b5 reductase *MCR1* were designed and integrated in the genome of the robust industrial strain GSE16, either alone or in combination, generating strains GSE16-*YAP1*, GSE16-*MCR1* and GSE16-*YAP1*-*MCR1* (Table 1). Simultaneous *YAP1* integration and *APJ1* deletion were obtained by inserting the *YAP1* overexpression cassette into the *APJ1* locus. The *MCR1* cassette was targeted to the YLR446W locus, a gene whose deletion has not affected the yeast performance during fermentation (Subtil and Boles 2012). A control strain in which both alleles of *APJ1* were deleted (GSE16- $\Delta\Delta$ APJ1) was included during the fermentations (Table 1).

The effect of each genetic modification was assessed during anaerobic batch fermentations of undiluted spruce hydrolysate. In order to have a medium composition similar

to large scale lignocellulose-based fermentations, spruce hydrolysate with limited nutrient supplementation was used (See Materials and methods). The xylose concentration was increased from 10 g.L⁻¹ to 20 g.L⁻¹ to allow a longer period for analysis of the xylose consumption phase. The initial composition of the fermentation medium consisted therefore of 11 g.L⁻¹ glucose, 17 g.L⁻¹ mannose, 4 g.L⁻¹ galactose, and 20 g.L⁻¹ xylose. The following inhibitors could also be identified: acetate (3.7 g.L⁻¹), HMF (0.96 g.L⁻¹) and furfural (0.78 g.L⁻¹). The strain robustness was first assessed by comparing lag phase duration, specific growth rate, and specific HMF and furfural conversion rates. Next, we evaluated the fermentation performance of the different strains in terms of glucose and xylose consumption rates, ethanol production rate, and product distribution. The carbon dioxide profile of all strains during the batch fermentations is presented in Additional file 1: Figure S1.

Impact of strain engineering on growth and furaldehyde conversion

During the anaerobic batch fermentations of 100% spruce hydrolysate, and with an initial biomass of 1 g.L⁻¹ cell dry weight (cdw), the control strain GSE16 showed a lag phase of 9 hours (Table 3). The strains overexpressing *YAP1* showed a consistent decrease in the duration of the lag phase, which lasted for 5.3 \pm 0.6 hours. The lag phase duration was not significantly affected by overexpression of *MCR1* while it was increased (by ~3 h) for the strain $\Delta\Delta$ APJ1 ($p = 0.01$). The specific growth rate was also altered in all the strains with the exception of $\Delta\Delta$ APJ1. GSE16-*YAP1* showed a specific growth rate of 0.21 h⁻¹, that is an increase of around 60% when compared to the control strain ($p = 0.08$). A similar increase was also displayed by GSE16-*MCR1* ($p = 0.15$) (Table 3). No additional increase was observed by the strain combining both modifications.

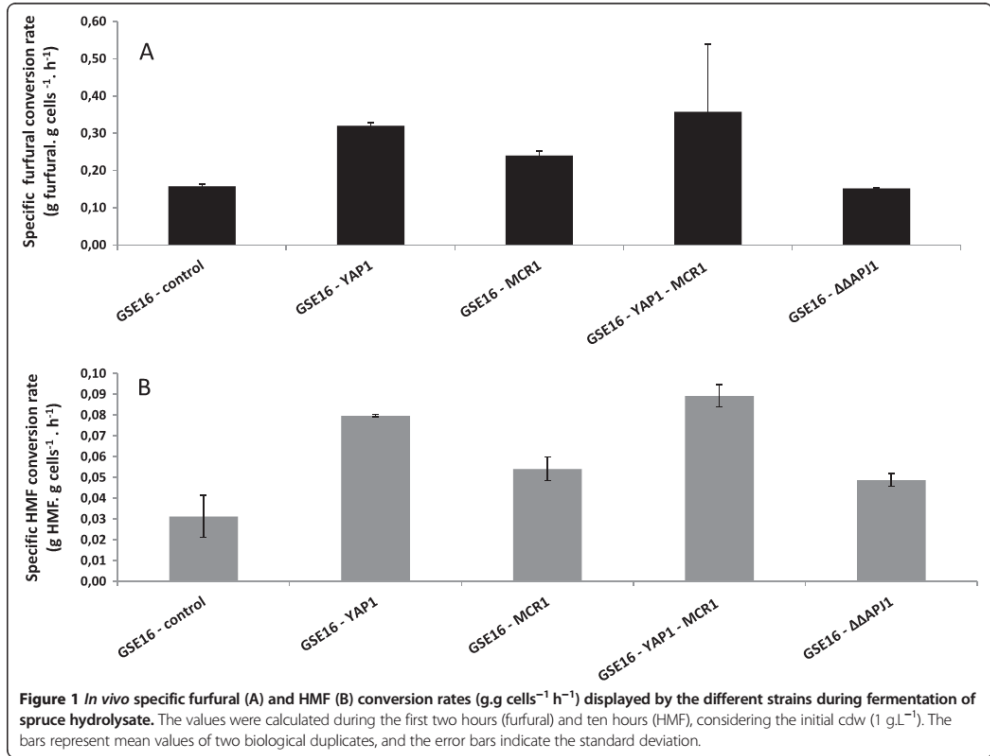
As for furaldehyde conversion, all strains exhibited a higher specific conversion rate (g.g cells⁻¹.h⁻¹) for furfural than for HMF (Figure 1). The control strain displayed a

Table 3 Lag phase duration and maximum specific growth rate during batch fermentation of spruce hydrolysate

Strain	Lag phase ^a (h)	Growth rate, μ ^b (h ⁻¹)
GSE16	8.96 \pm 0.08	0.13 \pm 0.02
GSE16 - <i>YAP1</i>	5.29 \pm 0.59	0.21 \pm 0.03
GSE16 - <i>MCR1</i>	7.65 \pm 1.06	0.20 \pm 0.04
GSE16 - <i>YAP1</i> - <i>MCR1</i>	5.49 \pm 0.83	0.21 \pm 0.02
GSE16 - $\Delta\Delta$ APJ1	12.04 \pm 0.48	0.12 \pm 0.02

Initial biomass concentration was 1 g.L⁻¹ cdw. The values show the mean and standard deviation of two biological replicates.

^aThe lag phase is defined as the time between inoculation and the onset of the increase on carbon dioxide production. ^bGrowth rates were calculated from the carbon dioxide production rates during the exponential phase on glucose.



furfural specific conversion rate of 0.16 g.g cells⁻¹.h⁻¹. GSE16-YAP1 showed the highest furfural specific conversion rate among all the strains (two times higher than the control strain, $p = 0.002$). Conversion of furfural was also improved when *MCR1* was overexpressed (Figure 1). Likewise, *in vivo* conversion of HMF was enhanced by over-expression of *YAP1* or *MCR1*. GSE16-YAP1 was able to convert the inhibitor around two times faster (0.08 g.g cells⁻¹.h⁻¹) than the parental strain ($p = 0.02$), and a 67% increase ($p = 0.11$) was observed for GSE16-MCR1 (Figure 1). As observed for the growth rate, overexpression of *YAP1* in combination with *MCR1* did not result in additional improvements neither in furfural nor in HMF conversion capacity. Neither was any significant difference in reduction capacity observed between the control strain and the *APJ1* double deletion mutant.

Impact of strain engineering on sugar metabolism

Cell dry weight (cdw) measured after 48 and 92 h of fermentation, suggested that all strains had a similar increase in biomass (data not shown). However, accurate determination of biomass concentration in the presence

of lignocellulosic hydrolysate was made difficult by the presence of solid particles in suspension in the medium or because of variations in the broth color (in the case of optical density measurement) over time. Therefore the strains were compared in terms of volumetric rates (of sugar and product formation) instead of specific rates.

The volumetric consumption rate of glucose was around 0.81 g.L⁻¹.h⁻¹ for the strains overexpressing *YAP1* or *MCR1*, which was at least 65% higher than the rate observed for the control strain (Table 4). And again, no additive effect was seen in the strains combining the over-expression of both genes. Deletion of *APJ1* resulted in a marginal increase in glucose utilization rate. Our analytical set-up did not allow accurate quantification of the mannose and galactose consumption rates. However, approximate determinations showed that mannose was consumed shortly after glucose was depleted from the medium. Co-consumption of mannose and xylose was observed during this phase. After mannose was exhausted from the medium, the relatively low amount of galactose (4 g.L⁻¹) was co-consumed together with xylose (data not shown). In contrast to glucose, the xylose consumption

Table 4 Volumetric consumption rate of glucose and xylose and volumetric production rate of ethanol during fermentation of spruce hydrolysate

Strain	Volumetric consumption and production rates (g.L ⁻¹ .h ⁻¹)				
	Glucose	Xylose	Ethanol (glucose phase)	Ethanol (xylose phase ^a)	Ethanol (overall)
GSE16	0.49 ± 0.01	0.22 ± 0.01	0.60 ± 0.13	0.13 ± 0.00	0.27 ± 0.04
GSE16 – YAP1	0.81 ± 0.15	0.13 ± 0.00	0.90 ± 0.00	0.09 ± 0.02	0.21 ± 0.00
GSE16 – MCR1	0.81 ± 0.12	0.24 ± 0.02	0.84 ± 0.08	0.13 ± 0.00	0.27 ± 0.01
GSE16 – YAP1- MCR1	0.84 ± 0.12	0.13 ± 0.00	0.90 ± 0.08	0.08 ± 0.00	0.20 ± 0.01
GSE16 – ΔΔAPJ1	0.64 ± 0.03	0.19 ± 0.02	0.57 ± 0.05	0.11 ± 0.01	0.28 ± 0.01

^aDuring this phase galactose is also consumed.

rate was found to be negatively affected by *YAP1* overexpression. While the control strain consumed 0.22 g xylose. L⁻¹.h⁻¹, a 40% reduction was observed for the two strains overexpressing the transcription factor ($p \leq 0.03$). Overexpression of *MCR1* alone and deletion of *APJ1* did not have a significant effect on xylose utilization (Table 4). Since the deletion of both alleles of *APJ1* in GSE16-ΔΔAPJ1 did not affect xylose consumption, it is very unlikely that the effect observed in the *YAP1*-overexpressing strains (carrying only one deleted allele) could be responsible for the lower xylose consumption rate observed in these strains.

The volumetric ethanol production rate was calculated both during the glucose and the xylose consumption phases (Table 4). As expected from the differences between the rates of consumption of the two sugars, the ethanol production rate measured during the xylose phase was considerably lower than the one observed during the glucose phase for all strains. For the control strain, for example, the ethanol production rate on xylose decreased by 78% as compared to the one measured during the glucose phase. When comparing the different strains on glucose, GSE16-YAP1 showed one of the fastest ethanol production rates (0.90 g ethanol.L⁻¹.h⁻¹, $p = 0.08$). More generally, the ethanol production rate calculated for the new constructs was between 18% and 53% higher than the one obtained for the control strain, thereby matching the increase in the glucose consumption rate. An exception to this correlation was GSE16- ΔΔAPJ1, for which the ethanol rate during the glucose phase was the same as for the control strain (Table 4). On xylose, both *YAP1*-overexpressing strains exhibited a 40% reduction in the ethanol production rate when compared to GSE16. For GSE16-MCR1 and GSE16- ΔΔAPJ1, the ethanol rate in the xylose phase was comparable with that of the control strain. When considering the total length of the fermentations (92 h), no substantial differences were observed between the strains in terms of the volumetric production rate of ethanol ($p > 0.1$) (Table 4).

All strains showed similar metabolite distributions, with the exception of xylitol which was around 2.4 times higher for the strains overexpressing *YAP1* ($p \leq 0.03$) (Table 5). The ethanol yields at the end of the fermentation

were very similar between all strains, and accounted for about 50-58% of the maximum theoretical yield (Table 5). These low ethanol yields are very likely a result of a significant rate of evaporation in the fermenter. As previously reported (Bengtsson et al. 2009; Wahlbom et al. 2001), the low boiling point of ethanol and the continuous sparging of the fermenters with nitrogen importantly affect the ethanol yields during prolonged fermentations. As mentioned earlier, the biomass yield was also similar for all strains as was the glycerol yield. The acetate yield was very low for all the strains and less than 0.01 g/g in all the cases (data not shown).

In summary, the deletion of *APJ1* in GSE16 was not detrimental for the strain performance when fermenting spruce hydrolysate. While it was not possible to evaluate the positive benefit of this deletion with the set-up used for the fermentations (expected concentrations of ethanol lower than 3% v/v), *APJ1* can be considered as a good candidate for integration of expression cassettes in strains to be used during very high gravity fermentations. In contrast, *YAP1* and *MCR1* had a positive effect on glucose fermentation in undetoxified hydrolysate but the effect was not cumulative. Finally, *YAP1* overexpression had an unexpected negative impact on xylose utilization.

YAP1 and xylose consumption in mineral medium

The positive effect of *YAP1* in relation to resistance to inhibitors was clearly shown by the improved fermentation rate of glucose during the anaerobic batch fermentations of spruce hydrolysate. The effect during the xylose phase, on the other hand, was not clear. In order to further investigate the effect of *YAP1* overexpression on xylose consumption and to prevent any interference caused by the complex matrix of spruce hydrolysate, the xylose consumption rate of strain GSE16-YAP1 was evaluated during anaerobic fermentation of mineral medium containing 5 g.L⁻¹ of glucose and 20 g.L⁻¹ xylose. Strain GSE16 was included as a control. With an initial biomass of 1 g.L⁻¹ (cdw), both strains consumed all the glucose within 5 hours. However, the negative effect of *YAP1* overexpression on xylose utilization was also observed in mineral medium, indicating that such effect was not

Table 5 Ethanol, glycerol, biomass and xylitol yields per gram of consumed sugars in anaerobic batch fermentation of spruce hydrolysate

Strain	Yields ¹ (g.g ⁻¹)			
	Y _{ethanol}	Y _{glycerol}	Y _{biomass}	Y _{xylitol}
GSE16	0.27 ± 0.08	0.05 ± 0.00	0.04 ± 0.01	0.05 ± 0.01
GSE16 – YAP1	0.28 ± 0.05	0.05 ± 0.01	0.04 ± 0.00	0.12 ± 0.00
GSE16 – MCR1	0.26 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.05 ± 0.01
GSE16 – YAP1- MCR1	0.25 ± 0.00	0.06 ± 0.00	0.04 ± 0.00	0.12 ± 0.01
GSE16 – ΔΔAPJ1	0.30 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.07 ± 0.02

Yields of ethanol, glycerol and biomass were calculated based on the total consumed sugars, while xylitol yields were calculated based on the consumed xylose only. The numbers reported are means ± standard deviation (n = 2).

related to the presence of inhibitors. After 54 h, the control strain GSE16 consumed 13.06 ± 0.46 g of xylose, while GSE16-YAP1 only consumed 3.17 ± 0.1 g xylose (Figure 2). As observed during the fermentations of hydrolysate, the final yields of biomass and extracellular metabolites were similar between the strains, except for xylitol. After 138 h, the xylitol yield of GSE16-YAP1 was 93% higher than that of the control strain (Table 6).

Discussion

In the present study two different genetic modifications that have been reported to improve strain tolerance in a laboratory strain background were introduced in the industrial *S. cerevisiae* strain GSE16 and assessed during

fermentation of undetoxified spruce hydrolysate. The objective was to test whether the overexpression of the selected genes would still be relevant in a strain with a high robust genetic background, to identify the most promising gene among the two candidates, and to assess any putative additive or synergistic effects.

During the glucose consumption phase, the overexpression of *YAP1* was shown to be relevant for inhibitor conversion, even in a robust genetic background. Similarly, the strain robustness in relation to spruce inhibitors was further increased when overexpressing *MCR1*, but no cumulative effect of combined overexpression was revealed. Slightly increased inhibitor tolerance was observed when overexpressing *YAP1* as compared to *MCR1*, which might be explained by the diversity of genes controlled by this transcription factor with relevant functions for detoxification (reductases, transporters, and oxidative stress-related enzymes among others) (Alriksson et al. 2010; Herrero et al. 2008; Toone and Jones 1999). Still, the explanation for the almost equivalent effect obtained by *MCR1* overexpression is less evident. Previous results showed that overexpression of *MCR1* resulted in better growth in mineral medium supplemented with 12 g.L⁻¹ acetic acid. (Signori et al., personal communication). Considering the role of the enzyme encoded by *MCR1* in maintaining the antioxidant D-erythroascorbic acid (EASC) in its reduced form (Lee et al. 2001), it is plausible that higher concentrations of this antioxidant may help counteracting the oxidative

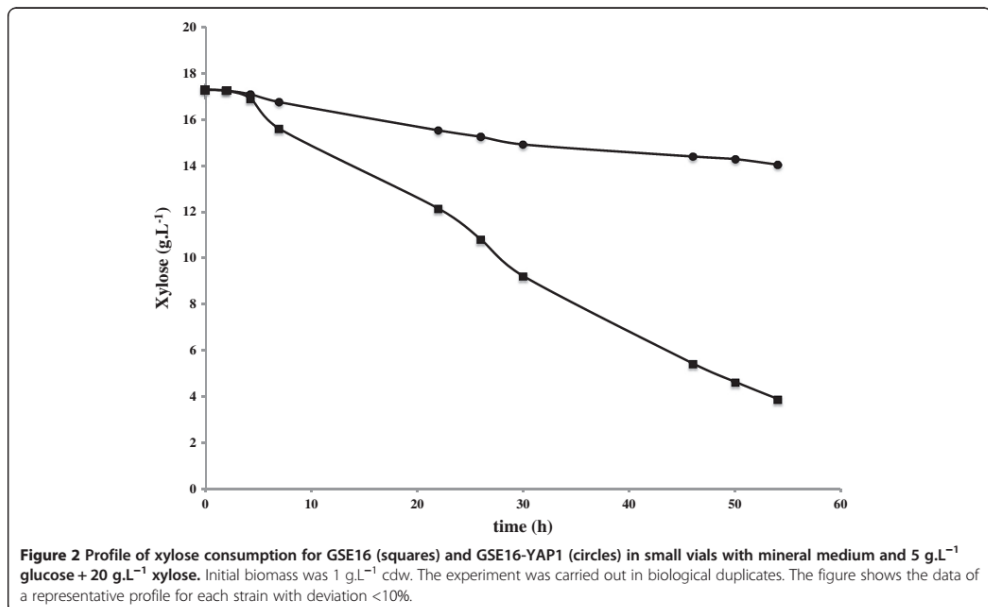


Table 6 Ethanol, glycerol, biomass, xylitol and acetate yields obtained during fermentations on mineral medium with 5 g.L⁻¹ glucose and 20 g.L⁻¹ xylose

Strain	Yield (g.g ⁻¹)				
	Y _{Ethanol}	Y _{Glycerol}	Y _{Biomass}	Y _{Xylitol}	Y _{Acetate}
GSE16	0.45 ± 0.03	0.04 ± 0.00	0.09 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
GSE16-YAP1	0.45 ± 0.02	0.04 ± 0.00	0.10 ± 0.00	0.06 ± 0.00	0.01 ± 0.00

Yields of ethanol, glycerol, biomass and acetate were calculated based on the total sugar consumed, while xylitol yields were calculated based on the consumed xylose only. The numbers reported are means ± standard deviation (n = 2).

effect exerted by the acetic acid present in the hydrolysate (Semchyshyn et al. 2011). In fact, the effect seen by *MCRI* overexpression in the presence of acetic acid could be similar to the effect obtained with the biosynthesis of ascorbic acid (ASC) reported by Branduardi (Branduardi et al. 2007). In this study the authors showed that in yeasts, biosynthesis of ASC -a molecule very similar in structure and properties to D-erythroascorbic acid (EASC) - conferred increased resistance to H₂O₂, low pH and organic acids. Moreover, the higher in vivo conversion rates of furaldehydes displayed by GSE16-MCRI also points towards an involvement of the enzyme in the reduction (by a yet unknown mechanism) of HMF and furfural probably into their less inhibitory alcohol forms (Liu et al. 2005). Given the specific location of this enzyme, this result supports previous observations that indicated a damaging effect of hydrolysate-derived inhibitors to the mitochondria (Allen et al. 2010; Nguyen et al. 2014). To the best of our knowledge this is the first report of a positive effect of *MCRI* overexpression on hydrolysate detoxification; and all together the results suggest that increased concentrations of NADH-cytochrome b5 reductase can improve the resistance of yeast to hydrolysate inhibitors and therefore increase the ethanol production rate on glucose.

On xylose, the much slower sugar consumption rate (as compared to glucose) in one of the best reported pentose fermenting industrial yeasts (Demeke et al. 2013b) and its derivatives emphasized that anaerobic pentose metabolism still requires further improvement, especially in the presence of lignocellulosic inhibitors. Recent comparisons of metabolic profiles between the glucose and xylose consumption phases of *S. cerevisiae* with (Wang et al. 2014; Ask et al. 2013) and without inhibitors (Bergdahl et al. 2012; Matsushika et al. 2013), highlighted the significant perturbations in the metabolic capacities of the yeast caused by xylose. In these metabolomics studies, such perturbations were ascribed to the depletion of key metabolites in glycolysis and cofactors (among other important metabolic variations) suggesting inefficient metabolic states such as carbon starvation, and diminished biosynthetic capacities (Bergdahl et al. 2012; Matsushika et al. 2013).

Our results also revealed a far more complex set of cellular responses deriving from the interactions between

xylose metabolism and *YAP1* overexpression. And although the evaluated physiological responses do not provide enough information for explaining the decrease in xylose consumption in *YAP1*-overexpressing strains, the integration of these observations with the results of previous studies could give some hints for further analysis.

First, higher concentrations of xylitol were obtained for *YAP1*-overexpressing strains, which may result from the unspecific reduction of xylose by the different reductases whose transcription is under the control of *YAP1* (Toone and Jones 1999). In fact, *YAP1*-overexpressing strains showed a higher xylose reductase activity than the control strain when cells extracts were used for reduction of xylose (data not shown). Possible inhibition of xylose isomerase (XI) by xylitol was considered, although the used XI originates from *C. phytofermentans*, and has been shown to be much less inhibited by xylitol than other previously expressed XIs in *S. cerevisiae* (Brat et al. 2009). Moreover, the lower xylose consumption for GSE16-YAP1 was observed from the beginning of the fermentation, i.e. when negligible levels of xylitol had been formed. Besides, the rate of consumption was almost constant during most of the process (except at the end of the fermentation when the low concentrations of xylose probably reduce the conversion rate for all strains). This implies that the consumption of xylose did not vary in relation to the increase of xylitol in the medium, i.e. xylitol was not inhibiting the conversion of xylose to xylulose by XI.

A second aspect was connected to the "history" of GSE16. Demeke and co-workers (Demeke et al. 2013b) reported that the diverse and complex paths followed during the development of GSE16 included unknown mutations that appeared to be linked to the high capacity of the strain to ferment xylose, but that such mutations could also be correlated with a possible detrimental effect in terms of inhibitor tolerance (Demeke et al. 2013b). The authors suggested that this mutually exclusive phenotype (good xylose fermentation – bad inhibitor tolerance and vice versa) could be either causally or structurally linked, i.e. genes responsible for improved xylose utilization could be functionally connected with genes responsible for the slower growth; or in the other case, such genes may be located close to each other in the genome (Demeke et al. 2013b). The results obtained in the current study with the *YAP1* overexpressing strains also point towards a mutually

exclusive phenotype, but in this case a structural link does not seem likely since the deletion of the locus used for *YAPI* integration (*APJ1*) did not cause a detrimental effect on xylose utilization.

With the exception of xylitol, comparable values were obtained for biomass and extracellular metabolites yields between the different strains in hydrolysate fermentations, which suggested that the overexpression of *YAPI* affected the rate of xylose metabolism but not the product distribution. As similar lower xylose uptake was obtained in mineral medium we could conclude that the negative interactions between xylose metabolism and overexpression of the Yap1 transcription factor were not dependent on the presence of inhibitors and their associated cell responses. When considering available omics data about the effects of overexpressing *YAPI* in *S. cerevisiae* in mineral medium, 17 transcripts were up-regulated by overexpression of the transcription factor (DeRisi et al. 1997) while around 55 proteins were present in higher concentrations (Jun et al. 2012). Although the differences in cultivation conditions do not permit to make any conclusion towards particular genes of interest, these two studies suggest that the overexpression of *YAPI* imposes a higher demand to the cells biosynthetic capacity. This, together with an impairment of biosynthetic capabilities on xylose (as presented in the metabolomics studies previously commented (Bergdahl et al. 2012; Matsushika et al. 2013)), and the high cell maintenance energy required during growth on this sugar (Feng and Zhao 2013) could explain the slower growth on xylose in *YAPI* overexpressing strains.

Nevertheless, the unexpected results seen with *YAPI*-overexpression during xylose assimilation require deeper analysis to further understand the biological responses that limit the development of robust xylose consuming strains. In this respect, we consider that the study at the molecular level of the cellular responses of *YAPI*-overexpression in GSE16 and other xylose consuming strains (for example expressing the fungal redox pathway) during glucose and xylose utilization would reveal important insights about limiting steps for xylose metabolism.

In conclusion, overexpression of the transcription factor *YAPI* and the mitochondrial reductase *MCR1* in the already robust strain GSE16 resulted in an even faster hexose catabolism in the presence of spruce hydrolysate-derived inhibitors, but the effect was not cumulative. The improved phenotype of *MCR1* overexpression seems to be related, at least in part, to a faster furaldehyde reduction, indicating that this reductase may have a wider substrate range than previously reported. Unexpected reduced xylose fermentation rate was observed in *YAPI* overexpressing strains and further studies are needed to elucidate the mechanisms behind this observation.

Additional file

Additional file 1: Table S1. Oligonucleotides used in the current work. Shows the sequence of the primers used during the study. **Figure S1.** Carbon dioxide profile of the strains GSE16 (parental), GSE16-*YAPI*, GSE16-*MCR1*, GSE16-*YAPI-MCR1* and GSE16- $\Delta\Delta$ APJ1 during anaerobic batch fermentations of spruce hydrolysate (only shown for the first 35 h). The figure shows the carbon dioxide profile of the different strains evaluated in the study during anaerobic batch fermentations of spruce hydrolysate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VWS participated in the design of the study, performed the experiments and wrote the manuscript. LS, YL, MA performed the experiments and commented on the manuscript. MB participated in the initial design of the study and commented the manuscript. DP and PB contributed in conceiving the study and revised the manuscript. MFM and JT participated in design of the study and revised the manuscript. MGG conceived the study and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This project was financed by the 7th European Commission Framework Project 222699 NEMO (Novel High performance Enzymes and Micro-organisms for conversion of lignocellulosic biomass to bioethanol) and SBO grants (IWT 90043) from IWT-Flanders. SEKAB, Sweden is gratefully acknowledged for the provision of the hydrolysate. VWS was co-financed by the Swedish Energy Agency (Energimyndigheten). LS acknowledges the doctoral fellowship of the University of Milano Bicocca. YL was supported by the China Scholarship Council. MB is financed by the Chalmers Energy Initiative.

Author details

¹Applied Microbiology, Department of Chemistry, Lund University, P.O. Box 124, SE-22100 Lund, Sweden. ²University of Milano Bicocca, Piazza della Scienza 2, 20126 Milan, Italy. ³Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, Leuven, KU, Belgium. ⁴Department of Molecular Microbiology, VIB, Kasteelpark Arenberg 31, Leuven, B-3001 Heverlee, Flanders, Belgium. ⁵Department of Chemical and Biological Engineering, Industrial Biotechnology, Chalmers University of Technology, SE-41296 Gothenburg, Sweden.

Received: 3 March 2014 Accepted: 19 June 2014

Published online: 22 July 2014

References

- Allen SA, Clark W, McCaffery JM, Cai Z, Lanctot A, Slinger PJ, Liu ZL, Gorsich SW (2010) Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. *Biotechnology for Biofuels* 3:2. doi:10.1186/1754-6834-3-2
- Almeida JRM, Bertilsson M, Gorwa-Grauslund MF, Gorsich S, Lidén G (2009a) Metabolic effects of furaldehydes and impacts on biotechnological processes. *Appl Microbiol Biotechnol* 82(4):625–638. doi:10.1007/s00253-009-1875-1
- Almeida JRM, Karhumaa K, Bengtsson O, Gorwa-Grauslund M-F (2009b) Screening of *Saccharomyces cerevisiae* strains with respect to anaerobic growth in non-detoxified lignocellulose hydrolysate. *Bioresour Technol* 100(14):3674–3677. doi:10.1016/j.biortech.2009.02.057
- Almeida JRM, Runquist D, Sánchez Nogue V, Lidén G, Gorwa-Grauslund MF (2011) Stress-related challenges in pentose fermentation to ethanol by the yeast *Saccharomyces cerevisiae*. *Biotechnol J* 6(3):286–299. doi:10.1002/biot.201000301
- Alriksson B, Horváth IS, Jönsson LJ (2010) Overexpression of *Saccharomyces cerevisiae* transcription factor and multidrug resistance genes conveys enhanced resistance to lignocellulose-derived fermentation inhibitors. *Process Biochem* 45(2):264–271. doi:10.1016/j.procbio.2009.09.016
- Ask M, Bettiga M, Duraiswamy V, Olsson L (2013) Pulsed addition of HMF and furfural to batch-grown xylose-utilizing *Saccharomyces cerevisiae* results in different physiological responses in glucose and xylose consumption phase. *Biotechnology for Biofuels* 6(1):181

- Bellissimi E, van Dijken JP, Pronk JT, van Maris AJA (2009) Effects of acetic acid on the kinetics of xylose fermentation by an engineered, xylose-isomerase-based *Saccharomyces cerevisiae* strain. *FEMS Yeast Res* 9(3):358–364. doi:10.1111/j.1567-1364.2009.00487.x
- Benatui L, Perez JM, Belk J, Hsieh CM (2010) An improved yeast transformation method for the generation of very large human antibody libraries. *Protein Eng Des Sel* 23(4):155–159. doi:10.1093/protein/gzq002
- Bengtsson O, Hahn-Hagerdal B, Gorwa-Grauslund M (2009) Xylose reductase from *Pichia stipitis* with altered coenzyme preference improves ethanolic xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Biotechnology for Biofuels* 2(1):9
- Bergdahl B, Heer D, Sauer U, Hahn-Hagerdal B, van Niel EW (2012) Dynamic metabolomics differentiates between carbon and energy starvation in recombinant *Saccharomyces cerevisiae* fermenting xylose. *Biotechnology for Biofuels* 5(1):34
- Branduardi P, Fossati T, Sauer M, Pagani R, Mattanovich D, Porro D (2007) Biosynthesis of vitamin C by yeast leads to increased stress resistance. *PLoS One* 2(10):e1092. doi:10.1371/journal.pone.0001092
- Brat D, Boles E, Wiedemann B (2009) Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 75(8):2304–2311. doi:AEM.02522-08
- Casey E, Sedlak M, Ho NWY, Mosier NS (2010) Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of *Saccharomyces cerevisiae*. *FEMS Yeast Res* 10(4):385–393. doi:10.1111/j.1567-1364.2010.00623.x
- Demeke MM, Dietz H, Li Y, Foulquie-Moreno MR, Mutturri S, Deprez S, Den Abt T, Bonini BM, Lidén G, Dumortier F, Verplaetse A, Boles E, Thevelein JM (2013a) Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. *Biotechnology for Biofuels* 6(1):89. doi:10.1186/1754-6834-6-89
- Demeke MM, Dumortier F, Li Y, Broeckx T, Foulquie-Moreno MR, Thevelein JM (2013b) Combining inhibitor tolerance and D-xylose fermentation in industrial *Saccharomyces cerevisiae* for efficient lignocellulose-based bioethanol production. *Biotechnology for Biofuels* 6(1):120. doi:10.1186/1754-6834-6-120
- DeRisi JL, Iyer VR, Brown PO (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 278(5338):680–686
- Feng X, Zhao H (2013) Investigating xylose metabolism in recombinant *Saccharomyces cerevisiae* via 13C metabolic flux analysis. *Microb Cell Fact* 12(1):114
- Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/S-DNA/PEG procedure. *Yeast* 11(4):355–360
- Gueldener U, Heinisch J, Koehler GJ, Voss D, Hegemann JH (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res* 30(6):e23. doi:10.1093/nar/30.6.e23
- Gueldener U, Heck S, Fiedler T, Beinbauer J, Hegemann JH (1996) A New efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* 24(13):2519–2524. doi:10.1093/nar/24.13.2519
- Hahne K, Hauke V, Ramage L, Schatz G (1994) Incomplete arrest in the outer membrane sorts NADH-cytochrome b5 reductase to two different submitochondrial compartments. *Cell* 79(5):829–839. doi:http://dx.doi.org/10.1016/0092-8674(94)90072-8
- Haitani Y, Tanaka K, Yamamoto M, Nakamura T, Ando A, Ogawa J, Shima J (2012) Identification of an acetate-tolerant strain of *Saccharomyces cerevisiae* and characterization by gene expression analysis. *J Biosci Bioeng* 114(6):648–651. doi:10.1016/j.jbiosc.2012.07.002
- Helle S, Cameron D, Lam J, White B, Duff S (2003) Effect of inhibitory compounds found in biomass hydrolysates on growth and xylose fermentation by a genetically engineered strain of *S. cerevisiae*. *Enzyme Microb Technol* 33(6):786–792. doi:10.1016/S0141-0229(03)00214-X
- Herrero E, Ros J, Belli G, Cabisco E (2008) Redox control and oxidative stress in yeast cells. *Biochim Biophys Acta* 1780(11):1217–1235. doi:10.1016/j.bbagen.2007.12.004
- Hill J, Donald KA, Griffiths DE (1991) DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res* 19(20):5791
- Jeppsson M, Johansson B, Jensen PR, Hahn-Hagerdal B, Gorwa-Grauslund MF (2003) The level of glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor sensitivity in recombinant *Saccharomyces cerevisiae* strains. *Yeast* 20(15):1263–1272. doi:10.1002/yea.1043
- Jun H, Kieselbach T, Jonsson L (2012) Comparative proteome analysis of *Saccharomyces cerevisiae*: a global overview of in vivo targets of the yeast activator protein 1. *BMC Genomics* 13(1):230
- Kim D, Hahn J-S (2013) Roles of Yap1 transcription factor and antioxidants in yeast tolerance to furfural and 5-hydroxymethylfurfural that function as thiol-reactive electrophiles generating oxidative stress. *Appl Environ Microbiol* 79(16):5069–5077. doi:10.1128/aem.00643-13
- Lee J-S, Huh W-K, Lee B-H, Baek Y-U, Hwang C-S, Kim S-T, Kim Y-R, Kang S-O (2001) Mitochondrial NADH-cytochrome b5 reductase plays a crucial role in the reduction of d-erythroascorbyl free radical in *Saccharomyces cerevisiae*. *Biochim Biophys Acta Gen Subj* 1527(1–2):31–38. doi:http://dx.doi.org/10.1016/S0304-4165(01)00134-9
- Liu Z (2011) Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. *Appl Microbiol Biotechnol* 90(3):809–825. doi:10.1007/s00253-011-3167-9
- Liu ZL, Slininger P, Gorsich S (2005) Enhanced biotransformation of furfural and hydroxymethylfurfural by newly developed ethanologenic yeast strains. *Appl Biochem Biotechnol* 121(1–3):451–460. doi:10.1385/abab:121:1-3:0451
- Ma M, Liu ZL (2010) Comparative transcriptome profiling analyses during the lag phase uncover *YAP1*, *PDR1*, *PDR3*, *RPN4*, and *HSF1* as key regulatory genes in genomic adaptation to the lignocellulose derived inhibitor HMF for *Saccharomyces cerevisiae*. *BMC Genomics* 11(1):660
- Martin C, Jönsson LJ (2003) Comparison of the resistance of industrial and laboratory strains of *Saccharomyces* and *Zygosaccharomyces* to lignocellulose-derived fermentation inhibitors. *Enzyme Microb Technol* 32(3–4):386–395. doi:http://dx.doi.org/10.1016/S0141-0229(02)00310-1
- Matsushika A, Nagashima A, Goshima T, Hoshino T (2013) Fermentation of xylose causes inefficient metabolic state. Due to carbon/energy starvation and reduced glycolytic flux in recombinant industrial *Saccharomyces cerevisiae*. *PLoS One* 8(7):e69005. doi:10.1371/journal.pone.0069005
- Meineke B, Engl G, Kemper C, Vasiljev-Neumejer A, Paulitschke H, Rapoport D (2008) The outer membrane form of the mitochondrial protein Mcr1 follows a TOM-independent membrane insertion pathway. *FEBS Lett* 582(6):855–860. doi:http://dx.doi.org/10.1016/j.febslet.2008.02.009
- Mira N, Palma M, Guerreiro J, Sa-Correia I (2010) Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. *Microb Cell Fact* 9(1):79
- Mumberg D, Müller R, Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156(1):119–122
- Nguyen TTM, Iwaki A, Ohya Y, Izawa S (2014) Vanillin causes the activation of Yap1 and mitochondrial fragmentation in *Saccharomyces cerevisiae*. *J Biosci Bioeng* 117(1):33–38. doi:http://dx.doi.org/10.1016/j.jbiosc.2013.06.008
- Parawira W, Tekere M (2011) Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. *Crit Rev Biotechnol* 31(1):20–31. doi:10.3109/07388551003757816
- Petersson A, Almeida JRM, Modig T, Karhumaa K, Hahn-Hagerdal B, Gorwa-Grauslund MF, Lidén G (2006) A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae ADH6* gene conveys HMF tolerance. *Yeast* 23(6):455–464. doi:10.1002/yea.1370
- Sambrook J, Russel D (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA
- Semchysyn HM, Abrat OB, Miedzobrodzki J, Inoue Y, Lushchak V (2011) Acetate but not propionate induces oxidative stress in bakers' yeast *Saccharomyces cerevisiae*. *Redox Rep* 16(1):15–23
- Subtil T, Boles E (2012) Competition between pentoses and glucose during uptake and catabolism in recombinant *Saccharomyces cerevisiae*. *Biotechnology for Biofuels* 5(1):14
- Sundström L, Larsson S, Jönsson L (2010) Identification of *Saccharomyces cerevisiae* genes involved in the resistance to Phenolic fermentation inhibitors. *Appl Biochem Biotechnol* 161(1–8):106–115. doi:10.1007/s12010-009-8811-9
- Swinnen S, Schaeferlaekens K, Pais T, Claesen J, Hubmann G, Yang Y, Demeke M, Foulquie-Moreno MR, Goovaerts A, Souvereyns K, Clement L, Dumortier F, Thevelein JM (2012) Identification of novel causative genes determining the complex trait of high ethanol tolerance in yeast using pooled-segregant whole-genome sequence analysis. *Genome Res* 22(5):975–984. doi:10.1101/gr.131698.111
- Toone WM, Jones N (1999) AP-1 transcription factors in yeast. *Curr Opin Genet Dev* 9(1):55–61. doi:http://dx.doi.org/10.1016/S0959-437X(99)80008-2

- van Dijken JP, Bauer J, Brambilla L, Duboc P, Francois JM, Gancedo C, Giuseppin MLF, Heijnen JJ, Hoare M, Lange HC, Madden EA, Niederberger P, Nielsen J, Parrou JL, Petit T, Porro D, Reuss M, van Riel N, Rizzi M, Steensma HY, Verrips CT, Vindelov J, Pronk JT (2000) An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb Technol* 26(9-10):706-714. doi:[http://dx.doi.org/10.1016/S0141-0229\(00\)00162-9](http://dx.doi.org/10.1016/S0141-0229(00)00162-9)
- Verduyn C, Postma E, Scheffers WA, van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts - a continuous culture study on the regulation of respiration and alcoholic fermentation. *Yeast* 8(7):501-517
- Wahlbom CF, Eliasson A, Hahn-Hägerdal B (2001) Intracellular fluxes in a recombinant xylose-utilizing *Saccharomyces cerevisiae* cultivated anaerobically at different dilution rates and feed concentrations. *Biotechnol Bioeng* 72(3):289-296. doi:[10.1002/1097-0290\(20010205\)72:3<289::aid-bit5>3.0.co;2-9](https://doi.org/10.1002/1097-0290(20010205)72:3<289::aid-bit5>3.0.co;2-9)
- Wang X, Jin M, Balan V, Jones AD, Li X, Li B-Z, Dale BE, Yuan Y-J (2014) Comparative metabolic profiling revealed limitations in xylose-fermenting yeast during co-fermentation of glucose and xylose in the presence of inhibitors. *Biotechnol Bioeng* 111(1):152-164. doi:[10.1002/bit.24992](https://doi.org/10.1002/bit.24992)

doi:10.1186/s13568-014-0056-5

Cite this article as: Wallace-Salinas et al.: Re-assessment of *YAP1* and *MCR1* contributions to inhibitor tolerance in robust engineered *Saccharomyces cerevisiae* fermenting undetoxified lignocellulosic hydrolysate. *AMB Express* 2014 4:56.

Submit your manuscript to a SpringerOpen® journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Immediate publication on acceptance
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com

Chapter II.

RESEARCH

Open Access

Effect of oxygenation and temperature on glucose-xylose fermentation in *Kluyveromyces marxianus* CBS712 strain

Lorenzo Signori¹, Simone Passolunghi^{1,3}, Laura Ruohonen², Danilo Porro¹ and Paola Branduardi^{1*}

Abstract

Background: The yeast *Kluyveromyces marxianus* features specific traits that render it attractive for industrial applications. These include production of ethanol which, together with thermotolerance and the ability to grow with a high specific growth rate on a wide range of substrates, could make it an alternative to *Saccharomyces cerevisiae* as an ethanol producer. However, its ability to co-ferment C5 and C6 sugars under oxygen-limited conditions is far from being fully characterized.

Results: In the present study, *K. marxianus* CBS712 strain was cultivated in defined medium with glucose and xylose as carbon source. Ethanol fermentation and sugar consumption of CBS712 were investigated under different oxygen supplies (1.75%, 11.00% and 20.95% of O₂) and different temperatures (30°C and 41°C). By decreasing oxygen supply, independently from the temperature, both biomass production as well as sugar utilization rate were progressively reduced. In all the tested conditions xylose consumption followed glucose exhaustion. Therefore, xylose metabolism was mainly affected by oxygen depletion. Loss in cell viability cannot explain the decrease in sugar consumption rates, as demonstrated by single cell analyses, while cofactor imbalance is commonly considered as the main cause of impairment of the xylose reductase (*KmXR*) - xylitol dehydrogenase (*KmXDH*) pathway. Remarkably, when these enzyme activities were assayed *in vitro*, a significant decrease was observed together with oxygen depletion, not ascribed to reduced transcription of the corresponding genes.

Conclusions: In the present study both oxygen supply and temperature were shown to be key parameters affecting the fermentation capability of sugars in the *K. marxianus* CBS712 strain. In particular, a direct correlation was observed between the decreased efficiency to consume xylose with the reduced specific activity of the two main enzymes (*KmXR* and *KmXDH*) involved in its catabolism. These data suggest that, in addition to the impairment of the oxidoreductive pathway being determined by the cofactor imbalance, post-transcriptional and/or post-translational regulation of the pathway enzymes contributes to the efficiency of xylose catabolism in micro-aerobic conditions. Overall, the presented work provides novel information on the fermentation capability of the CBS712 strain that is currently considered as the reference strain of the genus *K. marxianus*.

Keywords: *Kluyveromyces marxianus*, Glucose fermentation, Xylose fermentation, Ethanol production, Oxygen requirement, Xylose reductase, Xylitol dehydrogenase

* Correspondence: paola.branduardi@unimib.it

¹University of Milano Bicocca, Piazza della Scienza 2, 20126 Milan, Italy
Full list of author information is available at the end of the article

Background

Modern yeast biotechnology places great emphasis on developing new traits in already established yeast cell factories as well as in identifying yeast species with novel traits or properties [1]. *Saccharomyces cerevisiae* has been used in biotechnological processes for centuries and it is therefore the best known and established yeast workhorse. However, in the last years, modern genetic and molecular techniques are promoting and facilitating the so-called non-conventional yeasts being reconsidered as alternative cell factories (as discussed in [2,3]). Among the non-*Saccharomyces* or non-conventional yeasts with potential for industrial applications are those belonging to the genus *Kluyveromyces*.

Within this genus *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces aestuarii*, *Kluyveromyces dobzhanskii* and *Kluyveromyces wickerhamii* are highly related and appear clearly separated from the other *Kluyveromyces* species [4].

K. lactis is a model Crabtree-negative yeast that has been extensively investigated [5-7]. Since 1950s it has been used as a natural source of enzymes such as lactase/ β -galactosidase, [8] and as a protein supplement in food [7]. From 1980s onwards, its easiness to genetic manipulations was recognized, and subsequently, suitable genetic tools have been developed, rendering it an efficient host for recombinant production [7,9,10].

K. marxianus has up to now received less attention from the scientific community [11], in spite of some very interesting characteristics such as the highest specific growth rate among eukaryotic microbes [12], the ability to grow at temperature up to 45-52°C [13-16], and the capacity of metabolizing a wide range of substrates including glucose, mannose, galactose, lactose, but also the pentose sugars xylose and arabinose [17]. These features could make *K. marxianus* an alternative to *S. cerevisiae* as an ethanol producer from lignocellulosic sugars [17-20]. Currently, *S. cerevisiae* plays the major role in ethanol production due to its high ethanol productivity, tolerance and its efficient hexose fermentation [21,22]. However, its inability to ferment xylose and other C5 sugars constitutes a major obstacle to efficient conversion of lignocellulose to ethanol. Moreover, thermotolerant yeast applicable for high temperature fermentation are expected to have potential in reducing cooling costs, increasing saccharification and fermentation rates, facilitating continuous ethanol removal and minimizing contaminations [13,16,23]. Also in this respect *S. cerevisiae* displays limitations, due to its very low fermentation efficiency at high temperature (>35°C, [24]). Therefore, the natural ability of *K. marxianus* to metabolize xylose, which is the main C5 sugar present in lignocellulosic hydrolysates and the second most abundant fermentable material [25], and its remarkable thermotolerance are particularly relevant when lignocellulose is used as raw material.

Strains belonging to the *K. marxianus* species have been isolated from a great variety of habitats, resulting in a genetic polymorphism which has been the focus of several studies [26,27].

This great variety, together with lack of published research on physiology, metabolism and biochemistry are possible reasons as to why a *K. marxianus* industrial strain, which could constitute a real alternative to *S. cerevisiae* for ethanol production has not been developed yet.

The strain CBS712 is currently considered as the reference strain of the genus *K. marxianus* [3] being the most studied also at the genomic level, with about 20% of its genome randomly sequenced [28]. Despite its ethanol production yield being very low compared to that of other strains of the same species, the fact that its genome is partially available, offers the possibility of future *in silico* analyses based on additional wet lab data on its metabolic capabilities.

In the present study batch fermentations under different temperatures and oxygen supplies with *K. marxianus* CBS712 were performed: the potential for xylose utilization and ethanol production was investigated, together with quantitative measurements of biomass formation, substrate consumption and external metabolite accumulation. Cell viability and oxidative stress response to the process conditions were additionally monitored by flow cytometric analyses. It has been reported that *K. marxianus* CBS712 can assimilate xylose but its ability to produce ethanol from xylose is coupled to oxygen feed [25]. Interestingly, the *in vitro* activity measurements of xylose reductase (*KmXR*) and xylitol dehydrogenase (*KmXDH*) demonstrated that the more the oxygen supply was restricted, the more the respective activities diminished, but with no corresponding decrease of mRNA levels. Therefore, the cofactor imbalance of the *KmXR/KmXDH* pathway, particularly pronounced under oxygen limitation [29], seems not the only explanation for the limited capacity of xylose fermentation in the *K. marxianus* CBS712 strain.

Results

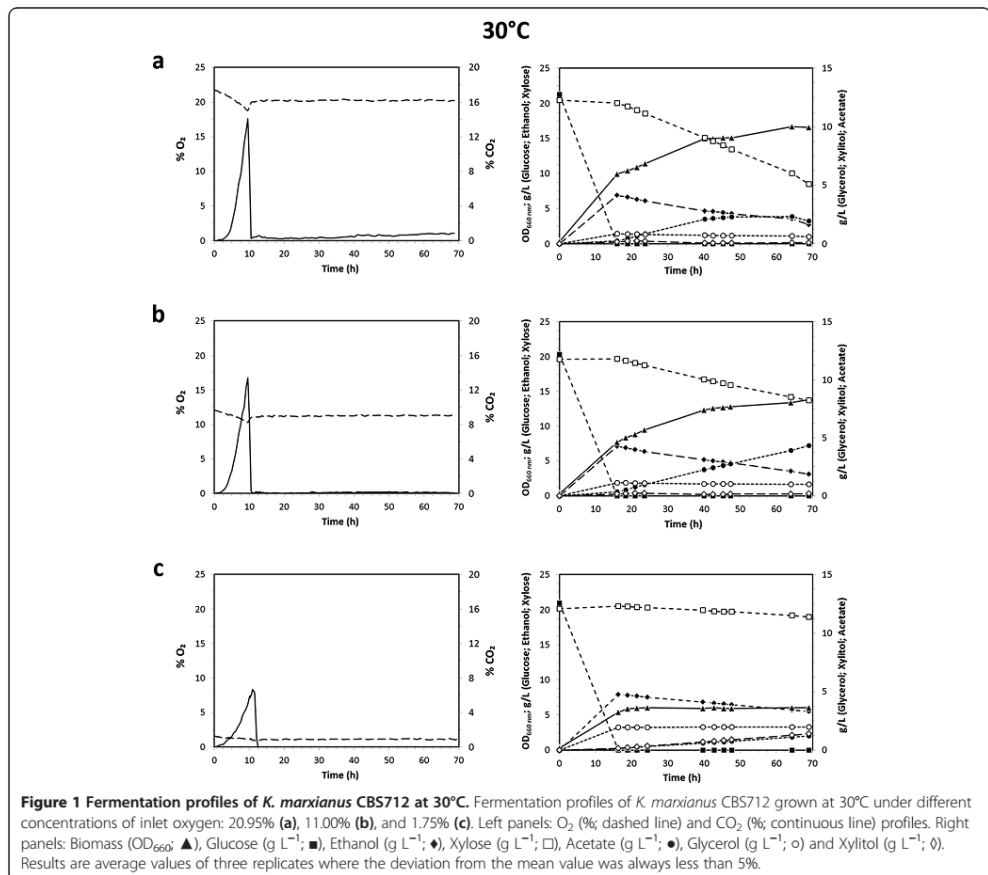
Growth and fermentation profiles of *K. marxianus* CBS712 at 30°C with different inlet oxygen concentrations on mixture of glucose and xylose

The *K. marxianus* strain CBS712 was cultivated in batch mode in minimal defined medium [30] with xylose (20 g L⁻¹) and glucose (20 g L⁻¹) as carbon and energy source to evaluate the eventual sugar co-consumption, highly desirable for lignocellulosic second-generation ethanol production. Fermentations were performed at 30°C, pH 5 and followed for 70 hours. The inlet gas flow rate was maintained constant at 0.2 vvm (corresponding to 0.3 L min⁻¹). The effect of three different concentrations of oxygen in the inlet gas flow, 20.95%, 11.00% and 1.75%, were monitored. For all the conditions studied, the dissolved oxygen fraction decreased to zero within

10 hours of growth. It has been reported that some strains of *K. marxianus* do not grow in anoxic conditions (as reviewed by [26]), while others do [31]. For this reason, at first it was verified that the model strain selected for the present study did not grow under anaerobic batch conditions (data not shown): therefore, fully anaerobic cultures were not assessed.

Figure 1 (left panels) shows the gas profiles of the three processes. The profiles of both O₂ and CO₂ over time were clearly related to the oxygen concentration of the inlet gas flow. Those of CO₂ deserve some attention. Indeed, not only the total amount of CO₂ produced, but also the maximum peak of production clearly appeared correlated with the amount of oxygen supplied in the gas flow. With the highest oxygen supply (Figure 1, left panel a), the peak of CO₂ production was reached after 9.5 ± 0.2 hours from the inoculum, but only after 11.3 ± 0.2 hours with the

lowest oxygen supply (left panel c). These differences were reflected on the profiles of sugar consumption and product accumulation as deduced by the measured samples, Figure 1, right panels. First of all, the production of biomass (closed triangles) was directly related to the oxygen supply, being proportionally lower for the lower concentrations of the inlet oxygen. Furthermore, and as already reported in literature, the CBS712 strain exhibited a delayed utilization of xylose (open squares), which was never significantly consumed until complete glucose (closed squares) exhaustion. Overall, also the xylose consumption appeared related to the concentration of the inlet oxygen, decreasing with the decrease of the oxygen supply: indeed, the amount of xylose consumed after 70 h of fermentation was about 12 g L⁻¹ and 5.9 g L⁻¹ with 20.95% and 11.00% of oxygen, respectively, and only about 1.5 g L⁻¹ with 1.75% of oxygen (right panels a, b, c). The peaks of CO₂



production (left panels) seemed to correlate with the onset of xylose consumption (or more precisely, with the glucose consumption being completed, see also below). Therefore, both biomass accumulation and the rate of sugar utilization were progressively decreased as the stringency of the oxygen supply increased (Table 1).

Ethanol production (closed diamonds) was in essence only observed during the exponential growth on glucose. Interestingly, similar amounts of ethanol were measured under the three different conditions. We can safely speculate that the highest level of ethanol accumulation correlated with the peak of CO₂ production (compare left with right panels).

As shown in Figure 1 (right panels), no noticeable ethanol production was detected during the xylose consumption phase of the batch fermentations. However, the CBS712 strain was able to produce ethanol from xylose, even if with very low yields, between 0.08 and 0.09 g g⁻¹. These experiments were performed in shake flasks with xylose (20 g L⁻¹) as the sole carbon source, both at 30°C and 41°C, with similar results (data not shown).

With the exhaustion of glucose and the onset of xylose consumption a decrease in the ethanol concentration was observed, being the trend of xylose and ethanol profiles very likely an indication of their co-consumption. In fact, evaporation tests (data not shown), performed under the same operative conditions demonstrated that stripping was not sufficient to explain the decrease in the ethanol concentration. This suggests that the CBS712 strain grows on ethanol after glucose exhaustion: in detail, it consumed ~1 g L⁻¹ and ~3 g L⁻¹ of ethanol with the lowest (1.75% O₂) and the highest (20.95%) oxygen supply, respectively.

The lower the oxygen supply, the higher was the glycerol (open circles) and xylitol (open diamonds) accumulation. Production of glycerol can be described as an alternative

way to ethanol of recycling NADH to NAD⁺ [32]. However, as already reported in literature for oxygen-limited conditions [29,33], the inefficient regeneration of NAD⁺ led also to xylitol accumulation. Acetate (closed circles) was produced in consistent amounts with high and medium oxygen supplies (Figure 1, right panels a and b), while its accumulation was limited under 1.75% oxygen inlet (panel c).

Growth and fermentation profiles of *K. marxianus* CBS712 at 41°C with different inlet oxygen concentrations on mixture of glucose and xylose

To evaluate the influence of temperature, the experiments described were repeated at 41°C, Figure 2. From a general point of view, the gas profiles showed similar trends (compare Figure 1 and Figure 2, left panels). However, at 41°C the CO₂ production rates (and therefore the glucose consumption rates) were markedly faster than at 30°C. Consequently, the onset and efficiency of xylose consumption was also more rapid at oxygen supply of 20.95% and 11.00% than at the lower temperature assessed. Once more, the kinetic profile suggests an apparent glucose-xylose co-consumption, very likely only a consequence of the sampling time, as also verified in shake flasks experiments (data not shown). Remarkably, at 41°C the biomass production (closed triangles) with high or medium oxygen supply was significantly lower after glucose exhaustion during the phase of xylose consumption.

As shown in Table 1, and as previously suggested by the CO₂ profiles (Figures 1 and 2, left panels), both the amount of oxygen supplied (1.75%, 11.00% and 20.95% of O₂) and temperature (30 and 41°C) affected the overall glucose consumption rate. In particular, at 41°C and independently from the oxygen supply, the glucose consumption rates were faster than at 30°C: at 41°C the lowest glucose consumption rate was ~2.80 g L⁻¹ h⁻¹ (1.75% of O₂) while at

Table 1 Sugars consumption rates and ethanol production rates

T ^a (°C)	O ₂ ^b (%)	Volumetric consumption and production rates (g L ⁻¹ h ⁻¹)							Yields (g g ⁻¹)			
		Glucose	Xylose	Ethanol	Biomass	Glycerol	Acetate	Xylitol	Biomass	Glycerol	Acetate	Xylitol
30°C	20.95	2,23	0,21	0,77	0,08	n.d. ^c	0,04	n.d. ^c	0,17	0,02	0,07	n.d. ^c
	11.00	2,13	0,11	0,79	0,07	0,01	0,07	n.d. ^c	0,17	0,03	0,16	n.d. ^c
	1.75	1,94	0,03	0,76	0,03	0,02	0,02	0,02	0,09	0,08	0,05	0,79
41°C	20.95	3,22	0,14	0,15	0,05	0,01	0,13	n.d. ^c	0,10	0,03	0,26	n.d. ^c
	11.00	3,16	0,15	1,18	0,05	0,01	0,11	n.d. ^c	0,11	0,04	0,24	n.d. ^c
	1.75	2,80	0,05	1,12	0,03	0,02	0,03	0,04	0,08	0,09	0,08	0,75

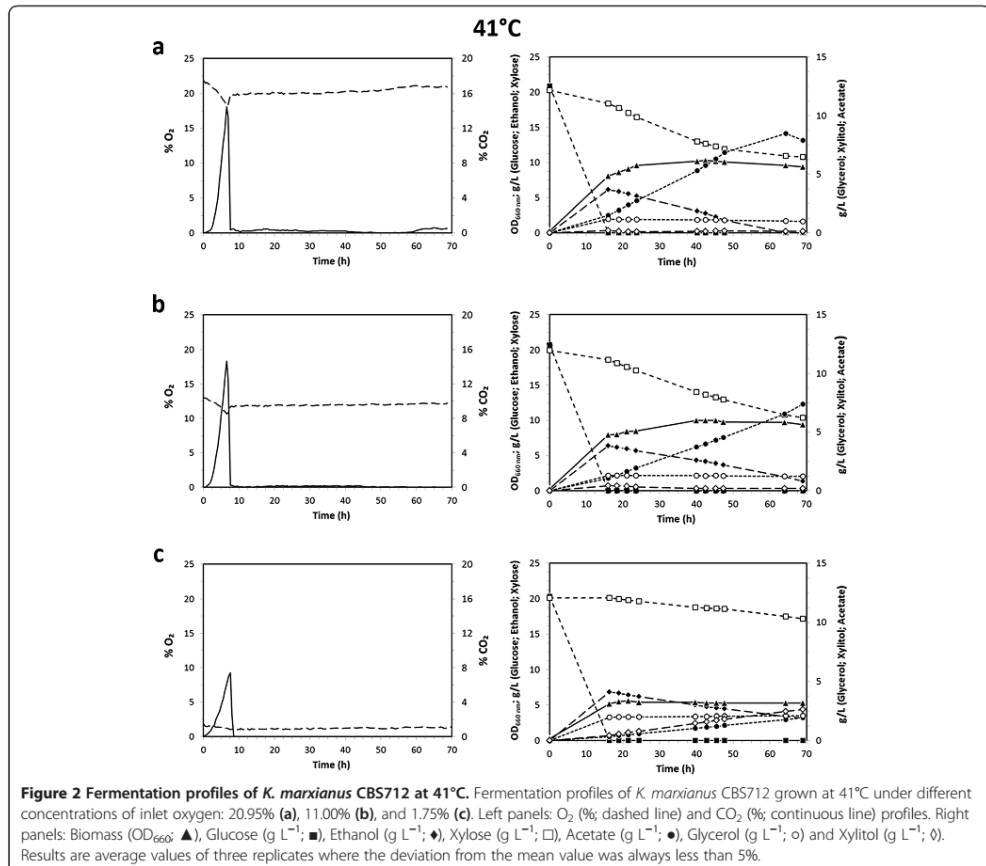
^aT, temperature.

^bO₂, inlet oxygen.

^cn.d., not detectable.

Sugar (glucose and xylose) consumption rates, production rates (biomass, ethanol, glycerol, acetate and xylitol) and yields (biomass, glycerol, acetate and xylitol) at different temperature and oxygen supply. Glucose consumption rates were calculated during the CO₂ production phase, while xylose consumption rates were calculated starting from the first point after glucose exhaustion until the last point of the fermentation. Ethanol production rates relate only to the glucose consumption phase since on xylose consumption phase, in all the tested conditions, no significant ethanol production was observed. Yields and volumetric production rates of biomass, glycerol and acetate were calculated based on the total consumed sugars, while xylitol yields and volumetric production rates were calculated based on the consumed xylose only.

The results shown are average values of three replicates where the deviation from the mean value was always less than 3%.



30°C the highest consumption rate was $\sim 2.23 \text{ g L}^{-1} \text{ h}^{-1}$ (20.95% of O₂). Overall, the glucose consumption rates were more influenced by changes in temperature than by changes in the oxygen supply.

Also at the higher temperature ethanol was produced only during the exponential growth on glucose and at similar levels. However, at 41°C the ethanol production rates were markedly faster than at 30°C, as expected by the observed glucose consumption rates (Table 1).

Interestingly, the amount of oxygen supplied did not significantly affect the ethanol production rates, even if at both temperatures (30 and 41°C) the same trend was observed: the lower ethanol production rates were observed with 1.75% of inlet oxygen ($\sim 0.76 \text{ g L}^{-1} \text{ h}^{-1}$ and $\sim 1.12 \text{ g L}^{-1} \text{ h}^{-1}$ at 30 and 41°C, respectively) while the highest production rates were observed with 11.00% of inlet oxygen ($\sim 0.79 \text{ g L}^{-1} \text{ h}^{-1}$ and $\sim 1.18 \text{ g L}^{-1} \text{ h}^{-1}$ at 30 and 41°C, respectively).

Also at 41°C, evaporation tests (data not shown) demonstrated that stripping, even if higher than that observed at 30°C, was not sufficient to explain the decrease in ethanol concentration(s), suggesting a co-consumption of ethanol and xylose.

Table 1 also reports the xylose consumption phase after glucose exhaustion: while at 30°C the xylose consumption rate was by far the highest with 20.95% of inlet oxygen ($\sim 0.21 \text{ g L}^{-1} \text{ h}^{-1}$), at 41°C significant differences were not observed between high or medium oxygen supply ($\sim 0.14 \text{ g L}^{-1} \text{ h}^{-1}$ and $\sim 0.15 \text{ g L}^{-1} \text{ h}^{-1}$, respectively).

These values underline once more that both the oxygen feeding and temperature are key parameters for the ability of CBS712 strain to consume xylose.

Like previously observed at 30°C, xylitol (open diamonds) and glycerol (open circles) accumulation increased at the lowest inlet of oxygen supply. Overall, the

highest concentration of xylitol was assessed at 41°C with 1.75% of inlet oxygen, with consumption of 3 g L⁻¹ of xylose and accumulation of 2.7 g L⁻¹ of xylitol, corresponding to a yield of 0.75 gg⁻¹. In the same condition the highest glycerol accumulation and yield (2.1 g L⁻¹ and 0.09 gg⁻¹, respectively) was also measured (Table 1).

Finally, at 41°C greater accumulation of acetate compared to the fermentations at 30°C was observed (Figures 2 and 1, closed circles, respectively). As an example, with 20.95% of oxygen, the highest acetic acid production and yield were 8.5 g L⁻¹ and 0.26 gg⁻¹ compared to 2.3 g L⁻¹ and 0.06 gg⁻¹ at 41°C and 30°C, respectively (Table 1).

Viability and intracellular ROS accumulation assessed by flow cytometric analyses

Cellular viability, or more generally active metabolism and cellular integrity, can strongly influence substrate uptake and production rates of biomass and metabolites. Therefore, a set of analyses at single cell level was performed to evaluate this parameter during the fermentation processes previously described. Cells were collected at different time points (T = 16, T = 40 and T = 64 hours after inoculation, see Figures 1, 2), stained with propidium iodide (PI) and analysed by flow-cytometry to detect severely damaged/dead cells ([34], see Materials and methods). In Figure 3 the percentages of severely damaged/dead cells in the different conditions tested are reported.

Generally speaking, the percentages of PI positive cells were very low, independent from the fermentation parameters. In particular, at 30°C such fraction never exceeded 5% of the whole population. At 41°C the percentages were a bit higher, slightly rising along the time and with the decreasing oxygen supply. Overall, the percentages of damaged cells appeared negligible in respect to the whole population, suggesting that the imposed process conditions did not severely affect the cell viability/integrity. Consequently, low viability does not seem to be the reason for the low xylose consumption rates.

Cells were also stained with dihydrorhodamine-123 (DHR-123) to detect intracellular accumulation of reactive oxygen species (ROS; [35], see Materials and methods, Figure 4). Surprisingly, at 30°C a high percentage of cells accumulated ROS under all the process conditions tested, Figure 4 left panels (being lower but still significant only during the first time point measured with the highest oxygen inlet). In contrast, at 41°C a progressive decrease in the comparably high intracellular ROS accumulation was observed, right panels. These trends were also confirmed by fluorescence microscopy analyses (data not shown). Data currently available are not sufficient to unambiguously explain either the high ROS accumulation both at 30°C and 41°C, or the decrease over time in ROS accumulation observed at 41°C. However, these results are further

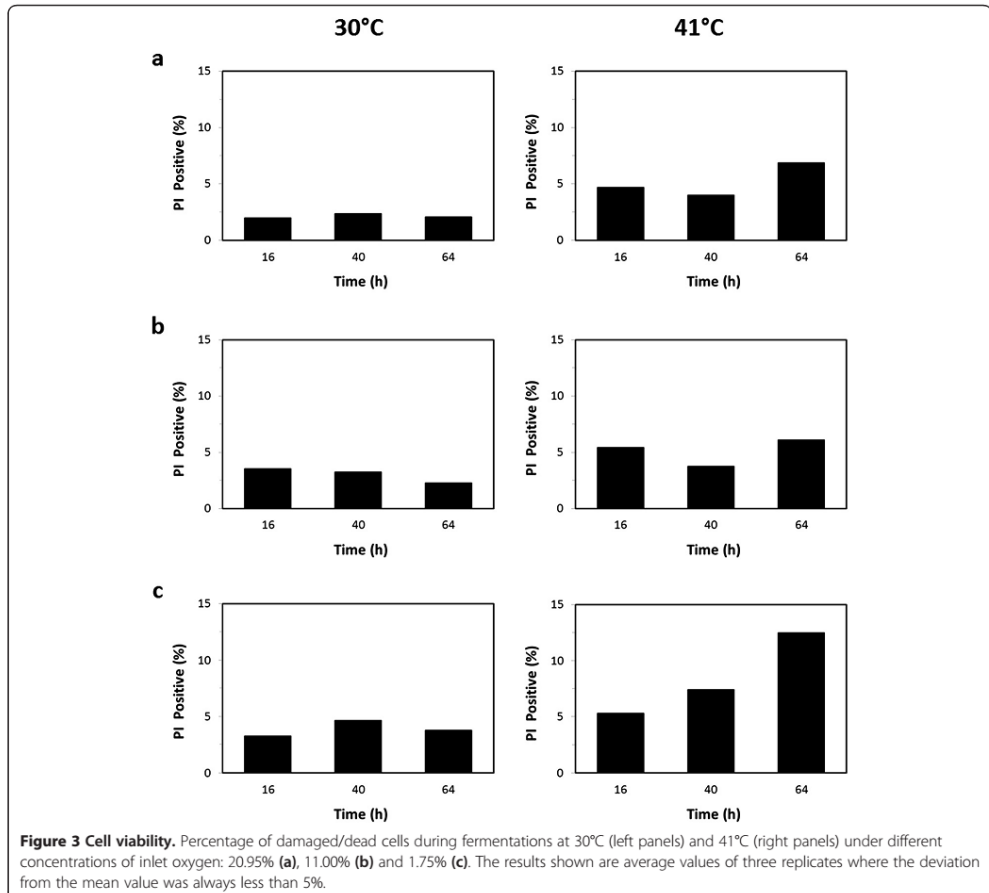
evidence of the different cellular metabolism at 30°C and 41°C, as previously commented (Figure 1 vs. Figure 2).

Activity and transcript level of xylose reductase (*KmXR*) and the xylitol dehydrogenase (*KmXDH*)

Xylose reductase (*KmXR*) and xylitol dehydrogenase (*KmXDH*) are the two key enzymes of xylose metabolism in *K. marxianus* [25,29,36], like in most other xylose-consuming yeast.

Aiming at further understanding of the xylose consumption pathway in the *K. marxianus* CBS712 strain, the activity of these two enzymes was determined during the xylose consumption phase of the batch cultures studied, Table 2. To simplify the interpretation of the data, only the two extreme conditions (20.95% and 1.75% inlet oxygen) are reported, being the data obtained with 20.95% and 11.00% inlet oxygen very similar. At both temperatures and all time points, the specific *KmXDH* activities were always higher than the specific *KmXR* activities. Overall, it can be noticed that for both activities, independently from the temperature, the higher values related to the higher inlet oxygen (Table 2). In detail, *KmXR* activity dropped almost to half when the inlet oxygen decreased from 20.95% to 1.75% at 30°C, while the reduction was less evident, if even present, at 41°C (where actually an increasing trend in the activity was observed). The inlet oxygen decrease (from 20.95% to 1.75%) determined a decrease also for the *KmXDH* activity. However, differently to what previously observed for the *KmXR* activity, the reduction was very dramatic at 41°C, approaching one order of magnitude. At 30°C, the initial decrease to less than one third (see data at early time point) was followed by an increase along the time, despite values never reaching the ones measured from samples collected from cultures at 20.95% of inlet oxygen.

It has to be highlighted that the enzymatic activities were assayed *in vitro*: this means that cofactor imbalance due to the different cofactor preferences of the two enzymes, which is commonly recognized as the limiting factor of this metabolic pathway under limited oxygen conditions [37], cannot be the explanation for the reduced activities measured, and the correlation with the fermentation profiles is not trivial. In fact, by looking once more at the data of the cultures at 30°C, both of the activities, in particular that of *KmXDH*, were lower at the two earlier time points assayed, but had increased at the last measurement point (40 h), while the xylose metabolism remained unchanged (see again Figure 1, open squares). On the other hand, from these data it can be concluded that *KmXDH* was the most affected enzyme by the oxygen supply, justifying in this case the previously described trends of xylitol accumulation (Figures 1 and 2 open diamonds): the

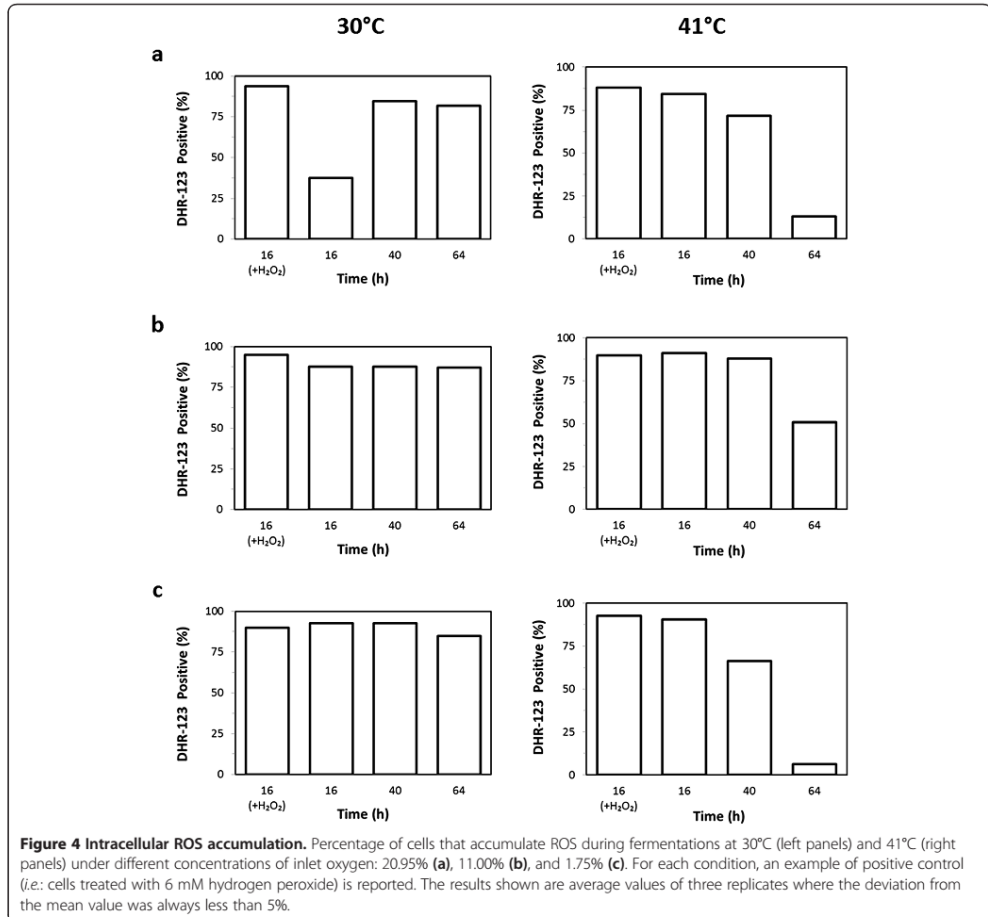


higher accumulation of xylitol corresponded to the lower *KmXDH* specific activity.

Differences in transcription profiles could explain the trend of the enzymatic activities observed. Thus, transcript levels were determined at different time points (T = 16, T = 40 and T = 64 h) during the fermentations described in Figures 1 and 2. However, the *KmXR* and *KmXDH* expression profiles showed no significant variation at any of the time points studied at the two different temperatures (data not shown). This suggests that, not excluding the impairment determined by cofactor imbalance, post-transcriptional and/or post-translational regulation of the *KmXR-XDH* xylose pathway plays a role in determining the efficiency of xylose metabolism in micro-aerobic conditions in the CBS712 strain of *K. marxianus*.

Discussion

The aim of the present study was to characterize the fermentation profiles of the *K. marxianus* CBS712 strain during growth in defined medium with glucose and xylose as carbon source, and with different oxygen supply mimicking the micro-aerobiosis conditions occurring in industrial processes. Moreover, to exploit the peculiar thermotolerance of this yeast, different operative temperatures were compared (see Materials and methods). These conditions, despite still far from a real process of second generation ethanol production, provide useful wet data for future investigations and *in silico* analyses. *K. marxianus* CBS712 strain was chosen even if its ethanol production yield is low compared to that of other strains of the same species because it represents the reference strain of the species and, to the best of our



information, is the only *K. marxianus* strain whose genome has been partially sequenced [28,38].

The data collected allowed describing the metabolic differences occurring by changing either the temperature or the inlet oxygen of the fermentation process. A number of these differences are more directly related to the ethanol production here reported or can be commented in the view of a lignocellulose-based process for ethanol production. They can be summarized as follows: *i*) independently from the temperature, the more the oxygen supplied was reduced, the more the metabolism was attenuated, and in particular that of xylose; *ii*) independently from the oxygen supply, *K. marxianus* CBS712 was able to grow both at 30 and 41°C, with a faster overall metabolism at 41°C, as confirmed by the CO₂

production rates, which were faster compared to those of fermentations performed at 30°C. As a possible consequence of an energy demanding condition, the biomass accumulation at 41°C was always lower than the corresponding biomass values reached at 30°C; *iii*) especially at the higher temperature, there was a significant increase in acetic acid accumulation that might be related to NADPH demand. In fact, it is well reported in literature that not only the pentose phosphate pathway, but also the acetaldehyde dehydrogenase can significantly contribute to the overall NADPH pool [39,40]. Additional data would be necessary to fully elucidate this point: however it should be noted that NADPH is the *KmXR* cofactor [41]. Moreover, since reducing power is required for the neutralization of reactive oxygen species

Table 2 Xylose reductase (*KmXR*) and xylitol dehydrogenase (*KmXDH*) activities

Inlet oxygen (%)	Time (h)	Enzyme activity (U mg ⁻¹)		Enzyme activity (U mg ⁻¹)	
		XR	XDH	XR	XDH
20.95	16	6,4	45,2	6,6	39,0
	23	7,4	43,9	6,1	37,9
	40	8,5	47,6	5,0	37,1
1.75	16	3,6	13,7	3,5	4,2
	23	2,3	22,3	4,6	6,3
	40	4,3	37,0	5,9	7,6

Xylose reductase (*KmXR*) and xylitol dehydrogenase (*KmXDH*) activities of *K. marxianus* CBS712 grown at 30°C and 41°C under different inlet oxygen concentrations: 20.95% (a) and 1.75% (b) are average values of two replicates where the deviation from the mean value was always less than 3%.

[42,43], NADPH formation through the acetate pathway might be required for counteracting ROS accumulation. This correlates with the overall ROS decrease observed at 41°C over time (Figure 4); *iv*) it is evident that, independently both from the oxygen supply and from the temperature values, the xylose fermentation always followed the glucose consumption (being slower with the lower inlet oxygen, see above). Considering that in the tested conditions the ethanol accumulation was evident only during the glucose phase, it can be concluded that no detectable ethanol production derived from xylose; *v*) in the tested conditions, cell viability did not seem to be an issue in *K. marxianus* CBS712, as demonstrated by single cell analysis. This point was addressed since previous reports on fermentation at high temperature evidenced a rapid decrease of cell viability [13]. However, it is important to remember that a very high percentage of cells showed positive in ROS detection; *vi*) remarkably, the overall ethanol production was very similar in all the tested conditions.

Considering the favourable physiological traits of *K. marxianus*, glucose-xylose co-fermentation appears as the most urgent to be addressed for lignocellulose-based production. In this respect, the biochemical data well supported how, also in *K. marxianus*, impairment in xylose metabolism under micro-aerobic condition can be ascribed to the difference in cofactor preference between *KmXR* and *KmXDH* [25,29,41]. Zhang and co-authors [44] overexpressed in *K. marxianus* a NADPH-preferring xylose reductase and engineered its cofactor preference to revert it to NADH. Data undoubtedly demonstrate the beneficial effect of a higher *KmXR* specific activity on xylose fermentation, while the advantage of cofactor exchange remains dubious. Not in contrast with that, here we demonstrated that additional regulation may occur at post-transcriptional level, since *in vitro* measured activities of both *KmXR* and *KmXDH* were reduced together with inlet oxygen reduction, despite the fact that the preferred cofactors were supplied within the reaction mix. Additionally, no coherent differences in transcription levels of the genes encoding these two enzymes were measured. An

alternative way of solving the problem was more recently proposed by Wang and co-authors [45], by substituting the native xylose pathway with the fungal one based on xylose isomerase, providing promising results. It would be very interesting to see if even in this engineered strain glucose represses xylose fermentation, as demonstrated in the native strain for different ratios of these two sugars [46]. Finally, it is interesting to underline once more the high or very high percentage of cells positive in ROS staining: this might prelude to the higher mortality registered when *K. marxianus* cells were grown in the presence of different pre-treated lignocellulose preparations [47,48], which are known to contain pro-oxidative inhibitors, such as acetic acid. In this respect, as already demonstrated for *S. cerevisiae* [49,50], improving strain robustness can certainly be seen as an additional manipulation target to be conducted for developing *K. marxianus* as a real possible alternative yeast for second generation processes of bio-production.

Conclusions

The results presented provide data indicating that sugars fermentation in the *K. marxianus* CBS712 strain is affected both by oxygen supply and temperature. Xylose fermentation, and more importantly glucose and xylose co-fermentation, under limited oxygen supply is of particular relevance for second generation bioethanol production. Therefore, fermentation profiles and biochemical data on the key metabolic pathway are desirable information for the development of an efficient cell factory. We showed a direct correlation between the decreased efficiency to consume xylose with the reduced specific activity of the two main enzymes (*KmXR* and *KmXDH*) involved in its catabolism. Therefore, it is possible to hypothesize that in the CBS712 strain the efficiency of xylose catabolism in micro-aerobic conditions is influenced not only by the cofactor imbalance, but also by post-transcriptional and/or post-translational regulation of the key enzymes of the pathway. Overall, the presented work provides novel information on the fermentation capability of the CBS712 strain that is currently considered as the reference strain of the genus *K. marxianus*.

Materials and methods

Yeast strain and inoculum preparation

The *K. marxianus* strain used in this study was CBS712 (<http://www.cbs.knaw.nl>, alias NBRC 10005). Strain maintenance: the strain was overnight shake flask cultured in YPX medium (1% wv^{-1} yeast extract, 2% wv^{-1} peptone, 2% wv^{-1} xylose). Sterile glycerol was added to a final concentration of 20% ($v v^{-1}$) and cells were stored in cryotubes at $-80^{\circ}C$. Pre-cultures were always prepared transferring some cells from these stock tubes to YPX agar plates and inoculating single colonies (visible after 24 h of incubation at $30^{\circ}C$), in defined mineral medium [30] with glucose (20 $g L^{-1}$) and xylose (50 $g L^{-1}$) as carbon sources. One colony was transferred into a 100 mL shake-flask with 25 mL medium and incubated 16 h at $30^{\circ}C$ with an agitation speed of 160 rpm. All 25 mL were transferred into a 250 mL shake-flask with 50 mL defined mineral medium with glucose (5 $g L^{-1}$) and xylose (20 $g L^{-1}$) as carbon sources, and incubated at $30^{\circ}C$ with an agitation of 160 rpm until exponential growth phase was reached.

Batch cultivations

Exponential phase shake-flasks cultures were used to inoculate 2 L bioreactors (Sartorius Stedim, BIostat B), with an operative volume of 1.5 L, to a final absorbance of OD_{660nm} 0.3 (CDW about 0.1 $g L^{-1}$) in defined mineral medium [30] with glucose (20 $g L^{-1}$) and xylose (20 $g L^{-1}$) as carbon and energy source. Stirrer speed was set at 250 rpm and the initial pH value at 5, maintained with 2 M NaOH. The inlet gas flow rate was 0.3 $L min^{-1}$ (0.2 vvm), adjusted with two mass flow controllers (Bronkhorst*High Tech- EL-FLOW*Select), one for air and the other for nitrogen (N_2). The two mass flow controllers were used to set the desired gas mixtures. Batch cultivations were carried out at 30 or $41^{\circ}C$ and three different concentration of inlet oxygen were tested: 20.95%, 11.00% and 1.75% of oxygen. Overall, six different fermentation protocols were run in triplicate varying temperature (30 or $41^{\circ}C$) and inlet oxygen (20.95%, 11.00% and 1.75% of oxygen).

The composition of the fermenter off-gas was on-line measured by a gas analyzer (Omnitec). The gas analyser was always calibrated 24 h before starting a cultivation using synthetic air containing a defined concentration of CO_2 (1%).

Samples (20 mL) were collected regularly from the bioreactor in vials; 1 mL was used for OD_{660nm} measurement, after appropriate dilution; 1 mL was centrifuged at $4^{\circ}C$, 14,000 rpm, for 5 min and supernatants were collected and stored at $-20^{\circ}C$ for later determination of metabolites concentrations.

The remaining liquid cultures were divided as described later and harvested by centrifugation (2000 rpm \times 10 min).

Pellets were stored at $-80^{\circ}C$ for subsequent enzymatic and RTq-PCR analysis.

Analytical methods

Cell growth was spectrophotometrically estimated as OD_{660nm} (Shimadzu UV-1800) and gravimetrically verified by measuring the cell dry weight (CDW $g L^{-1}$). For the CBS712 strain we established that a CDW of 1 $g L^{-1}$ correspond to an OD_{660nm} of 2.86. For dry weight determination, cells were filtered through a dried and pre-weighed 0.22 μm -pore membrane (Millipore, USA), washed twice with distilled water and dried in a microwave oven (180 W, 10 min). The sample volume filtered was 10 mL and the dry weight measurements were performed in duplicates for all samples.

Glucose, xylose, xylitol, glycerol, acetate, and ethanol concentrations were HPLC determined using a cation-exchange column (Bio-Rad Aminex* HPX-87H). The eluent was 5 mM H_2SO_4 pumped at 0.6 $mL min^{-1}$ and column temperature was $45^{\circ}C$. Separated components were detected by a refractive-index detector and peaks were identified by comparing with known standards (Sigma-Aldrich, St Louis, MO, USA).

Flow cytometric analyses

Dead or severely compromised cells were detected following Propidium Iodide (PI, Sigma-Aldrich CO., St. Louis, MO, USA) staining. Briefly, cell were washed twice with buffer (TrisHCl 50 mM, $MgCl_2$ 15 mM, pH 7.7), resuspended in a PI solution (0.23 mM), incubated in the dark on ice for 20 min and then analysed by flow-cytometry.

Reactive oxygen species (ROS) were detected by Dihydrorhodamine-123 (DHR-123, Sigma-Aldrich CO., St. Louis, MO, USA) as previously described by [35]. Briefly, cells were incubated for 2 h with Dihydrorhodamine-123 (5 $\mu g mL^{-1}$ from a 2.5-mg mL^{-1} , stock solution in ethanol), washed twice with PBS buffer and then analysed by flow-cytometry.

For both PI and DHR-123 staining positive and negative controls were performed. PI positive control was settled by killing cells with ethanol; ROS positive control was settled by adding to the medium H_2O_2 6 mM. Percentages shown in Figures 3 and 4 have been calculated by taking in account autofluorescence.

Samples were analysed using a Beckman Coulter FC-500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an Argon ion laser (excitation wavelength 488 nm, laser power 20 mW). The fluorescence emissions were measured through a 525–550 nm band pass filter (FL1 parameter) for DHR signals and through a 670 nm band pass filter (FL4 parameter) for PI signals. The sample flow rate during analysis did not exceed 500 cells s^{-1} . A total of 25,000 cells were measured for each sample. Data analysis was performed afterwards

Table 3 Primers list

Gene	Orientation	Oligonucleotide sequence
XR	Fwd	5'-CAGAGCCCTTGAGAAGTTGG-3'
	Rev	5'-AAGGATAGTGGGCCGAAACT-3'
XDH	Fwd	5'-GCTGTAGAGCCAGGTGTCC-3'
	Rev	5'-CAAATCCAGTACCCGGCAAGT-3'
ATC1	Fwd	5'-AGCACCCAGTTTGTGACC-3'
	Rev	5'-AGGAGAAACCGCGTAGATT-3'

Primers designed and used in the current study.

with *Cyflogic* software (PerttuTerho, Mika Korkeamäki, CyFlo Ltd).

Protein extraction and enzyme assays

For total protein extraction, 10 mL of liquid culture was harvested by centrifugation (2000 rpm × 10 min) and washed twice with 10 mL of ice-chilled distilled water. Subsequently, cells were resuspended in 1 mL of distilled water, transferred in FastPrep® tube and harvested by centrifugation (10000 rpm × 5 min). Total soluble protein were obtained by three treatments (30 sec, speed 6) of beads beating (*Savant Bio 101 FastPrep®*), interspersed by 30 sec on ice, in Tris-HCl buffer (0.1 M and pH7.5). After extraction, the debris was pelleted by centrifugation (10000 rpm × 5 min at 4°C). Supernatants, containing soluble proteins, were transferred to clean tubes and stored at -20°C until required. Protein concentration was measured according to Bradford [51], with bovine serum albumin as the standard.

KmXR activity was spectrophotometrically measured by monitoring the oxidation of NAD(P)H at 340 nm [52] in a reaction mixture (1.0 mL) with the following composition: 0.1 M sodium phosphate buffer (pH7), 0.2 M xylose and 0.15 mM NAD(P)H. *KmXDH* activity was measured by monitoring the reduction of NAD⁺ at 340 nm [52] in a reaction mixture (1.0 mL) with the following composition: 0.1 M TrisHCl (pH7), 1 mM MgCl₂, 50 mM xylitol and 5 mM NAD⁺. Reactions were started by the addition of substrate. The specific activities of the enzymes were expressed as U mg⁻¹.

One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of NADH/NAD(P)⁺ per min under the assay conditions [53].

Total RNA extraction, cDNA synthesis and RTq-PCR

For total RNA extraction, 5 mL of liquid culture was harvested by centrifugation (2000 rpm × 10 min) and washed twice with 5 mL of ice-chilled distilled water. Subsequently, cells were resuspended in 5 mL of distilled water and the required volume of cells was transferred into a 2 mL tube. Total RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad, USA) according to manufacturer's protocol. The concentration of total

RNA was spectrophotometrically measured (Shimadzu UV-1800; OD_{260nm}) using quartz cuvettes.

About 0.5 μg of total RNA was converted into cDNA by reverse transcription in a 20-μL reaction mixture using the iScriptcDNASynthesis Kit (Bio-Rad, USA) with the provided random primer mix (5X iScript reaction mix) following the recommended protocol.

The RTq-PCR was performed using the SsoAdvanced SYBR® Green Supermix (Bio-Rad, USA) according the manufacturer's instructions. For XR and XDH primer design, sequences deposited as GU574744 and GU574813 were used, respectively. Primer-3 (<http://primer3.ut.ee/>) was used to design PCR oligonucleotides, listed in Table 3.

The PCR amplification conditions were as follows: initial denaturation for 30 sec at 95°C, PCR amplification for 40 cycles with denaturation for 5 sec at 95°C, annealing temperature for 10 sec at 58°C, extension for 5 sec at 65°C in a MiniOpticon Real-Time PCR System (Bio-Rad; USA).

The relative expression levels of each targeted gene were normalized by subtracting the corresponding beta-actin threshold cycle (C_T) values and the fold increase (or decrease) was calculated through the 2^{-ΔΔCT} (Livak) method. Each sample was run in triplicate.

Abbreviations

KmXR: *Kluyveromyces marxianus* Xylose Reductase; *KmXDH*: *Kluyveromyces marxianus* Xylitol Dehydrogenase; CS sugar: Sugar with five carbon atoms; RNA: Ribonucleic acid; mRNA: Messenger Ribonucleic acid; cDNA: Complementary Deoxyribonucleic acid; vvm: Volume of gas per volume of batch per minute; NAD⁺: Nicotinamide adenine dinucleotide oxidized form; NADH: Nicotinamide adenine dinucleotide reduced form; NADP⁺: Nicotinamide adenine dinucleotide phosphate oxidized form; NADPH: Nicotinamide adenine dinucleotide phosphate reduced form; ROS: Reactive oxygen species; C_T: Threshold cycle; H₂O₂: Hydrogen peroxide; YPX: Medium containing Yeast extract, Peptone and Xylose; rpm: Revolutions per minute; PCR: Polymerase chain reaction; RTq-PCR: Quantitative reverse transcriptase polymerase chain reaction; CDW: Cell dry weight; OD: Optical density; HPLC: High pressure liquid chromatography; PI: Propidium iodide; DHR-123: Dihydrochlorodamine 123; H₂SO₄: Sulphuric acid; N₂: Nitrogen; NaOH: Sodium hydroxide; MgCl₂: Magnesium chloride solution; TrisHCl: Tris hydrochloride buffer; FL1: Filter n°1; FL4: Filter n°4; PBS: Phosphate-buffered saline.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LS carried out the fermentation experiments, the flow cytometric assays, the enzymatic and the transcriptional assays, participated in the evaluation of the data and in compiling the manuscript. SP carried out the fermentation and the flow cytometric assays, participated in the evaluation of the data. LR contributed to the data interpretation and manuscript revision. DP and PB conceived the study, participated in its design, data interpretation and compiled the manuscript. All the authors have read and approved the final manuscript.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under the project NEMO (Grant agreement no. 222699). The authors gratefully acknowledge Gabriele Erba for technical contribution.

Author details

¹University of Milano Bicocca, Piazza della Scienza 2, 20126 Milan, Italy. ²VTT Technical Research Centre of Finland, Espoo FI-02044 VTT, Finland. ³Current address: Sacco srl, Via Manzoni, 29/A, 22071 Cadorago Co, Italy.

Received: 10 January 2014 Accepted: 4 April 2014

Published: 8 April 2014

References

- Porro D, Branduardi P: Yeast cell factory: fishing for the best one or engineering it? *Microb Cell Factories* 2009, **8**:51.
- Lane MM, Morrissey JP: *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. *Fungal Biol Rev* 2010, **24**:7–26.
- Lane MM, Burke N, Karreman R, Wolfe KH, O'Byrne CP, Morrissey JP: Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*. *Antonie Van Leeuwenhoek* 2011, **100**:507–519.
- Belloch C, Querol A, Garcia MD, Barrio E: Phylogeny of the genus *Kluyveromyces* inferred from the mitochondrial cytochrome-c oxidase II gene. *Int J Syst Evol Microbiol* 2000, **50**:405–416.
- Gonzalez-Siso MI, Freire-Picos MA, Ramil E, Gonzalez-Dominguez M, Torres AR, Cerdan ME: Respirofermentative metabolism in *Kluyveromyces lactis*: insights and perspectives. *Enzyme Microb Technol* 2000, **26**:699–705.
- Schaffrath R, Breunig KD: Genetics and molecular physiology of the yeast *Kluyveromyces lactis*. *Fungal Genet Biol* 2000, **30**:173–190.
- van Ooyen AJJ, Dekker P, Huang M, Olsthoorn MMA, Jacobs DI, Colussi PA, Taron CH: Heterologous protein production in the yeast *Kluyveromyces lactis*. *FEMS Yeast Res* 2006, **6**:381–392.
- Lee YJ, Kim CS, Oh DK: Lactulose production by beta-galactosidase in permeabilized cells of *Kluyveromyces lactis*. *Appl Microbiol Biotechnol* 2004, **64**:787–793.
- Bianchi MM, Brambilla L, Protani F, Liu CL, Lievens J, Porro D: Efficient homolactic fermentation by *Kluyveromyces lactis* strains defective in pyruvate utilization and transformed with the heterologous *LDH* gene. *Appl Environ Microbiol* 2001, **67**:5621–5625.
- Breunig KD, Steensma HY: *Kluyveromyces lactis*: genetics, physiology and application. *Funct Genet Ind Yeasts Top Curr Genet* 2003, **2**:171–205.
- Fonseca GG, Gombert AK, Heinzele E, Wittmann C: Physiology of the yeast *Kluyveromyces marxianus* during batch and chemostat cultures with glucose as the sole carbon source. *FEMS Yeast Res* 2007, **7**:422–435.
- Groenewald P, Stouthamer AH, Westerhoff HV: Super life - how and why 'cell selection' leads to the fastest-growing eukaryote. *FEMS J* 2009, **276**:254–270.
- Anderson PJ, McNeil K, Watson K: High-efficiency carbohydrate fermentation to ethanol at temperatures above 40°C by *Kluyveromyces marxianus* var *marxianus* isolated from sugar mills. *Appl Environ Microbiol* 1986, **51**:1314–1320.
- Rouwenhorst RJ, Visser LE, Vanderbaan AA, Scheffers WA, Vandijken JP: Production, distribution, and kinetic properties of inulinase in continuous cultures of *Kluyveromyces marxianus* CBS 6556. *Appl Environ Microbiol* 1988, **54**:1131–1137.
- Steensma HY, Dejongh FCM, Linnekamp M: The use of electrophoretic karyotypes in the classification of yeasts: *Kluyveromyces marxianus* and *Kluyveromyces lactis*. *Curr Genet* 1988, **14**:311–317.
- Banat IM, Nigam P, Marchant R: Isolation of the thermotolerant, fermentative yeasts growing at 52°C and producing ethanol at 45°C and 50°C. *World J Microbiol Biotechnol* 1992, **8**:259–263.
- Rodussamee N, Lertwattanasakul N, Hirata K, Suprayogi Limtong S, Kosaka T, Yamada M: Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. *Appl Microbiol Biotechnol* 2011, **90**:1573–1586.
- Ballesteros M, Oliva JM, Negro MJ, Manzanares P, Ballesteros I: Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. *Process Biochem* 2004, **39**:1843–1848.
- Castro RC, Roberto IC: Selection of a thermotolerant *Kluyveromyces marxianus* strain with potential application for cellulosic ethanol production by Simultaneous Saccharification and Fermentation. *Appl Biochem Biotechnol* 2013, **3**:1553–1564.
- Goshima T, Tsuji M, Inoue H, Yano S, Hoshino T, Matsushika A: Bioethanol production from lignocellulosic biomass by a novel *Kluyveromyces marxianus* strain. *Biosci Biotechnol Biochem* 2013, **77**:1505–1510.
- Matsushika A, Inoue H, Kodaki T, Sawayama S: Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. *Appl Microbiol Biotechnol* 2009, **84**:37–53.
- Lin Y, Tanaka S: Ethanol fermentation from biomass resources: current state and prospects. *Appl Microbiol Biotechnol* 2006, **69**:627–642.
- Limtong S, Sringiew C, Yongmanitchai W: Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresour Technol* 2007, **98**:3367–3374.
- Suutari M, Liukkonen K, Laakso S: Temperature adaptation in yeasts: the role of fatty acids. *J Gen Microbiol* 1990, **136**:1469–1474.
- Zhang B, Zhang L, Wang D, Gao X, Hong J: Identification of a xylose reductase gene in the xylose metabolic pathway of *Kluyveromyces marxianus* NBRC1777. *J Ind Microbiol Biotechnol* 2011, **38**:2001–2010.
- Fonseca GG, Heinzele E, Wittmann C, Gombert AK: The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Appl Microbiol Biotechnol* 2008, **79**:339–354.
- Rocha SN, Abrahao-Neto J, Gombert AK: Physiological diversity within the *Kluyveromyces marxianus* species. *Antonie Van Leeuwenhoek* 2011, **100**:619–630.
- Llorente B, Malpertuy A, Blandin G, Artiguenave F, Wincker P, Dujon B: Genomic exploration of the hemiascomycetous yeasts: 12. *Kluyveromyces marxianus* var. *marxianus*. *FEMS Lett* 2000, **487**:71–75.
- Lulu L, Zhang L, Wang D, Gao X, Tamaki H, Kumagai H, Hong J: Identification of a xylitol dehydrogenase gene from *Kluyveromyces marxianus* NBRC1777. *Mol Biotechnol* 2013, **53**:159–169.
- Verduyn C, Postma E, Scheffers WA, Vandijken JP: Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* 1992, **8**:501–517.
- Sansonetti S, Hobbly TJ, Curcio S, Villadsen J, Sin G: Use of continuous lactose fermentation for ethanol production by *Kluyveromyces marxianus* for verification and extension of a biochemically structured model. *Bioresour Technol* 2013, **130**:703–709.
- Vandijken JP, Scheffers WA: Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol Rev* 1986, **32**:199–224.
- Winkelhausen E, Kuzmanova S: Microbial conversion of D-xylose to xylitol. *J Ferment Bioeng* 1998, **86**:1–14.
- Sasaki DT, Dumas SE, Engleman EG: Discrimination of viable and non viable cells using propidium iodide in 2 color immunofluorescence. *Cytometry* 1987, **8**:413–420.
- Madeo F, Frohlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Frohlich KU: Oxygen stress: A regulator of apoptosis in yeast. *J Cell Biol* 1999, **145**:757–767.
- Kotter P, Ciriacy M: Xylose fermentation by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 1993, **38**:776–783.
- Bruinenberg PM, Debat PHM, Vandijken JP, Scheffers WA: The role of redox balances in the anaerobic fermentation of xylose by yeasts. *Appl Microbiol Biotechnol* 1983, **18**:287–292.
- Jeong H, Lee D-H, Kim SH, Kim H-J, Lee K, Song JY, Kim BK, Sung BH, Park JC, Sohn JH, Koo HM, Kim JF: Genome sequence of the thermotolerant yeast *Kluyveromyces marxianus* var. *marxianus* KCTC 17555. *Eukaryot Cell* 2012, **11**:1584–1585.
- Blank LM, Lehmebeck F, Sauer U: Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. *FEMS Yeast Res* 2005, **5**:545–558.
- Salusjarvi L, Kankainen M, Solymani R, Pitkanen JP, Penttila M, Ruohonen L: Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*. *Microb Cell Factories* 2008, **7**:18.
- Yablochkova EN, Bolotnikova OI, Mikhallova NP, Nemova NN, Ginak AI: The activity of xylose reductase and xylitol dehydrogenase in yeasts. *Microbiology* 2003, **72**:414–417.
- Isabel Gonzalez Siso M, Garcia Leiro A, Tarrion N, Esperanza Cerdan M: Sugar metabolism, redox balance and oxidative stress response in the respiratory yeast *Kluyveromyces lactis*. *Microb Cell Factories* 2009, **8**:46.
- Saliola M, Tramonti A, Lanini C, Claffi S, De Biase D, Falcone C: Intracellular NADPH Levels Affect the Oligomeric State of the Glucose 6-Phosphate Dehydrogenase. *Eukaryot Cell* 2012, **11**:1503–1511.
- Zhang B, Li L, Zhang J, Gao X, Wang D, Hong J: Improving ethanol and xylitol fermentation at elevated temperature through substitution of xylose reductase in *Kluyveromyces marxianus*. *J Ind Microbiol Biotechnol* 2013, **40**:305–316.
- Wang R, Li L, Zhang B, Gao X, Wang D, Hong J: Improved xylose fermentation of *Kluyveromyces marxianus* at elevated temperature

- through construction of a xylose isomerase pathway. *J Ind Microbiol Biotechnol* 2013, **40**:841–854.
46. Dos Santos VC, Soares Braganca CR, Vieira Passos FJ, Lopes Passos FM: Kinetics of growth and ethanol formation from a mix of glucose/xylose substrate by *Kluyveromyces marxianus* UFV-3. *Antonie Van Leeuwenhoek* 2013, **103**:153–161.
 47. Oliva JM, Saez F, Ballesteros I, Gonzalez A, Negro MJ, Manzanares P, Ballesteros M: Effect of lignocellulosic degradation compounds from steam explosion pretreatment on ethanol fermentation by thermotolerant yeast *Kluyveromyces marxianus*. *Appl Biochem Biotechnol* 2003, **105**:141–153.
 48. Moreno AD, Ibarra D, Ballesteros I, Gonzalez A, Ballesteros M: Comparing cell viability and ethanol fermentation of the thermotolerant yeast *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* on steam-exploded biomass treated with laccase. *Bioresour Technol* 2013, **135**:239–245.
 49. Lee JS, Huh WK, Lee BH, Baek YU, Hwang CS, Kim ST, Kim YR, Kang SO: Mitochondrial NADH-cytochrome b(5) reductase plays a crucial role in the reduction of D-erythroascorbil free radical in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 2001, **1527**:31–38.
 50. Branduardi P, Fossati T, Sauer M, Pagani R, Mattanovich D, Porro D: Biosynthesis of Vitamin C by yeast leads to increased stress resistance. *PLoS One* 2007, **2**:e1092.
 51. Bradford MM: Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal Biochem* 1976, **72**:248–254.
 52. Smiley KL, Bolen PL: Demonstration of D-xylose reductase and D-xylitol dehydrogenase in *Pachysoles tannophilus*. *Biotechnol Lett* 1982, **4**:607–610.
 53. Twari MK, Moon H-J, Jeya M, Lee J-K: Cloning and characterization of a thermostable xylitol dehydrogenase from *Rhizobium etli* CFN42. *Appl Microbiol Biotechnol* 2010, **87**:571–581.

doi:10.1186/1475-2859-13-51

Cite this article as: Signori et al.: Effect of oxygenation and temperature on glucose-xylose fermentation in *Kluyveromyces marxianus* CBS712 strain. *Microbial Cell Factories* 2014 **13**:51.

**Submit your next manuscript to BioMed Central
and take full advantage of:**

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit



Chapter III.

Assessing an effective feeding strategy to optimize crude glycerol utilization as sustainable carbon source for lipid accumulation in *Rhodospiridium toruloides*, *Cryptococcus curvatus* and *Lipomyces starkeyi*

Lorenzo Signori¹, Diletta Ami^{1,2,3}, Riccardo Posterì¹, Andrea Giuzzi¹, Paolo Mereghetti⁴, Danilo Porro¹, Paola Branduardi^{1§}

¹ Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, Milano 20126 (Italy).

² Department of Physics, University of Milano-Bicocca, Piazza della Scienza 3, Milano 20126 (Italy).

³ Consorzio Nazionale Interuniversitario per le Scienze fisiche della Materia (CNISM) UdR Milano-Bicocca, Via R. Cozzi 53, Milano 20126 (Italy).

⁴ Center for Nanotechnology Innovation@NEST, Italian Institute of Technology, Piazza San Silvestro 12, Pisa 56127 (Italy).

§Corresponding author

Abstract

Background

Microbial lipids can represent a valuable alternative feedstock for biodiesel production in the context of a viable bio-based economy. This production can be driven by cultivating some oleaginous microorganisms on crude-glycerol, a 10% (w/w) by-product produced during the transesterification process from oils into biodiesel. Despite attractive, the perspective is still economically unsustainable, mainly because impurities in crude glycerol can negatively affect microbial performances. In this view, the selection of the best cell factory, together with the development of a robust and effective production process are primary requirements.

Results

The present work compared crude *versus* pure glycerol as carbon sources for lipid production by three different oleaginous yeasts: *Rhodospiridium toruloides* (DSM 4444), *Lipomyces starkeyi* (DSM 70295) and *Cryptococcus curvatus* (DSM 70022).

An efficient yet simple feeding strategy for avoiding the lag phase caused by growth on crude glycerol was developed, leading to high biomass and lipid production for all the tested yeasts. Flow-cytometry and Fourier Transform Infrared (FTIR) microspectroscopy, supported by Principal Component Analysis (PCA), were used as non-invasive and quick techniques to monitor, compare and analyze the lipid production over time. Gas chromatography (GC) analysis completed the quali-quantitative description.

Under these operative conditions, the highest lipid content (up to 60.9 % wt/wt) was measured in *R. toruloides*, while *L. starkeyi* showed the fastest glycerol consumption rate ($1.05 \text{ g L}^{-1}\text{h}^{-1}$). Being productivity the most industrially relevant feature to be pursued, under the presented optimized conditions *R. toruloides* showed the best lipid productivity (0.13 and $0.15 \text{ g L}^{-1} \text{ h}^{-1}$ on pure and crude glycerol, respectively).

Conclusions

Here we demonstrated that the development of an efficient feeding strategy is sufficient in preventing the inhibitory effect of crude glycerol, and robust enough to ensure high lipid accumulation by three different oleaginous yeasts.

Single cell and in situ analyses allowed depicting and comparing the transition between growth and lipid accumulation occurring differently for the three different yeasts. These data provide novel information that can be exploited for screening the best cell factory, moving towards a sustainable microbial biodiesel production.

Keywords: *Cryptococcus curvatus*, *Rhodospiridium toruloides*, *Lipomyces starkeyi*, Crude glycerol, Fatty acids methyl esters (FAME), Flow-cytometry, Fourier transform infrared (FTIR) microspectroscopy

BACKGROUND

The progressive depletion of natural oil reserves, together with associated environmental concerns about greenhouse gas (GHG) emissions, has stimulated the development of sustainable process for the production of materials, chemicals and fuels from renewable resources.

Biodiesel is a renewable, safe and non-toxic energy source and a possible substitute of petroleum-based diesel [1, 2]. It is mainly produced through the *trans*-esterification of renewable feedstock, such as vegetable oils and animal fats, into fatty acid methyl esters (FAMES).

However, to become an economically feasible alternative, biodiesel must compete in the market with petro-diesel fuel, but the actual high costs of production represent an obstacle for its commercialization. About 70-90 % of the overall costs depends on the feedstock price [3, 4]; furthermore, traditional oil-rich crops are limited by land availability, influenced by the climate and are in constant debate due to the food-*versus*-fuel issue [5].

An emerging potential alternative for biodiesel production is represented by microbial lipids, also referred as single-cell oils (SCOs; [6]), which could lead to a green and sustainable biodiesel production process, with no competition with the food supply chain.

Different oleaginous microorganisms have the ability to accumulate triacylglycerols (TAGs) as storage metabolites [7], with a fatty acid composition similar to that of many plant seed oils in terms of chain length and degree of unsaturation [8].

Among oleaginous microorganisms, yeasts exhibit advantages over bacteria, molds and algae, due to their higher growth rate, biomass and lipid

productivities [9, 10]. Moreover, in comparison to plants and open-pond grown algae, yeasts can be easily grown in bioreactors (therefore not affected by season nor by climate), and the process has an easier scale-up [11].

In oleaginous microorganisms lipid accumulation is critically affected by the carbon-to-nitrogen (C/N) ratio and typically occurs under nitrogen limited conditions and in the presence of high sugar content [1]. When nitrogen is limiting, triglycerides are stored within intracellular lipid bodies [12].

The main oleaginous yeast genera so far identified include species belonging to the genera *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces* [11]. Some oleaginous yeasts have been reported to accumulate lipids up to 80% of their total dry cell weight under appropriate conditions [7, 11, 13].

However, the production of biodiesel from microbial feedstock remains economically unsustainable if expensive and edible substrates are considered [14]. The implementation with renewable waste raw materials (e.g. whey, crude glycerol, lignocellulosic biomass), having zero or even negative costs, could make microbial lipid production economically feasible. Indeed crude glycerol is the main byproduct, about 10 % (w/w), of the conversion of oils into biodiesel. In other words, for every 3 mol of methyl esters produced, 1 mol of glycerol is obtained as a byproduct [15]. Considering the increasing demand for biodiesel, larger amounts of glycerol are expected of being accumulated as a byproduct [16]. Nowadays, in some countries, crude glycerol is treated as industrial wastewater or simply incinerated, making biodiesel a “grey” fuel rather a green fuel alternative [17].

Despite desirable, an efficient valorization of crude glycerol is difficult to achieve since it contains

several impurities such as residual methanol, NaOH, carry-over fat/oil, some esters, and minor amounts of sulfur compounds, proteins, and minerals [17]. Refined glycerol could be a valuable product, but once more the purification process is too costly and energy-intensive [18].

Nevertheless, crude glycerol has been tested in many studies as a substrate for the production of SCO or for other metabolic compounds (such as citric acid, acetic acid, polyols, etc.) by several eukaryotic microbial strains [19].

In this study, the oleaginous yeasts *Cryptococcus curvatus*, *Lipomyces starkeyi* and *Rhodospiridium toruloides* were chosen as three of the most promising cell factories for lipid production using crude glycerol as sole carbon source [5, 18, 20]. Furthermore, data concerning this topic in these strains are still scarce in literature [5, 18, 19, 21-24]. Here we demonstrate that the development of an efficient, yet simple, feeding strategy is sufficient to avoid the detrimental effects deriving from the impurities present in crude glycerol and to enhance the production of lipids. This fermentation strategy greatly increased cell density as well as the rate of lipid production.

The lipid-producing capability of the chosen yeasts was investigated through the application of different techniques. In particular, fluorescent microscopy, flow-cytometry and FTIR microspectroscopy analyses were performed. All these are relatively fast approaches that do not require lipid extraction and would be helpful in the initial screening phase as well as in the real time evaluation of the effective production over fermentation time [25]. To the best of our knowledge, this is the first time that a combination of non-invasive techniques are applied to monitor lipid accumulation from crude glycerol over fermentation time.

Finally, gas chromatography analysis (GC) was performed to preliminarily qualify the potential of the obtained microbial oil as biodiesel.

RESULTS AND DISCUSSION

Effect of crude glycerol on yeasts growth

It has been reported that different oleaginous yeast strains present different metabolic responses depending on the origin of the crude glycerol employed as carbon source [1]. To evaluate the growth of *R. toruloides* (DSM 4444), *C. curvatus* (DSM 70022) and *L. starkeyi* (DSM70295) on the crude glycerol stock adopted in this study, shake flasks experiments were performed.

Five different defined media containing different proportions of crude versus pure glycerol were prepared (from A to E; see Methods for details). For all formulations, the initial concentration of total glycerol was fixed at 100 g L⁻¹. Strains were cultivated in batch mode at 25°C, 220 rpm, pH 5.5 (buffered with citric acid) for about 200 hours, monitoring biomass (optical density and cell dry weight, Additional file 6) and lipid accumulation (flow-cytometry and fluorescence microscopy analyses, by Nile Red staining, Additional file 7).

As shown in Figure 1, medium formulated with 100 g L⁻¹ of pure glycerol (Medium A, dashed lines, open squares) well sustained the growth of all the strains selected for this study. On the contrary, no biomass increase was observed when crude glycerol concentrations of 50 (Medium D, closed diamonds) and 100 g L⁻¹ (Medium E, closed squares) were used. On Medium C (30 g L⁻¹ of crude glycerol; closed triangles) only *R. toruloides* showed a significant growth within 216 hours. Instead, with the lowest crude glycerol concentration tested (20 g L⁻¹; Medium B, closed

circles), all the yeasts were able to reach, after 216 h, biomass values similar to those obtained on Medium A.

Between the tested yeasts, *R. toruloides* proved to be the most tolerant yeast (Figure 1a). It also showed the highest biomass production, calculated as cell dry weight, within 216 h: about 30.3 g L⁻¹ against only about 19.9 g L⁻¹ and 25.2 g L⁻¹ reached with *C. curvatus* and *L. starkeyi*, respectively.

Overall, high crude glycerol concentrations inhibited yeasts growth. The inhibition has mainly affected the duration of the lag phase since the specific growth rates were similar or even higher of those observed on pure glycerol when crude glycerol was part of the medium composition (Data not shown). An acidification of the medium as possible cause of the inhibition was excluded by monitoring the pH immediately after sampling.

Feeding pure vs crude glycerol for lipid production

Feeding strategies have been often proven to be crucial for the control of microbial cultures and production in biochemical processes [26]. Therefore, here we assessed the efficacy of developing a fed-batch protocol for minimizing the growth inhibition caused by the use of crude glycerol.

Briefly, strains were cultivated at 25°C, pH 5.5 and monitored until glycerol exhaustion. The inlet gas flow rate was maintained constant at 1 vvm and stir was set in cascade to 25% of dissolved oxygen to ensure fully aerobic conditions.

The cultivation was designed to have three different phases: 1) initial phase on pure glycerol (from 0 to 28 h) to adapt cells to the operative conditions; 2) “feeding” phase (from 28 to 48 h) where the medium was fed to reach a final concentration of about 100 g L⁻¹ of glycerol (crude versus pure); 3)

“lipid accumulation” phase under nitrogen limitation and in excess glycerol (from 48 h to the time of glycerol depletion) (see Methods for details).

Figure 2 (left panels) shows the glycerol consumption profiles of *R.toruloides* (a), *C. curvatus* (b) and *L. starkeyi* (c) on both pure (dashed line) and crude (continuous line) glycerol. As reported in literature [27] the calculation of the optimal feeding rate is complex, since it has to match the requirements of the desired process of production. In this study, the defined dilution rate of 0.45 mL min⁻¹ was low enough to avoid culture dilution but at the same time as high as possible to sustain lipid accumulation.

From a general point of view, during the initial 24 h the glycerol utilization rate was low in all the three strains, associated with an adaptation to the operating conditions. With the start of the feeding (28 h) all the tested yeasts were able to grow on crude glycerol, demonstrating the efficacy and the robustness of the feeding strategy. The crucial role of the feeding strategy can be further appreciated considering the shake flasks experiments previously described, where none of the selected yeasts proved to be able to grow starting with 100 and 50 g L⁻¹ of crude glycerol. Same results have been also obtained in batch fermentations performed in bioreactors (data not shown).

In *C. curvatus* and *L. starkeyi* no significant differences were observed between the consumption profiles of pure and crude glycerol (Figure 1b and c, left panel). Surprisingly, in *R. toruloides* crude glycerol consumption was markedly faster than that of pure glycerol (~0.73 g L⁻¹ h⁻¹ and ~0.98 g L⁻¹ h⁻¹ on pure and crude glycerol, respectively) (Figure 1a, left panels).

Overall, the highest glycerol uptake rates were observed in *L. starkeyi* (~1.03 g L⁻¹ h⁻¹ and ~1.05 g

$L^{-1} h^{-1}$ on pure and crude glycerol, respectively), while the lowest uptake rates were measured for *C. curvatus* ($\sim 0.52 g L^{-1} h^{-1}$ and $\sim 0.48 g L^{-1} h^{-1}$ on pure and crude glycerol, respectively). This feature did not emerge during shake-flask batch experiments.

Table 1 reports a comparison of the cell mass and lipid productivities of the three yeasts growing on pure and crude glycerol. According to the data on substrates consumption, in *R. toruloides* also biomass production was markedly faster on crude glycerol compared to pure glycerol: at 144 h the dry cell weight on pure and crude glycerol was of $29.75 g L^{-1}$ and $39.55 g L^{-1}$, respectively (Table 1). These findings are in line with literature data reporting how crude glycerol can in some cases lead to higher biomass and lipid productivities when compared to pure substrate [18], due to the presence of macro elements, vitamins and other compounds that during the biodiesel production process can diffuse to the glycerol phase [28]. Possibly because of different nutritional requirements, biomass accumulation proceeded without significant differences between crude and pure glycerol cultures in *C. curvatus* and *L. starkeyi* (Table 1).

The highest biomass productions were achieved in *C. curvatus* ($\sim 43.7 g L^{-1}$ and $\sim 45.1 g L^{-1}$ on pure and crude glycerol, respectively) but, independently from the substrate (pure or crude glycerol), associated with the lowest productivity compared to the other strains. These data underline once more that both production and productivity can vary considerably depending on the selected cell factory. Regarding the production of interest, remarkably this process set-up assured in the three investigated yeasts a good lipid accumulation (Table 1). In particular, *R. toruloides* was found to be the most effective in terms of lipid productivity and overall

lipid content (on dry cell weight basis): on crude glycerol a lipid productivity of $0.15 g L^{-1} h^{-1}$, very close to the one obtained with pure glycerol ($0.13 g L^{-1} h^{-1}$), and almost the same lipid content (60.9 and 60.0 % on pure and crude glycerol, respectively) were obtained.

Table 2 presents an overview of literature data regarding total dry weight and microbial oil content achieved during cultivation of *R. toruloides*, *C. curvatus* and *L. starkeyi* yeast strains on glycerol.

In the case of *R. toruloides* strains, the CDW and SCO concentrations achieved in this study are among the highest reported in literature. To the best of our knowledge, on crude glycerol only in one case [23] a higher lipid productivity has been so far observed.

For *C. curvatus*, even if lipid content is in line than those of other reports, lipid productivity is lower than what reported in [5].

Finally, for *L. starkeyi* a scarce number of reports have indicated lipid accumulation based on crude glycerol consumption. Among these, the highest CDW and SCO concentrations as well as productivity have been achieved in this study (Table 2).

Nevertheless and for sake of clarity, it is important to notice that a direct comparison of the literature data is difficult: as shown in Table 2, various fermentation configurations, as well as nitrogen source and glycerol concentrations were tested.

Evaluation of lipid production in oleaginous yeasts by Nile Red staining

Nile Red (NR) is a red phenoxazine dye, present as a minor component of commercial preparations of the non-fluorescent stain Nile Blue, which selectively stains lipophilic substances.

During fed-batch experiments cells were collected at different time points (T=0, T=28, T=48, T=72,

T=144, T=192, T=240 h after inoculation, see Figure 3), stained with Nile Red and analyzed by flow-cytometry to evaluate the lipid content (see Methods for details). In Figure 3, the overlay histograms of *R. toruloides* (a), *C. curvatus* (b) and *L. starkeyi* (c) cells growing on both pure (left panels) and crude (right panels) glycerol are reported.

For all the tested strains, once started, lipid accumulation proceeded along with the fermentation as shown by an increase of fluorescence over time, with only slight variations from pure and crude glycerol cultures (Figure 3). This increase was negligible in the non-oleaginous yeast, *S. cerevisiae*, (data not shown). It is interesting to note that, at time points near to glycerol exhaustion, Nile Red fluorescence signals related to crude glycerol cultivations were higher compared to those of pure glycerol cultures.

Comparing the different yeasts, fluorescence signals were low at 28 h for *C. curvatus* and *R. toruloides* (Figure 3a and 3b), as expected. On the contrary, in *L. starkeyi* at 28 h fluorescence signals were higher (Figure 3b), suggesting that lipid accumulation was already started during the shake-flask cultivation (pre-inoculum phase). The Nile Red fluorescence signals collected immediately after the inoculum (T=0 h) were not showed since they were found to be very similar of those collected at 28 h. At 48 h in *R. toruloides* the Nile Red fluorescence signals were found to be higher compared to those related to 28 h, while a relevant increase of signal was observed only at 72 h from the inoculum for *C. curvatus* and *L. starkeyi*. Interestingly, a bimodal distribution was observed at 72 and 144 h in *L. starkeyi*, suggesting the existence of two distinct populations with different lipid content.

Overall, it can be noticed that, independently from the cultivation conditions (pure or crude glycerol), the higher fluorescence signals were observed in *R. toruloides* (Figure 3a).

Fluorescence microscopy images confirmed flow-cytometry observations: as example, at 28 h *R. toruloides* and *C. curvatus* showed very weak fluorescence signals, while small lipid droplets were already visible in *L. starkeyi* cells (Figure 4).

The direct observation of cells also enable the detection of differences in lipid bodies size, morphology and abundance among the different yeasts, as previously reported but for different growth conditions [29].

R. toruloides (Figure 4a) cells, with small lipid bodies at 72 h, modified into bigger cells (up to 5 μm) with one or two lipid bodies at 144 h. *C. curvatus* (Figure 4b) at 72 h showed numerous small lipid bodies (less than 1 μm diameter) contained in long rod-shaped cells. After 144 h of cultivation, the majority of the cells contained only one or two bigger lipid bodies (3-4 μm) and some smaller ones (less than 1 μm). *L. starkeyi* (Figure 4c) produced one or two large lipid bodies in their spherical cells (as shown in [30]). The small lipid bodies (less than 1 μm diameter), which were already visible in the cells at the beginning of the feeding phase (28 h), increased in size to more than 6 μm diameter after 144 h of cultivation.

Overall, in both dichroic and fluorescence microscopy images small lipid droplets, when present, were easily observed at early time points and they seemed to collapse into bigger structures over time.

Fluorescent microscopy analyses did not shed light on the formation in *L. starkeyi* of two sub-populations with different fluorescent signal, but overall they clearly depict the different transition between growth and lipid accumulation occurring

among the three yeasts and provide a useful semi-quantitative evaluation of their lipid accumulation over time.

FTIR microspectroscopy coupled to PCA for monitoring lipid accumulation in intact oleaginous yeast cells

Fourier Transform Infrared (FTIR) microspectroscopy has been demonstrated as a powerful, non-invasive and time-saving technique to monitor lipid accumulation in intact cells [25]. Indeed, this spectroscopic approach allows to obtain a biochemical fingerprint of the sample under investigation, giving information on its main biomolecule content (see Additional file 1) [31].

In this study we measured the infrared absorption of *R. toruloides*, *C. curvatus*, *L. starkeyi* and *S. cerevisiae* (as internal control) intact cells, at different times of growth (T=0, T=28, T=48, T=72, T=144, T=192, T=240 hours after inoculation). To evaluate the time course of lipid accumulation, in Figure 5a is reported the temporal evolution of the CH_x stretching band area, between 3050 and 2800 cm⁻¹ [32, 33], after normalization for the total protein content given by the amide I band area (see Additional file 1) [34]. We have found that lipid accumulation started approximately at 48 h of growth, with the exception of *L. starkeyi* (triangles) that, as detected also by Nile Red fluorescence (see Figure 3c and 4c), already started to accumulate in the pre-inoculum phase. Also FTIR analysis confirmed the highest lipid accumulation in *R. toruloides* (squares), under crude glycerol feeding. These results have been also confirmed by the temporal evolution of the ester carbonyl band area, between 1760 cm⁻¹ and 1730 cm⁻¹ [32, 33], again normalized for the total protein content (see Figure 5b), in agreement with Nile Red staining and analyses.

To better investigate the spectral features due to lipid accumulation and in particular to assign them to specific lipid molecules, we supported the infrared characterization with a multivariate analysis approach, namely the principal component analysis (PCA) [35]. In particular, we compared the IR response of the studied yeast strains with that of standard fatty acids, chosen among the most representative products of the oleaginous yeasts [1]. We performed the PCA on the raw spectra of samples taken at time 0 and 144 h, as representative of the yeast time-dependent behavior plus the raw spectra of the standard lipids. The spectral range was split into three ranges and the PCA was performed independently on each range. The Euclidean distance on the PCA score plot (Additional files 3, 4 and 5) among the lipids standards and a given oleaginous yeast was used as a measure to quantify the accumulation of a class of lipids in a specific yeast. The distance was computed for both time 0 and time 144 h, and a percentage change was then computed. The procedure was repeated for each yeast strain grown in pure glycerol as well as in crude glycerol and for each analyzed spectral range. Finally, an average percentage change across the ranges was computed and shown in Figure 6 (see Methods and Additional files for details about the procedure).

FTIR analysis of the cell wall modifications during lipid accumulation

The analysis of the second derivative spectra allowed to characterize the IR response of the yeast cell wall carbohydrates (Additional file 1) and in particular to investigate their modifications during the accumulation of lipids.

To this aim, we analyzed the spectral range between 1200-950 cm⁻¹, due to the overlapping absorption of C-O vibrations mainly from

carbohydrates, of O-P-O groups, and of C-O-P modes typical of phosphate esters [36, 37].

Firstly, in Figure 7a is reported the second derivative spectra of the control strain (*S. cerevisiae*) at

0 and 144 h. At time 0, the spectrum is dominated by the absorption of glucans and mannans, major components of the yeast cell wall [38]. In particular, the absorptions at $\sim 1156\text{ cm}^{-1}$, 1104 cm^{-1} and 1080 cm^{-1} can be mainly assigned to β 1 \rightarrow 3 glucans, while the band at $\sim 1024\text{ cm}^{-1}$ to β 1 \rightarrow 4 glucosidic bonds in chitin and/or in glucans linked to a N-acetylglucosamine molecule; moreover, the component at $\sim 998\text{ cm}^{-1}$ can be mostly due to β 1 \rightarrow 6 glucans [36, 37, 39]. It should be noted that the simultaneous presence of the three bands at 1156 cm^{-1} , 1080 cm^{-1} and 1024 cm^{-1} could be also indicative of the presence of glycogen [39]. Finally, the absorption at $\sim 1045\text{ cm}^{-1}$ is mainly due to mannans [36, 37]. These components were found to increase in intensity after 144 h of growth, as displayed. Moreover, some bands were found to shift in peak position, including the $\sim 1156\text{ cm}^{-1}$ that downshifted to $\sim 1152\text{ cm}^{-1}$, the $\sim 1024\text{ cm}^{-1}$ that upshifted to $\sim 1028\text{ cm}^{-1}$, and the $\sim 998\text{ cm}^{-1}$ that downshifted to $\sim 993\text{ cm}^{-1}$. Overall, these variations could reflect a modification of carbohydrate interactions with the surrounding molecules of the cell wall.

Analogously, we investigated possible variations of the cell wall features during the accumulation of lipids in the oleaginous yeasts. At time 0 the spectrum of *C. curvatus* grown in medium supplemented with crude glycerol is mainly characterized by the β 1 \rightarrow 3 glucan components at $\sim 1154\text{ cm}^{-1}$ and 1079 cm^{-1} (Figure 7b). Moreover, a very low intensity band was detected around 1041 cm^{-1} that can be attributed to the absorption of mannans and a weak absorption at $\sim 992\text{ cm}^{-1}$,

mostly due to β 1 \rightarrow 6 glucans, was also observed. Interestingly, these spectral components were found to dramatically increase in intensity at 144 h, reflecting a profound rearrangement of the cell wall architecture during lipid accumulation. Furthermore, new spectral features were observed. In particular, beside the upshift of the β 1 \rightarrow 3 glucans band up to $\sim 1156\text{ cm}^{-1}$, a new component at $\sim 1144\text{ cm}^{-1}$ was detected, again assigned to β 1 \rightarrow 3 glucans. The appearance of new spectral features in the β 1 \rightarrow 3 glucan absorption could be indicative of a modification of the physico-chemical properties of the complex network formed by glucans, which indeed interact with different cell wall components, including other carbohydrate polymers and/or proteins [38, 39]. In particular, modifications of glucan interactions with the surrounding wall components could lead to variations of their IR response. Moreover, a well resolved peak was observed at $\sim 1117\text{ cm}^{-1}$, negligible at T=0, that can be tentatively assigned to the O-H bending vibration of glycoproteins [40]. In addition, the component at $\sim 1055\text{ cm}^{-1}$, due to mannans, was also detected. Interestingly, the glycogen absorption at $\sim 1156, 1080, 1022\text{ cm}^{-1}$, negligible at T=0, was found at high intensity at T=144.

As also reported in the figure, very similar spectral features were observed when cells grew in medium supplemented with pure glycerol.

The analysis of cell wall carbohydrates in *R. toruloides* (Figure 7c) at T=0 in medium supplemented with crude as well as pure glycerol highlighted similar spectral features to those found for *C. curvatus* (Figure 7b). During the lipid accumulation up to 144 h, in particular the β glucan bands and the glycogen absorption were found to increase in intensity and their increase was slightly higher in the case of cells grown in medium

supplemented with pure glycerol. We should note that in *R. toruloides* the intensity variations of the β glucan bands were lower compared to those depicted in *C. curvatus* and also that the mannan absorption around 1041 cm^{-1} , observed in *C. curvatus*, was not detected in the case of *R. toruloides*. Moreover, the broad band at $\sim 1025\text{ cm}^{-1}$, that can be due to the overlapping absorption of glycogen and β 1 \rightarrow 4 glucosidic bonds, was found to increase in intensity up to 144 h, particularly in pure medium.

Interestingly, the spectral features of *L. starkeyi* cell wall carbohydrates appeared to be different compared to the other analyzed oleaginous yeasts. In fact, at T=0 the spectrum of *L. starkeyi*, grown in crude glycerol, is characterized firstly by a complex absorption band with three peaks at 1174 cm^{-1} , 1157 cm^{-1} , and 1142 cm^{-1} (Figure 7d). While the latter two are due to β 1 \rightarrow 3 glucans, the 1174 cm^{-1} band can be tentatively assigned to β glucans [41]. Furthermore, the glycoprotein component at $\sim 1117\text{ cm}^{-1}$ [40] is present in the T=0 spectrum, together with the β 1 \rightarrow 3 glucan absorption at $\sim 1080\text{ cm}^{-1}$. In addition, three low intensity bands were observed at $\sim 1045\text{ cm}^{-1}$, due to mannans, and at $\sim 1028\text{ cm}^{-1}$ and $\sim 993\text{ cm}^{-1}$, that can be respectively assigned to β 1 \rightarrow 4 glucosidic bonds and β 1 \rightarrow 6 glucans.

These spectral behaviors were found to change at 144 h, and in particular we observed a rearrangement of the β 1 \rightarrow 3 glucan components. Indeed, a new component at $\sim 1094\text{ cm}^{-1}$ appeared, accompanied by an intensity increase of the $\sim 1142\text{ cm}^{-1}$ absorption and a reduction of the $\sim 1080\text{ cm}^{-1}$ band. Moreover, a well resolved component at $\sim 1034\text{ cm}^{-1}$, that can be assigned mainly to mannans [41] was observed, instead of the two bands at $\sim 1045\text{ cm}^{-1}$ and $\sim 1028\text{ cm}^{-1}$ detected at T=0. These results, which have been also found for the growth

in medium added with pure glycerol, suggest that modifications of the cell wall physico-chemical properties occurred during lipid accumulation, involving the different carbohydrate components. However, in *L. starkeyi* we did not detect dramatic band intensity variations between 0 and 144 h, as instead seen for the other analyzed strains. This result could be due to the fact that cell wall modifications likely took place already in the pre-inoculum phase, when lipid accumulation has also started.

Gas chromatography analysis of fatty acid composition

The lipids produced by fed-batch experiments with *R. toruloides*, *C. curvatus* and *L. starkeyi* at 144 h were hydrolyzed and transmethylated. The resulting fatty acid methyl esters (FAME) were then analyzed by gas chromatography (Table 3, see Methods for details). Long-chain fatty acids were predominantly represented in all the strains and the four that accounted for over 90% of the total were palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic acid (C18:2). In agreement with PCA results, the oleic acid was the most produced, while the linolenic acid was accumulated in very small quantities by all yeasts. Nevertheless, the applied multivariate analysis was not sensitive enough to resolve FTIR spectra into the specific classes of fatty acids disclosed by GC analyses.

From a general point of view, the fatty acid profiles showed slight changes between the different culture conditions (pure vs crude glycerol). However, for all the tested yeasts, on crude glycerol the oleic acid content was slightly higher than that obtained from pure glycerol.

Table 3 details some interesting differences among the yeasts object of this study. In *R. toruloides* and *C. curvatus* at 144 h the percentage of 18:2 was

about twice the percentage observed in *L. starkeyi* and this independently from the glycerol employed (pure or crude). Also the percentage of oleic acid was significantly different: the highest percentage of 18:0 was observed in *L. starkeyi* (~ 39.4 % and ~ 40.9 % on pure and crude glycerol, respectively), while the lowest percentages were observed in *C. curvatus* (~ 27.6 % and ~ 31.2 % on pure and crude glycerol, respectively).

GC analysis of *R. toruloides* and *C. curvatus* were performed also just before the end of the fermentations (168 h and 240 h for *R. toruloides* and *C. curvatus*, respectively), finding that fatty acids profiles (% wt/wt) were basically unchanged compared to those observed at 144 h (data not shown).

The lipids chains of all the analyzed yeasts exhibit low degrees of unsaturation, which is desirable for their application in biodiesel production [42]. The highest percentages of polyunsaturated fatty acids (PUFAs) were observed in *R. toruloides* (~ 20.2 % and ~ 18.0 % on pure and crude glycerol, respectively), while the lower amounts were observed in *L. starkeyi* (~ 8.1 % and ~ 7.8 % on pure and crude glycerol, respectively).

It is reported in literature that comparing the fatty acids profiles of different oil feedstock [3], the biodiesel derived from yeast lipids are more saturated. It is also reported that yeast oil together with Palm oil and Jatropha oil have the highest percentages of monounsaturated fatty acids, which make them better sources for biodiesel production than soybean oil [21]. Overall, results reported in Table 3 are in line with these observations, showing in all the tested yeasts a higher content of saturated and monounsaturated fatty acids.

We have also observed that, for all the yeasts tested in this study, the percentages of 18:1 were lower than 20 % at day 2 (data not shown), but increased

over time reaching values reported in Table 3 by day 7 and were stable thereafter. Over time, the increase in the percentages of oleic acid (18:1) was followed by a decrease of the percentages of stearic acid (18:0): as example, in *R. toruloides* the percentages of 18:0 were ~ 50.0 % at day 2 but decrease to ~ 12.0 % by day 7. Percentages of linoleic acid (18:2) changed over time but with some differences among the different yeasts: in *R. toruloides* and *C. curvatus* the percentages decreased from day 2 to day 7 (~ 10.0 %) while in *L. starkeyi* percentages of 18:2 were very similar over time. Differently, percentages of 14:0, 16:1 and 18:3 were basically unchanged during the entire experimental time.

When considering whether biodiesel derived from yeast lipids is suitable for use as a fuel, several factors should be taken into account. The successful commercialization of biodiesel in many countries has been accompanied by the development of standards, as ASTM D6751 (ASTM=American Society for Testing and Materials) and the European standard EN, to ensure high product quality and user confidence [43]. In particular, ignition quality, heat of combustion, cetane number, oxidative stability, viscosity and lubricity are some of the properties of the biodiesel fuel influenced by fatty acids structure. Over the years predictive equations were developed and have generally proved successful in estimating the physical properties of oil composed of FAMES [44]. In the present study, the equations reported in [45] were employed to predict biodiesel properties, such as the viscosity and the cetane number. While cetane number for the tested yeasts was in the range between 59-62, meeting the international specifications [44], kinematic viscosity predicted values exceeded the limits of $3.5\text{--}5\text{ mm}^2\text{ s}^{-1}$ set by EN 14214.

However, as reported in [1], it is important to remember that the majority of the yeast oil-based biodiesel could be used as fuel for diesel engines only when they are blended with diesel fuel and the fatty acid profile that provides a fuel with all optimal parameters does not exist yet.

CONCLUSIONS

Valorization of waste raw materials represents a scientifically intriguing and socially relevant challenge in industrial biotechnology.

In this work we presented the oleaginous yeasts *R. toruloides*, *C. curvatus* and *L. starkeyi* as three of the most promising cell factories for biodiesel production starting from crude glycerol as substrate. In particular, we have demonstrated the possibility to design an efficient yet simple and robust feeding strategy useful in preventing the inhibitory effect of crude glycerol. The fermentation protocol is not trivial since, as well exemplified by our data on *L. starkeyi*, the transition between growth and lipid accumulation is species-specific.

With the herein described strategy all the selected yeasts were not only able to reach high biomass content but also to efficiently accumulate lipid over time, being high productivity one of the key parameter for the development of a viable process of production. The final lipid production on crude glycerol is similar if not higher than the one reached on pure glycerol. From the comparative analysis *L. starkeyi* showed the highest glycerol consumption rate, while *R. toruloides* the highest productivity and lipid titer.

FTIR microspectroscopy and flow cytometry analyses demonstrated to be efficient and time-saving methods for monitoring lipid accumulation

along the process within intact cells. However, GC analysis is still indispensable to accurately identify the lipids extracted from cells. Noteworthy, FTIR microspectroscopy revealed important changes occurring over time in the yeast cell wall composition. Considering that downstream processing procedures are costly and considering that one of the major issue with oleaginous yeast is cell breakage to rescue the product, these analyses will be relevant in guiding the choice of the cell factory, in designing the producing protocol, willing to develop a cost-effective process of production.

METHODS

Strains and media

R. toruloides (DSM 4444), *C. curvatus* (DSM 70022) and *L. starkeyi* (DSM70295) were purchased from DSMZ. Yeasts were stored in cryotubes at -80°C in 20% glycerol ($\text{v}\cdot\text{v}^{-1}$). The *S. cerevisiae* strain used in this study was GRF18U (MATa; *ura3*; *leu2-3,112*; *his3-11,15*; *cir⁺*, [46])

The composition of the inoculum and fermentation medium was (per liter): 1 g of yeast extract (0.114 g of nitrogen), 1.31 g of $(\text{NH}_4)_2\text{SO}_4$ (0.278 g of nitrogen), 0.95 g of Na_2HPO_4 , 2.7 g of KH_2PO_4 , 0.2 g of $\text{Mg}_2\text{SO}_4\cdot 7\text{H}_2\text{O}$, 0.04 g. After the pH was adjusted to 5.5 using NaOH 4 M, the medium was supplemented with a 100X trace mineral stock solution consisting of (per liter): 4 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$; 0.55 g $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$; 0.52 g citric acid; 0.10 g $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$; 0.076 g $\text{MnSO}_4\cdot \text{H}_2\text{O}$; and 100 μL 18M H_2SO_4 .

Yeast extract was provided by Biolife Italiana S.r.l., Milan, Italy. All the others reagents were provided by Sigma-Aldrich Co., St Louis, MO, USA.

Glycerol, both crude and pure, was used as carbon and energy source. Accordingly [47], for the calculations of the carbon-to-nitrogen ratio (C/N), a carbon content in glycerol of 39.1 %, and a nitrogen content of 21.2 % and 11.4 % in ammonium sulfate and yeast extract, respectively, were assumed.

Raw material

Crude glycerol derived from industrial biodiesel production out of palm oil. The stock had 80% content of glycerol and appeared as a dark brown liquid. In the text, reported crude glycerol concentrations (g L^{-1}) refer to the HPLC measured glycerol. Composition analyses of this stock were conducted: the original sample was diluted 50-fold and passed through 0.22 μm filter. Glycerol and methanol concentrations were determined by HPLC (see below for specifications). Crude glycerol was centrifuged at 6000 rpm for 20 min. The top, dark red layer, mainly consisting of free fatty acids (FFAs), was removed.

Batch cultures

Flasks experiments were conducted in 250 mL Erlenmeyer flasks containing 50 mL of liquid medium. As carbon and energy sources, glycerol stocks (pure and crude) were mixed at different ratio to a final concentration of 100 g L^{-1} (for crude glycerol, the calculation was done on the basis of the HPLC measurements). In particular, five different media (from Medium A to Medium E) were tested: 100 % of pure glycerol (Medium A), 80 % of pure and 20 % of crude glycerol (Medium B), 70 % of pure and 30 % of crude glycerol (Medium C), 50 % of pure and 50 % of crude glycerol (Medium D) and 100 % of crude glycerol (Medium E). The pH value of the medium was adjusted to 5.5 with NaOH 4M and was then

maintained using a citrate buffer solution pH5.5 at the final concentration of 0.1 M.

Shake flasks experiments were performed in triplicates on a rotary shaker at 25 °C and 220 rpm. The cultures were initiated upon 45 mL of the cultivation medium inoculated with 5 mL of the seed culture to a final absorbance (OD_{660}) of 3.0. Growth was monitored by regularly measuring optical density (OD_{660}) over time and by determining the cell dry weight (CDW) at 0 and 216 hours (final point). Samples for flow-cytometry and fluorescence microscopy analysis (1 mL) were taken every 24 h. The supernatant of these samples was used for the HPLC analysis.

Fed-batch cultures

Yeasts were revived from cryo-preserved stocks stored at -80 °C and grown on YP-Glycerol agar plates. One colony was used to inoculate 1 L flasks with 200 mL of the culture medium and seed cultures were placed on a rotary shaker at 25 °C and 220 rpm for 3 days. Exponential phase shake flasks cultures were used to inoculate bioreactors to a final optical density (OD_{660}) of 3.0. Briefly, cells were centrifuged at 6000 rpm for 5 min, washed twice with water, and finally resuspended in 20 mL of sterilized water.

The fed-batch experiments were conducted in 2.0 L bioreactors (Sartorius Stedim BIOSTAT® Bplus, Germany). For all bioreactor cultivations, the aeration rate, agitation, and temperature were set to 1 vvm, 300 rpm (in cascade to 25% of dissolved oxygen), and 25 °C, respectively. The pH was maintained by automatic pumping of 4 M NaOH.

The cultivation was designed to have three different phases: 1) initial phase on pure glycerol (from 0 to 28 h) to adapt cells to the operative conditions; 2) “feeding” phase (from 28 to 48 h) where the medium was fed to reach a final concentration of

about 100 g L⁻¹ of glycerol (crude versus pure); 3) “lipid accumulation” phase under nitrogen limitation and in excess glycerol (from 48 h to the time of glycerol depletion).

Bioreactor experiments were started with a working volume of 1.0 L and with 15 g L⁻¹ as initial concentration of pure glycerol, resulting in a medium with a balanced C/N ratio.

For each bioreactor the feeding consisted in a 540 mL solution containing concentrated pure or crude glycerol, so that the final glycerol concentration at the end of the feeding was ~ 100 g L⁻¹, and nutrients (salts and traces) to support cell growth. This led to a final working volume of 1.5 L (considering sampling).

The feeding rate was set as fast as possible for reaching the desired C/N ratio but based on the assumption that the biomass concentration should not decrease below the value measured at the beginning of the feeding. The lowest specific growth rate on pure glycerol was taken as reference to determine how long should the feed last. In our case, the (lowest) specific growth rate of 0.0247 h⁻¹ was observed with *C. curvatus*. Based on this value the minimal feed length was calculated to be of 20 h corresponding to a feeding rate of 0.45 mL min⁻¹. This feeding rate was applied in all fermentations to allow a direct comparison between the yeasts.

When needed, antifoam emulsion (Sigma-Aldrich, MO, USA) was added to prevent excess foam formation. Aliquots were collected at regular intervals to evaluate substrate concentration (HPLC analysis), optical density (OD₆₆₀), cell dry weight (CDW) and lipid content (flow-cytometry, gas chromatography, fluorescence microscopy and FTIR analysis).

Analytical methods

The optical density was measured at 660 nm (OD₆₆₀) with a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation). Samples collected at different times were centrifuged at 14000 rpm for 10 min. The supernatants were filtered (0.22 µm filter) and glycerol and methanol concentrations were HPLC determined using a Rezex ROA-Organic Acid (Phenomenex). The eluent was 0.01 M H₂SO₄ pumped at 0.5 mL min⁻¹ and column temperature was 35 °C. Separated components were detected by a refractive-index detector and peaks were identified by comparing with known standards (Sigma-Aldrich, St Louis, MO, USA).

Biomass was harvested by centrifugation of the culture samples at 4000 rpm for 10 min. The pellets were then washed twice with distilled water and dried at 40 °C (Concentrator 52301, Eppendorf, Germany) until a constant weight was obtained.

Additional biomass was also preserved for cellular lipids and fatty acids analysis.

Fluorescence microscopy and flow-cytometry analysis

Cell staining for lipids analysis was performed by using Nile Red (9-diethylamino-5-benzo[*a*]phenoxazinone) obtained from Sigma-Aldrich Co. (St Louis, MO, USA). As Nile Red is sensitive to light [48], it was always handled with reduced light conditions, and stored at 4 °C.

Briefly, a Nile Red stock solution (314 µM) was prepared by dissolving 0.1 mg of Nile Red in 1 mL acetone [29, 49]. Cells were washed twice with PBS buffer (0.05 M, pH 7.0) and Nile Red was then added at a final concentration of 31.4 µM in PBS. Before measurements, cells were incubated for 5 minutes in the dark at room temperature.

Flow-cytometry analysis were conducted using a Beckman Coulter FC-500 flow cytometer

(Beckman Coulter, Fullerton, CA, USA) equipped with an Argon ion laser (excitation wavelength 488 nm, laser power 20 mW). Upon excitation, NR exhibits intense yellow-gold fluorescence when dissolved in neutral lipids, and red fluorescence when dissolved in polar lipids [50]. The optical system used collect red light (>650 nm corresponding to polar lipids) in the FL3 channel. A total of 20,000 cells were measured for each sample using a log amplification of the fluorescent signal. Non-stained cells were used as auto-fluorescence control. Data analysis was performed afterwards with *Flowing software* (www.flowingsoftware.com) and *Cyflagic* (www.cyflagic.com).

Fluorescence microscopy studies were carried out with a Nikon Eclipse 90i (Nikon Instruments, Inc.). Nile Red fluorescence was registered using a 515- to 560-nm band-pass exciter filter. Images of stained cells were acquired both in dichroic and fluorescence mode.

Gas chromatography analysis

To determine the lipid content in yeast cells, lipids were extracted, based on the method of Bligh and Dyer [51] with modifications, and then analyzed through GC. Briefly, 10 OD (about 5×10^8 cells) of samples were centrifuged at 4000 rpm for 10 minutes and washed twice with 1 mL of distilled water. Pellets were then resuspended in 5 mL of MeOH/CHCl₃ (2:1) and mechanically disrupted twice using a French Press at 38,000 psi (Constant Cell Disruption System, Constant System Ltd). Then, 2 mL of citric acid and 3 mL of CHCl₃ were added to the samples. After mixing, the samples were centrifuged at 4000 rpm for 2 minutes and the upper phase was discarded. Derivation of methyl esters from fatty acids was as previously described [52]. Fatty acid methyl esters were analysed by gas

chromatography (DANI GC 1000. Alltech ECONO-CAPTM ECTM-WAX column, 30 m x 0.32 mm ID x 0.25 μ m). Starting from 100°C, the column temperature was headed to 200 at 10 °C min⁻¹, temperature was further increased to 245 °C at 5 °C min⁻¹ and then maintained for 1 min. Nonanoic acid was used as an internal standard. Fatty acids were identified by comparison of their retention times with those of standard (Sigma-Aldrich, St Louis, MO, USA), quantified based on their respective peak areas and normalized.

FTIR microspectroscopy

Yeast cells from *S. cerevisiae*, *C. curvatus*, *L. starkeyi* and *R. toruloides*, at different time points (T=0, T=28, T=48, T=72, T=144, T=168 and T=240 hours from the inoculum) were washed three times in distilled water to eliminate medium contamination. Approximately 3 μ L of the cell suspensions were then deposited onto an IR transparent BaF₂ support, and dried at room temperature for at least 30 minutes to eliminate the excess water. FTIR absorption spectra were acquired in transmission mode, between 4,000 and 700 cm⁻¹, by means of a Varian 610-IR infrared microscope coupled to the Varian 670-IR FTIR spectrometer (both from Varian Australia Pty Ltd), equipped with a mercury cadmium telluride (MCT) nitrogen-cooled detector. The variable microscope aperture was adjusted from approximately 60 μ m \times 60 μ m to 100 μ m \times 100 μ m). Measurements were performed at 2 cm⁻¹ spectral resolution; 25 KHz scan speed, triangular apodization, and by the accumulation of 512 scan co-additions. When necessary, spectra were corrected for residual water vapour absorption [31, 53].

Spectral analysis was conducted in the spectral range between 4,000 and 800 cm⁻¹. To this aim, second derivative spectra were obtained following

the Savitsky-Golay method (third-grade polynomial, 9 smoothing points), after a binomial 13 smoothing points of the measured spectra, using the GRAMS/32 software (Galactic Industries Corporation, USA).

To verify the reproducibility and reliability of the spectral results, more than three independent preparations were analyzed. In the Figures, reported data are representative of the independent experiments performed.

Principal component analysis of FTIR data

The PCA was independently performed on three ranges, 3050-2800, 1800-1350, and 1350-900. Since each range provides different and specific information, splitting the analysis on different ranges allows an easier interpretation of the PCA results. The correlation matrix was computed on standardized spectra (zero mean and standard deviation equal to 1) and diagonalized to get eigenvectors (loadings, v) sorted according to the magnitude of the corresponding eigenvalues (*Alvin Rencher and William F. Christensen, Method for Multivariate Data Analysis. ISBN*). In all cases, the first three eigenvectors already describe more than 90% of the total variance of the data. Principal components (scores) have been obtained projecting the original spectra on the orthogonal subspace defined by the first three eigenvalues. To quantify the distance from the lipids standards, the Euclidean distance in the three-dimensional principal components space was used:

$$d(\mathbf{p}_i^{K_t}, \mathbf{p}_j^S) = \sum_{m=1}^3 \sqrt{(p_{m,i}^{K_t} - p_{m,j}^S)^2}$$

Where \mathbf{p} indicates 3D principal component, K refers to the strains (*R. toruloides*, *C. curvatus*, *S. cerevisiae*, *L. starkeyi*), t corresponds to the time of growth (0 h or 144 h), m is the m -th principal

component and S indexes the standard lipids. Since each sample was measured multiple times the distance is computed between the i -th replica of sample K and the j -th replica of the standard S , obtaining the matrix $\mathbf{D}^{K_t, S} = [d(\mathbf{p}_i^{K_t}, \mathbf{p}_j^S)]$. An average value for the distance between K and S was obtained computing the median of all the $N(N-1)/2$ i - j pairs, $\tilde{d}(K_t, S) = \text{median}(\mathbf{D}^{K_t, S})$. Moreover to better quantify how much samples differ in terms of distance with the standard lipids, the percentage change between time 144 and 0 has been computed as :

$$\Delta(K_{144h,0h}, S) = \left[1 - \frac{\tilde{d}(K_{144h}, S)}{\tilde{d}(K_{0h}, S)} \right] \times 100$$

A positive value indicates that a given lipid standards contributes to the spectral profile changes of the sample at 144 hours compared to the time 0; this suggests that it is accumulated during growth. Since all the procedure is repeated for the three analyzed ranges, we indicate the percentage change as $\Delta(K_{144h,0h}, S)_R$, where R makes clear the range dependence. Finally, a unique average percentage change for each K - S pair is obtained averaging over the ranges. $\tilde{\Delta}(K_{144h,0h}, S) = \frac{1}{R} \sum_R \Delta(K_{144h,0h}, S)_R$.

Uncertainties have been estimated using a bootstrap procedure [54]. In particular, using 1000 bootstrap iterations, confidence intervals on the range specific percentage change, $\Delta(K_{144h,0h}, S)_R$, have been obtained using the bootstrap percentile method [55]. All data analyses were performed using the software package R (*Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>*), version 3.0.2. A pseudocode of the algorithm used for the bootstrap-PCA [54] we have implemented is given in the Additional files section (from Figure S3 to Figure S5).

List of abbreviations

C/N: carbon-to-nitrogen;

CDW: cell dry weight;

NR: Nile red;

FA: fatty acids;

FAME: fatty acids methyl esters;

FTIR: Fourier transform infrared;

GC: gas chromatography;

IR: infrared;

OD: optical density;

PBS: phosphate-buffered saline;

PCA: principal component analysis;

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LS carried out the shake-flasks and bioreactors experiments for lipid production and the fluorescent and GC analyses, participated in the evaluation of the data and in compiling the manuscript. RP carried out bioreactors experiments for lipid production and GC analyses. DA carried out the FTIR analyses, collaborated with the PCA analysis, participated in the evaluation of the data and in compiling the manuscript. AG carried out bioreactors experiments. PM performed the PCA analysis of the FTIR data. DP participated in the experimental work design and contributed to the data interpretation. PB conceived the study, participated in its design and compiled the manuscript. All the authors have read and approved the final manuscript.

Acknowledgments

D.A. acknowledges the University of Milano-Bicocca (Fondo Grandi Apparecchiature) for the acquisition of the FTIR spectrometer Varian 670-IR. The authors gratefully acknowledge Paolo Frati for his technical contribution. P.B. and D.P. acknowledge the support by FAR (Fondo di Ateneo per la Ricerca) of the University of Milano-Bicocca. L.S. acknowledges the PhD fellowship of the University of Milano-Bicocca.

Figure legends

Figure 1 – Growth profiles of *R. toruloides*, *C. curvatus* and *L. starkeyi* shake flasks cultivated with different concentrations of pure and crude glycerol

Growth curves (OD_{660 nm}) of *R. toruloides* (a), *C. curvatus* (b) and *L. starkeyi* (c) cells at 25°C and 220 rpm. Glycerol was used as sole carbon source at the final concentration of 100 g L⁻¹. Five different mix of pure and crude glycerol were evaluated: 100 % pure glycerol (A; dashed line and □), 80 % pure and 20% crude glycerol (B; continuous line and ●), 70 % pure and 30% crude glycerol (C; continuous line and ▲), 50 % pure and 50% crude glycerol (D; continuous line and ◆) and 100 % crude glycerol (E; continuous line and ■). Data are mean ± standard deviation (error bars) of three independent assays.

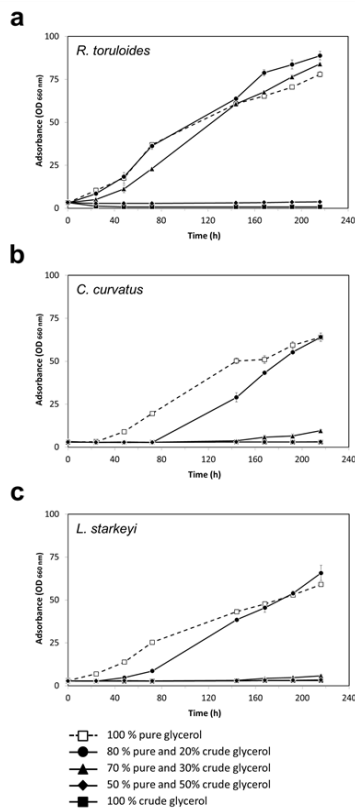


Figure 2 – Glycerol consumption and growth (CDW) profiles of *R. toruloides*, *C. curvatus* and *L. starkeyi* under fed-batch cultivation.

Glycerol consumption (g L^{-1}) and grown profiles (CDW; g L^{-1}) of *R. toruloides* (a), *C. curvatus* (b) and *L. starkeyi* (c) grown on pure (dashed line) and crude (continuous line) glycerol.

Data are mean \pm standard deviation (error bars) of three independent assays.

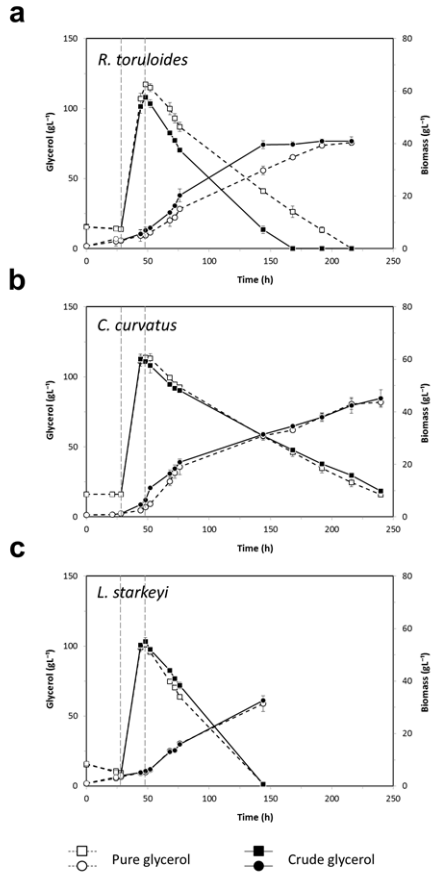


Figure 3 – Flow-cytometry analysis of *R. toruloides*, *C. curvatus* and *L. starkeyi*

Overlaid histograms of *R. toruloides* (a), *C. curvatus* (b) and *L. starkeyi* (c) cells grown on pure (left panels) and crude glycerol (right panels) stained with Nile Red and analyzed through flow-cytometry after 28, 48, 72, 144, 192 (only *R. toruloides* and *C. curvatus*) and 240 h (only *C. curvatus*). The fluorescence emission was measured in the FL3 channel (>650 nm corresponding to polar lipids). For each condition, an example of control (cells not stained) is reported.

Results shown are representative of three independent experiments, where the deviation from the X mean value was always less than 5 %.

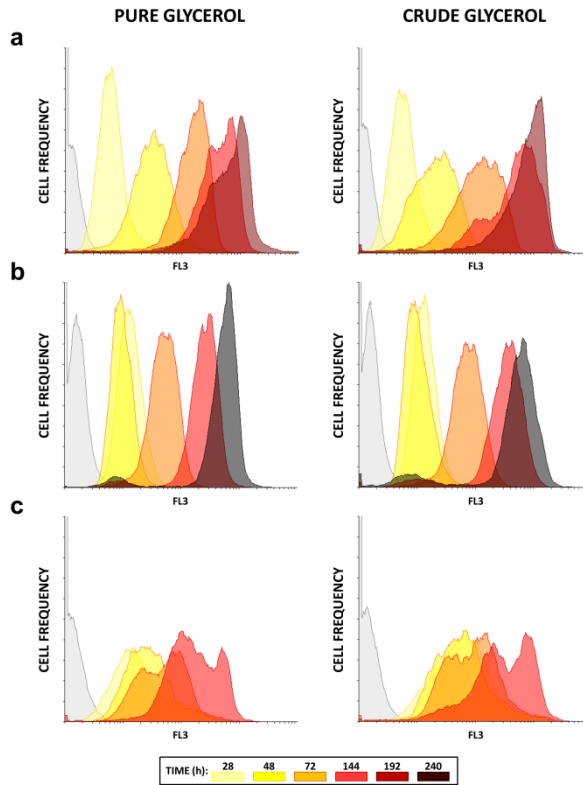


Figure 4 – Fluorescence microscope analysis of *R. toruloides*, *C. curvatus* and *L. starkeyi*

R. toruloides (a), *C. curvatus* (b) and *L. starkeyi* (c) cells were stained with Nile Red and observed under the microscope after 28, 72, 144 h. For each condition, fluorescence images and the corresponding dichroic image is reported. Since no significant differences in lipid bodies shape and number were observed between samples derived from pure and crude glycerol cultivations, the images here reported refers only to the first condition. Bar indicates 10 μm .

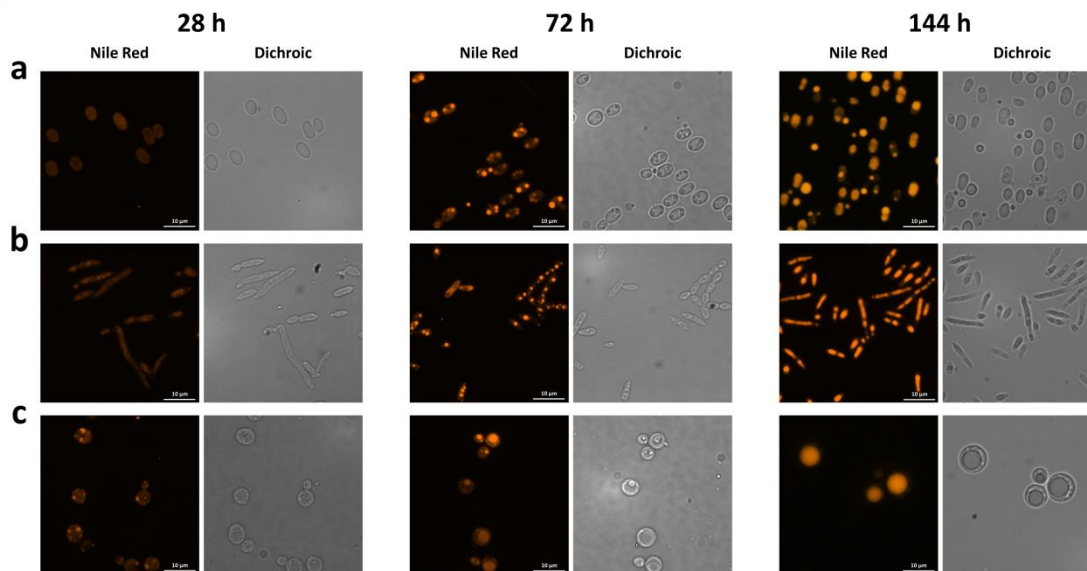


Figure 6 – Average percentage change.

Percentage change (Delta (144; 0 h, S), see Methods) are shown as averages across the three ranges, for *R. toruloides* (R.t.), *L. starkeyi* (L.s.), *C. curvatus* (C.c.) grown on puree glycerol (left panel) and crude glycerol (right panel). In addition, the average (across range) value of the gas chromatographic (GC) data is shown scaled in the range 0-100 for comparison. Error bars indicate the bootstrapped 95% confidence intervals.

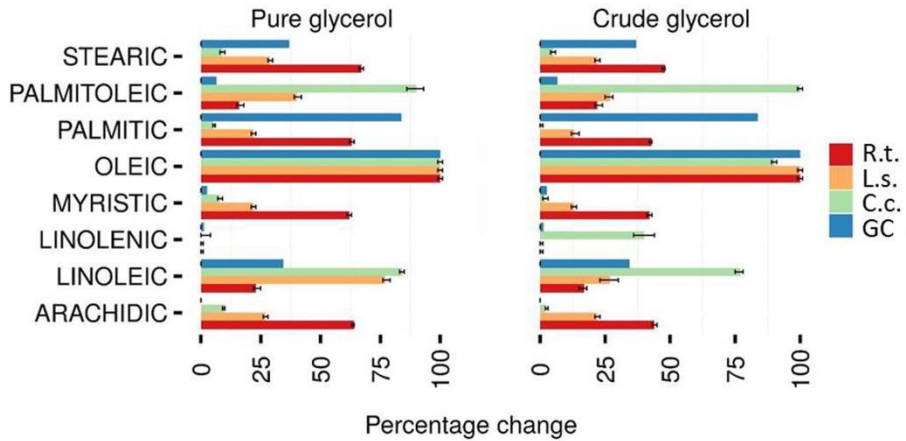


Figure 7 – FTIR analysis of yeast cell wall modifications during lipid accumulation.

The second derivatives of the FTIR spectra of yeast cells are reported in the spectral range comprised between 1200-950 cm^{-1} , mainly ascribable to the absorption of cell wall carbohydrates.

Spectra of control (a) and oleaginous yeasts *C. curvatus* (b) *R. toruloides* (c), and *L. starkeyi* (d) are displayed at time 0 and at 144 hours of growth in crude and pure glycerol. For comparison, the second derivative spectra have been normalized at the tyrosine band at $\sim 1516 \text{ cm}^{-1}$.

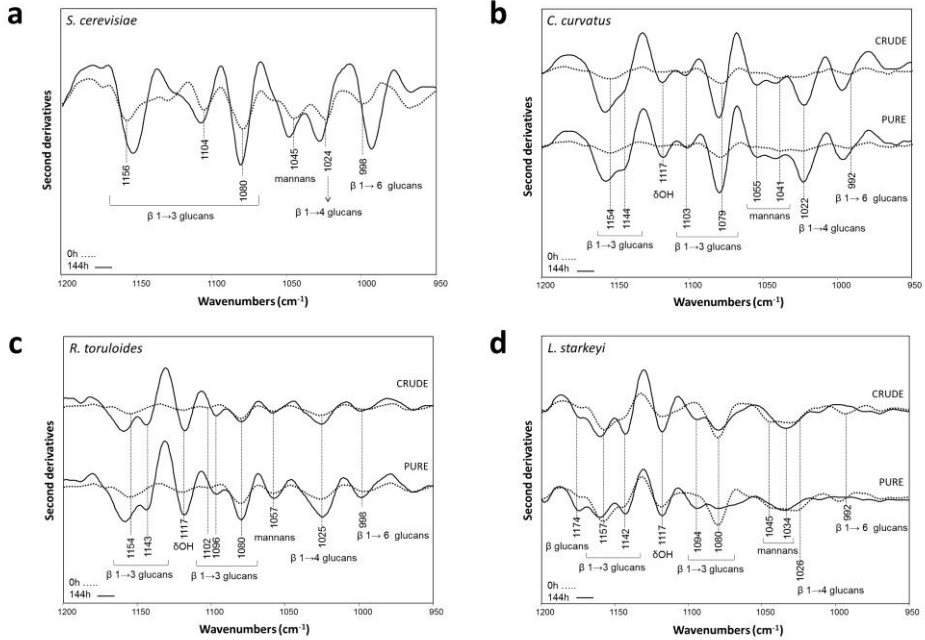


Table 1 – Comparison of parameters related to biomass and lipid production among *R. toruloides*, *C. curvatus* and *L. starkeyi* grown on pure and crude glycerol.

Yeast	Carbon source		Maximum DW (g L ⁻¹)	Biomass productivity (g L ⁻¹ h ⁻¹) ^a	Biomass Yield (g g ⁻¹)	Glycerol uptake rate (g L ⁻¹ h ⁻¹) ^a	Lipid (%)	Lipid productivity (g L ⁻¹ h ⁻¹)
<i>R. toruloides</i>	Pure Glycerol		40.4	0.21	0.33	0.73	60.9	0.13
	Crude Glycerol		41.0	0.24	0.36	0.98	60.0	0.15
<i>C. curvatus</i>	Pure Glycerol		43.7	0.21	0.38	0.52	46.9	0.10
	Crude Glycerol		45.1	0.18	0.37	0.48	50.9	0.09
<i>L. starkeyi</i>	Pure Glycerol		31.4	0.31	0.26	1.03	48.2	0.10
	Crude Glycerol		32.7	0.29	0.26	1.05	55.9	0.13

^aBiomass productivity as well as glycerol uptake rate were calculated starting to the end of the feeding.

Data shown are the mean of three independent experiments where the deviation from the mean value was less than 5 %.

Table 2. – Literature-cited results of *R. toruloides*, *C. curvatus* and *L. starkeyi* strains cultivated on various crude glycerol-based media during growth under various fermentation configurations.

Strain	Carbon source	Nitrogen source	Dry weight (g L ⁻¹)	Lipid (%)	Lipid productivity (g L ⁻¹ h ⁻¹)	Cultivation mode	Glycerol (g L ⁻¹)	Reference
R. toruloides Y4	Crude Glycerol	Hydrolysate from rapeseed meal	31.1	41.7	0.11	Fed-batch; 1-L bioreactor	-	[22]
			19.3	43.0	0.07	Batch; 1-L bioreactor	100	
	Pure Glycerol		35.3	46.0	0.14	Batch; 1-L bioreactor	100	
	43.0		45.8	0.17	Batch; 1-L bioreactor	100		
R. toruloides Y4	Crude Glycerol	Peptone and yeast extract	20.3	42.5	0.07	Flask	100	[24]
	Pure Glycerol		21.1	40.3	0.07	Flask	100	
R. toruloides AS2.1389	Crude Glycerol	(NH ₄) ₂ SO ₄ and Yeast extract	19.2	47.7	0.06	Flask	50	[18]
			20.1	42.9	0.05	Flask	50	
			26.7	69.5	0.09*	Batch; 5-L bioreactor	60	
			18.00	74.1	0.07*	Batch; 5-L bioreactor	60	
R. toruloides NRRL Y-27012	Crude Glycerol	Peptone and yeast extract	30.1	40.0	0.03	Flask	120	[19]
			23.8	47.0	0.04	Flask	95	
R. toruloides DSM 4444	Crude Glycerol	SFM hydrolysate	47.9	37.8	0.14	Fed-batch; 3.6-L bioreactor	-	[23]
		PSFM hydrolysate	37.4	51.3	0.17	Fed-batch; 3.6-L bioreactor	-	
C. curvatus ATCC 20508	Crude Glycerol	Corn steep liquor, baker's yeast autolysate and malt	50.4	45.0	0.17	Fed-batch; 30-L bioreactor	-	[5]
			58.9	43.0	0.19	Fed-batch; 6-L bioreactor	-	
			52.3	43.0	0.16	Fed-batch; 6-L bioreactor	-	

		extract	69.2	40.0	0.24	Fed-batch; 6-L bioreactor	-	
C. curvatus ATCC 20509	Crude Glycerol	Yeast extract	32.9	52.9	0.06	Two-stage fed-batch; 2-L bioreactor	-	[21]
C. curvatus ATCC 20509	Crude Glycerol	SFM hydrolysate	38.0	47.1	0.09	Fed-batch; 3.6-L bioreactor	-	[23]
		PSFM hydrolysate	34.6	50.0	0.11	Fed-batch; 3.6-L bioreactor	-	
L. starkeyi DSM 70296	Crude Glycerol	Peptone and yeast extract	34.4	35.9	0.03	Flask	120	[19]
			23.3	35.0	0.02	Flask	100	

* calculated considering fermentation time 195 h

Table 3 – Gas chromatography analysis of *R. toruloides*, *C. curvatus* and *L. starkeyi* lipids produced using crude and pure glycerol as a sole carbon source.

Peak areas less than 0.3% were considered insignificant

S, saturated; M, monounsaturated; P, polyunsaturated

Data are mean ± standard deviation of three independent assays

Yeast	Carbon source	Fatty acids composition (% wt/wt)											
		C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	C 18:3	Other	S	M	P	
<i>R. toruloides</i>	Pure Glycerol	1.5±0.1	27.9±0.1	2.1±0.1	12.3±0.1	35.3±0.1	17.4±0.2	2.8±0.1	0.7±0.1		42.4±0.2	37.4±0.1	20.2±0.2
	Crude Glycerol	1.5±0.1	27.5±0.1	2.0±0.3	12.5±0.2	37.8±0.1	15.8±0.2	2.2±0.1	0.7±0.1		42.2±0.1	39.8±0.4	18.0±0.3
<i>C. curvatus</i>	Pure Glycerol	1.6±0.3	29.6±0.1	3.7±0.3	18.6±0.1	27.6±0.2	14.9±0.1	1.9±0.2	2.1±0.6		51.9±0.1	31.3±0.1	16.8±0.2
	Crude Glycerol	1.6±0.3	30.6±0.1	4.0±0.3	15.4±0.1	31.2±0.1	14.5±0.1	1.6±0.1	1.1±0.1		48.7±0.4	35.2±0.4	16.1±0.1
<i>L. starkeyi</i>	Pure Glycerol	3.1±0.3	31.0±0.1	4.3±0.1	12.9±0.1	39.4±0.3	7.6±0.1	0.5±0.1	1.2±0.3		48.2±0.4	43.7±0.3	8.1±0.1
	Crude Glycerol	3.0±0.2	32.2±0.1	4.2±0.1	10.9±0.1	40.9±0.1	7.2±0.1	0.6±0.1	1.0±0.2		47.1±0.1	45.1±0.1	7.8±0.2

REFERENCES

1. Leiva-Candia DE, Pinzi S, Redel-Macias MD, Koutinas A, Webb C, Dorado MP: **The potential for agro-industrial waste utilization using oleaginous yeast for the production of biodiesel.** *Fuel* 2014, **123**:33-42.
2. Sitepu IR, Sestric R, Ignatia L, Levin D, German JB, Gillies LA, Almada LAG, Boundy-Mills KL: **Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species.** *Bioresource Technology* 2013, **144**:360-369.
3. Canakci M, Sanli H: **Biodiesel production from various feedstocks and their effects on the fuel properties.** *Journal of Industrial Microbiology & Biotechnology* 2008, **35**:431-441.
4. Meng X, Yang J, Xu X, Zhang L, Xian QN: **Biodiesel production from oleaginous microorganisms.** vol. 34. pp. 1-5. *Renewable Energy* 2008: 1-5.
5. Thiru M, Sankh S, Rangaswamy V: **Process for biodiesel production from *Cryptococcus curvatus*.** *Bioresource Technology* 2011, **102**:10436-10440.
6. Khot M, Kamat S, Zinjarde S, Pant A, Chopade B, RaviKumar A: **Single cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel.** *Microbial Cell Factories* 2012, **11**.
7. Galafassi S, Cucchetti D, Pizza F, Franzosi G, Bianchi D, Compagno C: **Lipid production for second generation biodiesel by the oleaginous yeast *Rhodotorula graminis*.** *Bioresource Technology* 2012, **111**:398-403.
8. Subramaniam R, Dufreche S, Zappi M, Bajpai R: **Microbial lipids from renewable resources: production and characterization.** *Journal of Industrial Microbiology & Biotechnology* 2010, **37**:1271-1287.
9. Freitas C, Nobre B, Gouveia L, Roseiro J, Reis A, da Silva TL: **New at-line flow cytometric protocols for determining carotenoid content and cell viability during *Rhodospiridium toruloides* NCYC 921 batch growth.** *Process Biochemistry* 2014, **49**:554-562.
10. Li Q, Du W, Liu D: **Perspectives of microbial oils for biodiesel production.** *Applied Microbiology and Biotechnology* 2008, **80**:749-756.
11. Ageitos JM, Vallejo JA, Veiga-Crespo P, Villa TG: **Oily yeasts as oleaginous cell factories.** *Applied Microbiology and Biotechnology* 2011, **90**:1219-1227.
12. Kosa M, Ragauskas AJ: **Lipids from heterotrophic microbes: advances in metabolism research.** *Trends in Biotechnology* 2011, **29**:53-61.
13. Li Y, Zhao Z, Bai F: **High-density cultivation of oleaginous yeast *Rhodospiridium toruloides* Y4 in fed-batch culture.** *Enzyme and Microbial Technology* 2007, **41**:312-317.
14. Koutinas AA, Chatzifragkou A, Kopsahelis N, Papanikolaou S, Kookos IK: **Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production.** *Fuel* 2014, **116**:566-577.
15. Zhou C-H, Beltramini JN, Fan Y-X, Lu GQ: **Chemoselective catalytic conversion of glycerol as a biorenewable source to valuable commodity chemicals.** *Chemical Society Reviews* 2008, **37**:527-549.
16. Celik E, Ozbay N, Oktar N, Calik P: **Use of biodiesel byproduct crude glycerol as the carbon source for fermentation processes by recombinant *Pichia pastoris*.** *Industrial & Engineering Chemistry Research* 2008, **47**:2985-2990.
17. Anand P, Saxena RK: **A comparative study of solvent-assisted pretreatment of biodiesel derived crude glycerol on growth and 1,3-propanediol production from *Citrobacter freundii*.** *New Biotechnology* 2012, **29**:199-205.
18. Xu J, Zhao X, Wang W, Du W, Liu D: **Microbial conversion of biodiesel byproduct glycerol to triacylglycerols by oleaginous yeast *Rhodospiridium toruloides* and the individual effect of some impurities on lipid production.** *Biochemical Engineering Journal* 2012, **65**:30-36.
19. Tchakouteu SS, Kalantzi O, Gardeli C, Koutinas AA, Aggelis G, Papanikolaou S: **Lipid production by yeasts growing on biodiesel-derived crude glycerol: strain selection and impact of substrate concentration on the fermentation**

- efficiency. *Journal of Applied Microbiology* 2015, **118**:911-927.
20. Oguri E, Masaki K, Naganuma T, Iefuji H: **Phylogenetic and biochemical characterization of the oil-producing yeast *Lipomyces starkeyi***. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* 2012, **101**:359-368.
 21. Liang Y, Cui Y, Trushenski J, Blackburn JW: **Converting crude glycerol derived from yellow grease to lipids through yeast fermentation**. *Bioresource Technology* 2010, **101**:7581-7586.
 22. Kiran EU, Trzcinski A, Webb C: **Microbial oil produced from biodiesel by-products could enhance overall production**. *Bioresource Technology* 2013, **129**:650-654.
 23. Leiva-Candia DE, Tsakona S, Kopsahelis N, Garcia IL, Papanikolaou S, Dorado MP, Koutinas AA: **Biorefining of by-product streams from sunflower-based biodiesel production plants for integrated synthesis of microbial oil and value-added co-products**. *Bioresource Technology* 2015, **190**:57-65.
 24. Yang X, Jin G, Gong Z, Shen H, Bai F, Zhao ZK: **Recycling biodiesel-derived glycerol by the oleaginous yeast *Rhodospiridium toruloides* Y4 through the two-stage lipid production process**. *Biochemical Engineering Journal* 2014, **91**:86-91.
 25. Ami D, Posterl R, Merighetti P, Porro D, Doglia SM, Branduardi P: **Fourier transform infrared spectroscopy as a method to study lipid accumulation in oleaginous yeasts**. *Biotechnology for Biofuels* 2014, **7**.
 26. Anschau A, Xavier MCA, Hernalsteens S, Franco TT: **Effect of feeding strategies on lipid production by *Lipomyces starkeyi***. *Bioresource Technology* 2014, **157**:214-222.
 27. Johnsson O, Andersson J, Liden G, Johnsson C, Hagglund T: **Feed rate control in fed-batch fermentations based on frequency content analysis**. *Biotechnology Progress* 2013, **29**:817-824.
 28. Saenge C, Cheirsilp B, Suksarogee TT, Bourtoom T: **Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids**. *Process Biochemistry* 2011, **46**:210-218.
 29. Kimura K, Yamaoka M, Kamisaka Y: **Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence**. *Journal of Microbiological Methods* 2004, **56**:331-338.
 30. Naganuma T, Uzuka Y, Tanaka K: **Using inorganic elements to control cell-growth and lipid-accumulation in *Lipomyces starkeyi***. *Journal of General and Applied Microbiology* 1986, **32**:417-424.
 31. Ami D, Natalello A, Doglia SM: **Fourier transform infrared microspectroscopy of complex biological systems: from intact cells to whole organisms**. *Methods in molecular biology (Clifton, NJ)* 2012, **895**:85-100.
 32. Casal HL, Mantsch HH: **Polymorphic phase-behavior of phospholipid-membranes studied by infrared-spectroscopy**. *Biochimica Et Biophysica Acta* 1984, **779**:381-401.
 33. Arrondo JLR, Goni FM: **Infrared studies of protein-induced perturbation of lipids in lipoproteins and membranes**. *Chemistry and Physics of Lipids* 1998, **96**:53-68.
 34. Barth A: **Infrared spectroscopy of proteins**. *Biochimica Et Biophysica Acta-Bioenergetics* 2007, **1767**:1073-1101.
 35. D. Ami PMA: **Multivariate Analysis for Fourier Transform Infrared Spectra of Complex Biological Systems and Processes**. *Multivariate Analysis in Management, Engineering and the Sciences: InTech*; 2013.
 36. Zimkus A, Misiunas A, Chaustova L: **Li+ effect on the cell wall of the yeast *Saccharomyces cerevisiae* as probed by FT-IR spectroscopy**. *Central European Journal of Biology* 2013, **8**:724-729.
 37. Galichet A, Sockalingum GD, Belarbi A, Manfait M: **FTIR spectroscopic analysis of *Saccharomyces cerevisiae* cell walls: study of an anomalous strain exhibiting a pink-colored cell phenotype**. *Fems Microbiology Letters* 2001, **197**:179-186.
 38. Latge J-P: **The cell wall: a carbohydrate armour for the fungal cell**. *Molecular Microbiology* 2007, **66**:279-290.
 39. Naumann D: **Infrared Spectroscopy in Microbiology**. R.A. Meyers (Ed.) edition. *Encyclopedia of Analytical Chemistry* 2000.

40. Wang TD, Triadafilopoulos G, Crawford JM, Dixon LR, Bhandari T, Sahbaie P, Friedland S, Soetikno R, Contag CH: **Detection of endogenous biomolecules in Barrett's esophagus by Fourier transform infrared spectroscopy.** *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**:15864-15869.
41. Michell AJ, Scurfiel G: **An assessment of infrared spectra as indicators of fungal cell wall composition.** *Australian Journal of Biological Sciences* 1970, **23**:345-&.
42. Reis EM, Coelho RS, Grimaldi R, Anschau A, Cacia Ferreira Lacerda LM, Chaar J, Franco TT: **In Situ Transesterification from Oleaginous Yeast Biomass.** *Ibic2014: 4th International Conference on Industrial Biotechnology* 2014, **38**:319-324.
43. Knothe G: **Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters.** *Fuel Processing Technology* 2005, **86**:1059-1070.
44. Tanimura A, Takashima M, Sugita T, Endoh R, Kikukawa M, Yamaguchi S, Sakuradani E, Ogawa J, Shima J: **Selection of oleaginous yeasts with high lipid productivity for practical biodiesel production.** *Bioresource Technology* 2014, **153**:230-235.
45. Chandrasekar SSaS: **Predictive Equations for Viscosity and Cetane Number of Biodiesel.** vol. Vol. 1. pp. pp. 23-28: German journal of renewable and sustainable energy research (Gjrser); 2013: pp. 23-28.
46. Brambilla L, Bolzani D, Compagno C, Carrera V, van Dijken JP, Pronk JT, Ranzi BM, Alberghina L, Porro D: **NADH reoxidation does not control glycolytic flux during exposure of respiring *Saccharomyces cerevisiae* cultures to glucose excess.** *Fems Microbiology Letters* 1999, **171**:133-140.
47. Braunwald T, Schwemmlin L, Graeff-Hoenninger S, French WT, Hernandez R, Holmes WE, Claupein W: **Effect of different C/N ratios on carotenoid and lipid production by *Rhodotorula glutinis*.** *Applied Microbiology and Biotechnology* 2013, **97**:6581-6588.
48. Greenspan P, Fowler SD: **Spectrofluorometric studies of the lipid probe, Nile red.** *Journal of Lipid Research* 1985, **26**:781-789.
49. Greenspan P, Mayer EP, Fowler SD: **Nile red - A selective fluorescent stain for intracellular lipid droplets.** *Journal of Cell Biology* 1985, **100**:965-973.
50. Shapiro HM, Shapiro HM: **Practical flow cytometry, Third edition.** *Practical flow cytometry, Third edition* 1995:xxxviii+542p-xxxviii+542p.
51. Bligh EG, Dyer WJ: **A rapid method of total lipid extraction and purification.** *Canadian journal of biochemistry and physiology* 1959, **37**:911-917.
52. Holub BJ, Skeaff CM: **Nutritional regulation of cellular phosphatidylinositol.** *Methods in Enzymology* 1987, **141**:234-244.
53. Natalello A, Ami D, Doglia SM: **Fourier transform infrared spectroscopy of intrinsically disordered proteins: measurement procedures and data analyses.** *Methods in molecular biology (Clifton, NJ)* 2012, **895**:229-244.
54. Timmerman ME, Kiers HAL, Smilde AK: **Estimating confidence intervals for principal component loadings: A comparison between the bootstrap and asymptotic results.** *British Journal of Mathematical & Statistical Psychology* 2007, **60**:295-314.
55. Davison AC, Hinkley, D.V.: **Bootstrap Methods and Their Application.** Cambridge University; 1997.

Chapter IV.

ENHANCED TOLERANCE TO LIGNOCELLULOSE-DERIVED INHIBITORS IN *Lipomyces starkeyi* BY ADAPTIVE LABORATORY EVOLUTION

Keywords: *L. starkeyi*, adaptive laboratory evolution (ALE), inhibitors, acetic acid, furfural, hydroxymethylfurfural (HMF), pretreated material.

BACKGROUND

Lignocellulosic biomass is the most relevant renewable resource for biofuels and chemicals production. However, presence of different carbon sources (e.g. glucose, mannose, arabinose, xylose) as well as the presence of inhibitory compounds derived from biomass pretreatment (e.g. HMF, furfural, acetic acid) makes lignocellulosic hydrolysates challenging substrates for the growth of many microorganisms.

Several researches have been conducted to find suitable microbial cell factories that can directly grow on lignocellulosic hydrolysates, converting the sugars into the value-added compounds of interest.

In this context, the oleaginous yeast *L. starkeyi* represents an interesting microbial cell factory for biorefinery applications. This yeast is able to efficiently convert glucose as well as the pentoses xylose and arabinose. Moreover, it is able to accumulate lipids over 70 % of its dry cell weight [1]. However, its limited tolerance against high inhibitors concentration still constitutes an obstacle for an efficient conversion of lignocellulosic biomasses.

Application of rational metabolic engineering approaches to increase *L. starkeyi* robustness is limited by the absence of a published genome sequence as well as of

efficient transformation protocols. Moreover, a rational approach requires a deep understanding of the pathways and mechanisms behind the phenotype of interest [2, 3].

The application of non-targeted strategies such as adaptive laboratory evolution (ALE) represents a possible alternative approach. ALE has successfully been performed to increase the robustness of *S. cerevisiae* to both hydrolysates [4] as well as specific inhibitors such as furfural [5] and HMF [6].

The aim of the current work was to increase the robustness of *L. starkeyi* by improving the tolerance towards lignocellulose-derived inhibitors through an adaptive laboratory evolution approach.

In particular, here we describe the generation and selection of tolerant isolates.

RESULTS AND DISCUSSION

Evaluation of *L. starkeyi* tolerance to inhibitory compounds

It has been reported that different yeasts have inherent different mechanisms to counteract the negative impact of inhibitory compounds. To evaluate the tolerance of *L. starkeyi* (DSM70295) to inhibitors derived from biomass pretreatment, preliminary shake flasks experiments were performed.

The first set of experiments was performed using a defined mineral medium supplemented with the main inhibitory compounds derived from biomass pretreatment: acetic acid, furfural and HMF. In particular, according to concentrations reported in literature [7] different cultivation media were formulated: Medium A (acetic acid 2 g L⁻¹, furfural 0.15 g L⁻¹ and HMF 0.33 g L⁻¹), Medium B (acetic acid 2 g L⁻¹, furfural 0.25 g L⁻¹ and HMF 0.75 g L⁻¹), Medium C (acetic acid 2 g L⁻¹, furfural 0.5 g L⁻¹ and HMF 1.5 g L⁻¹) and Medium D (acetic acid 4 g L⁻¹,

furfural 0.25 g L^{-1} and HMF 0.75 g L^{-1}). The second set of experiments was performed using defined media containing different percentage of the liquid fraction of steam-pretreated *Arundo donax*: Medium E (pretreated material 10 %), Medium F (pretreated material 20 %) and Medium G (pretreated material 50 %). Higher percentages of pretreated material were also evaluated and proved to be strongly inhibitory for cell growth (data not shown).

For all formulations, the initial concentration of sugars was fixed at 20 g L^{-1} (10 g L^{-1} of glucose and 10 g L^{-1} of xylose). Strains were cultivated in batch mode at $30 \text{ }^{\circ}\text{C}$, 220 rpm and pH 5.5 (adjusted with NaOH and buffered with citric acid), monitoring biomass over time (optical density).

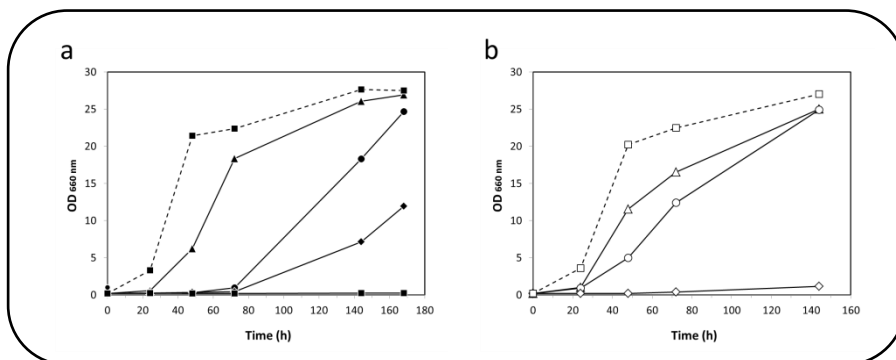


Figure 1 – Growth profiles of *L. starkeyi* shake flasks cultivated in defined medium added with different concentrations of inhibitors (a) or different percentages of pretreated lignocellulosic material (b).

Growth curves (OD_{660 nm}) of *L. starkeyi* cells at $30 \text{ }^{\circ}\text{C}$ and 220 rpm. Glucose and xylose were used as carbon source at the final concentration of 10 g L^{-1} each. Different concentrations of inhibitors or different percentages of pretreated material were evaluated: (a) Medium A (acetic acid 2 g L^{-1} , furfural 0.15 g L^{-1} and HMF 0.33 g L^{-1} ; filled triangles, continuous line); Medium B (acetic acid 2 g L^{-1} , furfural 0.25 g L^{-1} and HMF 0.75 g L^{-1} ; filled circles, continuous line); Medium C (acetic acid 2 g L^{-1} , furfural 0.5 g L^{-1} and HMF 1.5 g L^{-1} ; filled diamonds, continuous line); Medium D (acetic acid 4 g L^{-1} , furfural 0.25 g L^{-1} and HMF 0.75 g L^{-1} ; filled squares, continuous line). (b) Medium E (pretreated material 10 %; empty

triangles, continuous line); Medium F (pretreated material 20 %; empty circles, continuous line); Medium G (pretreated material 50 %; empty diamonds, continuous line). During these experiments a control condition with defined mineral media without inhibitors was also performed: (a) Control A (filled squares, dashed line) and (b) Control B (empty squares, dashed line).

As shown in Figure 1a, in Medium A (acetic acid 2 g L^{-1} , furfural 0.15 g L^{-1} and HMF 0.33 g L^{-1}) *L. starkeyi* was able to grow even if with a lag phase of almost 20 h. An increased concentrations of HMF and furfural until 0.5 and 1.5 g L^{-1} , respectively, resulted in an extension of the lag phase (Medium B and C; Figure 1a). A further increase in the concentration of acetic acid from 2 to 4 g L^{-1} (Medium D) resulted in the suppression of the cell growth. Those reported in Figure 1a are representative growth curves. Additional kinetics were performed to evaluate the effect of single inhibitors as well as of intermediate concentrations: low acetic acid concentrations (around about 1 g L^{-1}) were able to stimulate cell growth and higher concentration of furfural and HMF, compared to those used in Medium C, suppressed cell growth (data not shown).

In Figure 1b it is possible to observe that 20 % of pretreated material (Medium F) was sufficient to produce a lag phase of almost 20 h and 50 % caused a suppression of cell growth (Medium G). This is interesting if we consider that concentrations of acetic acid, furfural and HMF used in Medium G were lower than those of Medium B. This suggests the presence in the pretreated material of others inhibitory compounds and underline once more the importance of the synergic effect of inhibitors.

Overall, these preliminary experiments were useful in establishing the starting operative condition for the evolution strategies.

Shake flask evolution

The evolution process in shake flasks was performed by adopting two different strategies. For the first strategy the medium was changed when cells reached the early exponential growth phase (~ 24 h), while for the second strategy the transfer was operated during the exponential growth phase (see Material and Methods for details). Consequently, in both strategies cells were systematically exposed to non-metabolized forms of the inhibitors (e.g. non-reduced forms of the furaldehyde inhibitors) and this was expected to aid the development of inhibitor tolerance. Starting from this condition, the selection pressure (concentration of inhibitors or percentage of pretreated material) was progressively increased to select for improved phenotypes.

A significant difference between the two strategies was related to the nature of the inhibition: in the first strategy, cells were grown in minimal medium added with inhibitory compounds (acetic acid, furfural and HMF) at different concentration, while in the second strategy the mineral medium was directly supplemented with different percentages of the liquid fraction of steam-pretreated *Arundo donax*. On the one hand the use of a mineral media with the addition of the inhibitory compounds of interest allows to evaluate the effect of every single compound and fine-tuning the inhibition. On the other hand, the use of pretreated material addresses the need to develop industrial yeasts strains that are tolerant to the cocktail of inhibitory compounds arising from biomass pretreatment.

The majority of the batches was started with an initial OD₆₆₀ of 0.2. Some batches were started with a lower inoculum size (OD₆₆₀ ~ 0.1 or ~ 0.05). This because, as reported in literature [8], not only the concentrations of inhibitors but also the concentration of the initial biomass has a direct effect on the inhibitor conversion rate. Therefore a lower inoculum was used to select for cells with better detoxification capacity and/or cells with an improved tolerance [6].

In both strategies already after 4 to 6 batches a reduction of the lag phase was observed. It was established that a marked reduction of the lag phase was taken as the signal to increase the selection pressure, by increasing either the concentration of inhibitors or the percentage of pretreated material. The first rounds of division after the transferring were characterized by a longer lag phase and a decrease of the growth rate, indicating the effect of severe conditions of stress for the cells. However, usually after 2 to 4 batches it was possible to restore the starting growth parameters.

The first strategy (S1) was started using 2 g L^{-1} of acetic acid, 0.15 g L^{-1} of furfural and 0.3 g L^{-1} of HMF and ended after 68 round of evolution with 3 g L^{-1} , 0.33 g L^{-1} and 1 g L^{-1} of acetic acid, furfural and HMF, respectively. In the second strategy (S2), after 46 round of evolution the percentage of pretreated material was increased from 25 % to 45 %.

At the end of each batch, spot assays were performed on plates with minimal medium and pretreated material or acetic acid, furfural and HMF. Once a week, these spot assay were used also to compare intermediate results of the two evolution strategies (Figure 2), as well as for storing samples in order to preserve a backup of intermediate evolution steps. The control strain was not included since as observed in preliminary experiments (data not shown) it was not able to grow on plates used for these comparative experiments.

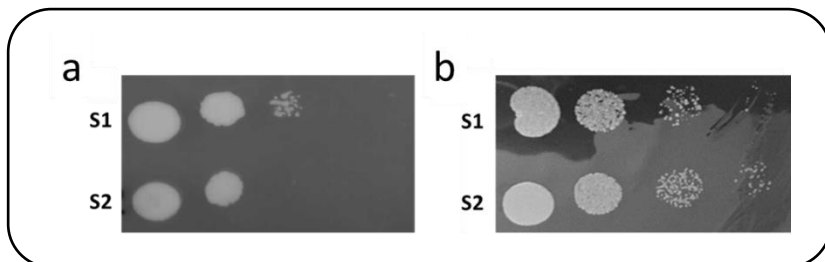


Figure 2. - Spots assay of cells deriving from the two evolution strategies.

Spot assay of cells deriving from S1 and S2 after 27 and 10 batch of evolution, respectively. (a) Plate with defined medium containing acetic acid 1 g L^{-1} , furfural 0.25 g L^{-1} and HMF 0.5 g L^{-1} . (b) Plate with defined medium containing pretreated material 30 %;

In Figure 2 it is possible to observe that the strains deriving from the two evolution strategies were able to grow on both plates but with some differences depending on the adopted selective pressure.

In particular, on plates with pretreated material (Figure 2b), cells derived from the S1 grew more slowly compared to cells of S2. Results were instead the opposite when cell were grown on plates with the addition of acetic acid, furfural and HMF (Figure 2a).

This result underline how the experimental set up directly affects cells adaptation.

After a total of 68 and 46 sequential batch cultivations, for the first and the second strategy, respectively, the experiment was ended and the pertinence and progress of the adaptation was evaluated.

Evaluation of isolates deriving from repeated batch in shake flasks

Upon conclusion of the adaptive evolution experiments, two isolates for every strategy (S1 and S2) were chosen for further analysis aimed to evaluate the results of adaptation.

Three different comparison methods (from Method A to Method C; see Materials and Methods for details) were chosen to evaluate the performance of the isolated strains in the presence of inhibitory compounds. The comparison methods differed only in the increased number of re-inoculum in medium without inhibitory compound, aimed at discriminating between adaptation and a robust genetic inherited robustness.

The first set of experiments was performed with strains isolated from the first evolution strategy. Both the tested clones showed a significant variability in their performance during biological replicates (data not shown). In general, with Method A the adapted strains were always able to grow at similar levels, while the parental strain could not resume growth. Instead, a decreased growth capability was often observed with Method B and the growth profiles were similar to that of the parental strain with Method C. Because of this, they were not considered for further characterization.

The second set of experiments was performed with strains isolated from the second evolution strategy. Comparison experiments were performed on plate with different percentages of pretreated materials.

Also in this case the increase in re-inoculum steps in defined media without inhibitory compounds lead to a decrease of the growth capability once strains were inoculated in presence of pretreated material. However, even after two or more re-inocula the adapted strains showed a better growth capability compared to the parental strain.

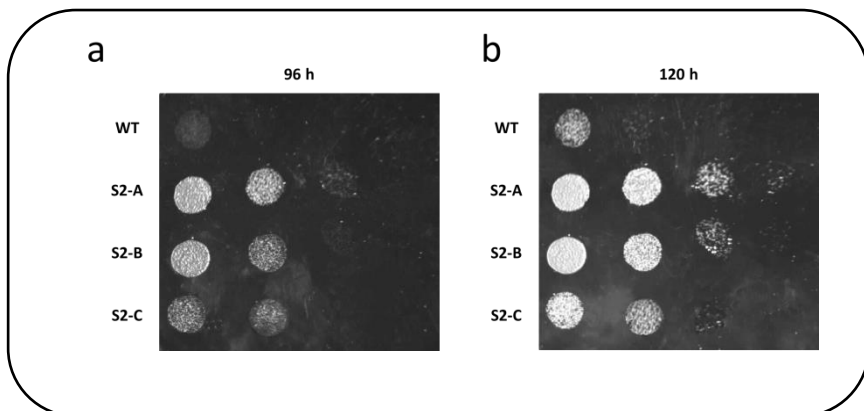


Figure 3. - Spot assay for the evaluation of the strain isolated through S2.

Spot assay of the parental strain (WT) and the adapted strain isolated from S2 after 92 (a) and 120 h (b). The adapted strain was cultivated according Method A (S2-A), Method B (S2-B) and Method C (S2-C). Plates were formulated with 20 % of pretreated material.

As shown in Figure 3 after 96 and 120 h of incubation the strain derived from Method A showed a remarkable growth advantage compared to the parental strain. Increasing the number of re-inocula, the growth advantage despite significant, decreased.

What is more important is that the same trend was observed during replicate experiments and also increasing the steps without inhibitors the growth advantage compared to the parental strain was always relevant.

Despite interesting, these results underline how the selection protocol of the evolved strains was not adequate and how, in this experimental set up, it was adaptation to play a key role.

Evolution on plate

During shake flasks cultivations under batch mode it was possible to increase progressively the selection pressure. However, we observed that a targeted selection of the more robust clone was difficult to achieve. Therefore, cells originating from the S2 strategy were subjected to further evolution and selection on plate. In particular, three different evolution protocols (from SP1 to SP3) were tested (see Material and Methods for details).

For the SP2 and SP3 strategies the growth on plate was preceded by a cultivation step in media without inhibitors. The aim was to select strains whose increased robustness was directly linked to genotypic mutations and not to an environmental adaptation.

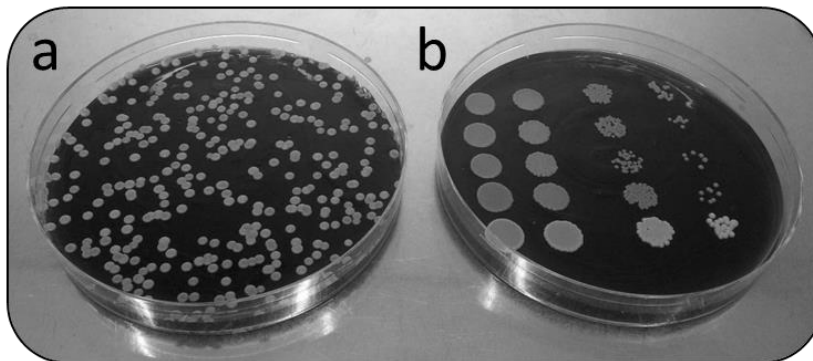


Figure 4. - Representative image of plates deriving from the application of the different strategies.

Cells deriving from SP3 (a) and SP2 (b) strategies after 144 h of incubation on plates with 25 % of pretreated material.

In Figure 4 it is possible to observe a representative image of plates deriving from the application of the SP3 (a) and SP2 (b) strategies after 144 h of incubation. Cells were selected on agar plates containing 25 %, 35 % and 45 % (v/v) of pretreated material. The biggest colonies deriving from the harsh experimental condition were used for the next round of evolution.

Independently from the applied strategy, after one round of evolution no growth was observed on plates with 35 % and 45 % of pretreated material. After 8 rounds of evolution it was possible to isolate a consistent number of colonies growing on plates with 35 % of pretreated material (SP1, SP2 and SP3) and few colonies growing on 45 % of pretreated material (SP2).

It is important to notice that a direct comparison between shake flasks and plates experiments was not possible even if the same amount of pretreated material was used. In general, during plate experiments a stronger inhibitory effect of pretreated material was observed. This may be due to the lower oxygen availability and consequently affect cells detoxification capability.

After 8 rounds we started evaluating the progress of evolution on plate.

Evaluation of isolates on plates

The best clones deriving from the three evolution strategies on plate (SP1, SP2 and SP3), stored at $-80\text{ }^{\circ}\text{C}$, have been evaluated for their growth capability in shake flask experiments.

Comparison experiments were performed in 250 mL flasks (50 mL of liquid medium) at $30\text{ }^{\circ}\text{C}$, 220 rpm and pH 5.5 (buffered with citric acid), monitoring biomass over time (optical density).

In particular, the best clone deriving from every strategy (named SP1a, SP2a and SP3a) was plated, together with the parental strain, into solid mineral medium. A single colony of each strain was used to inoculate shake flasks without inhibitors. Cells of the parental and adapted strains in exponential phase were then used to inoculate liquid cultures with 30 and 40 % (v/v) of pretreated material and their growth was followed over time (Figure 5).

The same viability at 0 h was verified by a PI staining performed just after the shake flask inoculum (data not shown).

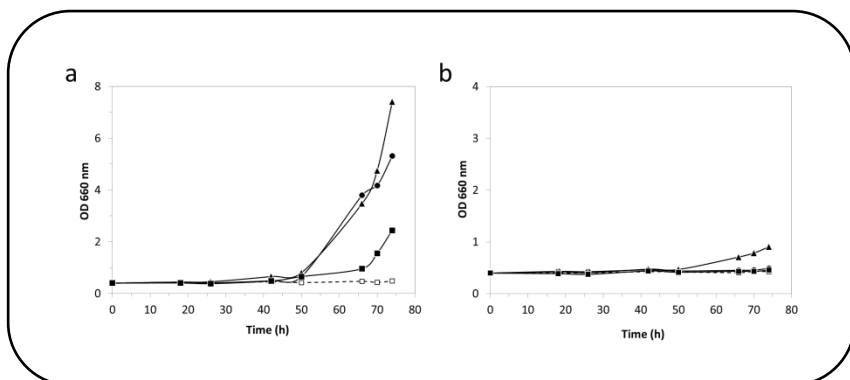


Figure 5 – Growth profiles of *L. starkeyi* parental and evolved strains cultivated with different percentages of pretreated material.

Growth curves (OD_{660 nm}) of *L. starkeyi* parental and evolved strains during shake flasks experiments on 30 (left panel) and 40 % (right panel) of pretreated material. During these experiments the parental strain (dashed line, empty squares) and the best clones deriving from SP1 (SP1a; continuous line, filled circles), SP2 (SP2a; continuous line, filled triangles) and SP3 (SP3a; continuous line, filled squares) were examined.

As shown in Figure 5a with 30 % of pretreated material all the adapted clones were able to grow, while the parental strain could not resume growth even after 74 h of incubation. In particular, after 74 h clones deriving from the SP1, SP2 and SP3 strategies were able to reach an OD of 5.3, 7.4 and 2.4, respectively.

Instead, with 40 % of pretreated material (Figure 5b) only the clone derived from the SP2 strategy was able to grow, even if it was able to reach only an OD₆₆₀ of 0.91 after 74 h of incubation.

To conclude, preliminary results suggests that all the tested clones derived from evolution experiments on plate have an higher tolerance to the inhibitory compounds contained in the pretreated material compared to the parental strain.

CONCLUSION

Through an adaptive laboratory evolution approach it was possible to isolate three *L. starkeyi* strains with an enhanced tolerance towards inhibitory compounds derived from the lignocellulosic biomass pretreatment.

Comparison experiments as well as screening for more tolerant isolates are still ongoing.

Since pretreated biomass contains a complex mixture of inhibitors, adaptation of the evolved strains could be a result of enhanced resistance to one or more of the inhibitors.

Because of new technologies including transcriptional profiling and massive next-generation DNA sequencing (NGS), phenotype-genotype correlations could be obtained by whole genome resequencing (WGS), despite a good genome assembly is needed. Transcriptomic analysis could help in elucidate the mechanisms for enhanced tolerance in the isolated adaptive mutants.

In conclusion, *L. starkeyi* natural capability to utilize a wide range of sugars associated with an increased tolerance to inhibitory compounds gained through adaptive evolution may contribute to render it a reliable microbial host for sustainable production of industrially relevant compounds.

MATERIALS AND METHODS

Strains and media

L. starkeyi (DSM70295) was purchased from DSMZ. The yeast was stored in cryotubes at -80°C, in 20% glycerol (v^v-1).

Shake flasks experiments were carried out in minimal medium containing (per liter): 1 g of yeast extract (0.114 g of nitrogen), 1.31 g of (NH₄)₂SO₄ (0.278 g of nitrogen), 0.95 g of Na₂HPO₄, 2.7 g of KH₂PO₄, 0.2 g of Mg₂SO₄·7H₂O, 0.04 g. After the pH was adjusted to 5.5 using NaOH 4 M, the medium was supplemented with a 100X trace mineral stock solution consisting of (per liter): 4 g CaCl₂·2H₂O; 0.55 g FeSO₄·7H₂O; 0.52 g citric acid; 0.10 g ZnSO₄·7H₂O; 0.076 g MnSO₄·H₂O; and 100 μL of 18M H₂SO₄. Glucose and xylose were used as carbon and energy sources at the final concentration of 10 g L⁻¹ each. For the evolution experiments the inhibitor cocktail (acetic acid, furfural and HMF) or the pretreated material (*Arundo donax*) were added to minimal media to achieve the desired concentrations.

Yeast extract was provided by Biolife Italiana S.r.l., Milan, Italy. All the others reagents were provided by Sigma-Aldrich Co., St Louis, MO, USA.

Pretreated material

Arundo donax was obtained from Mossi & Ghisolfi (Italy) and consisted of the non-detoxified liquid fraction of steam-pretreated *Arundo donax*.

The pretreated material was diluted to different concentrations (% v/v) with defined mineral medium according to the experimental requirements.

The major inhibitory compounds of the hydrolysate before dilution were (g L⁻¹): acetic acid 4.15, furfural 0.27 and HMF 0.25. The pH of the material was 3.9 and was adjusted to 5.5 with 8 M NaOH prior to use.

Shake flask evolution experiment: cultivation conditions

Starting from a fresh YPD agar plate, a single colony of the parental strain was used to inoculate a 100 mL flask with 20 mL of defined medium. The pre-culture was grown overnight and once exponential growth phase was reached the appropriate volume from the pre-culture was used to inoculate the evolution flasks at the OD₆₆₀ of 0.2.

Evolution experiments were conducted in 100 mL (strategy one) or 250 mL (strategy two) Erlenmeyer flasks containing 50 mL or 20 mL of liquid medium, respectively.

For all the shake flasks cultivations the temperature was set at 30 °C and stir at 220 rpm. The pH of the medium was adjusted to 5.5 with 8 M NaOH and then maintained using a citrate buffer solution pH 5.5 at the final concentration of 0.1 M. Cell growth was followed by measuring every day the OD of the culture broth at 660 nm.

Possible contamination of the cultivation was checked by ocular inspection at the microscope.

After each round, spot assays were performed and cell samples were withdrawn and stored in glycerol vials at - 80°C for further evaluation.

Shake flask evolution experiment: strategy one (S1)

According to preliminary experiments, the initial inhibitors concentration was set at 2 g L⁻¹ of acetic acid, 0.15 g L⁻¹ of furfural and 0.3 g L⁻¹ of HMF.

The first strategy consisted in serial transfer of the cells every 24 h, when cells were in the early exponential growth phase (corresponding to the beginning of the growth phase in which cells divided as fast as possible, according the experimental conditions.).

Depending on the yeast growth, the concentration of inhibitors was further increased when an adaptive event (reduced lag phase and/or increased specific growth rate) was observed.

With this strategy, every 24 h the appropriate volume of culture was used to inoculate fresh medium (with inhibitors) to a target OD₆₆₀ of 0.2. Due to the impossibility to follow the cells during the weekend, after four serial transfer (one every 24 h) cells were used to inoculate at 0.2 OD₆₆₀ three shake flasks with different concentrations of inhibitors. The flasks were then incubated over-weekend and after 72 h the flask with the highest amount of inhibitors and in which the cells were able to grow was used as inoculum for the new week of evolution.

This experiment was conducted for 68 round.

Shake flask evolution experiment: strategy two (S2)

According to preliminary experiments, the initial amount of pretreated material was set at 20 % (v/v). In this strategy cells were serially transferred to subsequent shake flasks after the exponential phase was reached (corresponding to the growth phase in which cells divided as fast as possible, according to the experimental conditions).

Depending on the yeast growth capability, the percentage of pretreated material was further increased when an adaptive event (reduced lag phase and/or increased specific growth rate) was observed.

With this strategy, every 24 to 72 h the appropriate volume of culture was used to inoculate fresh medium (with pretreated material) to a target OD₆₆₀ of 0.2.

This experiment was conducted for 46 round.

Evolution on plate

Evolution on plate was performed only for cells derived from the S2. In particular, three different methods (from SP1 to SP3) were followed.

Method SP1: this method started from the last spot assay performed with the S2. Single colonies were used to inoculate sterile tubes with 4 mL of defined medium. The tubes were then incubated for ~ 24 h at 30 °C and 220 rpm. Cells in exponential growth phase were utilized to perform a spot assay on plates with 25, 35 and 45 % of pretreated material. The single colonies deriving from the most stressful condition were used to repeat the method.

Method SP2: this method is similar to the method SP1. However, instead of inoculate colonies deriving from the spot assay in sterile tubes with fresh medium, the colonies are re-suspended in water and used to perform a new spot assay on plates with 25, 35 and 45 % of pretreated material.

As for the previous method, single colonies deriving from the most stressful condition were used to repeat the method.

Method SP3: also this method started from the last spot assay performed with the S2. Single colonies were used to inoculate sterile tubes with 4 mL of defined medium. The tubes were then incubated at 30 °C and 220 rpm. After 8 h the OD660 was measured, normalized at 0.5, serial 1 to 10 dilutions were performed and finally 200 µL were plated on solid mineral medium with different percentages of pretreated material (25, 35 and 45 %). The single colonies deriving from the most stressful condition were used to repeat the method.

Evaluation of adaptive mutant strains

Three different comparative methods (from A to C) were performed to evaluate the robustness to inhibitory compounds (or pretreated material) of the strains derived from the different evolution strategies compared to the parental strain.

Method A: cells of the parental and of the evolved strains were used to inoculate at OD660 0.2 flasks containing defined medium and defined medium with inhibitors (or pretreated material), respectively (step one). After 24 h of incubation this step was repeated (step two). After the second step, the appropriate volume of cells was used to inoculate at OD660 0.2 flasks containing inhibitors (or pretreated material) (step three).

Method B: the first step was same of method A (step one). Cells of both evolved and parental strains were then used to inoculate at OD660 0.2 flasks containing defined medium (step two). After 24 h of incubation the appropriate volume of cells was used to inoculate at OD660 0.2 flasks containing inhibitors (or pretreated material) (third step).

Method C: this method was almost identical to method B. The only difference was that the second step consisting of 24 h of cultivation on defined medium was repeated twice.

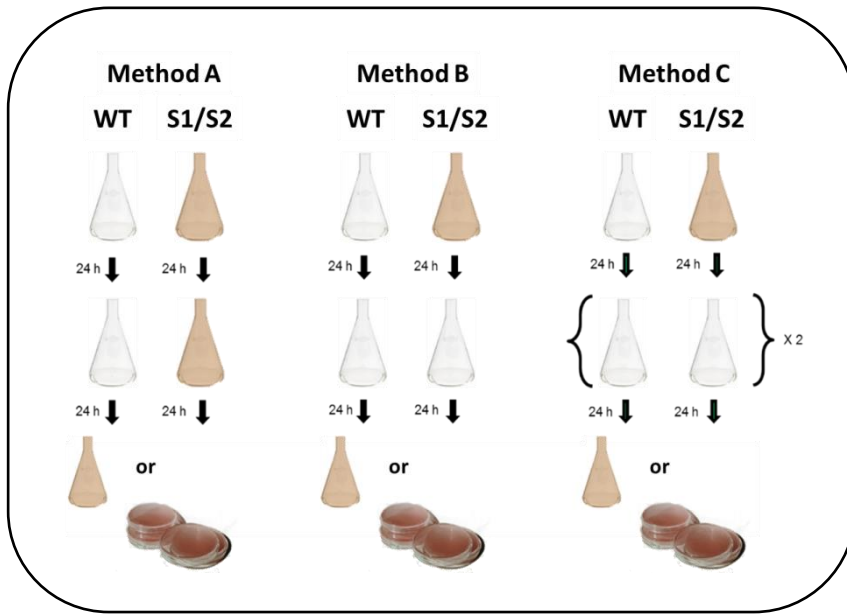


Figure 6. - Schematic representation of the comparison methods.

Methods used to compare the parental strain (WT) and isolates from the evolution strategy 1 and 2 (S1 or S2). White flasks: flasks with mineral medium and without inhibitors. Brown flasks: flasks with mineral medium and inhibitory compounds (acetic acid, furfural and HMF) or pretreated material.

During these experiments cell growth was followed by measuring the OD over time and obtained values were used to determine the duration of the lag phase and the specific growth rate for each condition. Moreover, before the last step of every method, cells were collected and stained with propidium iodide (PI) to detect severely damaged/dead cells by flow cytometer (see below).

The same comparison methods were also performed by changing the final step in flask with spot assay on plates containing inhibitory compounds or pretreated material.

Analytical methods

The optical density was measured at 660 nm (OD_{660}) with a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation). Samples collected at different times were centrifuged at 14000 rpm for 10 min. Glucose and xylose concentrations were HPLC determined using a Rezex ROA-Organic Acid (Phenomenex). The eluent was 0.01 M H_2SO_4 pumped at 0.5 mL min^{-1} and column temperature was $35 \text{ }^\circ\text{C}$. Separated components were detected by a refractive-index detector and peaks were identified by comparing with known standards (Sigma-Aldrich, St Louis, MO, USA). Analysis of HMF and furfural was performed using a C-18 column. The eluent was a gradient of mqH_2O at pH 3.5 (using HCl 6 M) and Acetonitrile.

Biomass was harvested by centrifugation of the culture samples at 4000 rpm for 10 min. The pellets were then washed twice with distilled water and dried at $40 \text{ }^\circ\text{C}$ (Concentrator 52301, Eppendorf, Germany) until a constant weight was obtained.

Flow cytometric analysis

Dead or severely compromised cells were detected by Propidium Iodide (PI, Sigma-Aldrich CO., St. Louis, MO, USA) staining. Briefly, cell were washed twice with buffer (TrisHCl 50 mM, $MgCl_2$ 15mM, pH 7.7), resuspended in a PI solution (0.23 mM), incubated in the dark on ice for 20 min and then analyzed by flow-cytometry. Samples were analyzed using a Beckman Coulter FC-500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an Argon ion laser (excitation wavelength 488 nm, laser power 20 mW). The fluorescence emission was measured through a 670 nm band pass filter (FL4 parameter) for PI signal. The sample flow rate during analysis did not exceeded 500 cells/s. A total of 25.000 cells were measured for each sample. Data analysis was performed afterwards with *Cylogic* software (PerttuTerho, Mika Korkeamäki, CyFlo Ltd).

REFERENCES

1. Angerbauer C, Siebenhofer M, Mittelbach M, Guebitz GM: **Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production.** *Bioresource Technology* 2008, **99**:3051-3056.
2. Oud B, van Maris AJA, Daran J-M, Pronk JT: **Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast.** *Fems Yeast Research* 2012, **12**:183-196.
3. Cebollero E, Gonzalez-Ramos D, Tabera L, Gonzalez R: **Transgenic wine yeast technology comes of age: is it time for transgenic wine?** *Biotechnology Letters* 2007, **29**:191-200.
4. Almario MP, Reyes LH, Kao KC: **Evolutionary engineering of *Saccharomyces cerevisiae* for enhanced tolerance to hydrolysates of lignocellulosic biomass.** *Biotechnology and Bioengineering* 2013, **110**:2616-2623.
5. Heer D, Sauer U: **Identification of furfural as a key toxin in lignocellulosic hydrolysates and evolution of a tolerant yeast strain.** *Microbial Biotechnology* 2008, **1**:497-506.
6. Wallace-Salinas V, Gorwa-Grauslund MF: **Adaptive evolution of an industrial strain of *Saccharomyces cerevisiae* for combined tolerance to inhibitors and temperature.** *Biotechnology for Biofuels* 2013, **6**.
7. Almeida JRM, Modig T, Petersson A, Hahn-Hagerdal B, Liden G, Gorwa-Grauslund MF: **Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*.** *Journal of Chemical Technology and Biotechnology* 2007, **82**:340-349.
8. Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW: **Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran.** *Journal of Industrial Microbiology & Biotechnology* 2004, **31**:345-352.

Chapter V.

Assessing physio-macromolecular effects of lactic acid on *Zygosaccharomyces bailii* cells during microaerobic fermentation

Nurzhan Kuanyshhev¹, Diletta Ami^{1,2,3*}, Lorenzo Signori, Danilo Porro, John P. Morrissey⁴ and Paola Branduardi^{1*}

¹Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 2, Milano 20126 (Italy).

²Department of Physics, University of Milano-Bicocca, Piazza della Scienza 3, Milano 20126 (Italy).

³Consorzio Nazionale Interuniversitario per le Scienze fisiche della Materia (CNISM) UDR Milano-Bicocca, Via R. Cozzi 53, Milano 20126 (Italy).

⁴School of Microbiology, University College Cork, Cork, Ireland

*Corresponding authors

ABSTRACT

The ability of *Zygosaccharomyces bailii* to grow at low pH and in the presence of considerable amounts of weak organic acids, lethal condition for *Saccharomyces cerevisiae*, increased the interest in the biotechnological potential of the yeast. To understand the mechanism of tolerance and growth effect of weak acids on *Z. bailii*, we evaluated the physiological and macromolecular changes of the yeast exposed to sub lethal concentrations of lactic acid. Lactic acid represents one of the important commodity chemical which can be produced by microbial fermentation. We assessed physiological effect of lactic acid by bioreactor fermentation using synthetic media at low pH in the presence of lactic acid. Samples collected from bioreactors were stained with propidium iodide (PI) which revealed that, despite lactic acid negatively influence the growth rate, the number of PI positive cells is similar to that of the control. Moreover, we have performed Fourier Transform Infra-Red (FTIR) microspectroscopy analysis on intact cells of the the same samples. This technique has been never applied before to study *Z. bailii* under this condition. The analyses revealed lactic acid induced macromolecular changes in the overall intracellular protein secondary structures, and alterations of cell wall and membrane physico-chemical properties.

Keywords: *Zygosaccharomyces bailii*, lactic acid, FTIR, bioreactor, fermentation

INTRODUCTION

The yeast *Zygosaccharomyces bailii* is well-known to be responsible for major spoilage losses in the food and beverage industry (Fleet 2007). This spoilage nature is explained by its remarkable ability to grow in harsh environments such as high osmotic pressure, low pH, low water activity, and high concentration of weak organic acids (Thomas and Davenport 1985; James and Stratford 2011). These same traits have drawn attention to *Z. bailii* as a potential cell factory for production of biomolecules (Branduardi *et al.*, 2004; Sauer *et al.*, 2004; Vigentini *et al.*, 2005). Although initial studies focused on understanding tolerance to weak organic acids with a view to controlling the yeast in food spoilage, more recent work aims to dissect the mechanisms of tolerance to acids such as acetic and lactic acid to facilitate exploitation for biotechnological applications. Lactic acid is of particular relevance as it represents an important commodity chemical that can be produced by microbial fermentation as reviewed by (Sauer *et al.*, 2010; Becker *et al.* 2015).

Most of our understanding of the toxicity of weak organic acids to yeast cells, and the cellular response, comes from studies on the model yeast *Saccharomyces cerevisiae*. The lipophilic nature of most undissociated organic weak acids (Lambert and Stratford 1999) allows them to diffuse across the semi-permeable cell membrane. The ratio of undissociated:dissociated acid, and hence degree of diffusion into the cell, increases with decrease of external pH. Once inside of the cell where the cytoplasmic pH is close to neutral, the weak acid dissociates to a proton and respective anion. The presence of proton acidifies the internal pH causing inhibition of most metabolic processes, and the released anions may have additional toxicity

causing programmed cell death (Ullah *et al.*, 2012; Zhao *et al.*, 2008; Russel *et al.*, 1992). Various multiple responses is detected in *S. cerevisiae* to tolerate weak acid toxicity, most pronounced among them is activation of H⁺-ATPases present in the plasma and vacuolar membranes to stabilize of internal pH, cell wall and membrane remodeling to decrease fluidity thus preventing/decreasing undissociated weak acid diffusion, alteration in central carbon metabolism to increase ATP pool, anion extrusion through MDR-MFS transporters. The mechanism of acetic acid toxicity, model organic acid for stress response studies, has been extensively studied in the yeast *S. cerevisiae* (Paiva *et al.*, 2004; Mira *et al.*, 2010; Nygård *et al.*, 2013). Remarkably, different weak acids may have different physiological and morphological responses (Stratford *et al.*, 2013), differing also among diverse yeasts.

There have been several studies in *Z. bailii*, focused on acetic acid tolerance due to its common use as a weak acid preservative and ubiquitous inhibitor in industrial fermentation (Lindberg *et al.*, 2013; Sousa *et al.*, 1998; Guerreiro *et al.*, 2012; Rodrigues *et al.*, 2012). The reports suggest diverse weak acid toxicity responses of *Z. bailii*, during acetic acid exposure. Among them the cell membrane modulation to decrease weak acid diffusion and acid uptake through active transport, allowing dose dependent uptake of acetic acid, thus preventing oversaturation of acetic acid in the cell and its high toxicity (Sausa *et al.*, 1996; Lindberg *et al.*, 2013; Rodrigues *et al.*, 2012). In addition, *Z. bailii* can utilize acetic acid as an extra carbon source in the presence of glucose, suggesting that under certain conditions *Z. bailii* may even benefit from the presence of the acid. This simultaneous co-consumption of glucose and acetic acid possible because *Z. bailii* acetyl-CoA synthetase is not

subject to glucose repression (Guerreiro *et al.*, 2012; Rodrigues *et al.*, 2004., 2012).

Being the product of interest in industrial fermentation and food acidulant, lactic acid represents great interest. Lactic acid possess general weak acid inhibition effect in yeast, affecting internal cell pH, acidification, and ROS accumulation. However, low amount of lactic acid has beneficial effect due to its buffering capacity, but with increase of concentration the effect disappears (Dang *et al.*, 2009; Nugroho *et al.*, 2015).

In this study we investigated the physiological and macromolecular responses of *Z. bailii* exposed to lactic acid stress at low aeration and pH. Bioreactor fermentation profile, propidium iodide staining and Fourier Transform Infrared microspectroscopy (FTIR) analysis were chosen to study the growth performance and the cellular response of *Z. bailii* to lactic acid at various time points. The study revealed significant phenotypic response of *Z. bailii* to lactic acid, moreover exposure to lactic acid induce macromolecular changes, which are augmented by time.

MATERIALS AND METHODS

Cell cultivation

The *Z. bailii* strains ATCC36947, ATCC60483, ATCC8766 and ATCC for convenience named Zb1, Zb2, Zb3 and Zb4 were used. The *S. cerevisiae* laboratory CEN.PK113-7D (obtained from Kötter P, Institut für Mikrobiologie der Johann Wolfgang Goethe Universität, Frankfurt, Germany) and commercial alcohol yeast (Dry Ethanol Red®; Fermentis, Marcq-en-Baroeul, France) strains were used. The cells were stored at -80°C in YPD glycerol stock. For liquid cultivation,

the cells were pre-grown on YPD plates (20 gL⁻¹ peptone, 10 gL⁻¹ yeast extract, 20 gL⁻¹ glucose, 20 gL⁻¹ agar). Phosphate – citrate buffer at pH 3 was used for buffering the flask fermentation. All liquid cultures were grown on at 30°C on synthetic minimal Verduyn medium (Verduyn *et al.*, 1992). Pre-culture for microaerobic bioreactor batch cultivation was prepared by transferring one full loop of cells from YPD plate to 20 mL Verduyn medium in 125 mL flask. The flasks were incubated at 160 rpm at 30°C overnight. The culture from first flask pre-culture was re-inoculated into 50mL Verduyn medium in 250 mL flask at OD_{660nm} 1.5 and let it grow for 4-5 hours until OD_{660nm} 4. The inoculum for the bioreactor cultivation was harvested at 3000g for 3 min at 20°C and resuspended in 20 mL of sterile ddH₂O, and aseptically added to the bioreactor.

Microaerobic bioreactor cultivation

Bioreactor experiment were performed in 2L volume bioreactors (BIOSTAT B, Sartorius AG, Germany) with operative volume of 1.5L. Zb2 was cultivated in 2x Verduyn medium containing 40 gL⁻¹ glucose with 40 gL⁻¹ lactic acid or no lactic acid. The cells were grown to mid exponential phase and inoculated to the bioreactor, to final absorbance of OD₆₆₀ 0.1. The temperature was maintained at 30°C, pH at 3 by addition of 4M NaOH and the stirrer speed was setup to 400 rpm. The inlet gas flow was adjusted by two mass flow controllers (Bronkhornst@High Tech- EL-FLOW@Select). The mass flow was setup to mixture N₂ and air with final concentration of inlet oxygen 5%. The mixture was sparged at 0.75 vvm. Antifoam (Antifoam 204, Sigma Aldrich) was used for foaming control. A minimum of 3 independent cultivations were performed per each condition (Table 1).

The concentration of produced CO₂ was monitored by on-line gas analyzer (Omnitec). The gas analyzer was always calibrated 24 h before starting the cultivation using synthetic air containing defined concentration of CO₂.

Samples (20 mL) were collected regularly from the bioreactor in vials; 1 mL was used for OD_{660nm} measurement, after appropriate dilution; 1 mL was centrifuged at 4°C, 14,000 rpm for 5 min and supernatants were collected and stored at -20°C for later determination of extracellular metabolites concentrations.

Dry cell weight

The dry weight of the cell mass was measured per each sampling by washing samples in ddH₂O and pelleting the cells. The cell pellets were dried in vacuum concentrator using default mode (Concentrator 5301, Eppendorf, Germany) before measuring.

Extracellular metabolite quantification

Residual glucose, ethanol and lactic acid were determined via high-performance liquid chromatography (HPLC, Model 1100, Agilent Technologies) using Aminex HPX-87H ion exchange column 300 mm × 7.8 mm (Bio-Rad) thermostated at 45°C. The mobile phase was 5 mM sulphuric acid with a flow of 0.5 mlmin⁻¹. Lactic acid was detected with an UV-detector at 210 nm. Glucose and Ethanol were detected with a RI detector.

Propidium iodide staining and flow cytometry

For identification of dead/severely compromised cells, cells were washed three times (Tris-HCl 50 mM, MgCl₂ 15 mM, pH 7.7) and resuspended in propidium iodide (PI, Sigma-Aldrich CO., St. Louis, MO, USA) solution 0.23 mM, incubated on

ice for 20 min. Positive and negative controls were also prepared. In particular, positive control was prepared by killing cells with ice cold ethanol (percentage? Time?). Samples were then analyzed using a CYTOMICS FC 500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an Argon laser (excitation wavelength 488 nm, laser power 20 mW). The fluorescence emission was measured through a 670 nm long pass filter (FL4 parameter) for PI signal. The sample flow rate during analysis did not exceed 600–700 cells/s. Threshold settings were adjusted so that the cell debris was excluded from the data acquisition; 25000 cells were measured for every sample. Data analysis was performed afterwards with Cyflogic 1.2.1 software (©Perttu Terho & ©CyFlo Ltd).

FTIR microspectroscopy analysis

The bioreactor cultivated intact cells of *Z. bailii* were collected at 18, 24 and 40 hours. The cells were washed three times in distilled water to eliminate medium contamination. Approximately 3 µl of the cell suspensions were then deposited onto an IR transparent BaF₂ support, and dried at room temperature for at least 30 minutes to eliminate the excess water.

FTIR absorption spectra were acquired in transmission mode, between 4000 and 700 cm⁻¹, by means of a Varian 610-IR infrared microscope coupled to the Varian 670-IR FTIR spectrometer (both from Varian Australia Pty Ltd), equipped with a mercury cadmium telluride (MCT) nitrogen-cooled detector. The variable microscope aperture was adjusted to ~ 100 µm × 100 µm. Measurements were performed at 2 cm⁻¹ spectral resolution; 25 KHz scan speed, triangular apodization, and by the accumulation of 512 scan co-additions.

Second-derivatives spectra were obtained following the Savitsky-Golay method (third-grade

polynomial, 9 smoothing points), after a binomial 13 smoothing points of the measured spectra (Susi and Byler 1986), using the GRAMS/32 software (Galactic Industries Corporation, USA).

To verify the reproducibility and reliability of the spectral results, three independent preparations were analyzed and for each preparation at least ten spectra for sample were measured.

In the Figures, reported data are representative of the independent experiments performed.

RESULTS AND DISCUSSION

Establishing the suitable lactic acid concentration to investigate the effects on *Z. bailii* cells

Four *Z. bailii* strains (see Materials and Methods) obtained from ATCC collection were tested by plate assay experiment using different combinations of weak acids at pH 3 on Verduyn minimal medium to identify the best strain to be further studied for lactic acid tolerance. In addition, laboratory and industrial *S. cerevisiae* strains were included for comparison. The cells were pre-grown on YPD media until exponential phase and drop plated at 10 , 10^{-1} , 10^{-2} , 10^{-3} dilution. No difference in yeast growth were observed in control plates without weak acid addition (data not shown). When evaluated for lactic acid tolerance (Figure 1A and 1B), other than Zb4 all strains were only minimal affected by 40 gL^{-1} of lactic acid. At 80 gL^{-1} lactic acid only Zb2 and Zb3 showed growth, and even those strains were strongly inhibited by this condition. Next, the effects of acetic acid and a combination of acetic acid and lactic acid were assessed (Figure 1C and 1D). At 5 gL^{-1} of acetic acid, growth of *Z. bailii* Zb4 and the two *S. cerevisiae* strains was completely inhibited,

whereas *Z. bailii* strains Zb1, Zb2 and Zb3 were unaffected. No synergistic or additive inhibitory effects of 40 gL^{-1} lactic acid and 3 gL^{-1} acetic acid on any of the strain were observed (Figure 1, D). Overall, these results highlight the superior weak acid tolerance relative to *S. cerevisiae* of *Z. bailii* strains to weak organic acids and suggest that different mechanisms are likely to be involved in tolerance to specific acids (compare the profile of Zb1 with Zb2 or Zb3). Strain Zb2 showed high levels of tolerance and this particular strain was previously reported to be amenable to genetic manipulation (Dato *et al.*, 2010; Passolunghi *et al.*, 2010), Zb2 was therefore chosen for subsequent detailed tests on the effects of lactic acid. Preliminary shake flask fermentations of Zb2 using Verduyn medium at pH 3 with different concentrations of lactic acid were carried out to assess sensitivity to the stressor in liquid medium (Supplementary material, Figure S1). Growth was not significantly impaired at 40 gL^{-1} lactic acid but was strongly reduced at 60 or 80 gL^{-1} lactic acid. Interestingly, growth was actually stimulated by 20 gL^{-1} lactic acid. This may be attributed to a buffering effect of the weak organic acid, which may mitigate acidification of the growth medium caused by yeast growth. At higher concentrations, the toxic effect of the acid would dominate over this mild buffering. In addition, we performed propidium iodide staining analysis to evaluate the percentage of damaged cells under these test conditions (Supplementary material, Figure S2). In comparison to the control condition, there was only little difference in the percentage of PI positive cells at 18 and 22 hours (5-15%) for cultures grown with 20 gL^{-1} and 40 gL^{-1} lactic acid, indicating little cellular damage, whereas cells treated with 60 gL^{-1} and 80 gL^{-1} lactic acid show a high percentage of PI positive cells, which is a clear evidence these or

higher concentrations significantly affect the cell viability. Based on these data, 40 gL⁻¹ lactic acid was selected as the optimum sub-lethal lactic acid concentration for further tests. It should be noted that as the pKa of lactic acid is 3.86, according to Henderson-Hasselbalch equation the total concentration of undissociated lactic acid in the medium was approximately 34.5 gL⁻¹.

Effects of lactic acid on the growth and metabolic profiles of *Z. bailii* during microaerobic bioreactor fermentation

Bioreactor cultivation, in addition of allowing a precise monitoring and control of fermentation parameters, better represents industrial conditions. Therefore experiments to assess the effect of 40 gL⁻¹ lactic acid on strain Zb2 were performed in a 2L bioreactor (see Material and Methods for growth conditions). Cells were inoculated from overnight cultures at OD 0.1 and growth, as monitored by the production of CO₂, commenced after 5-8 hours of adaptation to the new environment (Figure 2). The similar lag phase, regardless of the presence/absence of lactic acid, suggests that a specific pre-adaption to lactic acid is not required. Nevertheless, it was noted that there was an effect on growth rate and yield (Figure 2), detailed with biomass and metabolite profiles in Figure 3. Cells grown in presence of lactic acid (40 gL⁻¹) exhibited a 25% reduction in growth rate (0.19h⁻¹ ±0.012 vs 0.14h⁻¹±0.017) and a 15% reduction in final biomass titer (5.833gL⁻¹±0.16 vs 5.54gL⁻¹±0.21). Also the specific glucose consumption rate decreased of 13% reduction in lactic acid treated cells in comparison to control (1.38g gDCW⁻¹h⁻¹±0.05 vs 1.15g gDCW⁻¹h⁻¹±0.07). The reduction in growth rate and yield under bioreactor conditions is consistent with the described effects of other organic acids in yeast and in part may be

attributable to the energetic cost of maintaining pH homeostasis by pumping H⁺ ions from the cytoplasm using the plasma membrane ATPase, which requires energy for its activity in one proton per ATP rate (van der Rest *et al.*, 1995; Stratford and Anslow 1996). However we should not exclude other intracellular effects of lactic acid, which may contribute to overall growth inhibition. Indeed, the reduction in the glucose consumption and growth rate can be else ascribed to a general conservative response, including a decrease in protein synthesis and inhibition of glycolytic enzymes activity in yeast (Pearce *et al.*, 2001). Despite the difference in biomass yield, the presence of lactic acid under our experimental setting did not influence ethanol fermentation. In other words, weak acid stressed cells prefer fermentative carbon utilization, which may be due to higher energy demand at O₂ limitation and/or lactate induced oxidative stress which affect mitochondria (Table 2) (Sousa *et al.* 2012). After glucose depletion, the residual ethanol was slowly consumed, contributing to slight increase of biomass observed starting from 28 hours on, in both conditions.

S. cerevisiae is able to consume lactic acid through mitochondrial L-lactate ferricytochrome c oxidoreductase (L-LCR) and D-lactate ferricytochrome c oxidoreductase (D-LCR) activities (Lodi and Ferrero 1992), encoded by genes that are targets for glucose repression and highly depended on aeration. Comparable studies are not present in literature for *Z. bailii*. In our experimental setting, only a slight decrease of lactic acid concentration was detected (Figure 3), possibly because of lactic acid influx into the cell, even when glucose was exhausted in the medium. At high aeration condition, Zb2 consumed lactic acid after glucose depletion (data not shown). The consumption of lactic acid only under conditions of

high aeration and glucose depletion suggests that comparable mechanisms are involved in *Z. bailii* and *S. cerevisiae*. During fermentation, control cultures used around 30 mL of NaOH base for pH maintenance, while cells grown with lactic acid barely used 1 mL NaOH. This confirms the indication deriving from the shake flask experiments (Supplementary material, Figure S1) of the lactic acid buffering effect. As with the shake flask experiment, growth is not promoted in media with 40 gL⁻¹ lactic acid because of the more dominant inhibitory effects. Interestingly, no acetic acid accumulation was observed in both conditions at low aeration, which is very likely in agreement with previous reports describing *Z. bailii* for its ability to consume acetic acid even at presence of glucose and microaerobic condition (Sousa *et al.*, 1998, Rodrigues *et al.*, 2012).

The viability/integrity of cells recovered from bioreactors with and without lactic acid treatment was assessed using propidium iodide (PI) staining (Figure 4). There were no major differences in the percentage of PI positive cells between control and treatment conditions, confirming the data obtained in shake flasks (Supplementary material, Figure S1) and supporting the explanation that an increased energy burden rather than a toxic effect is responsible for the slight reduction in growth rate and yield seen in treated cultures (Figure 3). The minor differences in PI positivity measured at 12h, were no longer apparent at 18 h. There was an increase in the percentage of damaged cells from ~12% to ~20% between 18 h and 42 h and this is likely to be due to glucose starvation and ethanol exposure.

FTIR microspectroscopy analysis of *Z. bailii* reveals gradual macromolecular changes during exposure to lactic acid

The results obtained from PI staining indicate that 40gL⁻¹ lactic acid has no obvious cell damaging property on this strain, which may be a consequence of some cellular adaptation.

To investigate possible effects of lactic acid on the cell molecular composition and structure, we used Fourier transform infrared (FTIR) microspectroscopy. Intact *Z. bailii* cells were collected at 18, 24 and 40 hours after inoculation, respectively, corresponding to the early, late exponential, and the stationary phases of growth, and analyzed by FTIR microspectroscopy. As an example, in Figure 5 we reported the measured absorption spectrum of *Z. bailii* cells, grown in the absence of lactic acid, at 24 hours after the inoculation. As illustrated, the spectrum is due to the overlapping absorption of multiple components representing the different specific cellular macromolecules. Therefore, to better resolve the absorption bands, an essential prerequisite for the identification of peak positions and for their assignment to the different biomolecules (Susi *et al.*, 1986), we analyzed the second derivative spectra. The amide I band (Figure 6a), between 1700 and 1600 cm⁻¹, principally gives information on the whole cell protein secondary structures and aggregation (Tamm and Tatulian 1997; Barth 2007). In early exponential phase, there are no differences observed between the treated and untreated cells. The spectra are dominated by a band at ~1657 cm⁻¹, mainly due to alpha-helix and random-coil structures, and by a band at ~1638 cm⁻¹, due to intramolecular native beta-sheets. Moreover, two minor absorption at ~1692 cm⁻¹ and ~1685 cm⁻¹ were present, respectively due to beta-sheet and beta-turn structures (Tamm and Tatulian 1997; Barth 2007). As the cells entered later exponential and stationary phase there were only minor changes to the whole cell protein structure

seen in the untreated cells. Indeed, in the late exponential phase we observed only the appearance of two well resolved absorption at $\sim 1690\text{ cm}^{-1}$ and $\sim 1680\text{ cm}^{-1}$, respectively assigned to beta-sheets and beta-turns. More pronounced changes were instead evident in the treated cells. In late exponential phase, we detected a minor but significant reduction in the intensity of the alpha helix/random coil and of the native beta-sheet absorption, accompanied by the appearance of a shoulder around 1627 cm^{-1} , mostly due to intermolecular beta-sheets, typical of protein aggregates (Seshadri, S., Khurana, R., Fink, A.L. 1999). Moreover, the upshift of the $\sim 1690\text{ cm}^{-1}$ beta-sheet absorption to $\sim 1694\text{ cm}^{-1}$ again indicates the presence of proteins with intermolecular beta-sheets. These changes were even more evident in the stationary phase consistent with a progressive effect of the exposure to lactic, and possibly ethanoloic stress on protein structure and folding.

Next we explored the IR response between $1500\text{--}1200\text{ cm}^{-1}$ (Figure 6b), mainly due to the absorption of the lipid hydrocarbon tails and head groups. In particular, the second derivative spectrum of the unchallenged cells is characterized by several bands mostly due to CH_3 and CH_2 deformations arising from lipid hydrocarbon tails (Casal and Mantsch 1984.; Arrondo and Goñi 1998). In addition, two spectral components were present at $\sim 1397\text{ cm}^{-1}$ and $\sim 1387\text{ cm}^{-1}$, respectively due to the CH_3 bending vibration of the $\text{N}(\text{CH}_3)_3$ head group of phosphatidylcholine (PC) and to the CH_3 deformation mainly arising from ergosterol (Levchuk 1968; Casal and Mantsch 1984). Finally, a broad band at $\sim 1248\text{ cm}^{-1}$ was also observed, due to the PO_2^- stretching mode of phospholipids and nucleic acids (Casal and Mantsch 1984; Banyay *et al.*, 2003). There were no major alterations in the

lipid components of lactic acid – treated cells in the early exponential phase but in the later exponential and stationary phase some important changes were detected. Lactic acid induced an important reduction in the intensity of the $\sim 1400\text{ cm}^{-1}$ band, marker of PC (phosphatidylcholine). In later growth, that was accompanied by a slight increase of the ergosterol component at $\sim 1386\text{ cm}^{-1}$, though this change was less evident in stationary phase.

Next, we analyzed the spectral range between $3050\text{--}2800\text{ cm}^{-1}$ (Figure 6c) that is mainly due to the stretching vibrations of the lipid hydrocarbon tails (Casal and Mantsch 1984; Arrondo and Goñi 1998). In particular, the spectrum of cells grown in absence of lactic acid is characterized by four well resolved bands due to the CH_2 (at $\sim 2921\text{ cm}^{-1}$ and 2851 cm^{-1}) and CH_3 (at $\sim 2958\text{ cm}^{-1}$ and 2872 cm^{-1}) absorption. In addition, a low intensity band was detected at $\sim 3007\text{ cm}^{-1}$, due to the olefinic $=\text{CH}$ groups in acyl chains (Casal and Mantsch 1984). Lactic acid treated cells displayed very similar spectral features to those detected in not exposed cells, suggesting that membrane lipids were not significantly affected by the exposure to the stressing agent in the exponential phase of growth. In stationary phase cells exposed to lactic acid displayed a slightly lower intensity of the lipid hydrocarbon tail CH_2 bands ($\sim 2920\text{ cm}^{-1}$ and $\sim 2851\text{ cm}^{-1}$) compared to cells grown in absence of lactic acid, which could reflect a decrease of the acyl chain length on lipids.

We finally analyzed the complex range between $1200\text{--}900\text{ cm}^{-1}$ (Figure 6d), dominated by the absorption of carbohydrates, with additional overlapping contributions of phosphate groups mainly from phospholipids and nucleic acids (Casal and Mantsch 1984; Kačuráková and Mathlouthi 1996; Banyay *et al.*, 2003). The analysis of this spectral range can provide information on cell wall

properties that involve in particular the yeast envelope carbohydrate composition (Galichet *et al.*, 2001; Zimkus *et al.*, 2013). The second derivative spectrum of *Z. bailii* cells not exposed to lactic acid is characterized in particular by the simultaneous presence of three absorption at $\sim 1156\text{ cm}^{-1}$, $\sim 1081\text{ cm}^{-1}$ and $\sim 1022\text{ cm}^{-1}$, altogether marker of glycogen (Naumann 2000). We should note that $\beta(1-3)$ glucans, as well as absorbing at $\sim 1103\text{ cm}^{-1}$, can have overlapping contributions with glycogen at $\sim 1156\text{ cm}^{-1}$ and at $\sim 1081\text{ cm}^{-1}$ (Galichet *et al.*, 2001; Zimkus *et al.*, 2013). The two bands at $\sim 1042\text{ cm}^{-1}$ and $\sim 966\text{ cm}^{-1}$ are mainly assigned to mannans (Galichet *et al.*, 2001; Zimkus *et al.*, 2013). Even in early exponential phase, these spectral features were partly found to change in cells challenged with lactic acid, and in particular an important reduction of glycogen occurred. This result suggests that lactic acid treated cells have faster glycogen turnover, possibly due to energy demand required to maintain cell homeostasis (François and Parrou 2001). By late exponential phase, in lactic acid-treated cells there was a reduction in the intensity of the absorption mainly due to glycogen ($\sim 1156\text{ cm}^{-1}$, 1081 cm^{-1} , 1023 cm^{-1}). In addition, a slight reduction of the $\beta 1\rightarrow 3$ glucan absorption ($\sim 1156\text{ cm}^{-1}$, $\sim 1104\text{ cm}^{-1}$, $\sim 1081\text{ cm}^{-1}$) and of the $\beta 1\rightarrow 6$ glucans at $\sim 997\text{ cm}^{-1}$ was also detected, suggesting that the exposure to the stressing agent induced a reorganization of the cell wall components. The changes were even more profound in the stationary phase. Interestingly, glycogen was not detectable in either untreated or treated cells, but dramatic changes in the carbohydrate components of the cell wall were observed. Lactic acid-treated cells displayed a significant reduction in the intensity of the $\beta 1\rightarrow 3$ and $\beta 1\rightarrow 6$ glucan bands, and of the mannan band at 1046 cm^{-1} (Zimkus *et al.*, 2013) compared to cells in

absence of lactic acid. The spectrum of cells grown in the absence of lactic acid was characterized by a new band, not observed in the other phases of growth, at $\sim 1028\text{ cm}^{-1}$ that can be assigned to $\beta 1\rightarrow 4$ glucosidic bonds (Naumann 2000). Interestingly, this component almost disappeared in cells challenged with lactic acid. These results indicate that lactic acid led to a dramatic rearrangement of the cell wall properties - mainly involving the carbohydrate components - that started in the early exponential phase and continued progressively to the stationary phase.

Summarizing the insights obtained from the FTIR analysis the lactic acid treatment results in four major types of cellular changes. (1) An increase in the level of protein aggregation caused by lactic acid and ethanol stress, which suggests protein misfolding; (2) a reduction in glycogen, possibly caused by energy requirements for homeostasis; (3) modification of lipids; and (4) carbohydrate cell wall remodeling. Some of these alterations are consistent with other studies on organic/lactic acid stresses in *Z. bailii* or other yeasts. The reduction of PC and the simultaneous slight increase in ergosterol could account for a reduction of membrane fluidity (Fajardo *et al.*, 2011) that in turn could lead to the observed increased resistance to lactic acid. Lipidomic profiling of *Z. bailii* under acetic acid stress reported a high basal level of sphingolipids (Lindberg *et al.*, 2013). Since the infrared response of sphingolipids (ceramide, glycolipids, etc) is very complex and covers the whole mid IR range, we could not find by second derivative analysis unambiguous variations of vibrational modes of the sphingolipid heads. The main reason may be due to overlapping absorption of lipid heads with other biomolecules absorption and no significant variations of their content. The decrease detected in acyl chain length in the

stationary phase of growth could also contribute to lower membrane fluidity. Changes in cell wall in response to weak acids has previously been reported in *S. cerevisiae* where exposure to weak acids induced formation of a more rigid cell wall resistant to zymolyase digestion (Simoes *et al.*, 2006). Although the mechanism of response may be different in *Z. bailii*, the common response of cell wall modification suggests that this is an important tolerance mechanism in yeasts.

In conclusion, the knowledge gathered during the study will help to better understand the weak acid tolerance of *Z. bailii* in the view of further ameliorating its biotechnological potential.

Acknowledgements

This work was supported by European Union FP7 Marie Curie Programme [YEASTCELL - 7PQ MARIE CURIE (12-4-2001100-40)] and partially by the SYSBIO - Centre of Systems Biology (SysBioNet, Italian Roadmap for ESFRI Research Infrastructure).

Figure 1 Spotting growth assay for weak acid tolerance screening of various *Z. bailii* and *S. cerevisiae* strains. Cells of the indicated *Z. bailii* and *S. cerevisiae* strains were cultivated until mid-exponential phase and spotted to Verdun minimum medium plate (2% glucose) at pH 3, added with different concentration of lactic and acetic acid. Cells were 10-fold serially diluted and incubated at 30°C for 2 days.

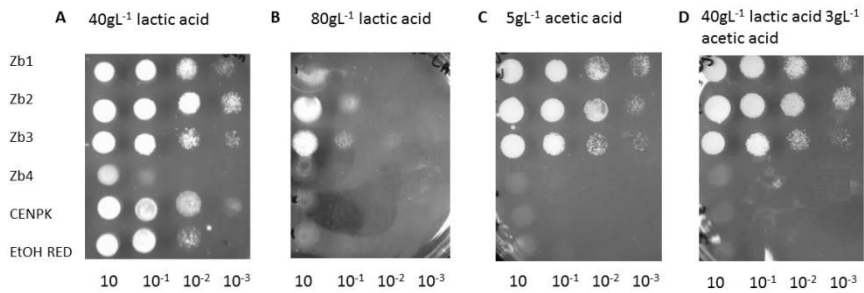


Figure 2 CO₂ profile of the *Z. bailii* bioreactor batch fermentation. Cells were cultivated in bioreactor. Gas samples were taken every 10 minutes. Solid line (control): Zb2 without lactic acid, dash line: Zb2 with 40 gL⁻¹ lactic acid. Results are average values of three replicates.

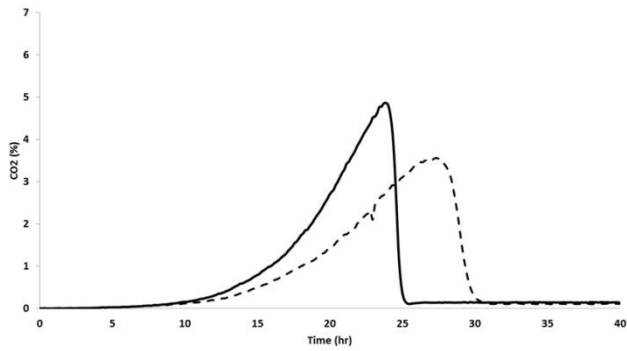


Figure 3 Fermentation performance of Zb2 with and without lactic acid. Cells were cultivated in bioreactor using Verduyn minimum medium (40 gL⁻¹glucose) under controlled condition (5% inlet oxygen, pH 3). Solid line (control): Zb2 without lactic acid. Dash line: Zb2 with 40 gL⁻¹ lactic acid. **A.** Optical density at 660nm **B.** Lactic acid concentration **C.** Ethanol production rate **D.** Glucose consumption rate. Results are average values of three replicates. Error bars represent standard deviation from three independent fermentations.

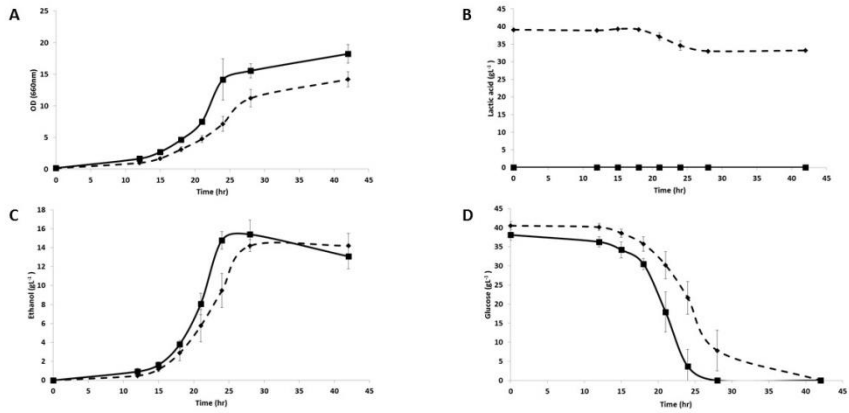


Figure 4 Propidium Iodide (PI) staining of Zb2 cells during course of bioreactor fermentation. Cells were cultivated in bioreactor. Samples were taken at 0, 12, 18 and 42 hours and stained with PI. Damaged/dead cells, positive for the staining, were detected using flow cytometry. The columns represent percentage of damaged/dead cells measured by fluorescence emission at 670nm (FL3). Black columns (control): Zb2 without lactic acid. Grey columns: Zb2 with 40 gL⁻¹ lactic acid. Error bars represent standard deviation from at least three independent experiments.

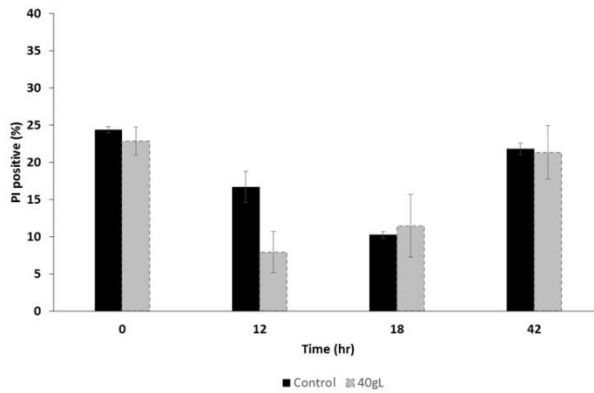


Figure 5 FTIR spectrum of *Z. bailii* intact cells. FTIR absorption spectrum of *Z. bailii* cells, grown in Verduyn minimum medium in the absence and in the presence of 40 g/L of lactic acid (LA). FTIR analysis was performed at 24 hours after the inoculation, corresponding to the late exponential phase of growth. The assignment of selected bands to the main biomolecules is reported.

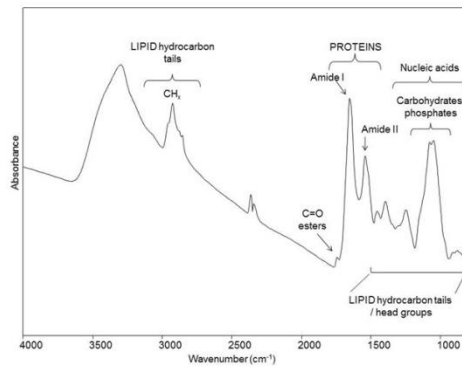


Figure 6 Second derivatives of the FTIR absorption spectra of *Z. bailii* cells, in the absence and in the presence of lactic acid. Cells were grown in Verdun minimum medium in the absence (control) and in the presence of 40 g/L of lactic acid (LA). FTIR analysis was performed at 18 hours, 24 hours and 42 hours after the inoculation, corresponding to the i: early exponential phase; ii: mid exponential phase; iii: stationary phase of growth. **a**: amide I band; **b**: vibrational modes mainly due to lipid hydrocarbon tails and head groups, as well as to phosphate groups; **c**: stretching modes from lipid hydrocarbon tails; **d**: spectral range dominated by the absorption of the cell wall carbohydrates. In **a**, **b**, and **d** derivative spectra have been normalized to the tyrosine band at ~ 1516 cm⁻¹, while in **c** spectra have been normalized at the CH₃ band at ~ 2959 cm⁻¹.

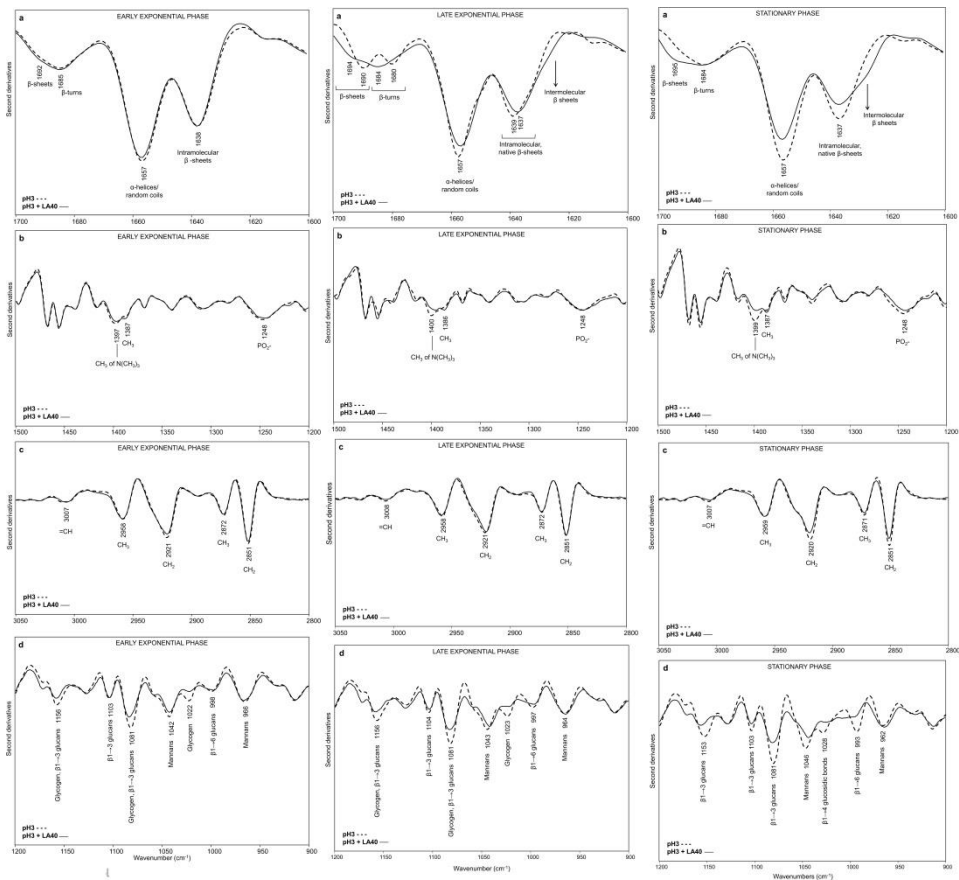


Table 1 Bioreactor settings used in this study. The settings were modified to closely resemble industrial organic acid batch fermentation, where low pH and aeration is essential.

	Control	Lactic Acid
Medium	Verdyun, 40 gL ⁻¹ Glucose	Verdyun, 40 gL ⁻¹ Glucose, 40 gL ⁻¹ Lactic acid
pH	3, controlled with NaOH	3, controlled with NaOH
Air flow rate, (vvm)	0,75	0,75
Stirring speed, (rpm)	400	400
Inlet oxygen, (%)	Set to 5%, not controlled	Set to 5%, not controlled
Initial OD _{660nm}	0,1	0,1
Temperature, (°C)	30	30

Table 2 Physiological data obtained from microaerobic batch fermentation. Zb2 cells were cultured in minimal medium using bioreactors. The results were calculated from at least three biological replicates, and are given as the means with corresponding standard deviation.

	Control	Lactic Acid
Specific growth rate μ , h^{-1}	0,19 \pm 0.012	0,14 \pm 0.017
Biomass yield x/s (g g^{-1})	0,13 \pm 0,01	0,11 \pm 0,007
Ethanol yield EtOH/s (g g^{-1})	0,41 \pm 0,05	0,4 \pm 0,07
Specific glucose consumption rate ($\text{g g DCW}^{-1}\text{h}^{-1}$)	1,38 \pm 0.05	1,15 \pm 0.07

References

- Aguilar-Uscanga B, François JM. A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. *Lett Appl Microbiol* 2003b;**37**:268–74.
- Ami D, Natalello A, Doglia S. Fourier Transform Infrared Microspectroscopy of Complex Biological Systems: From Intact Cells to Whole Organisms. In: Uversky VN, Dunker AK (eds.). *Intrinsically Disordered Protein Analysis SE - 7*. Vol 895. Humana Press, 2012, 85–100.
- Ami D, Natalello A, Zullini A *et al*. Fourier transform infrared microspectroscopy as a new tool for nematode studies. *FEBS Lett* 2004;**576**:297–300.
- Ami D, Posterl R, Mereghetti P *et al*. Fourier transform infrared spectroscopy as a method to study lipid accumulation in oleaginous yeasts. *Biotechnol Biofuels* 2014;**7**:12.
- Arrondo JLR, Goñi FM. Infrared studies of protein-induced perturbation of lipids in lipoproteins and membranes. *Chem Phys Lipids* 1998;**96**:53–68.
- Banyay M, Sarkar M, Gräslund A. A library of IR bands of nucleic acids in solution. *Biophys Chem* 2003;**104**:477–88.
- Barth A. Infrared spectroscopy of proteins. *Biochim Biophys Acta - Bioenerg* 2007;**1767**:1073–101.
- Becker J, Lange A, Fabarius J *et al*. Top value platform chemicals: bio-based production of organic acids. *Curr Opin Biotechnol* 2015;**36**:168–75.
- Boumann HA, Gubbens J, Koorengel MC *et al*. Depletion of phosphatidylcholine in yeast induces shortening and increased saturation of the lipid acyl chains: evidence for regulation of intrinsic membrane curvature in a eukaryote. *Mol Biol Cell* 2006;**17**:1006–17.
- Branduardi P, Valli M, Brambilla L *et al*. The yeast *Zygosaccharomyces bailii*: A new host for heterologous protein production, secretion and for metabolic engineering applications. *FEMS Yeast Res* 2004;**4**:493–504.
- Branduardi P. Molecular cloning and sequence analysis of the *Zygosaccharomyces bailii* HIS3 gene encoding the imidazole glycerolphosphate dehydratase. *Yeast* 2002;**19**:1165–70.
- Carmelo V, Bogaerts P, Sá-Correia I. Activity of plasma membrane H⁺-ATPase and expression of PMA1 and PMA2 genes in *Saccharomyces cerevisiae* cells grown at optimal and low pH. *Arch Microbiol* 1996;**166**:315–20.
- Casal HL, Mantsch HH. Polymorphic phase behaviour of phospholipid membranes studied by infrared spectroscopy. *Biochim Biophys Acta - Rev Biomembr* 1984;**779**:381–401.
- Casal HL, McElhaney RN. Quantitative determination of hydrocarbon chain conformational order in bilayers of saturated phosphatidylcholines of various chain lengths by Fourier transform infrared spectroscopy. *Biochemistry* 1990;**29**:5423–7.
- Castillo Martínez FA, Balcunas EM, Salgado JM *et al*. Lactic acid properties, applications and production: A review. *Trends Food Sci Technol* 2013;**30**:70–83.
- Choo LP, Wetzel DL, Halliday WC *et al*. In situ characterization of beta-amyloid in Alzheimer's diseased tissue by synchrotron

- Fourier transform infrared microspectroscopy. *Biophys J* 1996;**71**:1672–9.
- Dang TDT, Vermeulen A, Ragaert P *et al.* A peculiar stimulatory effect of acetic and lactic acid on growth and fermentative metabolism of *Zygosaccharomyces bailii*. *Food Microbiol* 2009;**26**:320–7.
- Dato L, Branduardi P, Passolunghi S *et al.* Advances in molecular tools for the use of *Zygosaccharomyces bailii* as host for biotechnological productions and construction of the first auxotrophic mutant. *FEMS Yeast Res* 2010;**10**:894–908.
- Fajardo VA, McMeekin L, Leblanc PJ. Influence of phospholipid species on membrane fluidity: A meta-analysis for a novel phospholipid fluidity index. *J Membr Biol* 2011;**244**:97–103.
- Fleet GH. Yeasts in foods and beverages: impact on product quality and safety. *Curr Opin Biotechnol* 2007;**18**:170–5.
- François J, Parrou JL. Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews*. Vol 25. 2001, 125–45.
- Galichet A, Sockalingum GD, Belarbi A *et al.* FTIR spectroscopic analysis of *Saccharomyces cerevisiae* cell walls: Study of an anomalous strain exhibiting a pink-colored cell phenotype. *FEMS Microbiol Lett* 2001;**197**:179–86.
- Guerreiro JF, Mira NP, Sá-Correia I. Adaptive response to acetic acid in the highly resistant yeast species *Zygosaccharomyces bailii* revealed by quantitative proteomics. *Proteomics* 2012;**12**:2303–18.
- James SA, Stratford M. *The Yeasts*. Elsevier, 2011.
- Kačuráková M, Mathlouthi M. FTIR and laser-Raman spectra of oligosaccharides in water: characterization of the glycosidic bond. *Carbohydr Res* 1996;**284**:145–57.
- Lambert RJ, Stratford M. Weak-acid preservatives: Modelling microbial inhibition and response. *J Appl Microbiol* 1999;**86**:157–64.
- Lindberg L, Santos AXS, Riezman H *et al.* Lipidomic Profiling of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* Reveals Critical Changes in Lipid Composition in Response to Acetic Acid Stress. *PLoS One* 2013;**8**:1–12.
- Lodi T, Ferrero I. Isolation of the DLD gene of *Saccharomyces cerevisiae* encoding the mitochondrial enzyme D-lactate ferri-cytochrome c oxidoreductase. *Mol Gen Genet* 1993;**238**:315–24.
- Mira NP, Teixeira MC, Sa I. Adaptive Response and Tolerance to Weak Acids in *Saccharomyces cerevisiae*: A Genome-Wide View. 2010;**14**, DOI: 10.1089/omi.2010.0072.
- Naumann D. Infrared Spectroscopy in Microbiology. *Encycl Anal Chem* 2000:102–31.
- Nugroho RH, Yoshikawa K, Shimizu H. Metabolomic analysis of acid stress response in *Saccharomyces cerevisiae*. *J Biosci Bioeng* 2015;**xx**, DOI: 10.1016/j.jbiosc.2015.02.011.
- Nygård Y, Mojzita D, Toivari M *et al.* The diverse role of *Pdr12* in resistance to weak organic acids. *Yeast* 2014;**31**:219–32.
- Paiva S, Devaux F, Barbosa S *et al.* Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. *Yeast* 2004;**21**:201–10.

- Passolunghi S, Riboldi L, Dato L *et al.* Cloning of the *Zygosaccharomyces bailii* GAS1 homologue and effect of cell wall engineering on protein secretory phenotype. *Microb Cell Fact* 2010;**9**:7.
- Pearce AK, Booth IR, Brown AJP. Genetic manipulation of 6-phosphofructo-1-kinase and fructose 2,6-bisphosphate levels affects the extent to which benzoic acid inhibits the growth of *Saccharomyces cerevisiae*. *Microbiology* 2001;**147**:403–10.
- Rodrigues F, Sousa MJ, Ludovico P *et al.* The Fate of Acetic Acid during Glucose Co-Metabolism by the Spoilage Yeast *Zygosaccharomyces bailii*. *PLoS One* 2012;**7**:1–7.
- Rodrigues F, Zeeman AM, Cardoso H *et al.* Isolation of an acetyl-CoA synthetase gene (ZbACS2) from *Zygosaccharomyces bailii*. *Yeast* 2004;**21**:325–31.
- Sauer M, Branduardi P, Valli M *et al.* Production of L-ascorbic acid by metabolically engineered *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*. *Appl Environ Microbiol* 2004;**70**:6086–91.
- Sauer M, Porro D, Mattanovich D *et al.* 16 Years Research on Lactic Acid Production With Yeast - Ready for the Market? *Biotechnol Genet Eng Rev* 2010;**27**:229–56.
- Sauer M, Sauer M, Valli M *et al.* Production of. *Society* 2004b;**70**:6086–91.
- Seshadri S, Khurana R, Fink AL. *Amyloid, Prions, and Other Protein Aggregates*. Elsevier, 1999.
- Sousa MJ, Ludovico P, Rodrigues F *et al.* Stress and Cell Death in Yeast Induced by Acetic Acid. In: Dr. Paula Bubulya (Ed.) *Cell Metabolism - Cell Homeostasis and Stress Response*: InTech, 2012, DOI: 10.5772/27726. Available from: <http://www.intechopen.com/books/cell-metabolism-cell-homeostasis-and-stress-response/stress-and-cell-death-in-yeast-induced-by-acetic-acid>
- Sousa MJ, Rodrigues F, Côrte-Real M *et al.* Mechanisms underlying the transport and intracellular metabolism of acetic acid in the presence of glucose in the yeast *Zygosaccharomyces bailii*. *Microbiology* 1998;**144**:665–70.
- Stratford M, Anslow PA. Comparison of the inhibitory action on *Saccharomyces cerevisiae* of weak- acid preservatives, uncouplers, and medium-chain fatty acids. *FEMS Microbiol Lett* 1996;**142**:53–8.
- Stratford M, Steels H, Nebe-von-Caron G *et al.* Extreme resistance to weak-acid preservatives in the spoilage yeast *Zygosaccharomyces bailii*. *Int J Food Microbiol* 2013;**166**:126–34.
- Susi H, Byler DM. Resolution-Enhanced Fourier Transform Infrared Spectroscopy of Enzymes. *Methods Enzym* 1986;**130**:290–311.
- Tamm LK, Tatulian SA. Infrared spectroscopy of proteins and peptides in lipid bilayers. *Q Rev Biophys* 1997;**30**:365–429.
- Thomas DS, Davenport RR. *Zygosaccharomyces bailii* — a profile of characteristics and spoilage activities. *Food Microbiol* 1985;**2**:157–69.
- Ullah A, Orij R, Brul S *et al.* Quantitative analysis of the modes of growth inhibition by weak organic acids in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 2012;**78**:8377–87.
- Vigentini I, Brambilla L, Branduardi P *et al.* Heterologous protein production in *Zygosaccharomyces bailii*: Physiological

effects and fermentative strategies. *FEMS Yeast Res* 2005;**5**:647–52.

Zimkus A, Misiūnas A, Chaustova L. Li⁺ effect on the cell wall of the yeast *Saccharomyces cerevisiae* as probed by FT-IR spectroscopy. *Cent Eur J Biol* 2013;**8**:724–9.

Chapter VI.

PHYSIOLOGICAL AND METABOLIC CHARACTERIZATION OF THE YEAST *Candida lignohabitans*

Keywords: *C. lignohabitans*, glucose, xylose, hydrolysate, batch, chemostat cultivation

BACKGROUND

One of the major goals of the bio-economy is the development of economically feasible and sustainable production processes, mainly based on biotransformation of fresh biomasses [1]. The development of such processes requires first of all the identification of suitable cell factories that are able to convert all the sugars derived from lignocellulosic material into value-added chemicals.

As reported in Bellasio and coworkers [2], *C. lignohabitans* is a promising microbial host for production of organic acids from lignocellulosic material. Indeed, this yeast proved to be able to grow on several hexose (glucose, mannose and galactose) and pentose (xylose and arabinose) sugars. Moreover, it also proved a good tolerance towards low pH and lignocellulose-derived inhibitors.

However, still very few information regarding its physiology and metabolism are available in literature. Moreover, all cultivations described to date were performed in shake flasks, which are systems known not to offer the possibility to control and regulate chemical-physical parameters as bench-top bioreactor [3].

Here, batch and chemostat cultivations were performed in bench-top bioreactor to deeply investigate the metabolism of *C. lignohabitans*. In particular, this work was aimed at the identification of parameters that can allow to control in *C.*

lignohabitans the transition from a fully respiratory to a respiro-fermentative metabolism.

Be able to provide the optimal oxygen supply would be crucial for the process control since many fermentation products are produced under microaerobic or anaerobic conditions, in which oxygen is undetectable by dissolved oxygen probe.

RESULTS AND DISCUSSION

Batch cultivations

For the characterization of the yeast *C. lignohabitans* the identification of a suited defined mineral medium was a primary requirement.

Three different defined mineral medium were evaluated: Verduyn media (modified from [4]), MeOL (modified from [5]) and B15 (medium suited for *Yarrowia lipolytica* cultivations). The rich media, YPD, was used as reference since in Bellasio and coworkers [2] it proved to guarantee high biomass production in shake flasks experiments.

For preliminary experiments, glucose was used as sole carbon and energy source at the concentration of 50 g L⁻¹. Cultivations were performed at 30°C, pH 6.5 and followed for 44 h. Full aerobic conditions were maintained by setting a set point of 30 % of dissolved oxygen controlled in cascade with stir and air flow (see Table 1 for details).

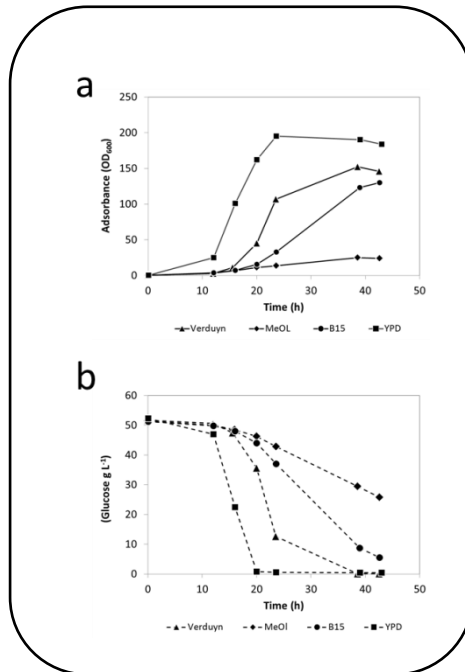


Figure 1. – Growth and glucose consumption profiles of *C. lignohabitans* batch cultivations with YPD and three different defined mineral medium.

Growth curves (a; continuous lines) and glucose consumption profiles (b; dashed lines) of *C. lignohabitans* cells cultivated at 30°C and pH 6.5. Batch cultivations were performed in aerobic conditions: 30 % of dissolved oxygen maintained through a cascade (Table 1). Glucose was used as sole carbon source at the final concentration of 50 g L⁻¹. In addition to YPD medium (filled squares), three different defined mineral medium were evaluated: Verdun (filled triangles), MeOL (filled circles) and B15 (filled diamonds).

As shown in Figure 1, four different growth trend were obtained. As expected, the faster growth rate was observed on YPD medium where glucose was completely exhausted after 24 h (Figure 1b). On MeOL and B15 medium glucose consumption was very slow and after 24 h of cultivation only 8.7 and 14.1 g L⁻¹ of glucose were

consumed, respectively. On Verduyn media sugar consumption was faster compared to the others defined medium but still slower compared to YPD medium: after 24 h of cultivation, 39.1 g L⁻¹ of glucose were consumed.

However, since Verduyn was by far the most promising defined mineral medium, triplicate experiments were performed taking samples every two hours (data not shown). A specific growth rate (μ) of 0.25 h⁻¹ was calculated during the exponential growth phase, almost identical to that calculated on YPD medium. From these experiments emerged that the slower glucose consumption on Verduyn media was related only to a longer lag phase.

Overall, Verduyn medium was able to meet the nutritional growth requirements of *C. lignohabitans* and was therefore chosen as defined mineral medium for the next experiments.

Once identified a suitable defined mineral medium the metabolic capabilities of *C. lignohabitans* were evaluated in aerated liquid cultures with Verduyn media supplemented with: 5 % of glucose, 5 % of xylose, 3 % of glucose and 2 % of xylose, and 25 % of hydrolysate. Since in the hydrolysate the amount of sugars can vary depending on the hydrolysis process, when hydrolysate was used the final concentration of sugars was adjusted in order to have 3 % of glucose and 2 % of xylose.

Operative condition were maintained as those described in batch experiments.

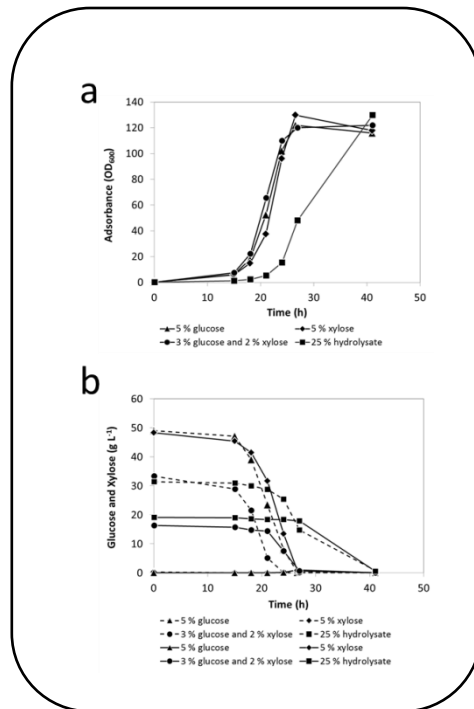


Figure 2. – Growth and sugar consumption profiles of *C. lignohabitans* batch cultivations in aerobic conditions.

Growth curves (a; continuous lines), glucose (b; dashed lines) and xylose (b; continuous lines) consumption profiles of *C. lignohabitans* cells cultivated at 30°C and pH 6.5. Batch cultivations were performed in aerobic conditions: 30 % of dissolved oxygen maintained through the cascade (Table 1). Four different experimental settings were evaluated: Verduyn media with 5 % glucose (filled triangles), Verduyn media with 5 % xylose (filled diamonds), Verduyn media with 3 % glucose and 2 % xylose (filled circles) and Verduyn media with 25 % of hydrolysate (filled squares).

As shown in Figure 2a in all the tested conditions a high amount of biomass reaching similar maximum values was accumulated ($21.0 \pm 1 \text{ g L}^{-1}$). Almost

identical growth profiles and biomass yields were observed for cultivations in defined mineral medium supplemented with glucose and/or xylose. Instead, a longer lag phase was observed during cultivation with 25 % of hydrolysate (Figure 2a).

During cultivations with both glucose and xylose, the latter was never used until complete glucose consumption (Figure 2b).

The production of metabolites during these aerated liquid cultures was also monitored at different time points (data not shown). In general, no ethanol production, or at least not at detectable level, was observed during aerobic batch cultivations.

To evaluate the influence of oxygenation the experiments just described were repeated in micro-aerobic conditions (Figure 3). During these cultivations stir was fixed at 400 rpm, air flow at 2 sL h^{-1} and no control was set on the percentage of dissolved oxygen. The cultivations were started in aerobiosis but a micro-aerobic environment was rapidly reached in each bioreactor with the increase of the biomass.

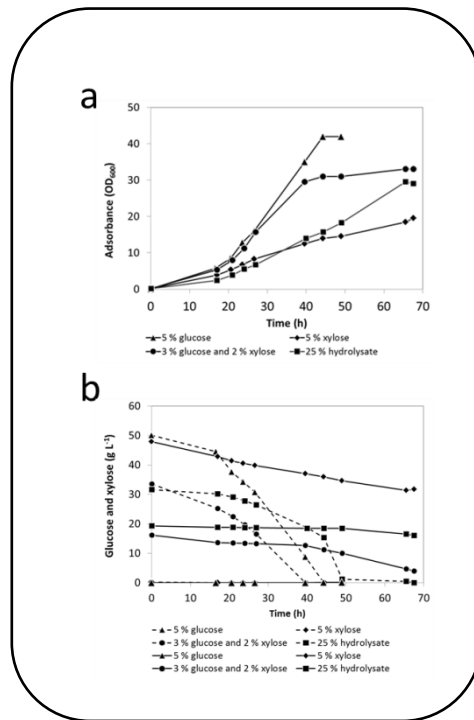


Figure 3. – Growth and sugar consumption profiles of *C. lignohabitans* batch cultivations in microaerobic conditions.

Growth curves (a; continuous lines), glucose (b; dashed lines) and xylose (b; continuous lines;) consumption profiles of *C. lignohabitans* cells at 30°C and pH 6.5. Batch cultivations were performed in microaerobic conditions (400 rpm and 2 sL h⁻¹). Four different experimental settings were evaluated: Verduyn media with 5 % glucose (filled triangles), Verduyn media with 5 % xylose (filled diamonds), Verduyn media with 3 % glucose and 2 % xylose (filled circles) and Verduyn media with 25 % of hydrolysate (filled squares).

As expected, oxygenation played a key role in the biomass production. As shown in Figure 3a the highest biomass production was observed when glucose was present as

sole carbon source: after 44 h glucose was exhausted leading to the accumulation of 7.6 g L⁻¹ of biomass (compared to 21.0 g L⁻¹ of biomass in aerobic conditions).

An even lower biomass production was observed in the other conditions and this was due to the very slow xylose consumption capability: after 68 h only 16.8 g L⁻¹ of xylose were consumed starting from 47.9 g L⁻¹.

the production of metabolites was monitored Also during micro-aerobic liquid cultures at different time points (data not shown). Ethanol was detected when glucose was used as carbon source. As example, starting from 50 g L⁻¹ of glucose 14.4 g L⁻¹ of ethanol were accumulated.

No ethanol production, or at least not at significant levels, was observed from xylose. Instead, up to 4.5 g L⁻¹ of xylitol were accumulated from less than 10 g L⁻¹ of xylose.

In these conditions, *C. lignohabitans* also showed to accumulate significant amounts of glycerol. A maximum titer of 2.8 g L⁻¹ was obtained after the consumption of 50 g L⁻¹ of glucose. Glycerol is a known by-product of fermentation of sugars to ethanol, contributing to the maintenance of the redox balance in the cell in oxygen-limited cultures [6].

The ability of *C. lignohabitans* to grow aerobically on Verduyn medium supplemented with 50 and 100 % of hydrolyzed lignocellulosic material was also investigated (Figure 4). During these experiments the total amount of sugars was 50 g L⁻¹ (40 g L⁻¹ glucose and 10 g L⁻¹ xylose) and 100 g L⁻¹ (80 g L⁻¹ glucose and 20 g L⁻¹ xylose) for 50 and 100 % hydrolysate, respectively. In parallel, batch cultivations with the same amount of sugars but without hydrolysate were also performed as control.

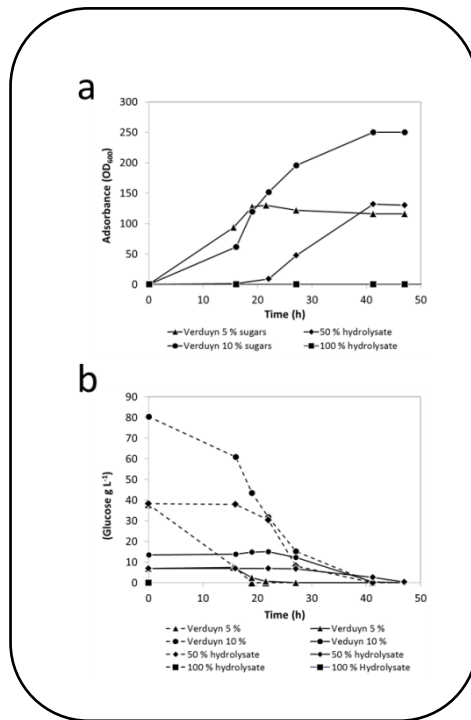


Figure 4. – Growth and sugar consumption profiles of *C. lignohabitans* batch cultivations in aerobic conditions with 50 and 100 % of hydrolysate.

Growth curves (a; continuous lines), glucose (b; dashed lines) and xylose (b; continuous lines) consumption profiles of *C. lignohabitans* cells at 30°C and pH 6.5. Batch cultivations were performed in aerobic conditions: 30 % of dissolved oxygen maintained through the cascade (Table 1). Four different experimental settings were evaluated: Verduyn media with 4 % glucose and 1 % xylose (filled triangles), Verduyn media with 50 % of hydrolysate (filled diamonds), Verduyn media with 8 % glucose and 2 % xylose (filled circles) and Verduyn media with 100 % of hydrolysate (filled squares).

As shown in Figure 4 high hydrolysate concentrations severely affect *C. lignohabitans* growth capability. In particular, when Verduyn medium was supplemented with 50 % of hydrolysate a long lag phase (around 20 h) was

observed. However, after 41 h of cultivation sugars were completely consumed leading to a biomass production similar to that of the respective control: 22.4 and 22.9 g L⁻¹ of biomass were obtained in cultivations with and without hydrolysate, respectively.

Instead, when Verduyn medium was supplemented with 100 % of hydrolysate no growth was observed even after 88 h of cultivation. In the respective control, 100 g L⁻¹ of glucose were exhausted after 41 h leading to a maximum biomass production of 41.1 g L⁻¹.

In general, high amount of hydrolysate are inhibitory for *C. lignohabitans*. However, it must be considered that the starting biomass was low compared to those usually used in cultivations with 100 % of hydrolysate [7]. Therefore, the experiment with 100 % of hydrolysate should be repeated starting with a higher initial biomass.

Chemostat cultivations

Chemostat cultures can be used in physiological studies to specifically investigate the effect of individual culture parameters [8]. In particular, as reported in literature [9-11] during chemostat cultivation several physiological and transcriptional responses to the availability of oxygen and/or glucose have been identified.

The goal of our study was to investigate the dynamic adaptation of *C. lignohabitans* to the transition from the aerobic, sugar-limited and respiratory growth to the micro-aerobic, sugar-limited and fermentative condition. In particular, during this transition we were interested in a deeper understanding of the changes in redox values and in respiration activity parameters (OTR, CTR, RQ).

Aerobic sugar-limited chemostat cultures grown at a moderate specific growth rate (0.1 h⁻¹) were exposed to sequential perturbations. Every time a steady-state was reached and proved to be stable for at least 12 h, a change in the percentage of dissolved oxygen or in the percentage of inlet oxygen was applied. This resulted in a

total of 8 different conditions tested for each chemostat (see Material and Methods for details). Also the dilution rate was changed over time in order to avoid a wash-out and to help reaching the new steady-state.

Overall, three different chemostat cultivations were performed. These differed only in the carbon source: glucose (setup 1), a mix of glucose and xylose (setup 2) and xylose (setup 3). The chemostat cultivations were constantly monitored (Biomass and HPLC measurements, RQ, OTR and CTR calculations) and lasted 480 h.

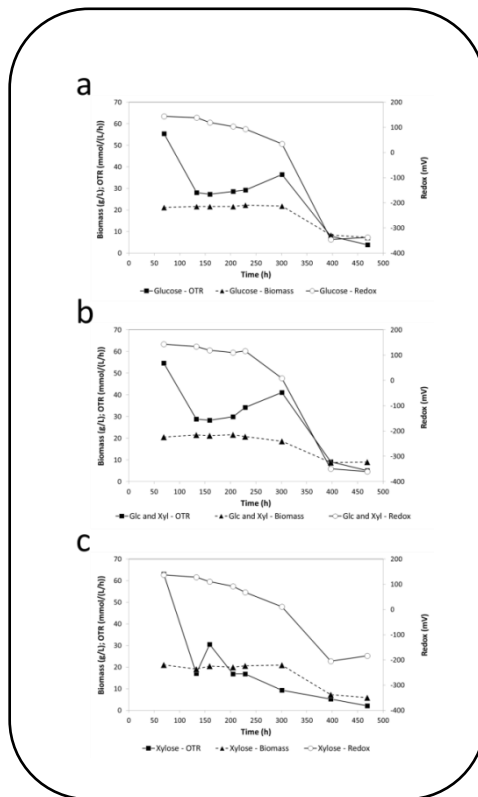


Figure 5. – Redox, OTR and biomass representative values during the chemostat cultivations.

Redox (continuous line; empty circles), OTR (continuous line; filled squares) and biomass (dashed line; filled triangles) values during 480 h of chemostat cultivations performed with different carbon sources: glucose (a; setup 1), glucose and xylose (b; setup 2) and xylose (c; setup 3). During the chemostat cultivations for every steady-state reached representative redox, OTR and biomass value was reported.

In Figure 5 it is possible to observe some representative redox, OTR and biomass values that were extrapolated from the obtained data. In general, independently from the carbon source, biomass values were constant until 300 h. This indicating that the decrease of the percentage of dissolved oxygen (from 30 % to 1.25 %) did not affect greatly the system and the achievement of a new steady-state. Instead, with the decrease of the percentage of inlet oxygen (from 21 % to 10 %) it is clearly possible to observe a loss in biomass. This decreased continued until a new steady-state was reached. With the further decrease of the percentage of inlet oxygen (from 10 % to 5 %) it was also necessary to reduce the dilution rate in order to avoid a wash out and make the achievement of a new steady-state possible. This drastic decrease of the biomass was an indicator of the shift form a fully respiratory to a respiro-fermentative metabolism.

A further prove of this was provided from redox values. As shown in Figure 5, redox values decreased progressively all along the chemostat cultivations. However, while the decrease was in the range of 100-200 mV during the first 300 h, the decrease was in the range of 200-400 mV during the next 100 h. Since, as reported in literature [12] redox potential reflects overall electron transfer and redox balance involved in intracellular metabolism, obtained values are a further indication of the shift to a respiro-fermentative metabolism.

An additional indication of this transition was obtained from the calculation of OTR, CTR and RQ. In particular, the respiratory quotient (RQ) it is well known to be an indicator of the metabolism. Independently from the carbon source, until 300 h the

RQ was around 1 (data not shown), as expected during aerobic respiration of carbohydrates. Instead, after 300 h of cultivation on glucose or glucose and xylose the RQ values increased to 2-3. As expected, this increase in RQ values was not observed on xylose since, as previously described, this was not fermented (at least not to a significant level) to ethanol during micro-aerobic conditions.

Overall, all these data provide a solid indication of the range of values in which the transition occurred.

CONCLUSION

Here, the metabolic capabilities of the yeast *C. lignohabitans* were deeply investigated.

On the one hand, the ability to rapidly consume xylose in aerobic conditions as well as the good tolerance to inhibitory compounds make *C. lignohabitans* a reliable microbial host for production of chemicals from lignocellulosic material. On the other hand, the very slow xylose consumption capability in micro-aerobic conditions as well as the inability to grow on 100 % of hydrolysate represent potential obstacles for the development of an efficient second generation production process. Further investigations are therefore necessary in order to highlight limits and potentials of this yeast.

Currently, many fermentation products are produced under microaerobic or anaerobic conditions, in which oxygen is undetectable by dissolved oxygen probe. Therefore, provide the optimal oxygen supply represents a challenge for process monitoring and control.

In this study, during the chemostat cultivations both redox potential and respiratory quotient emerged as suitable control parameters for optimum oxygen supply in *C. lignohabitans*.

Obtained data will be used to write a program script for the control of the *C. lignohabitans* fermentations.

To conclude, the development of an efficient production process will be crucial for the success of *C. lignohabitans* as fermentative host for the production of organic acids.

MATERIALS AND METHODS

Strain and media

The strain *C. lignohabitans* CBS 10342 was obtained from the CBS-KNAW (Fungal Biodiversity Centre, The Netherlands). Cells were maintained at -80 °C in YPD medium (1 % yeast extract, 2 % soy peptone, 2 % glucose) supplemented with 10 % (w/v) glycerol.

According to [4], the composition of the inoculum and fermentation medium was (per liter): 5 g of $(\text{NH}_4)_2\text{SO}_4$, 3 g of KH_2PO_4 and 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. After the pH was adjusted to 6.5 using NaOH 4 M, the medium was supplemented with a 1000X trace mineral stock solution and a 1000X vitamins stock solution. The trace mineral stock solution consist of (per liter): 15 g EDTA, 4.5 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.3 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 4.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g H_2BO_3 and 0.1 g KI. The vitamins stock solution consist of (per liter): 0.05 g D-biotin, 1 g Ca D(+)panthotenate, 1 g Nicotinic acid, 25 g Myo-inositol, 1 g thiamine hydrochloride, 1 g pyridoxol hydrochloride and 0.2 g p-aminobenzoic acid.

Glucose and/or xylose were used as carbon and energy source.

Preparation of lignocellulosic hydrolysates

Straw, Miscanthus, sawdust, shrub cuttings and wood chips containing 30 % (w/w) spruce and 70 % (w/w) beech were chosen as lignocellulosic biomass. A steam explosion treatment (15 min at 121 °C, 2 bar) was performed and the samples were stored at -20 °C until further treatment. The dry matter content of the lignocellulosic samples was determined and adjusted to 20 % with water. Cellic Ctec2[®] (Novozymes[®], USA) was added according to the manufacturer's recommendations and the enzymatic digestions were performed in shake flasks at 50 °C, 220 rpm, for 72 h. The liquid fraction resulting from the digestion was separated from the solid residues by centrifugation, filter-sterilized and used as stock for preparation of media.

Batch cultures

For the batch cultivations a bioreactor system (DASGIP AG, Jülich, Germany) with four parallel bioreactors was used. The reactors were sterilized by autoclaving at 121 °C for 20 min. The culture medium was supplemented with 5 % pure carbon sources or with steam explosion treated, cellulose digested lignocellulosic material and was added by sterile filtration into the reactor.

Starting from a fresh YPD agar plate, a single colony of the parental strain was used to inoculate a 500 mL flask with 50 mL of defined medium. The pre-culture was grown overnight and once exponential growth phase was reached the appropriate volume from the pre-culture was used to inoculate 700 mL culture medium at the OD₆₀₀ of 0.1.

Cultivations were carried out in aerobic or micro-aerobic conditions with O₂ and N₂ gassing (DASGIP AG, Jülich, Germany). The starting stirring speed was 400 rpm, the temperature set to 30.0 °C and the pH controlled at 6.5 with 4 M KOH. pH, pO₂ and redox reduction potential values were monitored with sensors provided by the

bioreactor system. During aerobic cultivations a cascade was applied in order to optimize biomass production (Table 1).

Table 1. – Range of values (min and max) of agitation speed, flow rate and inlet oxygen during batch cultivations in aerobic conditions.

	AGITATION SPEED		OXYGEN		FLOW RATE	
min	0%	400 rpm	0%	21%	5%	2 sLh ⁻¹
max	30%	1200 rpm	100%	21%	20%	40 sLh ⁻¹

During batch cultivations twelve milliliters of samples were taken at regular intervals throughout the whole cultivation duration.

Chemostat cultures

For the chemostat cultivations a bioreactor system (DASGIP AG, Jülich, Germany) with four parallel bioreactors was used. The reactors were sterilized by autoclaving at 121 °C for 20 min and the culture medium was added by sterile filtration into the reactor. The starting stirring speed was 400 rpm, the temperature set to 30.0 °C and the pH controlled at 6.5 with 4 M KOH. pH, pO₂ and redox reduction potential values were monitored with sensors provided by the bioreactor system.

Starting from a fresh YPD agar plate, a single colony of the parental strain was used to inoculate a 500 mL flask with 50 mL of defined medium. The pre-culture was grown overnight and once exponential growth phase was reached the appropriate volume from the pre-culture was used to inoculate 400 mL culture medium at the OD₆₀₀ of 0.1.

The culture volume was kept constant by a mass-controlled pump: a specific script was used to control the bioreactor volume via the DASGIP software.

Three different experimental conditions differing only in the carbon source were investigated: glucose (setup 1), glucose and xylose (setup 2) and xylose (setup 3). The feed was Verduyn medium with 5 % glucose for setup 1, 3 % glucose and 2 % xylose for setup 2 and 5 % xylose for setup 3. Tween and Ergosterol for growth in microaerobic/anaerobic conditions were added. The feed medium was prepared in 2 L bottles and sterilized by filtration through a 0.2 μm membrane filter. A specific system was designed for the exchange of the bottles every 2 to 4 days. Cultivations were started in aerobic conditions. Growth in batch mode was performed until the exhaustion of the carbon source. Subsequently, the flow of fresh substrate was turned on. The flow rate was chosen to give appropriate dilution rate. At least three change of volume were necessary before the steady state was reached. The dilution rate, the percentage of dissolved oxygen and the inlet percentage of oxygen were changed over time as shown in Table 2. Every time one of these operative values was changed three change of volume were necessary to reach a new steady state.

Table 2. – Dilution rate, percentage of dissolved oxygen and inlet percentage of oxygen during chemostat cultivations.

SETUP 1			SETUP 2			SETUP 3		
Inlet O ₂ (%)	Dilution rate (h ⁻¹)	Dissolved O ₂ (%)	Inlet O ₂ (%)	Dilution rate (h ⁻¹)	Dissolved O ₂ (%)	Inlet O ₂ (%)	Dilution rate (h ⁻¹)	Dissolved O ₂ (%)
21	0.1	30	21	0.1	30	21	0.1	30
21	0.05	30	21	0.05	30	21	0.05	30
21	0.05	10	21	0.05	10	21	0.05	10
21	0.05	5	21	0.05	5	21	0.025	5
21	0.05	2.5	21	0.05	2.5	21	0.025	2.5
21	0.05	1.25	21	0.05	1.25	21	0.025	1.25
10	0.05	1.25	10	0.05	1.25	10	0.025	1.25
5	0.025	1.25	5	0.025	1.25	5	0.02	1.25

For the first six configurations was applied the cascade described in Table 1 while in the last two configuration stir was fixed at 400 rpm and flow rate at 2 sL h⁻¹

Measurements and Calculations

In continuous culture, the dilution rate (D) depends of the flow rate (F) and the volume of the fermentor (V) according the equation:

$$D = F/V$$

The dilution rate was chosen according to *C. lignohabitans* specific growth rate (μ) calculated during exponential growth phase and considering that if μ is kept $< \mu_{\max}$ then dilution rate D can be varied over a fairly wide range without wash-out.

A DASGIP Off-gas Analyzer GA4 (DASGIP BioTools, LLC, Shrewsbury, MA) was connected to bioreactor off-gas streams. Differences between oxygen composition in the inlet and outlet gas represent consumption in the bioreactor by the equation [13]:

$$OTR = c/V*(F^{in}*C^{in} - F^{out}*C^{out}) = OUR$$

wherein OTR is the oxygen transfer rate in mmol=(L*h), c is the standard molar volume constant of 44.64 mmol=standard L, V is the working volume of the bioreactor in L, F is the total gas flow rate in standard L=h and C is the percentage of oxygen in the gas stream. Cell-specific OUR was calculated by first fitting a curve to the viable cell density time course. Dividing the local OUR by the respective cell density results in the cell-specific OUR value.

Biomass quantification

Biomass was quantified by measuring the optical density of culture samples at 600 nm and by determining the cell dry weight (CDW). A correlation between OD600 and dry biomass content was established. Interestingly, the correlation is: OD600/5.546 = g L⁻¹ dry biomass during the initial exponential growth phase on sugars, but decreases to OD600/9.703 = g L⁻¹ dry biomass during the later growth

phase on fermentation products (no significant change in morphology can be observed).

HPLC measurements

The concentrations of d-glucose, d-xylose, glycerol, ethanol and acetic acid in the culture were determined by HPLC analysis (Shimadzu, Korneuburg, Austria). Samples were prepared for the analysis adding H₂SO₄ to a final concentration of 4 mM; the samples were filtrated and 10 µL were injected in a Rezex ROA-Organic Acid H⁺ column (300 × 7.8 mm, Phenomenex, USA). The column was operated with 4 mM H₂SO₄ as mobile phase, with 1.0 mL min⁻¹ flow rate at 60 °C. A refraction index detector (RID- 10A, Shimadzu, Korneuburg, Austria) was used for quantification of sugars.

REFERENCES

1. Kavscek M, Strazar M, Curk T, Natter K, Petrovic U: **Yeast as a cell factory: current state and perspectives.** *Microbial Cell Factories* 2015, **14**.
2. Bellasio M, Mattanovich D, Sauer M, Marx H: **Organic acids from lignocellulose: *Candida lignohabitans* as a new microbial cell factory.** *Journal of Industrial Microbiology & Biotechnology* 2015, **42**:681-691.
3. Hsu W-T, Aulakh RPS, Traul DL, Yuk IH: **Advanced microscale bioreactor system: a representative scale-down model for bench-top bioreactors.** *Cytotechnology* 2012, **64**:667-678.
4. Verduyn C, Postma E, Scheffers WA, Vandijken JP: **Effect of benzoic-acid on metabolic fluxes in yeasts - a continuous-culture study on the regulation of respiration and alcoholic fermentation.** *Yeast* 1992, **8**:501-517.
5. Liang Y, Cui Y, Trushenski J, Blackburn JW: **Converting crude glycerol derived from yellow grease to lipids through yeast fermentation.** *Bioresource Technology* 2010, **101**:7581-7586.
6. Vandijken JP, Scheffers WA: **Redox balances in the metabolism of sugars by yeasts.** *Fems Microbiology Letters* 1986, **32**:199-224.
7. Wallace-Salinas V, Signori L, Li Y-Y, Ask M, Bettiga M, Porro D, Thevelein JM, Branduardi P, Foulquie-Moreno MR, Gorwa-Grauslund M: **Re-assessment of *YAP1* and *MCR1* contributions to inhibitor tolerance in robust engineered**

- Saccharomyces cerevisiae* fermenting undetoxified lignocellulosic hydrolysate.** *AMB Express* 2014, **4**:56-56.
8. van den Brink J, Daran-Lapujade P, Pronk JT, de Winde JH: **New insights into the *Saccharomyces cerevisiae* fermentation switch: Dynamic transcriptional response to anaerobicity and glucose-excess.** *Bmc Genomics* 2008, **9**.
 9. Kwast KE, Lai LC, Menda N, James DT, Aref S, Burke PV: **Genomic analyses of anaerobically induced genes in *Saccharomyces cerevisiae*: Functional roles of Rox1 and other factors in mediating the anoxic response.** *Journal of Bacteriology* 2002, **184**:250-265.
 10. ter Linde JJM, Liang H, Davis RW, Steensma HY, van Dijken JP, Pronk JT: **Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*.** *Journal of Bacteriology* 1999, **181**:7409-7413.
 11. Tai SL, Snoek I, Luttk MAH, Almering MJH, Walsh MC, Pronk JT, Daran J-M: **Correlation between transcript profiles and fitness of deletion mutants in anaerobic chemostat cultures of *Saccharomyces cerevisiae*.** *Microbiology-Sgm* 2007, **153**:877-886.
 12. Liu C-G, Xue C, Lin Y-H, Bai F-W: **Redox potential control and applications in microaerobic and anaerobic fermentations.** *Biotechnology Advances* 2013, **31**:257-265.
 13. Gilbert A, McElearney K, Kshirsagar R, Sinacore MS, Ryll T: **Investigation of Metabolic Variability Observed in Extended Fed Batch Cell Culture.** *Biotechnology Progress* 2013, **29**:1519-1527.

Chapter VII.

Yeast

Yeast 2013; 30: 365–378.

Published online 30 July 2013 in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/yea.2969

Research Article

Different response to acetic acid stress in *Saccharomyces cerevisiae* wild-type and L-ascorbic acid-producing strains

Francesca Martani[†], Tiziana Fossati[†], Riccardo Posterì, Lorenzo Signori, Danilo Porro* and Paola Branduardi
University of Milano Bicocca, Milano, Italy

*Correspondence to:

D. Porro, University of Milano
Bicocca, Piazza della Scienza
2, 20126 Milano, Italy.
E-mail: danilo.porro@unimib.it

[†]Francesca Martani and Tiziana
Fossati contributed equally to this
study.

Abstract

Biotechnological processes are of increasing significance for industrial production of fine and bulk chemicals, including biofuels. Unfortunately, under operative conditions microorganisms meet multiple stresses, such as non-optimal pH, temperature, oxygenation and osmotic stress. Moreover, they have to face inhibitory compounds released during the pretreatment of lignocellulosic biomasses, which constitute the preferential substrate for second-generation processes. Inhibitors include furan derivatives, phenolic compounds and weak organic acids, among which acetic acid is one of the most abundant and detrimental for cells. They impair cellular metabolism and growth, reducing the productivity of the process: therefore, the development of robust cell factories with improved production rates and resistance is of crucial importance. Here we show that a yeast strain engineered to endogenously produce vitamin C exhibits an increased tolerance compared to the parental strain when exposed to acetic acid at moderately toxic concentrations, measured as viability on plates. Starting from this evidence, we investigated more deeply: (a) the nature and levels of reactive oxygen species (ROS); (b) the activation of enzymes that act directly as detoxifiers of reactive oxygen species, such as superoxide dismutase (SOD) and catalase, in parental and engineered strains during acetic acid stress. The data indicate that the engineered strain can better recover from stress by limiting ROS accumulation, independently from SOD activation. The engineered yeast can be proposed as a model for further investigating direct and indirect mechanism(s) by which an antioxidant can rescue cells from organic acid damage; moreover, these studies will possibly provide additional targets for further strain improvements. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: *Saccharomyces cerevisiae*; L-ascorbic acid (L-AA); acetic acid; reactive oxygen species (ROS); cell factory; robustness

Received: 8 February 2013

Accepted: 5 July 2013

Introduction

Yeasts are nowadays regarded as effective cell factories for the production of metabolites and bio-based products of relevant economic impact as bulk and fine chemicals, protein drugs and biofuels (for recent reviews see, among others, Porro *et al.*, 2011; Hong and Nielsen, 2012).

During industrial fermentations, yeasts are subjected to different stresses, associated with either the operating parameters of the process and/or the presence of compounds that, according to

their concentrations and effects, turn out to be toxic. Yeast cells counteract these adverse conditions by triggering a stress response, with the aim of adapting to the new environment. However, under severe stress(es) the defence mechanisms may become insufficient to guarantee an efficient metabolism. This very often strongly limits the production, productivity and yield of the process itself (Mattanovich *et al.*, 2004). Furthermore, cell death may occur.

Yeasts are also considered an important resource in fundamental research, where *Saccharomyces*

cerevisiae appears as one of the most-used and versatile eukaryotic cell models, given its simplicity, feasibility of manipulation and genetic tractability. Indeed, *S. cerevisiae* has been the first sequenced eukaryotic organism (Goffeau, 1996). Current knowledge on cellular stress response and programmed cell death (PCD) in higher eukaryotes and yeasts confirms that, despite some differences in signalling pathways, several steps are phylogenetically conserved. Therefore, *S. cerevisiae* can be considered an ideal model system to study the molecular pathways underlying these processes (Munoz et al., 2012).

As a consequence of various stress conditions, different reactive oxygen species (ROS) may accumulate within the cells and are regarded as key mediators of stress response in *S. cerevisiae* (Madeo et al., 1999; Farrugia and Balzan, 2012). *S. cerevisiae* counteracts ROS accumulation through several antioxidant defence mechanisms, aiming to detoxify ROS and maintaining the intracellular redox environment in a reduced state. Yeast antioxidant defences include non-enzymatic compounds, such as glutathione (GSH) and erythroascorbate (EAA), as well as a number of protective enzymes, including superoxide dismutase, catalase, several peroxidases and thioredoxin (Huh et al., 1998; Herrero et al., 2008). Among the enzymatic systems, superoxide dismutases (SODs), which catalyse the disproportionation of superoxide anion to hydrogen peroxide, and catalases, which reduce hydrogen peroxide to water, play a relevant role (Fridovich, 1975; Switala and Loewen, 2002).

Acetic acid represents one of the most limiting by-products released from the hydrolysis of lignocelluloses, contributing to reduce ethanol yield and productivity when these waste materials are intended to be used as substrates for biofuels production (Maiorella et al., 1983; Palmqvist and Hahn-Hägerdal, 2000). The undissociated form of acetic acid is freely membrane-permeable and therefore enters the cell by simple diffusion. Once in the cytosol, where the pH is near-neutral, it dissociates, leading to the release of protons (H^+) and acetate (CH_3COO^-) (Guldfeldt and Arneborg, 1998). Proton accumulation determines the intracellular acidification, which inhibits many metabolic activities, while acetate may cause turgor pressure and free radicals production, inducing severe oxidative stress (Pampulha and Loureiro-Dias, 1990; Piper et al., 2001; Semchyshyn et al., 2011; Ullah et al., 2012). Acetic acid has also

been shown to induce programmed cell death (defined as AA-PCD) in *S. cerevisiae* cells characterized by chromatin condensation, TUNEL-positive phenotype and ROS accumulation, resulting in turn in lipid peroxidation, protein oxidation and carbonylation and genetic damages (Ludovico et al., 2001; Guaragnella et al., 2007; Morano et al., 2012; Rego et al., 2012).

L-Ascorbic acid (L-AA) is an important ROS scavenger in animals and plants and there is evidence that it is effective in exerting a protective function in yeast under oxidative stress: survival of *S. cerevisiae* cells, challenged either with the oxidative agents paraquat or H_2O_2 , was found to be enhanced upon treatment with exogenous L-AA in the range 5–50 μM (Saffi et al., 2006; Amari et al., 2008). This effect was maximal when L-AA was added before the stressing agent, suggesting that prompt intracellular L-AA availability may have a key role in optimizing its antioxidant action.

On the other hand, it has been reported that L-AA can also act as an oxidant, depending upon the environment where the molecule is present (Halliwell, 1999; Paolini et al., 1999) and, in one case (to the best of our knowledge), it may even increase *S. cerevisiae* cell mortality, due to extracellular auto-oxidation and production of H_2O_2 (Poljsak et al., 2005). The pro-oxidant effect can vary, depending on the ability of L-AA to interact with different cell culture media and to produce H_2O_2 at different rates, leading to different conflicting results obtained using ascorbate in cultured cell assays (Clément et al., 2001).

We have previously developed *S. cerevisiae* strains metabolically engineered to endogenously produce L-AA, thanks to the insertion of the necessary steps of the plant biosynthetic pathway (Branduardi et al., 2007; Fossati et al., 2011). As a consequence, the L-AA-producing strains became more tolerant to various stressful agents, including H_2O_2 and weak organic acids such as acetic, formic and lactic acid. Interestingly, the intracellular production of even a small quantity of L-AA (approximately 1.2 μM) was more effective in protecting cells from stress than the exogenous addition of higher amounts (up to 60 mg/l, i.e. 340 μM) of this antioxidant, as reported in our previous studies (Branduardi et al., 2007).

The aim of the present study was to better characterize the robustness exhibited by recombinant

strains under acetic acid stress, with particular attention to cell viability and ROS accumulation. The correlation between L-AA production and the main endogenous cellular defences was also investigated.

Materials and methods

Yeast strains, media, growth conditions

The *S. cerevisiae* genetic background used in this study was GRF18U (*MATa; ura3; his3-11,15; leu2-3,112; cir⁺*) (Brambilla *et al.*, 1999). The strains used were GRF18Uc [*MATa; ura3; his3-11,15; leu2-3,112*; (pYX012; pYX022; pYX042; pZ₅)] and GRF18U L-AA-producing [*MATa; ura3; his3-11,15; leu2-3,112*; (p012bTAfME AtMIP; p022AtLGDH; p042ScALO; pZ₅ArVTC2)] (Branduardi *et al.*, 2007; Fossati *et al.*, 2011) and the respective strains in which *SOD1* was disrupted. To delete the *SOD1* gene, the *sod1::hphMX4* cassette was amplified from the plasmid pAG26 (Goldstein and McCusker, 1999), using the oligonucleotides 5'-ATG GTT CAG CAG TCG CAG TGT TAA AGG GTG ATG CCG GTG TCT CTC GCC AGA TCT GTT TAG TAG CTT G-3' and 5'-TTA GTT GGT TAG ACC AAT GAC ACC AGG CTG GTC TTG GAC CGG CGA GCT CGT TTT CGA CAC TGG-3'. The *sod1::hphMX4* cassette was used to replace the *SOD1* gene in both GRF18U and GRF18U L-AA-producing strains. Yeast transformations were performed according to the LiAc/PEG/ss-DNA protocol (Gietz and Woods, 2002). Transformed clones were selected on YPD agar plates supplemented with the antibiotic hygromycin B at the final concentration of 2 mg/ml (Roche). Gene disruption was confirmed by PCR analyses.

Yeast cultures were grown in minimal synthetic medium (0.67% w/v YNB medium; cat. no. 919-15, Difco Laboratories, Detroit, MI, USA) with 2% w/v D-glucose as carbon source. The amino acids histidine, uracil, leucine, lysine, isoleucine and valine (Sigma) and the antibiotic nourseotricine sulphate (cloNAT, Werner BioAgents, Germany) were added to a final concentration of 50 mg/l. Δ *sod1* cells were grown in minimal synthetic medium supplemented with 100 mg/l lysine and methionine. Growth curves were obtained by inoculating yeast cells at an initial optical density of 0.1 (660 nm) and then the optical density was

measured at specific time intervals over at least 78 h from the inoculum. Each experiment was repeated at least three times. All strains were grown in shake flasks at 30 °C and 160 rpm and the ratio of flask volume:medium was 5:1.

Acetic acid treatment

Exponential phase cells ($OD_{660} = 0.5-0.8$) grown in minimal medium were harvested and resuspended (10^7 cells/ml) in minimal medium (pH 3.0, set with HCl) containing different concentrations of acetic acid (40–120 mM). Cells were also resuspended in minimal medium pH 3.0 without acetic acid. Cell viability was determined by measuring colony-forming units (cfu) after 2 days of growth on the plate at 30 °C; 100% of cell survival corresponds to cfu at time zero.

Cycloheximide (Sigma-Aldrich) dissolved in water was added at a final concentration of 50 µg/ml to yeast cells cultures grown to OD_{660} 0.5–0.7 and the cultures were then incubated for 30 min before acetic acid treatment. The absence of cytotoxicity by cycloheximide at this concentration was assessed after 200 min of incubation, by cfu (data not shown).

Acid adaptation was performed by incubating the cells in minimal medium pH 3.0 for 30 min before acetic acid treatment as described in Giannattasio *et al.* (2005).

Fluorescence microscopy analyses

Intracellular ROS production was detected with 2,7-dichlorodihydrofluorescein diacetate (H₂-DCF-DA; Sigma) and dihydroethidium (DHE). H₂-DCF-DA, dissolved in dimethylformamide (DMF), was added (as reported by Guaragnella *et al.*, 2007) to yeast cell cultures at the final concentration of 10 µg/ml, both 30 min before and during treatment, with or without acetic acid. DHE, dissolved in dimethyl sulphoxide (DMSO), was added (as reported by Guaragnella *et al.*, 2007) during acetic acid treatment, at a concentration of 5 µg/ml, 10 min before collecting the cells. In both cases, 10^8 cells were collected and resuspended in phosphate-buffered saline (PBS; NaH₂PO₄ 53 mM, Na₂HPO₄ 613 mM, NaCl 75 mM). Cells were then observed in a Nikon ECLIPSE 90i fluorescence microscope (Nikon) equipped with a ×100 objective. Emission fluorescence due to oxidation of H₂-DCF-DA or DHE was detected using a B-2A (EX 450–490 DM 505 BA520) or

G-2A (DX 510–560 DM 575 BA 590) filter (Nikon), respectively. Digital images were acquired with a CoolSnap CCD camera (Photometrics), using MetaMorph 6.3 software (Molecular Devices).

Flow-cytometric analyses

Phosphatidylserine exposure was evaluated using the ApoAlert Annexin V-FITC Apoptosis Kit (Clontech Laboratories). Cells were harvested and washed with 35 mM phosphate buffer, pH 6.8, containing 0.5 mM MgCl₂ and 1.2 M sorbitol. Cell walls were digested with 0.2 mg/ml zymolyase T100 (MP Biomedicals) in the above-mentioned buffer for 20 min at 37 °C. Protoplasts were then washed twice with binding buffer supplemented with sorbitol (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂, 1.2 M sorbitol), 4 µl annexin V-FITC (20 µg/ml) or 5 µl PI (50 µg/ml) were added to 40 µl cell suspension (1 × 10⁷ cells) and incubated for 20 min at room temperature. Cells were then washed once and resuspended in 1 ml binding/sorbitol buffer containing 0.1% BSA (Sigma). Fluorescence intensity was measured in FL1 and FL3 detectors (see below) for annexin V-FITC and PI signal, respectively.

Mitochondrial superoxide anion was detected using MitoSox (Molecular Probes). Briefly, cells were washed once in PBS, pH 6.8, and incubated in PBS with 5 µM MitoSox for 20 min at 30 °C. The cells were then washed twice in PBS and fluorescence intensity in FL3 was assessed.

All measurements of fluorescence intensity were performed on a Cytomics FC 500 flow cytometer (Beckman Coulter) equipped with an argon laser (excitation wavelength 488 nm, laser power 20 mW). The fluorescence emissions were measured through a 525 band-pass filter (FL1 parameter) and through a 620 nm short-pass filter (FL3 parameter). A total of 30 000 cells was measured for each sample. Data analysis was performed afterwards using WinMDI 2.8 software.

Catalase and superoxide dismutase activity assay

Exponential phase (OD₆₆₀ = 0.5–0.8) cells grown in minimal medium were harvested and resuspended (1 × 10⁷ cells/ml) in minimal medium (pH 3.0, set with HCl) containing 100 mM acetic acid. At different times, 5 × 10⁸ cells were harvested by centrifugation at 4000 rpm for 10 min, washed once with 1 ml ice-cold water and resuspended in

350 µl 50 mM sodium phosphate buffer, pH 7.0, for catalase assay, or Tris-HCl, pH 7.5, for SOD assay, containing 0.5 mM PMSF and protease inhibitors (complete Protease Inhibitor Cocktail, Roche). Cells were then disrupted with glass beads (425–600 µm; Sigma) in a homogenizer (BIO101/Savant FastPrep FP120) by two cycles of 20 s at speed 6.0. Lysates were collected and centrifuged for 15 min at 4 °C and the obtained supernatants were used as cell-free extracts.

Catalase activity was measured discontinuously by sampling H₂O₂ at appropriate time intervals. Briefly, 30 µl 2.2 mM H₂O₂ was added to 300 µl cell-free extract and rapidly mixed to give an initial H₂O₂ concentration of 200 µM. Every 2 min between 0 and 10 min, 50 µl aliquots of the incubation mixture were removed and rapidly mixed with 950 µl FOX reagent [ammonium ferrous sulphate (Sigma-Aldrich) 250 µM, xylenol orange (Sigma-Aldrich) 100 µM, sorbitol 100 mM, H₂SO₄ 25 mM]. The mixtures were centrifuged for 3 min at 12 000 rpm after 30 min of incubation at room temperature. The supernatants were then read at 560 nm. The H₂O₂ concentration in the sample was calculated using a standard calibration curve made with different standard samples of known concentrations of H₂O₂. Activities were calculated based on a reference curve, obtained with serial dilutions of a catalase standard (catalase from bovine liver, Sigma-Aldrich, cat. no. C30).

SOD activity was assayed using the Sigma SOD assay kit (19160), following the manufacturer's instructions.

Enzymatic activities were expressed as U/mg protein. The total protein concentration was determined as described by Bradford (1976), using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as the standard.

Results and discussion

Wild-type and L-AA-producing strains exhibit a marked different viability under acetic acid stress

Exponentially growing *S. cerevisiae* wild-type and recombinant L-AA-producing strains were incubated in the presence of increasing concentrations (40–120 mM) of acetic acid in minimal medium at pH 3.0. Cell viability was estimated through the determination of cfu at *T* = 0, 50, 100, 150 and 200 min after acetic acid addition (Figure 1A). In

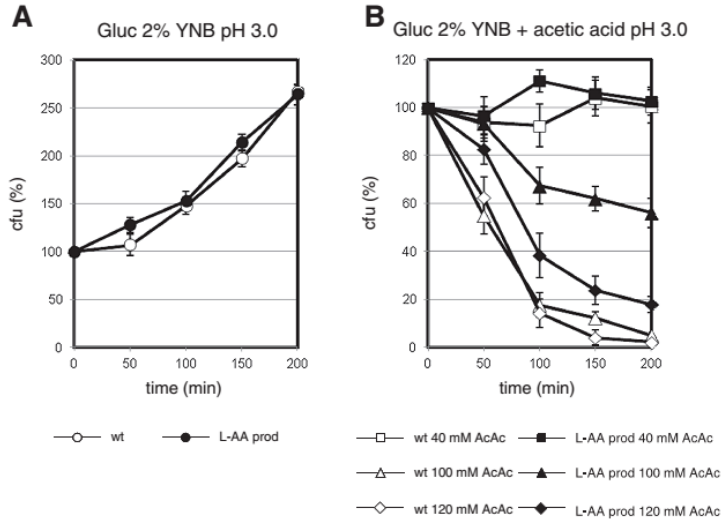


Figure 1. Viability of exponentially growing wild-type and L-AA-producing cells incubated in the absence (A) or presence (B) of increasing concentrations of acetic acid. Cells, grown in minimal medium up to $OD_{660} = 0.7-0.8$, were harvested and exposed to 0, 40, 100 and 120 mM acetic acid in minimal medium, pH 3.0. At the indicated times, cell viability was analysed by measuring colony-forming units (cfu) after 2 days of growth on plates at 30 °C; 100% corresponds to cfu at time zero. Error bars correspond to SDs from three independent experiments

the absence of acetic acid (control condition), cell viability rose to 260% during 200 min of growth for both strains (Figure 1A, circles; 100% corresponds to the cfu value at $T=0$). At low concentration (40 mM), acetic acid exerted a cytostatic effect on both wild-type and L-AA-producing cells, inhibiting cell growth (Figure 1B, squares). Cell death was instead progressively induced when cells were exposed to increasing concentrations of acetic acid (100 and 120 mM; Figure 1B, triangles and diamonds). Remarkably, under these severe conditions, the L-AA-producing strain exhibited a higher viability compared to the wild-type. In particular, when cells were challenged with 100 mM acetic acid for 200 min, cell viability was found to be 56% in the L-AA-producing strain (Figure 1B, closed triangles) against 5% in the wild-type (Figure 1B, open triangles). The same trend was highlighted when cells were treated with 120 mM acetic acid, even though this condition more severely affected the cell viability of both strains (Figure 1B, diamonds). Acetic acid concentrations > 120 mM severely caused cell death in both strains, even 30 min after the stressful treatment (data not shown).

The kinetics of the death process induced by 100 mM acetic acid was considered suitable for further characterization of the stress response in the strains.

It has been reported that in the yeast *S. cerevisiae* the cell death process mediated by moderately toxic concentrations of acetic acid (up to 100 mM) is at least partially an active process dependent on *de novo* protein synthesis, designated 'acetic acid-induced programmed cell death' (AA-PCD) (Ludovico *et al.*, 2001). To address whether the higher viability in the L-AA-producing strain under acid acetic stress may correlate with protection from AA-PCD, both wild-type and L-AA-producing strains were grown in the presence of cycloheximide, an inhibitor of protein synthesis, which is traditionally used to prevent PCD and therefore to evaluate its onset (Mattson and Furukawa, 1997; Ludovico *et al.*, 2001, 2003).

As clearly shown in Figure 2, with cycloheximide cell death was partially prevented in both strains: survival was enhanced from 5% to 47% in wild-type (Figure 2, open circles vs open squares) and from 56% to 74% in L-AA-producing cells (Figure 2,

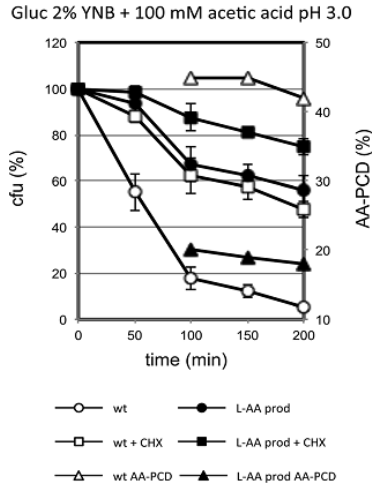


Figure 2. Viability of exponentially growing wild-type and L-AA-producing cells exposed to 100 mM acetic acid, pH 3.0, in the absence or presence of cycloheximide (50 μ g/ml). At the indicated times, cell viability was analysed by measuring colony-forming units (cfu) after 2 days of growth on plates at 30 °C; 100% corresponds to cfu at time zero. Error bars correspond to SDs from three independent experiments. Estimation of cells that undergo programmed cell death (AA-PCD, secondary axis) was calculated at 100, 150 and 200 min as the difference between the percentages of non-viable cells in the absence and in the presence of cycloheximide. SD does not exceed 7%

closed circles vs closed squares) after 200 min of acetic acid treatment, indicating that AA-PCD occurs in both strains but to a different degree. The percentage of cells that underwent AA-PCD was simply estimated by subtracting the fraction of non-viable cells determined in the presence from that measured in the absence of cycloheximide (Figure 2, triangles). Starting from 100 min after acetic acid exposure, the fraction of cells undergoing AA-PCD in the L-AA-producing strain was about 20% (closed symbols), while it was about double (43–45%) in the wild-type strain (open symbols), suggesting that the intracellular production of L-AA in the recombinant strain effectively takes part in preventing AA-PCD. To better elucidate this aspect, wild-type and L-AA-producing strains were stained with annexin V-FITC, which evidences phosphatidylserine exposure on the outer side of cell membranes, as an early marker of apoptosis. Cells were also stained with PI, which evidences

severely damaged/dead cells. Flow-cytometric analysis of the two strains after acetic acid and cycloheximide treatments (as described in Material and methods) are shown in Figure 3. When stained with annexin V-FITC in the absence of cycloheximide (Figure 3A, upper panels), the fraction of annexin V-FITC-positive cells progressively increased with time in both strains, much more in the wild-type. Indeed, only a small fraction of cells in the L-AA-producing strain is annexin V-FITC-positive (as a clear example, see the bimodal distribution after 180 min of acetic acid treatment), while most of the wild-type cells appear annexin V-FITC-positive.

In contrast, cycloheximide treatment (Figure 3A, lower panels) decreased the fraction of annexin V-FITC-positive cells. In this case, the difference between the two strains was even more evident. Hence, the data shown support the hypothesis that L-AA at least partially prevents AA-PCD.

The same trend was observed when cells were stained with PI (Figure 3B): the L-AA-producing strain displayed only a minor fraction of PI-positive cells in the absence of cycloheximide (upper panels) compared to wild-type cells. Cycloheximide treatment strongly prevented the accumulation of dead cells in both strains (lower panels), as previously evidenced in Figure 2.

Wild-type and L-AA-producing strains exhibit different ROS content

ROS production is also a key cellular event of AA-PCD in *S. cerevisiae* (Guaragnella *et al.*, 2007, 2011; Farrugia and Balzan, 2012). To better understand the effect of the endogenous production of L-AA on ROS content under acetic acid stress, cells were stained with the fluorescent probes 2,7-dichlorofluorescein diacetate (H₂-DCF-DA) and dihydroethidium (DHE) during exposure to 100 mM acetic acid at pH 3.0 (Figure 4).

H₂-DCF-DA permeates into the cell, where it is hydrolysed by cellular esterases to DCFH, which is then oxidized, primarily by H₂O₂ but also by other reactive species such as HO[•], ROO[•] and reactive nitrogen species (RNS) such as [•]NO and ONOO⁻, to the green fluorescent DCF (Gomes *et al.*, 2005). DHE has been used to detect superoxide anion (O₂^{•-}) due to its reported relative specificity for this ROS, which has been described to be associated with cells committed to death (Gomes *et al.*, 2005; Guaragnella *et al.*, 2007).

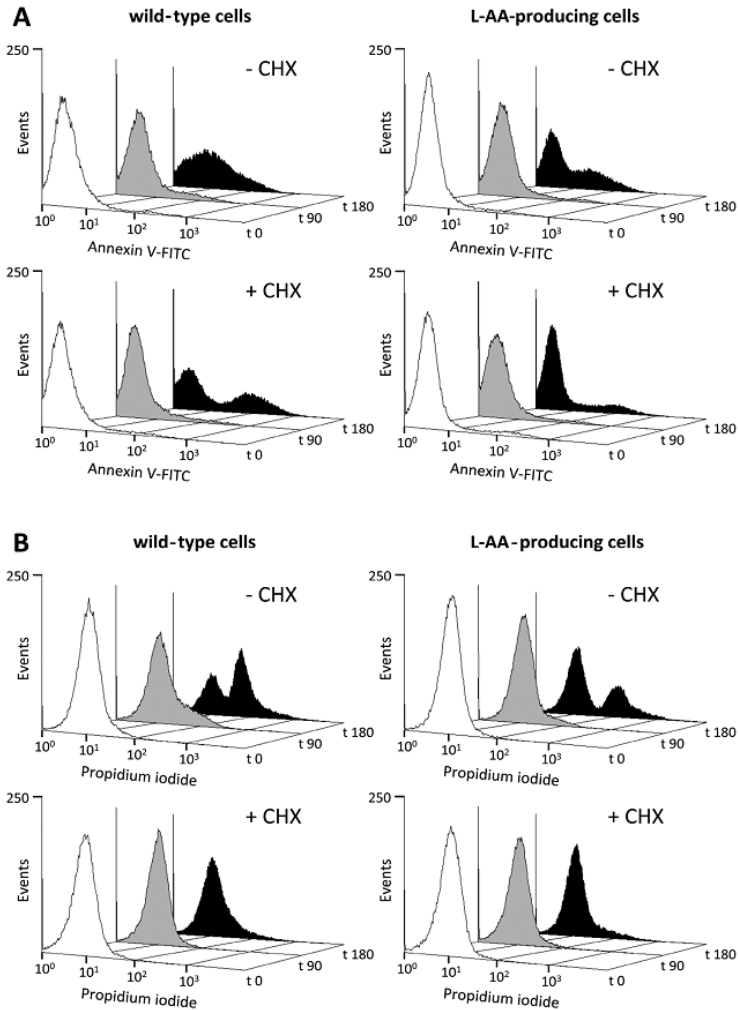


Figure 3. Flow-cytometric analysis of wild-type and L-AA-producing cells stained with annexin V-FITC (A) or propidium iodide (B). Exponentially growing cells were incubated in the absence (–CHX) or presence (+CHX) of cycloheximide (50 µg/ml) for 30 min and then exposed to 100 mM acetic acid, pH 3.0. Cells were stained at 0, 90 and 180 min after acetic acid exposure. The fluorescence distributions of wild-type (left) and L-AA-producing (right) cells (annexin V-FITC and propidium iodide) are represented

No fluorescence signal associated with both H₂-DCF-DA and DHE was detected when cells were incubated in minimal medium at pH 3.0 (data not shown).

Consistently with the literature data (Guaragnella *et al.*, 2007), fluorescence signal of H₂-DCF-DA

was detected during the first 45 min of acetic acid treatment and not at later times (Figure 4A). DHE signal was instead detected after 60 min of acetic acid treatment and not before (Figure 4B). The L-AA-producing strain exhibited a lower percentage of both H₂-DCF-DA- and DHE-positive cells

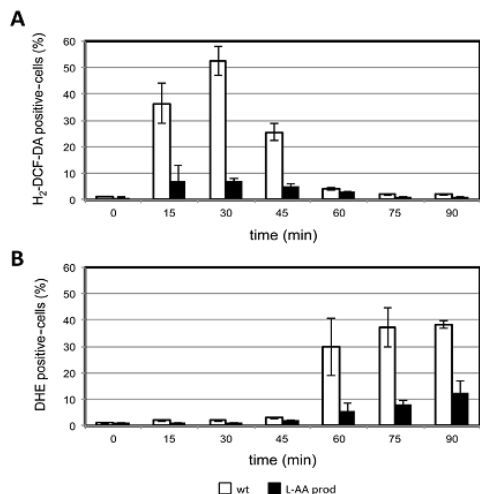


Figure 4. (A) H₂-DCF-DA and (B) DHE-positive cells (%) during acetic acid treatment in wild-type and L-AA-producing cells. To determine the percentage of cells positive to the dyes, at least 400 cells were evaluated for each sample and for each time and analysed in three independent experiments. Error bars correspond to SDs from three independent experiments

compared with wild-type at all the tested times (Figure 4A, B). The lower intracellular ROS accumulation correlates with the higher viability of the L-AA-producing strain compared with the wild-type strain.

To further support this conclusion, the MitoSox probe was used to monitor mitochondrial superoxide anion. The data obtained showed that the percentage of cells not accumulating O₂⁻ in the mitochondrial matrix after 100 min of acetic acid exposure was higher in the L-AA-producing compared to the wild-type strain (45% vs 36%), confirming the better detoxification occurring in the engineered strain.

Acetic acid stress induces high SOD activity in the wild-type but not the L-AA-producing strain

As stated in the Introduction, the yeast antioxidant defences include, among others, GSH, EAA and enzymes such as SODs and catalases, contributing to modulate ROS levels (Fridovich, 1975; Switala and Loewen, 2002). H₂O₂ and O₂⁻ levels in any single cell are therefore the result of their generation, counteracted by the efficiency of their detoxification.

To further characterize the difference in ROS accumulation observed between the wild-type and L-AA-producing strains, the SOD activity was determined. We focused our attention on the first 60 min of stress, where a good fraction (at least 50%) of cells is still viable in both strains (Figure 1B, triangles).

In the absence of acetic acid, a 'basal' SOD activity was detected in the wild-type strain that remained essentially constant over time. Also, in the L-AA-producing strain, the SOD activity remained essentially constant, but at a significantly lower (eight-fold) level (Figure 5A).

After exposure to acetic acid, SOD activity increased significantly in the wild-type strain with time, while interestingly, it remained substantially negligible in the L-AA-producing strain (Figure 5B). This data indicates that L-AA may possibly avoid the triggering of SOD, in both the absence and presence of acetic acid, conferring a pronounced difference between the two strains. It is important to underline once more that SOD generally represents the first line of defence in the wild-type strain in the early phases of oxidative stress exposure. Indeed, it has been shown that *sod1* and *sod2* yeast mutants, defective in cytosolic and mitochondrial SOD, respectively, turned out to be hypersensitive to oxidative stress induced by various pro-oxidant agents (Outen *et al.*, 2005; Auesukaree *et al.*, 2009; Kwolek-Mirek *et al.*, 2011). However, literature sources have already suggested that a triggering of the sole SOD activity is, in any case, not enough to overcome a severe stress (Harris *et al.*, 2005; Guaragnella *et al.*, 2008), but should at least be coupled with the overexpression of *CCSI*, which encodes for a chaperone responsible for Sod1 apoprotein activation (Brown *et al.*, 2004; Harris *et al.*, 2005). Indeed, a high SOD activity in the wild-type strain after 60 min (Figure 5B) is not enough to prevent the death process, a process which is much avoided in the L-AA-producing strain (Figure 1).

To better elucidate the role of SOD, we deleted the *SOD1* gene in the wild-type and L-AA-producing strains. Deleted strains were grown in liquid medium in the absence or in the presence of 40 mM acetic acid (Figure 6). Methionine and lysine (100 mg/l) were added to the medium, as *SOD1* deletion has been reported to cause auxotrophy for both amino acids when grown aerobically (Liu *et al.*, 1992). With and without acetic acid stress, the *sod1*-deleted

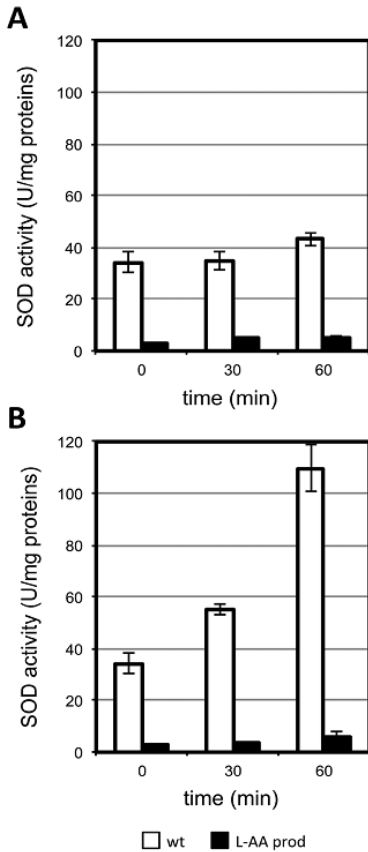


Figure 5. SOD activity in wild-type and L-AA-producing strains. Both strains were grown in minimal medium until exponential phase ($OD_{660} = 0.7\text{--}0.8$) and then inoculated in minimal medium, pH 3.0, in the absence (A) or presence (B) of 100 mM acetic acid. SOD activity was assayed at 0, 30 and 60 min. Error bars correspond to SDs from three independent experiments

wild-type strain did not start growing up to 78 h (Figure 6A, B, open squares), while growth of L-AA-producing *sod1*-deleted strain (Figure 6A, closed squares) was only slightly affected compared to wild-type and L-AA-producing strains (open and closed circles). The results clearly indicate, once more, that Sod1 activity appears to be strictly necessary in the wild-type strain, even in the absence of stress. However, it should be underlined that, even if low, SOD activity (Figure 5) is also required by

the L-AA-producing strain. In fact, the L-AA-producing strain grew better than the deleted one in the presence of acetic acid (Figure 6B; cf. closed circles and closed squares). Clearly, deeper investigations will be necessary to elucidate this aspect, with a particular glance to the other known functions of Sod1 (Culotta *et al.*, 1995).

Under acetic acid stress, the catalase response is similar in wild-type and L-AA-producing strains

Cells were challenged with acetic acid, as described for Figure 5. The catalase activity measured in minimal medium at pH 3.0 increased over time in both strains, after 60 min reaching a specific activity of 1 U/mg proteins for the wild-type and 1.3 U/mg proteins for the L-AA-producing strain (Figure 7A). This observation confirms that the simple exposure to low pH values modulates this activity, as previously described by Giannattasio *et al.* (2005). When cells were pretreated with cycloheximide for 30 min before being exposed at pH 3.0, we did not observe any increase of the catalase activity (data not shown), indicating that the observed increase shown in Figure 7A can not be due to a generic catalytic activation by post-translational mechanisms.

In the presence of acetic acid, the catalase activity was hardly detectable in both wild-type and L-AA-producing cells (measured activity of about 0.2 U/mg proteins) and did not change over time (Figure 7B), suggesting that under the tested conditions the enzyme may not be activated or remain inactive, as previously proposed by Giannattasio *et al.* (2005).

Viability enhancement caused by low pH pretreatment implies a different involvement of SOD and catalase enzymes in wild-type and L-AA-producing strains

Many organisms become more tolerant to severe doses of a stress agent as a consequence of an initial exposure to a mild dose of the same or of another type of stress. This phenomenon, termed 'acquired stress resistance', has been observed in microbes, such as bacteria and yeasts, as well as in multicellular organisms, including plants, mammals and even humans (Davies *et al.*, 1995; Durrant and Dong, 2004; Hecker *et al.*, 2007; Kensler *et al.*, 2007; Matsumoto *et al.*, 2007). This adaptive response relays upon the *de novo* protein synthesis occurring during the initial mild pretreatment, but

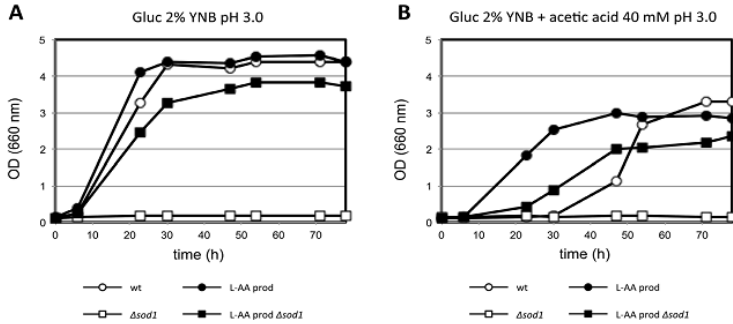


Figure 6. Growth curves of wild-type, $\Delta sod1$, L-AA-producing and L-AA-producing $\Delta sod1$ cells inoculated in minimal medium supplemented with lysine and methionine (100 mg/l) in the absence (A) or presence (B) of 40 mM acetic acid at pH 3.0

not during the severe stress treatment (Berry and Gasch, 2008; Lewis *et al.*, 2010). It has been previously demonstrated that acid stress adaptation of *S. cerevisiae* (pre-incubation of cells in minimal medium at pH 3.0 for 30 min prior to the exposure to acetic acid) protects cells from subsequent AA-PCD, and this correlates with a high catalase activity (Giannattasio *et al.*, 2005). Consistently, overexpression of *CTT1*, encoding for the cytosolic form of catalase, leads to reduced oxidative stress and increased cell resistance to various stress conditions (Guaragnella *et al.*, 2008; Abbott *et al.*, 2009; Collinson *et al.*, 2011).

Cell survival of wild-type and L-AA-producing strains was assayed after pre-incubation in minimal medium at pH 3.0 for 30 min prior to acetic acid exposure (Figure 8).

Acid stress adaptation significantly enhanced cell viability, in particular 5% to 41% in wild-type (Figure 8, open circles vs open squares) and 45% to 69% in L-AA-producing cells (Figure 8, closed circles vs closed squares) after 200 min of acetic acid exposure. Hence, acquisition of acetic acid tolerance through pre-adaptation at low pH occurs more evidently in wild-type cells, but also plays a relevant role in the L-AA-producing strain, suggesting that the endogenous defences may usefully cooperate with L-AA to further increase cell robustness.

To better investigate this hypothesis, SOD and catalase activities were measured in the pre-adapted strains. SOD activity was substantially not influenced by the low pH treatment (Figure 9A), as it remained constant in both strains for 30 min of pretreatment. After 30 min of acetic acid exposure, it was

subsequently induced in both strains, even though in the L-AA-producing strain it remained very low, resembling the trend obtained in the absence of acetic acid treatment (Figure 9A).

On the contrary, catalase activity was influenced by the low pH treatment, as it was found to be increased in both strains upon pre-incubation for 30 min at pH 3.0 (Figure 9B, time points -30 and 0 min), consistently with the literature data (Giannattasio *et al.*, 2005). Moreover, the activity went on to increase up to 30 min of acetic acid treatment in the wild-type strain, while it remained essentially constant and significantly lower in the L-AA-producing strain (Figure 9B, time points 30 and 60), reaching after 60 min a specific value of 1.7 U/mg proteins in the first and 1.1 U/mg proteins in the second. We can therefore argue that catalase action is at least in part responsible for the increased survival under acetic acid after low pH pretreatment in both strains (see again Figure 8, closed symbols), while SOD activity seems required only in the wild-type strain.

It has been previously shown that the addition of exogenous L-AA to yeast cells challenged with paraquat enhanced cell viability and reduced catalase activity compared with cells treated with the sole stressing agent (Saffi *et al.*, 2006). Our results seem to confirm this trend, as catalase, even if induced, remained significantly lower in the L-AA-producing strain, suggesting that also this activity (as previously shown in the case of SOD) can be 'economized' without affecting the better performance of the said strain under acetic stress conditions. We therefore suggest that the L-AA-producing strain is favoured under stress conditions, by both the direct intracellular

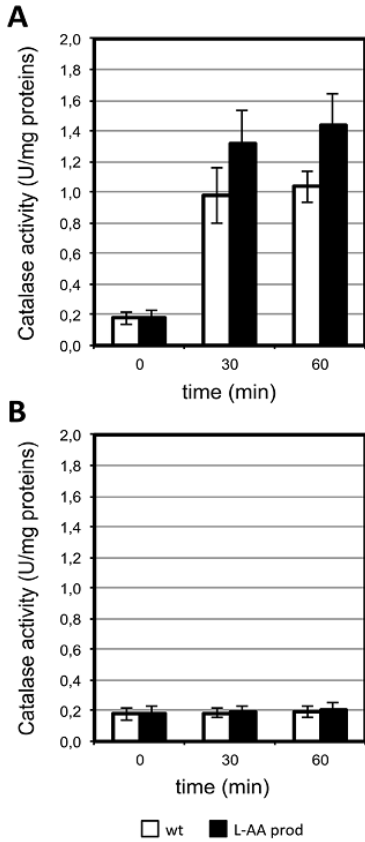


Figure 7. Catalase activity in wild-type and L-AA-producing strains. Both strains were grown in minimal medium until exponential phase ($OD_{660} = 0.7-0.8$) and then inoculated in minimal medium, pH 3.0, in the absence (A) or presence (B) of 100 mM acetic acid. Catalase activity was assayed at 0, 30 and 60 min. Error bars correspond to SDs from three independent experiments

availability of L-AA and the indirect energy saving of endogenous defences. This consideration is additionally supported by the observation that L-AA-producing cells directly exposed to acetic acid (negligible SOD and catalase activities) showed a viability comparable to that of adapted wild-type cells (high SOD and catalase activities) (Figure 8, closed circles vs open squares).

On the other hand, catalase induction in the pre-adapted L-AA-producing strain, and the consequent

Gluc 2% YNB + 100 mM acetic acid pH 3.0

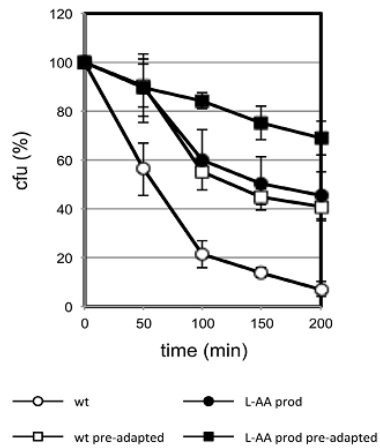


Figure 8. Viability of exponentially growing wild-type and L-AA-producing cells exposed to 100 mM acetic acid, pH 3.0, with or without pretreatment in minimal medium at pH 3.0. At the indicated times, cell viability was analysed by measuring colony-forming units (cfu) after 2 days of growth on plates at 30 °C; 100% corresponds to cfu at time zero. Error bars correspond to SDs from three independent experiments

further increase of stress tolerance, demonstrate that L-AA and catalase effects are only partially overlapping and that the stress response is a complex trait. It is clear that the interaction of L-AA with these and other cellular defences (the GSH system, among others) still need to be elucidated more deeply.

Moreover, as a second consideration, it turns out that SOD and catalase enzymes do not necessary act in sequence, as it may be expected. We could measure catalase activity even in the absence of SOD activity, as in the case of the pre-adapted L-AA-producing strain. In this sense, our results corroborate the hypothesis of catalase involvement in the acquisition of tolerance and in the adaptation response to the stress, as previously suggested (Izawa *et al.*, 1996). Further investigations are still necessary to fully explore the effective role of catalase in the stress response and its relations with the GSH system, as suggested by previous studies (Grant *et al.*, 1998).

In conclusion, here we show that the higher viability of the L-AA-producing strain exposed to acetic acid in comparison with the wild-type strain correlates with a minor to null activation of SOD and, following pretreatment at pH 3.0, with a minor

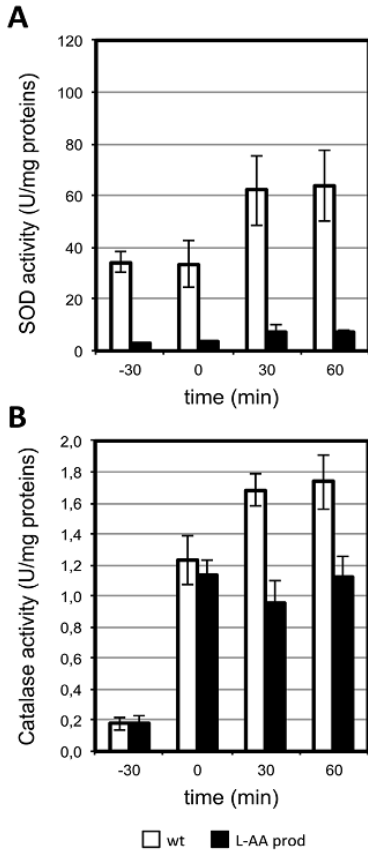


Figure 9. SOD (A) and catalase (B) activity in wild-type and L-AA-producing strains. Exponentially growing cells were incubated in minimal medium, pH 3.0, for 30 min and then exposed to 100 mM acetic acid, pH 3.0. Enzymatic activities were assayed at -30 (corresponding to the time 0 of incubation in minimal medium at pH 3.0), 0, 30 and 60 min. Error bars correspond to SDs from three independent experiments

induction of catalase. Moreover, in the engineered strain there is a significantly lower fraction of cells undergoing an active mechanism of death, correlating with a lower accumulation of ROS. Last but not least, a relevant difference in SOD activation between the two strains is also detected in the absence of stress conditions, and a consequence on growth is visible also at a non-lethal concentration of acetic acid (40 mM, Figure 6).

Finally, we could speculate that the effects derived from endogenous vitamin C production allow the cells to save energy, and these features may be desirable for a cell factory which requires energy to be devoted to growth and biomass/metabolites accumulation/production.

For these reasons, the engineered yeast is an interesting model for further studying how, and to which extent, intracellular antioxidants can prevent cellular damage and its consequences, and at the same time for further suggesting how to improve a cell factory.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under the project NEMO (Grant Agreement No. 222699). This work was supported by Regione Lombardia, Fondo per la Promozione di Accordi Istituzionali, Project No. BIOGESTECA 15083/RCC. The authors gratefully acknowledge Giovanna Tengatini for technical contributions.

References

- Abbott DA, Suir E, Duong GH, et al. 2009. Catalase overexpression reduces lactic acid-induced oxidative stress in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **75**: 2320–2325.
- Amari F, Fettouche A, Samra MA, et al. 2008. Antioxidant small molecules confer variable protection against oxidative damage in yeast mutants. *J Agric Food Chem* **56**: 11740–11751.
- Auesukaree C, Damnernsawad A, Kruatrachue M, et al. 2009. Genome-wide identification of genes involved in tolerance to various environmental stresses in *Saccharomyces cerevisiae*. *J Appl Genet* **50**: 301–310.
- Berry DB, Gasch AP. 2008. Stress-activated genomic expression changes serve a preparative role for impending stress in yeast. *Mol Biol Cell* **19**: 4580–4587.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.
- Brambilla L, Bolzani D, Compagno C, et al. 1999. NADH reoxidation does not control glycolytic flux during exposure of respiring *Saccharomyces cerevisiae* cultures to glucose excess. *FEMS Microbiol Lett* **171**: 133–140.
- Branduardi P, Fossati T, Sauer M, et al. 2007. Biosynthesis of vitamin C by yeast leads to increased stress resistance. *PLoS One* **2**: e1092.
- Brown NM, Torres AS, Doan PE, O'Halloran TV. 2004. Oxygen and the copper chaperone CCS regulate posttranslational activation of Cu,Zn superoxide dismutase. *Proc Natl Acad Sci USA* **101**: 5518–5523.
- Clément MV, Ramalingam J, Long LH, Halliwell B. 2001. The *in vitro* cytotoxicity of ascorbate depends on the culture medium

- used to perform the assay and involves hydrogen peroxide. *Antioxid Redox Signal* **3**: 157–163.
- Collinson EJ, Wimmer-Kleikamp S, Gerega SK, et al. 2011. The yeast homolog of heme oxygenase-1 affords cellular antioxidant protection via the transcriptional regulation of known antioxidant genes. *J Biol Chem* **286**: 2205–2214.
- Culotta VC, Joh HD, Lin SJ, et al. 1995. A physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. *J Biol Chem* **270**: 29991–29997.
- Davies JM, Lowry CV, Davies KJ. 1995. Transient adaptation to oxidative stress in yeast. *Arch Biochem Biophys* **317**: 1–6.
- Durrant WE, Dong X. 2004. Systemic acquired resistance. *Annu Rev Phytopathol* **42**: 185–209.
- Farrugia G, Balzan R. 2012. Oxidative stress and programmed cell death in yeast. *Front Oncol* **2**: 64.
- Fossati T, Solinas N, Porro D, Branduardi P. 2011. L-Ascorbic acid producing yeasts learn from plants how to recycle it. *Metab Eng* **13**: 177–185.
- Fridovich I. 1975. Superoxide dismutases. *Annu Rev Biochem* **44**: 147–159.
- Giannattasio S, Guaragnella N, Corte-Real M, et al. 2005. Acid stress adaptation protects *Saccharomyces cerevisiae* from acetic acid-induced programmed cell death. *Gene* **354**: 93–98.
- Gietz RD, Woods RA. 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* **350**: 87–96.
- Goffeau A. 1996. A vintage year for yeast and *Yeast*. *Yeast* **12**: 1603–1605.
- Goldstein AL, McCusker JH. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15**: 1541–1553.
- Gomes A, Fernandes E, Lima JL. 2005. Fluorescence probes used for detection of reactive oxygen species. *J Biochem Biophys Methods* **65**: 45–80.
- Grant CM, Perrone G, Dawes IW. 1998. Glutathione and catalase provide overlapping defenses for protection against hydrogen peroxide in the yeast *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* **253**: 893–898.
- Guaragnella N, Antonacci L, Giannattasio S, et al. 2008. Catalase T and Cu,Zn-superoxide dismutase in the acetic acid-induced programmed cell death in *Saccharomyces cerevisiae*. *FEBS Lett* **582**: 210–214.
- Guaragnella N, Antonacci L, Passarella S, et al. 2007. Hydrogen peroxide and superoxide anion production during acetic acid-induced yeast programmed cell death. *Folia Microbiol (Praha)* **52**: 237–240.
- Guaragnella N, Antonacci L, Passarella S, et al. 2011. Achievements and perspectives in yeast acetic acid-induced programmed cell death pathways. *Biochem Soc Trans* **39**: 1538–1543.
- Guldfeldt LU, Arneborg N. 1998. Measurement of the effects of acetic acid and extracellular pH on intracellular pH of nonfermenting, individual *Saccharomyces cerevisiae* cells by fluorescence microscopy. *Appl Environ Microbiol* **64**: 530–534.
- Halliwell B. 1999. Vitamin C: poison, prophylactic or panacea? *Trends Biochem Sci* **24**: 255–259.
- Harris N, Bachler M, Costa V, et al. 2005. Overexpressed Sod1p acts either to reduce or to increase the lifespans and stress resistance of yeast, depending on whether it is Cu²⁺-deficient or an active Cu,Zn-superoxide dismutase. *Aging Cell* **4**: 41–52.
- Hecker M, Pané-Farré J, Völker U. 2007. SigB-dependent general stress response in *Bacillus subtilis* and related Gram-positive bacteria. *Annu Rev Microbiol* **61**: 215–236.
- Herrero E, Ros J, Bellí G, Cabisco E. 2008. Redox control and oxidative stress in yeast cells. *Biochim Biophys Acta* **1780**: 1217–1235.
- Hong KK, Nielsen J. 2012. Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries. *Cell Mol Life Sci* **69**: 2671–2690.
- Huh WK, Lee BH, Kim ST, et al. 1998. D-Erythroascorbic acid is an important antioxidant molecule in *Saccharomyces cerevisiae*. *Mol Microbiol* **30**: 895–903.
- Izawa S, Inoue Y, Kimura A. 1996. Importance of catalase in the adaptive response to hydrogen peroxide: analysis of acatalasaemic *Saccharomyces cerevisiae*. *Biochem J* **320**(1): 61–67.
- Kensler TW, Wakabayashi N, Biswal S. 2007. Cell survival responses to environmental stresses via the Keap1–Nrf2–ARE pathway. *Annu Rev Pharmacol Toxicol* **47**: 89–116.
- Kwolek-Mirek M, Bartosz G, Spickett CM. 2011. Sensitivity of antioxidant-deficient yeast to hypochlorite and chlorite. *Yeast* **28**: 595–609.
- Lewis JA, Elkon IM, McGee MA, et al. 2010. Exploiting natural variation in *Saccharomyces cerevisiae* to identify genes for increased ethanol resistance. *Genetics* **186**: 1197–1205.
- Liu XF, Elashvili I, Gralla EB, et al. 1992. Yeast lacking superoxide dismutase. Isolation of genetic suppressors. *J Biol Chem* **267**: 18298–18302.
- Ludovico P, Sansonetti F, Silva MT, Côte-Real M. 2003. Acetic acid induces a programmed cell death process in the food spoilage yeast *Zygosaccharomyces bailii*. *FEMS Yeast Res* **3**: 91–96.
- Ludovico P, Sousa MJ, Silva MT, et al. 2001. *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiology* **147**: 2409–2415.
- Madeo F, Fröhlich E, Ligr M, et al. 1999. Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biol* **145**: 757–767.
- Maiorella B, Blanch HW, Wilke CR. 1983. By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **25**: 103–121.
- Matsumoto H, Hamada N, Takahashi A, et al. 2007. Vanguarders of paradigm shift in radiation biology: radiation-induced adaptive and bystander responses. *J Radiat Res* **48**: 97–106.
- Mattanovich D, Gasser B, Hohenblum H, Sauer M. 2004. Stress in recombinant protein producing yeasts. *J Biotechnol* **113**: 121–135.
- Mattson MP, Furukawa K. 1997. Anti-apoptotic actions of cycloheximide: blockade of programmed cell death or induction of programmed cell life? *Apoptosis* **2**: 257–264.
- Morano KA, Grant CM, Moye-Rowley WS. 2012. The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics* **190**: 1157–1195.
- Munoz AJ, Wanichthanarak K, Meza E, Petranovic D. 2012. Systems biology of yeast cell death. *FEMS Yeast Res* **12**: 249–265.
- Outten CE, Falk RL, Culotta VC. 2005. Cellular factors required for protection from hyperoxia toxicity in *Saccharomyces cerevisiae*. *Biochem J* **388**: 93–101.
- Palmqvist E, Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresour Technol* **74**: 25–33.

- Pampulha ME, Loureiro-Dias MC. 1990. Activity of glycolytic enzymes of *Saccharomyces cerevisiae* in the presence of acetic acid. *Appl Microbiol Biotechnol* **34**: 375–380.
- Paolini M, Pozzetti L, Pedulli GF, et al. 1999. The nature of prooxidant activity of vitamin C. *Life Sci* **64**: 273–278.
- Piper P, Calderon CO, Hatzixanthis K, Mollapour M. 2001. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* **147**: 2635–2642.
- Poljsak B, Gazdag Z, Jenko-Brinovec S, et al. 2005. Pro-oxidative vs antioxidant properties of ascorbic acid in chromium(VI)-induced damage: an *in vivo* and *in vitro* approach. *J Appl Toxicol* **25**: 535–548.
- Porro D, Gasser B, Fossati T, et al. 2011. Production of recombinant proteins and metabolites in yeasts: when are these systems better than bacterial production systems? *Appl Microbiol Biotechnol* **89**: 939–948.
- Rego A, Costa M, Chaves SR, et al. 2012. Modulation of mitochondrial outer membrane permeabilization and apoptosis by ceramide metabolism. *PLoS One* **7**: e48571.
- Saffi J, Sonogo L, Varela QD, Salvador M. 2006. Antioxidant activity of L-ascorbic acid in wild-type and superoxide dismutase deficient strains of *Saccharomyces cerevisiae*. *Redox Rep* **11**: 179–184.
- Semchyshyn HM, Abrat OB, Miedzobrodzki J, et al. 2011. Acetate but not propionate induces oxidative stress in bakers' yeast *Saccharomyces cerevisiae*. *Redox Rep* **16**: 15–23.
- Switala J, Loewen PC. 2002. Diversity of properties among catalases. *Arch Biochem Biophys* **401**: 145–154.
- Ullah A, Orij R, Brul S, Smits GJ. 2012. Quantitative analysis of the modes of growth inhibition by weak organic acids in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **78**: 8377–8387.

- Nobody ever figures out what life is all about, and it doesn't matter.

Explore the world. Nearly everything is really interesting

if you go into it deeply enough. -

