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Metabolic Opportunities Offered by Wild-Type and Engineered *Saccharomyces cerevisiae* Strains for Biofuels Production

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Selfishly.....to me and only to me!

II

Follow through
Make your dreams come true
Don't give up the fight
You will be alright
Cause there's no one like you in the universe
Don't be afraid
What your mind conceives
You should make a stand
Stand up for what you believe
And tonight
We can truly say
Together we're invincible
During the struggle
They will pull us down
But please, please
Lets use this chance
To turn things around
And tonight
We can truly say
Together we're invincible
Do it on your own
It makes no difference to me
What you leave behind
What you choose to be
And whatever they say
Your souls unbreakable
During the struggle
They will pull us down
But please, please
Let use this chance
To turn things around
And tonight
We can truly say Together we're invincible
Together we're invincible
During the struggle
They will pull us down
Please, please
Let use this chance
To turn things around
And tonight
We can truly say
Together we're invincible
Together we're invincible
Invinsible - Black Holes And Revelations (2006) -Muse

Abstract

Human society has always been dependent on biomass-derived carbon and energy for nutrition and survival. Recently, we have also become dependent on petroleum-derived carbon and energy for commodity chemicals and fuels. However, the nonrenewable nature of petroleum stands in stark contrast to the renewable carbon and energy present in biomass. Thus, there is an increasing demand to develop and implement strategies for production of commodity chemicals and fuels from biomass instead of petroleum. In the past few decades, efforts in the development of bioethanol as an alternative transportation fuel have led to significant success [40]- [44], with over 17 billion gallons of worldwide production reported for 2008 (http:// ethanolrfa.org/resource/fact/trade/). However, ethanol fails the requirement for compatibility with existing fuel infrastructure mainly because of its tendency to adsorb water. Currently, the focus is on higher alcohols which have a higher energy density, lower vapour pressure and are less hygropscopic and corrosive than ethanol. Among these advanced biofuels, n-butanol represents the most attractive alternative to gasoline thanks to its similar energy density. n-butanol is naturally produced by many *Clostridium* strains. The butanol biosynthetic pathway consists of condensing two acetyl CoA molecules (catalyzed by a thiolase) and then reducing the product to butanol (requiring four reductase and one dehydrogenase). In contrast to the fermentative pathway, the nonfermentative short-chain alcohol pathway consists of carboxylating ketoacid intermediates from aminoacid biosynthesis and then reducing the resulting compound to a correspondent alcohol. In particular n-butanol is derived from the non-natural aminoacid L-norvaline through the αketovaleric acid formation.
The principle impediment to the massive production of these biofules is the lack of an efficient, high-yielding, commercially feasible process. Thus, in order for a bio-based process to compete with existing (petroleum-based) precesses, the target chemical must be produced at high yield, titer and productivity. These goals can be difficult to attain with naturally-occuing microrganisms, which are able to produce some of these potential biofuels as a side-products. Therefore, the hosts with these desired traits are still to be developed. In this sense, metabolic engineering offers the platform for the *"directed improvement of production, formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology"* [1].
Compare with other commonly used industrial production microrgan-

isms like *E. coli*, *S. cerevisiae* is more robust against toxic inhibitors and fermentation products and is able to ferment sugars at low pH values, minimizing the contamination risk. Hence, yeast is already used today in food and beverage industry as well as for industrial production of speciality chemicals [9], [16], [17]. All these advantages together with our lab-existing knowledges drove the choice of the host towards this microrganism as target of our study aimed at developing a *S. cerevisiae* strain for the biofuels production. In particular, we focused our attention on ethanol and n-butanol because of their potential key roles as petroleum substitutes.

Initially our efforts were focused on the improvement of the glycolytic flux. Glycolysis is the main pathway involved in the sugars degradation and its intermediates are the precursors for most of the cell biosynthesis/products. Therefore, assuming that an increase in the glycolytic flux could lead to an increase in a growth/ethanol production rate (the two main "products" of the sugar yeast respiro-fermentative metabolism), we modulated the glucose transport which was indicated as one of the determining steps for the sugar metabolism [53], overexpressing the hexose transporter genes (*HXT*). Glucose transport in *S. cerevisiae* relies on a multi-factorial uptake system and in particular it is control led by multiple hexose transporter (Hxts) encoded by at least 20 *HXT* genes. These transporters differ considerably in substrate specificity and affinity and *HXT* gene inactivation has shown that the hexose carriers Hxt1 to Hxt7 are mainly involved in glucose transport [31]. Therefore, we modulated the glucose uptake rate overexpressing the lowest and the highest glucose affinity transporters, Hxt1 and Hxt7 respectively, in two different yeast backgrounds. The coding sequences were cloned into an integrative plasmid and the resulting vectors were used to transform the GRF18U [57] and CEN.PK [70] strains. The effects of this modification were analyzed by shake-flask batch growing the transformed cells in minimal medium with glucose as carbon source. Despite their biochemical differences, the overxpression of *HXT1* or *HXT7* genes has led to a very similar results in the tested conditions. In particular, the glucose consumption rate and the ethanol production and productivity were improved in the two different yeast backgrounds analyzed. Considering the improved product titers and productivities obtained, we also tested the effects of this modification on an industrial developed yeast able to produce lactic acid [10]. Based on the similar results provided by the two transporters, the industrial strain was transformed only with the *HXT1* gene. In this case, no improvement in biomass production was observed, while the measured glucose consumption was faster leading to an increase in the lactic acid production (about 15% more than in the

control straim) and also in the productivity (about 1.4 times faster than the control). These findings shows the relevance of this strategy for the industrial application.
We deeper analyzed the HXT strain phenotype by an *in silico* study. In particular, we inferred the intracellular state from growth rate (GR), glucose consumption (GUR) and ethanol (ESR) secretion rate measured for wild-type and *Sc*HXT1 overexpressing strains. Data obtained indicated that the yeast metabolism supported higher ethanol secretion rate than the experimentally determined flux. Thus, it is possible to speculate that this optimality could be reached by working on the process conditions. Furhtermore, the results shown that perturbation induced by our manipulation had widespread effects beyond just altering the glucose metabolism. In particular, the oxidative metabolism seemed to play a key role in determining the engineered strain features.
Considering the ethanol limits as a biofuel [7], we also focused our efforts on the development of a *S. cerevisiae* strain able to produce n-butanol. To realize this, two different approaches were followed: the transfer of the fermentative pathway from the natural producer and the exploration of the aminoacidic yeast metabolism which is reported to be involved in the biosynthesis of the higher alcohols and other flavor compounds during the wine production [14], [15].
Also in this case, we evaluated the expression of the *Clostrium* pathway in two different yeast backgrounds: CEN.PK [70] and BY4741 [71]. Moreover, different isoenzymes from different microrganisms were tested. Initially, the effect of the single expression of each *Clostridium* gene was analyzed growing the yeast transformed with each gene individually in minimal synthetic medium (YNB) with glucose as carbon source. Data obtained indicated that the single expression of all these genes did not affect the yeast growth. Furthermore, the *in vitro* functionality of the first three enzymes of the heterologous pathway was tested spectrophotometrically. The results obtained indicated the functionality of these enzymes. Finally, the n-butanol production was evaluated growing each strains in minimal (YNB) medium with glucose as carbon source. Considering the crucial role of cofactors such as coenzymeA and NADH/NAD+ in the desired production, the two biosynthetic precursors (panthothenic and nicotinic acid respectively) were additionally supplied. For each strain and condition, cell growth, glucose consumption, ethanol, glycerol and n-butanol production were measured every 24h. Despite no n-butanol was detected in any conditions/strains tested, the differences between producer and control strains in the ethanol and the glycerol released, suggested a possible interaction between the heterologous pathway and the yeast metabolism. For these reason, an *in*

silico analysis of the n-butanol production is in progress in order to identify the best medium conditions that lead to produce the desired alcohol. In the same time, we investigated the aminoacid yeast metabolism in order to identify some precursors of the n-butanol. Considering the wide range of alcohols and esters which are accumulated during wine production, we initially tested different wine strains. Cells were grown in minimal medium in the presence or not of αketovaleric acid or L-Norvaline. Yeast growth seemed not to be affected by the presence of these two substrates, which were converted into n-butanol in a strain-dependent manner. After that, we tested also the laboratory strain CEN.PK. Also this lab strain was able to produce n-butanol from the two precursors tested even if in a less efficient manner than the wine strains.
Villas-Boas *et al.* [79] reported the presence in *S. cerevisiae* of the αketovalerate as intermediate of the glycine metabolism. For this reason, we also tested this aminoacid as n-butanol precursor growing the CEN.PK strain in a minimal medium with glucose or galactose as carbon source and glycine as main nitrogen source. Data obtained indicated that glycine was converted into n-butanol but also into isobutanol in a environment-dependent manner. Once identified the potential precursors of the desired alcohol, we tried to improve the yield/productivity of the conversion by two different strategies: first the constitutive expression of an additional copy of the permease Gap1 from *S. cerevisiae* was performed, second, the heterologous expression of an high affinity αketovalerate dehydrogenase (PaOxoDH) was evaluated. The effects of the single or coupled expression of these enzymatic activities on n-butanol production from the previously selected compounds were analyzed.
Gap1 is an integral membrane protein able to transport all of the naturally occuring L-aminoacids found in proteins and related compouds [84]. Despite many of them also have their specific transporter, no specific transporter is described for glycine. Thus, Gap1 represented the only transporter able to internalize it [84]. Therefore, Gap1 functionality was evaluated growing the cells in minimal medium with glycine as sole nitrogen source and monitoring the glycine uptake rate. Both the strains expressing Gap1 individually and coupled with PaOxoDH consumed glycine faster than the control.
The αketoacid dehydrogenase from *Pseudomonas aeruginosa* (PaOxoDH) is a complex formed by two catalytic subunits which directly interact with the substrate, and two accessory subunits that are involved in the interaction with the cofactors. *In vitro* studies indicated a high affinity for the αketoacids and in particular for the αketovalerate [85], [78]. Its functionality in yeast was tested by an *in vitro* spectrophotometric

assay on protein extracts. Data obtained suggested the activity of this complex in the yeast intracellular environment.
Once veried the functionality of the dehydrogenase and obtained an increase in the glycine uptake, the effects of their expression on the desired conversion were analyzed. The transformed strains and the control were grown in minimal medium supplied with the three selected precursors. Both these modifications improved the n-butanol production from each precursor tested, but further experiments are needed to identify the endogenous enzymatic activities involved in the desired conversion in order to improve the yield.
Clearly, the results obtained during this project are far to be of interest and attractive for the industrial world. But this project suggests the key role of the substrate transport as production/productivity determinant. Moreover, we have shown how the tightly connectivity of the yeast metabolism can be used to produce the desired compound and that is not possible to *a priori* predict how a microrganism metabolizes e particular substrate.

Riassunto

La società umana ha sempre avuto un rapporto di dipendenza dal carbonio e dall'energia derivante dalle biomasse per la propria nutrizione e sopravvivenza. Recentemente, tale dipendenza si é estesa all'utilizzo del carbonio e dell'energia derivante dal petrolio per la produzione di composti chimici e combustibili. La natura non rinnovabile del petrolio si scontra peró con l'inesauribilità del carbonio e dell'energia ottenibile dalle biomasse. Nasce quindi la necessità di sviluppare e attuare strategie per la produzione di composti chimici e di combustibili derivanti da fonti alternative. Negli ultimi decenni, gli sforzi finalizzati all'utilizzo del bioetanolo come alternativa ai carburanti hanno portato ad un significativo successo [40]- [44], con una produzione mondiale di oltre 17 bilioni di galloni nel 2008 (http:// ethanolrfa.org/resource/fact/trade/). La sua tendenza ad adsorbire l'acqua lo rende peró incompatibile con le insfrastrutture esistenti, limitandone dunque l'utilizzo. Per questo motivo, l'attenzione attuale é rivolta verso gli alcoli superiori caratterizzati da una densità energetica maggiore, da una pressione di vapore inferiore e da una minor igroscopicità e quindi corrosività rispetto all'etanolo. Tra questi "nuovi" biocombustibili, l'n-butanolo rappresenta l'alternativa migliore al gasolio grazie ad una densità energetica simile. n-butanolo viene naturalmente prodotto dai Clostridi attraverso un pathway biosintetico che comporta la condensazione di due molecole di acetilCoA (catalizzata dalla tiolasi) e la successiva riduzione dell'intermedio a butanolo (attraverso quattro reduttasi e una deidrogenasi). Diversamente, la via non fermentativa comporta la carbossilazione dei ketoacidi derivanti dalla biosintesi degli aminoacidi, seguita dalla riduzione ad alcolo dell'intermedio derivante. Nello specifico, l'n-butanolo deriva dall'aminoacido non-naturale L-norvalina attraverso la formazione intermedia dell'αketovalerato.

Il principale ostacolo alla produzione massiva dei biocarburanti é la mancanza di un efficacie processo produttivo con elevate rese di conversione e che pertanto sia economicamente sostenibile. Infatti, perché un bio-processo sia competitivo rispetto a quelli esistenti, deve essere in grado di produrre i composti target con elevate rese, produzioni e produttivitá. Il raggiungimento di un tale traguardo puó essere limitato dall'impiego di microrganismi naturali in grado di produrre tali molecole solo come metaboliti secondari. Nasce quindi la necessità di sviluppare l'ospite con le caratteristiche produttive desiderate. In questo contesto, l'ingegneria metabolica offre la possibilità di migliorare in modo

diretto la produzione e particolari proprietà cellulari attraverso la modifica di specifiche reazioni biochimiche o l'introduzione di nuove mediante l'utilizzo della tecnologia del DNA ricombinante [1].
Confrontato con gli altri microrganismi utilizzati industrialmente come *E. coli*, *S. cerevisiae* é caratterizzato da una maggior robustezza verso inibitori tossici e i prodotti di fermentazione. Inoltre é capace di fermentare gli zuccheri a bassi pH, minimizzando in questo modo i rischi di contaminazione. Attualmente, questo lievito viene comunemente utilizzato nell'industria alimentare, oltre che per la produzione di composti chimici ad elevato valore aggiunto [9], [16], [17]. Tutti questi vantaggi insieme alle conoscenze di base del nostro laboratorio, hanno guidato la scelta proprio verso questo microrganismo che é stato utilizzato come modello per lo sviluppo di un lievito capace di produrre biocarburanti. Considerando il potenziale ruolo chiave come sostituti del petrolio rispetto alle alternative esistenti, i nostri sforzi sono stati rivolta alla produzione di etanolo e n-butanolo.
Inizialmente la nostra attenzione é stata rivolta alla possibilitá di incrementare il flusso glicolitico. La glicolisi rappresenta infatti il principale pathway coinvolo nella degradazione degli zuccheri ed i suoi intermedi sono i precursori per la maggior parte delle vie biosintetiche e dei prodotti rilasciati dalla cellula. Per questo, é possibile ipotizzare che un incremento del flusso attraverso questo via si traduca in un aumento della velocitá di crescita/produzione di etanolo (i due principali "prodotti" del metabolismo respiro-fermentativo di lievito). A tale fine, abbiamo modulato la capacità della cellula di trasportare il glucosio (indicato come uno dei rate-determining step della glicolisi [53]) attraverso l'overespressione dei geni codificanti i trasportatori per gli esosi (*HXT*). In *S. cerevisiae*, il trasporto del glucosio coinvolge diverse proteine caratterizzate da differenti affinità e velocità massima di trasporto, codificate da almeno 20 geni. Studi basati sull'inattivazione genica hanno dimostrato come i carriers dall' Hxt1 al Hxt7 rappresentino quelli maggiormente rilevanti dal punto di vista fisiologico [31]. Sulla base di quanto descritto in letteratura, abbiamo deciso di modulare il trasporto del glucosio attraverso l'overespressione dei geni *HXT1* e *HXT7*, codificanti per le proteine caratterizzate rispettivamente, dalla piú bassa e piú alta affinità per il glucosio. Le sequenze codificanti sono state clonate in plasimidi integrativi e i vettori risultanti sono stati utilizzati per trasformare due diversi ceppi di *S. cerevisiae*: GRF18U [57] e CEN.PK [70]. L'effetto determinato da questa modifica é stato analizzato mediante cinetica di crescita in terreno minimo utilizzando il glucosio come fonte di carbonio. Nonostante le differenze biochimiche che li caratterizzano, l'overespressione dei geni *HXT1* e*HXT7* porta a risultati simili, nelle

condizioni testate. Nello specifico, in entrambi i ceppi la modifica introdotta determina un incremento sia della velocità di consumo del glucosio che della produzione di etanolo. Sulla base di quanto osservato in ceppi di laboratorio, l'effetto é stato analizzato anche in un ceppo industriale sviluppato e selezionato per la produzione di acido lattico [10]. Sulla base dei risultati simili ottenuti con i due trasportatori, é stato analizzato solo l'effetto determinato dall'espressione del gene *HXT1*. In questo caso nessun incremento sulla velocità di crescita é stato rilevato, mentre la velocità di consumo di glucosio risulta aumentata. Inoltre, sia la produzione di acido lattico che la sua produttività risultano migliorate (del 15% e di 1.4 volte rispettivamente rispetto al controllo). Nell'insieme questi risultati mostrano l'applicabilità di questa strategia a livello indutriale.
Successivamente abbiamo analizzato i fenotipi determinati dall'over-espressione dei geni *HXT* attraverso un'analisi *in silico*. Nello specifico, abbiamo dedotto i flussi intracellulari a partire dalla velocità di crescita, di consumo del glucosio e di produzione di etanolo misurati per il ceppo wild-type e per il trasformato con l'*HXT1*. I risultati ottenuti mostrano come il metabolismo di lievito sia in grado di produrre etanolo ad una velocità maggiore rispetto a quella misurata sperimentalmente. Per questo motivo é possibile ipotizzare che un ulteriore incremento nella produttività dell'etanolo puó essere ottenuto modulando le condizioni di crescita/processo. Inoltre, l'analisi mostra come la perturbazione determinata dall'ingegnerizzazione effettuata non si limiti al solo metabolismo del glucosio ma sia in realtà molto piú diffusa. In particulare, il metabolismo ossidativo sembra rivestire un ruolo importante nel determinare le caratteristiche del ceppo trasformato.
Considerando i limiti legati all'utilizzo dell'etanolo come biocarburante [7], parte di questo progetto é volto allo sviluppo di ceppi di *S. cerevisiae* capaci di produrre n-butanolo. A tal fine, sono stati elaborati due diversi approcci: il trasferimento del pathway fermentativo dal produttore naturale e l'analisi del metabolismo aminoacidico di lievito, coinvolto nella biosintesi degli alcoli superiori durante la produzione del vino [14], [15]. Anche in qusto caso, l'espressione del pathway di *Clostridium* é stata analizzata in due diversi background genici: CEN.PK [70] e BY4741 [71]. Inoltre, é stata valutata l'espressione di diversi isoenzimi come alternative alle attività del produttore naturale. Inizialmente, l'effetto determinato dall'espressione singola di ognuno dei geni d'interesse sulla crescita di lievito é stata valutata attraverso cinetiche di crescita in terreno minimo (YNB) con il glucosio come fonte di carbonio. I dati ottenuti indicano che nessuno dei geni, espressi singolarmente, influenza la capacitá di *S.cerevisiae* di crescere. Successivamente, la funzionalità

in vitro delle prime tre attività del pathway é stata verificata spettrofotometricamente. Infine, la produzione di n-butanolo é stata analizzata attraverso cinetiche di crescita in terreno minimo (YNB) con il glucosio come fonte di carbonio. Considerando il ruolo chiave di cofattori come il coenzima A e il NADH/NAD+ per la produzione d'interesse, i due precursori biosintetici (l'acido pantotenico e nicotinico, rispettivamente) sono stati addizionati al medium. Per ogni ceppo e condizione, la crescita cellulare, il consumo di glucosio e la produzione di etanolo, glicerolo e butanolo é stata monitorata ogni 24 ore. Nonostante l'n-butanolo non sia stato rilevato in nessuna condizione/ceppo analizzata, le differenze osservate tra ceppo produttore e il suo controllo nell'accumulo di etanolo e glicerolo suggeriscono una possibile interazione tra il pathway eterologo e il metabolismo di *S. cerevisiae*. Per questo motivo, sono in corso delle analisi *in silico* della produzione di n-butanolo in modo da elaborare le condizioni migliori che consentano la produzione dell'alcolo desiderato in lievito.
Parallelamente, abbiamo analizzato il metabolismo aminoacidico di *S. cerevisiae* al fine di identificare precursori alternativi del n-butanolo. Considerando l'ampio range di alcoli ed esteri accumulati durante la produzione del vino, abbiamo inizialmente analizzato diversi ceppi vinari. Le cellule sono state fatte crescere in terreno minimo addizionato o no con αketovalerato o L-norvaline. La capacità di crescere delle cellule non sembra essere influenzata dalla presenza di questi composti che vengono entrambi convertiti nell'alcolo d'interesse in modo ceppo-dipendente. Sulla base di quanto osservato, abbiamo successivamente testato il ceppo di laboratorio CEN.PK. Anche questo ceppo é in grado di produrre n-butanolo a partire da entrambi i substrati testati, anche se in modo meno efficiente rispetto ai ceppi vinari.
Villas-Boas *et a.* [79] hanno descritto la presenza in *S. cerevisiae* dell'αketovalerato come intermedio del metabolismo della glicina. Sulla base di queste evidenze abbiamo testato anche questo amminoacido come potenziale precursore del n-butanolo attraverso cinetiche di crescita in terreno minimo con glucosio o galattosio come fonti di carbonio e glicina come fonte di azoto. I risultati ottenuti mostrano come la glicina fornita venga utilizzata sia per produrre n-butanolo che isobutanolo in modo dipendente dalle condizioni di crescita. Una volta selezionati i potenziali precursori per la produzione d'interesse, abbiamo tentato di incrementare yield/produttività della conversione desiderata attraverso due differenti strategie: la prima riguarda l'espressione costitutiva di una copia aggiuntiva del gene endogeno codificante per la permeasi generica Gap1; la seconda comporta l'espressione eterologa di un'attività ketoacido deidrogenasica caratterizzata da un'elevata affinità per l'αketova

lerato (PaOxoDH) [85], [78]. L'effetto dell'espressione singola o accoppiata di queste attività é stato analizzato rispetto alla produzione di n-butanolo.
Gap1 é una proteina integrale di membrana coinvolta nel trasporto di L-aminoacidi naturalmente presenti nelle proteine e altri composti ad essi associati [84]. Nonostante molti di questi composti abbiano uno specifico trasportatore, nessuna proteina esclusivamente dedicata al trasporto della glicina é stata finora descritta [84]. Gap1 risulta pertanto l'unico trasportatore coinvolto nell'uptake di questo aminoacido. La funzionalità di Gap1 nei ceppi ingegnerizzati é stata quindi valutata monitorando il consumo di glicina fornita come unica fonte di azoto durante cinetiche di crescita in terreno minimo. Entrambi i ceppi trasformati con Gap1 singolarmente o accoppiato a PaOxoDH consumano la glicina piú velocemente del relativo controllo.
αketoacido deidrogenasi di *Pseudomonas aeruginosa* (PaOxoDH é un complesso enzimatico formato da due subunità catalitiche coinvolte nella diretta interazione con il substrato e due accessorie responsabili invece del reclutamento dei cofattori. La sua funzionalità in lievito é stata testata mediante analisi spettrofotometrica *in vitro* effettuata sugli estratti proteici. I dati ottenuti evidenziano la funzionalità dell'attività eterologa espressa nell'ambiente intracellulare di lievito.
Una volta verificata la funzionalità della deidrogenasi d'interesse e incrementato l'uptake della glicina, é stato analizzato l'effetto delle ingegnerizzazioni effettuate sulla produzione di n-butanolo. I ceppi trasformati e il relativo controllo sono stati fatti crescere in terreno minimo addizionato con i tre precursori precedentemente selezionati. Entrambe le modifiche portano ad un incremento nella produzione d'interesse ma ulteriori esperimenti sono necessari al fine di verificare le attivitá enzimatiche endogene coinvolte nella regione del metabolismo considerata al fine di migliorare ulteriormente la resa.
Chiaramente, i risultati ottenuti nel corso di questo progetto sono lontano dall'essere interessanti per il panorama industriale, ma suggeriscono il ruolo chiave che il trasporto del substrato riveste in ogni processo produttivo. Inoltre, abbiamo mostrato come l'elevata connettività che caratterizza il metabolismo di lievito possa essere sfruttata per produrre composti d'interesse, dimostrando come non sia possibile predirre *a priori* il modo con cui un microrganismo converte un particolare substrato.

Contents

1 Introduction **1**

1.0.1 Why *Saccharomyces cerevisiae?* 3

1.0.2 The effects of *HXT* overexpression on the production of natural and non-natural compounds 4

1.0.3 Model-driven characterization of *Sc*HXT strain phenotypes 5

1.0.4 Construction of n-Butanol producing yeast: The heterologous expression of the ABE*Clostridium* pathway 6

1.0.5 Re-routing of the aminoacid metabolism for n-Butanol production 8

2 Analysis of the effects of an increased glycolytic flux on yeast metabolism **11**

2.1 Results 13

2.1.1 Effect of *HXT*1 and *HXT*7 overexpression in naturally ethanol producing yeasts 13

2.1.2 Effect of *HXT*1 overexpression in yeast engineered for lactic acid production 15

2.1.3 Effect of *HXT1* overexpression in homolactic yeasts 17

2.2 Discussion 17

2.3 Methods 20

2.3.1 Yeast strains, transformation, media and cultivation 20

2.3.2 Gene amplification and plasmids construction . . . 21

2.3.3 Metabolite determination 22

3 Model-driven characterization of intracellular state of *Saccharomyces cerevisiae* strains improved in glucose uptake **23**

3.1 Results 24

3.1.1 Predicting optimal phenotypes 24

3.1.2 The intracellular states: from phenotypes to intracellular behavior 26
3.2 Discussion 30
3.3 Methods 32
3.3.1 Constraining the iMM904 network 32
3.3.2 Random Sampling of Solution Space 33

4 Construction of Butanol-producing yeast through the expression of *Clostridium* pathway 35
4.1 Results 36
4.1.1 Construction of butanologenic *S. cerevisiae* strains 36
4.1.2 Enzyme assays for n-butanol synthetic enzymes expressed in *S. cerevisiae* 37
4.1.3 n-Butanol production by butanolgenic *S.cerevisiae* strains 38
4.2 Discussion 40
4.3 Methods 41
4.3.1 Strains and Media 41
4.3.2 Plasmids construction 41
4.3.3 Yeast transformation and strain construction . . . 43
4.3.4 Yeast cultivation 43
4.3.5 Enzymatic assay 43
4.3.6 Metabolite detection 44

5 Evaluation of yeast conversion capability for biofuels production 45
5.1 Results 47
5.1.1 Analysis of wild-type *S. cerevisiae* metabolic versatility 47
5.1.2 Optimization of wild-type yeast bioconversion performance 54
5.2 Discussion 59
5.3 Methods 60
5.3.1 Strains, Media and Culture Conditions 60
5.3.2 Sequences amplification and cloning strategies . . . 60
5.3.3 Enzymatic assay 62
5.3.4 Metabolite Determination 62

6 Conclusions 63

A Additional File 67

Chapter 1

Introduction

The whole collective metabolic diversity found in biological systems is remarkable in its specificity and efficiency with respect to biochemical conversions. For this reason, it is potentially possible to isolate a class of microrganism capable to naturally produce each kind of desired compound. Even subtle genetic differences can lead to large functional changes and, with the ongoing and completed genome-sequencing projects, a detailed and broad perspective of biodiversity and metabolic diversity is being explored. The diverse metabolic capabilities analyzed could be utilized in metabolic engineering and natural-products research to develop bioprocesses that are difficult or impossible to reproduce by chemical synthesis methods. One specific area where the employment of biological and metabolic diversity has the potential to dramatically impact society is in biofuels and bulk chemicals productions. In this context the ideal microrganism that expresses all of the desirable biofuel-producing properties probably does not exist, as the combination of features required for industrial conversion is so distant from native conditions that the evolution of optimal properties for industrial bioprocessing does not naturally occur. Therefore, metabolic engineering approaches are necessary.

Metabolic engineering is an applied science focusing on the development of new or improving existing cell factories [1]- [4]. Several definitions exist, but most of these are consistent with: *the use of genetic engineering to perform directed genetic modifications of cell factories with the objective to improve their properties for industrial application* [5]. In this definition the word "improve" indicates also the insertion of completely new pathways with the purpose of producing a heterologous compound in a given host cell factory. The use of advanced analytical tools for

the identification of appropriate targets for genetic modification and possibly even the use of mathematical models to perform *in silico* design of optimized cell factories, allow to distinguish this field of research from applied genetic engineering [5]. As described by Nielsen J. [3], metabolic engineering could be seen as a cyclic process, where during the design phase an appropriate target is identified based on the analysis of the cell factory. The target is then experimentally implemented and the resulting strain is analyzed again. Thus, metabolic engineering involves a continuos iteration between design and experimental work. It is clear how mathematical models could support the design phase and increase the efficiency of the metabolic engineering cycle.
Recently, the number of heterologous expressions of complete biosynthetic pathways is rapidly increasing. In this way, it is possible to introduce new functionalities which are not naturally shown in the selected host. In general, the insertion of heterologous pathways for the production of valuable compounds does not by itself imply in high-level production of the desired product. In order to increase the yield and/or productivity, it is generally required to improve the supply of the precursor metabolites and the cofactors required for the biosynthesis of the product. Furthermore, the tight connection that characterizes the microbial metabolism imposes a major constraint when the desired objective is to increase the flux towards a specific metabolite. Also in this sense, mathematical models play a crucial role in understanding how the complete network operates.
During this work we touched all of these issues: from the improvement of carbon uptake to the *in silico* analysis of the effects of this modification on the global metabolic network; from the heterologous expression of an entire pathway to the evaluation of the natural productive potential by modifying the environmental conditions. In particular, we followed different approaches with the aim to transform *S. cerevisiae* in a "gas station". We focused our attention on the two main biofuels: ethanol and n-butanol [6]. The former is today the most produced biofuels with over 17 billion gallons produced worldwide (http:// ethanolrfa.org/resource/fact/trade/); the latter represents the most attractive alternative to the petroleum derivates for the transport usage as a consequence of its chemical properties similar to those of gasoline [7].
Keeping in mind our goal, we initially modulated the yeast ability to transport glucose, overexpressing the *HXT1* and *HXT7* genes [8], two of the twenty yeast hexose transporters. This modification improved the glucose uptake rate leading to a higher growth and ethanol production rate. We also tested the effect of this overexpression on industrial production in order to verify the effectiveness of this strategy at every

production level (from lab to large-scale). Considering the history of our laboratory, we chose the lactic acid production, overexpressing the *HXT1* in an *ad hoc* developed strain able to produce the desired acid [9], [10], [11], [12]. Also in this case we observed an increase in the glucose consumption rate and in the lactic acid production.
Considering the ethanol limits as a biofuel [7], we also focused our efforts on the development of a *S. cerevisiae* strain able to produce n-butanol. To realize this, two different approaches were followed: the transfer of the pathway from the natural producer and the exploration of the aminoacidic yeast metabolism which is reported to be involved in the biosynthesis of higher alcohols (which present more than four carbons) and other flavor compounds during the wine production [14], [15]. The heterologous expression did not lead to a detectable amount of n-butanol but exploring the yeast metabolism, we identified some potential targets which will be manipulated to optimize the n-butanol production. Clearly this study is only the beginning in the construction of a butanolgenic *S. cerevisiae* but we hope that the approaches and the knowledge obtained during this project could represent a starting point for the future yeast optimizations which will lead to produce a competitive amount of biofuels.

1.0.1 Why *Saccharomyces cerevisiae?*

The relevance of *S. cerevisiae* in the industrial biotechnology scenario can be easily understood considering the number of related articles that are written every year. This was a direct consequence of the familiarity of molecular biologists with this yeast, combined with the deep knowledge about its genetics, biochemistry, physiology and fermentation technologies. All of this make it a very attractive platform for metabolic engineering. The last decade has seen several examples of how this yeast could be manipulated to improve its performance in terms of substrate/product range, yields, productivity and robustness. In particular, *S. cerevisiae* was engineered to produce, in an efficient manner, flavonoids [16], isoprenoids and polyunsatured fatty acids [17] , API (active pharmaceutical ingredients) [18] and organic acids [9]. Another issue that must be considered in the strain development is the tolerance to a number of the adverse and harmful conditions typical of the large-scale production. This aspect is becoming more and more important considering the biofuels production from biomass materials [19]. In this case, both the substrate and the product are toxic for cells and affect their viability. Our laboratory has grown addressing these issues. We

participated in the development of yeast strains able to produce up to 65 g/L of lactic acid [10], [9], [11], [12]. Moreover, we faced the tolerance issue with an innovative approach of metabolic engineering. Following the plant world example, the biosynthetic pathway of one of the main antioxidant, L-ascorbic acid (L-AA, that naturally lacks in yeast), was expressed in *S. cerevisiae* [20]. As a consequence, the recombinant yeasts became more tolerant to a number of stressful conditions, displaying an increased cell viability. After that, the L-AA production was enhanced by using again the plant model and the plant recycle system was inserted [21].
Considering the lab existing knowledges, the extensive fundamental research carried out on *S. cerevisiae* and the substantial industrial interest in this organism as a cell factory, this study was performed on this yeast.

1.0.2 The effects of *HXT* overexpression on the production of natural and non-natural compounds

The glycolytic pathway and its individual enzymes are conserved during evolution, although the mechanisms controlling carbon and energy metabolism have adapted to the needs of each species or cell type. Each glycolytic intermediate is the precursor for the biosynthesis of other small metabolites and macromolecules [22]. Thus, it is possible to speculate that an increase in the glycolytic flux could lead to an increase in the production rate of different compounds. Both experimental and *in silico* analysis suggested the role of the glucose transport as a rate-determining step of the glycolytic pathway [23], [24]. The uptake of glucose into *S. cerevisiae* is controlled by multiple hexose transporters (Hxts) [25], which have different substrate specificity and affinity and are expressed under different overlapping conditions [8]. Analyses of the effect of *HXT* gene inactivation have shown that the hexose carriers Hxt1 to Hxt7 are the main transporters [8]. In this respect, it has been already shown that the ethanol (and CO_2) productivity and yield (grams of ethanol produced per gram of glucose consumed) can be improved by overexpression of *HXT1* transporter in *S. cerevisiae* [26]- [29].
Based on these evidences, we analyzed the effects of the overexpression of the *HXT1* and *HXT7* which are respectively, the lowest and the highest affinity transporter (K_m[glucose] = 107+/-49 and 1.3+/-0.3 respectively, [31]) on the production of natural (ethanol) and non natural (lactic acid) compounds. As subsequently described, the ethanol importance is increasing in the last years and in the same way the ef-

forts to produce it at high yield and titer [19]. Also the lactic acid relevance is increasing mainly due to two emerging products, polylactic acid for biodegradable plastics and the environmentally friendly solvent ethyl lactate [6]. Moreover, this acid can be applied in food, cosmetic, tanning industry and as an intermediate in pharmaceutical processes. Wild-type yeast is not able to produce it. Thus, we tested the effects of *HXT1* overexpression on the lactic acid production in the engineered host strain CEN.PK m850, *ad hoc* constructed for being a low-pH homolactic producing yeast [10].

1.0.3 Model-driven characterization of *Sc*HXT strain phenotypes

The functioning of cells is based on complex networks of interacting chemical reactions carefully organized in space and time. These biochemical reaction networks produce observable cellular functions. Considering the complexity of the metabolism, the need of bioinformatic tools to analyze and understand cell behavior became clear.
"Omics" technologies are rapidly generating high amounts of data at different levels of biological detail which are then compiled and stored into databases [32], [33]. This, together with the increasing number of sequenced genomes available, has provided the basis for the assembly of genome-scale metabolic networks for various organisms [32], [33]. These network reconstructions represent both a manually curated knowledge base of biological information and mathematical representations of biochemical components and interactions specific to each organism. Thus, these genome-scale network reconstructions are a structured collection of genes, proteins, biochemical reactions and metabolites described to exist and operate within a particular organism which can be converted into predictive models that enable *in silico* simulations of allowable network states. A wide range of constraint-based methods have been developed and applied in order to analyze network metabolic capabilities which rely on the theory that evolution selects for fitness-optimizing organisms, a concept crystallized with the development of flux balance analysis (FBA) [34]. FBA involves optimization of a network for a given objective function, often a "biomass" reaction, to predict *in silico* flux values and/or growth. This optimization process outputs an optimal set of metabolic flux values that are consistent with maximization (or minimization) of the chosen objective. Another class of constraint-based approaches, called uniform random sampling of steady-state flux distributions, allows statistical analysis of a large range of possible alternative

solutions determined by constraints imposed on the network [35]. Both these approaches are applied to analyze the differences observed between *Sc*HXT strain and its relative control.
Metabolite release by a cell reflects its internal metabolic state and metabolite composition varies in response to genetic and environmental perturbations due to changes in intracellular pathway activities involved in the consumption/production of extracellular metabolites. Thus, variations in intracellular flux distributions can be also analyzed by changes in the extracellular profiles. For these reason, experimental data related to growth rate, glucose consumption and ethanol secretion rates were applied as additional exchange flux constraints that define the observed metabolic behavior. By using both FBA and a sampling-based network approach and statistical methods [36], differences in phenotype were linked to systemic intracellular flux perturbations. The inferred perturbations were analyzed using a reporter metabolite approach [36] in order to identify the dominant metabolic features that are perturbed.

1.0.4 Construction of n-Butanol producing yeast: The heterologous expression of the ABE*Clostridium* pathway

The supplementation and eventual replacement of petroleum fuels with renewable biologically produced fuels has been recognized as a critical goal by both the U.S. Department of Energy [37] and the European Commission Biofuels Research Advisory Council [38]. The last two years have seen great advances in the development of renewable biofuels. Ethanol produced from starch remains the most produced biofuel in the US, with nine billion gallons produced in 2008 and with a worldwide production of 17 billion gallons (http://www.ethanolrfa.org/industry/outlook). However, ethanol is not the ideal biofuel. Corrosivity and hygroscopicity make it incompatible with existing fuel storage and distribution infrastructures [38] and the construction of novel infrastructures for an ethanol economy would cost hundreds of billions of dollars [7]. Furthermore, despite its high octane number (116), ethanol contains only 70% of the energy content of gasoline [38]. The challenge, therefore, is to produce advanced biofuels that have high energy content and are compatible with storage and transportation infrastructures designed for petroleum based products, but which are also economically feasible to be produced on an industrial scale. In the near future, advanced biofuels will be required to have very similar properties to current transportation fuels. This will allow for maximal compatibility with existing engine

design, distribution systems and storage infrastructure. Butanol, a C4 alcohol, is a biosynthetic alternative to gasoline. It has an energy density of 29.2 MJ/L, which is comparable to that of gasoline (32 MJ/L), an octane number of 87, and can be mixed with gasoline at any percentage or even completely replace it [39]. In addition, butanol high hydrophobicity may allow the use of existing fuel transportation and storage infrastructures without major modifications.
The highest butanol production reported a concentration of 19.7 g/L

Table 1.1: **Maximal n-butanol productions obtained in different optimized hosts** - *atoB*: *E. coli* thiolase, *hbd*: Clostridium 3-hydroxybutyrylCoA dehydrogenase, *bcd/etfAB*: Clostridium butyrylCoA dehydrogenase/ electron transfer flavoprotein, *adhE1,adhE2*: Clostridium alcohol dehydrogenase, *ldhA*: lactate dehydrogenase, *frdBC*: fumerate reductase, *adhE*: alcohol dehydrogenase, *pta*: phpsphate acetyltransferase, *fnr*: gene product regulates expression of gene encoding PDH, *thl*: Clostridium thiolase, *fdh1*: formate dehydrogenase, *gapA*: glyceraldehyde-3-phosphate dehydrogenase, *erg10*: *S. cerevisiae* thiolase

HOST	Modifications	n-Butanol Production	References
C. beijerinckii		19.6 g/L	[40]
E. coli	*atoB, hbd, crt, bcd/etfAB adhE2* expressed in 2 vectors, Δ*ldhA,* Δ*frdBC,* Δ*adhE,* Δ*pta,* Δ*fnr*	373 mg/L	[41]
E. coli	*thl, hbd, crt, bcd/etfAB, adhE2* expressed in a single vector	1.2 g/L/ 60 hr	[42]
E. coli	*thl, hbd, crt, bcd/etfAB, adhE1, fdh1, gapA* expressed in two vectors - polycistronic vs individual expression that leaded to the highest value	580 mg/L	[43]
S. cerevisiae	*erg10, hbd, crt, bcd/etfAB, adhE2*	2.5 mg/L	[44]

produced by *Clostrdium beijerinckii*, which is one of the natural butanol producers belonging to a Clostridia specie. Despite this innate ability, Clostridia are not ideal for industrial scale production because of the limited genetic tools to manipulate their metabolism, their slow growth, their intolerance to n-butanol above 2% and to oxygen and finally their production of butyrate, acetone and ethanol as byproducts. To overcome these problems and reach higher yields, in the last two years the *Clostridium* n-butanol pathway has been heterologously expressed in well-characterized hosts such as *E. coli* and *S. cerevisiae*. The *Clostridium* pathway requires 1 mol of glucose and 4 mol of NADH to produce 1 mol of n-butanol. During the course of this project, different groups reported different butanol productions in these two hosts using a cyclic

metabolic engineering approach (Table 1.1). Based on these evidence we also expressed the *Clostridium* native pathway in two different *S. cerevisiae* backgrounds.

1.0.5 Re-routing of the aminoacid metabolism for n-Butanol production

S. cerevisiae is commonly used for wine production mostly for its ability to produce a wide range of minor but sensorially important volatile metabolites that gives wine its vinous character [48]. These volatile metabolites, which are derived from the sugar and amino acid metabolism of wine yeast, include esters, carbonyls, volatile fatty acids, sulphur compounds and higher alcohols. Thus a number of alcohols can be produced from aminoacid metabolism intermediates [14], [15]. Briefly, the aminoacid biosynthetic pathway can generate a number of ketoacid intermediates. The yeast *S. cerevisiae* converts the ketoacids through the leucine, valine, isoleucine (also called Branched-chain aminoacids), phenylalanine, tryptophan, and methionine pathway into "fusel" alcohols as byproducts of fermentation [46]. For example, isobutanol is produced by wild-type *S. cerevisiae* as a degradation product of valine metabolism [81]. Various companies (DuPont, Gevo and Butalco) are working on different metabolic engineering strategies to improve this natural production and these efforts leaded to several patent applications. One of the strategy used suggested the yeast cytosolic overexpression of heterologous enzymes of the valine pathway, in particular the overexpression of ketoacid decarboxylase and alcohol dehydrogenase. To avoid cofactor imbalances between glycolytically derived NADH and NADPH requirements of the isobutanol pathway, engineering of cofactor specificities of the involved oxidoreductase was proposed. Ethanol formation can be blocked by deletion of pyruvate decarboxylase genes. The resulting cytosolic acetyl CoA requirements can be overcome by the expression of a formate dehydrogenase (PCT/EP2009/000181). In a similar way Atsumi *et al.* reconstituted the pathway to produce n-butanol from aminoacids [47] by expressing only two activities in *E. coli*. In this way, they obtained a mixture of several alcohols (1- propanol (31 mg/L), 2-methyl-butanol (2MB, 68 mg/L), isobutanol (389 mg/L), 3-methyl-butanol (3MB, 132 mg/L), 2-phenylethanol (40 mg/L), and 1-butanol (16 mg/L). Then, the authors deleted each gene coding for activities involved in the formation of undesired alcohols, increasing the butanol but also the propanol titers (1.2 g/L).

These examples suggest that the aminoacidic metabolism can be covered to obtain higher alcohols. During this project we firstly evaluated which aminoacid can be used as n-butanol precursor. After that we tried to increase the efficiency of the desired conversion modulating the aminoacid uptake overexpressing the general permease Gap1. Finally we improved the specificity of the conversion of interest by the overexpression of a high affinity-αketovalerate dehydrogenase.
Our strategy led to a slightly increase in the efficiency and the specificity of the conversion of the selected precursor into n-butanol but the yield obtained is far from the industrial interest also because the production does not start from glucose. Clearly, further engineering steps will be necessary. Data obtained suggested the centrality of the αketovaleric acid and the activities involved in its conversion. Therefore the enzyme(s) involved in the biosynthesis of this intermediate will be firstly identified and overexpressed in order to improve the αketovalerate pool. Moreover, the aminoacid metabolism is tightly regulated *via* feedback inhibition of its intermediates; deregulation of these feedback mechanisms leads to an increase in alcohol production [47]. Finally, the promiscuity of the decarboxylase and dehydrogenase to transform ketoacids to alcohols leads to obtain a mixture of them. In this sense, despite to some extend also a mixture of different superior alcohols could be of interest, the identification of the yeast specific activity involved in the conversion of αketovalerate into n-butaol represents a necessary step towards an increased n-butanol yield, allowing to delete all the competitor activities which produce undesired alcohols. In this respect, the pyruvate decarboxylases will represent certainly the main targets of future studies.

Chapter 2

Analysis of the effects of an increased glycolytic flux on yeast metabolism

Glucose, either derived from starch and/or cellulosic materials, is the main carbon and energy source today available. An economically sustainable bioprocess requires a high yield (grams of product obtained per grams of substrate), high production titer (g/L) and high productivity (g/L/hr). In the last decade many examples have shown how high yields and high production titers can be obtained by recombinant redirection of the carbon flow towards the desired compound. In this respect, an increase in the glycolytic flux could represent a further strategy to improve the efficiency of bioprocesses, considering the centrality of this pathway. Even recognizing that in baker's yeast one determining step for the glucose catabolism is the sugar uptake [53], this has never been experimentally analyzed and conceived to improve the metabolite(s) productivity. Glucose transport in S. cerevisiae relies on a multi-factorial uptake system and in particular it is controlled by multiple hexose transporter (Hxts) encoded by at least 20 HXT genes. These transporters differ considerably in substrate specificity and affinity and HXT gene inactivation has shown that the hexose carriers Hxt1 to Hxt7 are the main glucose transporters. HXT1 and HXT7, which encode for the two hexose transporters having the lowest and the highest affinity for glucose respectively, were selected for this study in order to evaluate the effects of the expression of an

additional copy of one of these transporters on the ethanol and the lactic acid production. **From: "Effect of *HXT1* and *HXT7* hexose transporter overexpression on wild-type and lactic acid producing *Saccharomyces cerevisiae* cells - Rossi G. Sauer M., Porro D., Branduardi P. - MCF 2010: 9:15**

2.1 Results

2.1.1 Effect of *HXT*1 and *HXT*7 overexpression in naturally ethanol producing yeasts

First, the effect of the overexpression of the two different hexose transporters in two different yeast genetic backgrounds was studied. The strains GRF18U (the model yeast strain used in our laboratory) and CEN.PK (a generally accepted reference yeast strain) were both transformed with the integrative plasmids p022HXT1 or p022HXT7, respectively carrying the HXT1 and HXT7 genes under the control of the glycolytic *Sc*TPI promoter (Figure 2.1: for each transformation, at least three independent transformants were analysed in three independent experiments). The natural abilities of the control and of the HXT1 or

Strains	Genotype	Plasmid*
GRF18U	*MATa, ura3, his3, leu2*	**p022** (*Sc*TPI; -; *HIS3*)
GRF18U [*HXT1*]	*MATa, ura3, his3, leu2*	**p022HXT1** (*Sc*TPI; *HXT1*; *HIS3*)
GRF18U [*HXT7*]	*MATa, ura3, his3, leu2*	**p022HXT7** (*Sc*TPI; *HXT7*; *HIS3*)
CEN.PK	*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2*	**p022** (*Sc*TPI; -; *HIS3*)
CEN.PK [*HXT1*]	*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2*	**p022HXT1** (*Sc*TPI; *HXT1*; *HIS3*)
CEN.PK [*HXT7*]	*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2*	**p022HXT7** (*Sc*TPI; *HXT7*; *HIS3*)
CEN.PK [LDH]	*MATa, ura3-52, his3-11, TRP1, MAL2-8c, SUC2*	**p022** (*Sc*TPI; -; *HIS3*) **p212LDH** (*Sc*TPI; LDH; *LEU2*)
CEN.PK [*HXT1*] [LDH]	*MATa, ura3-52, his3-11, TRP1, MAL2-8c, SUC2*	**p022HXT1** (*Sc*TPI; *HXT1*; *HIS3*) **p212LDH** (*Sc*TPI; LDH; *LEU2*)
CEN.PK [*HXT7*] [LDH]	*MATa, ura3-52, his3-11, TRP1, MAL2-8c, SUC2*	**p022HXT7** (*Sc*TPI; *HXT7*; *HIS3*) **p212LDH** (*Sc*TPI; LDH; *LEU2*)
CEN.PKm850 [LDH][50]	*MATa, pdc1::loxP, pdc5::loxP, pdc6::loxP, ura3-52*	**YEpLpLDH**[50] (*Sc*TPI; LDH; *URA3*) **p022KMX4** (*Sc*TPI; -; $G418^R$)
CEN.PKm850 [*HXT1*] [LDH]	*MATa pdc1::loxP pdc5::loxP pdc6::loxP ura3-52*	**YEpLpLDH**[50] (*Sc*TPI; LDH; *URA3*) **p022KMX4HXT1** (*Sc*TPI; *HXT1*; $G418^R$)
CEN.PKm850 [*HXT7*] [LDH]	*MATa pdc1::loxP pdc5::loxP pdc6::loxP ura3-52*	**YEpLpLDH**[50] (*Sc*TPI; LDH; *URA3*) **p022KMX4HXT7** (*Sc*TPI; *HXT7*; $G418^R$)

Figure 2.1: **Transformed strains used in this study.** * Plasmid name. In brackets are reported the promoter, the harboured gene and the selection marker respectively.

HXT7 transformed yeasts to produce ethanol were compared in order to analyze the effects on yeast metabolism.

Figure 2.2 and Figure 2.3 show the shake-flask batch growth kinetics in defined YNB-2% glucose medium of transformed GRF18U (Figure 2.2, see also Figure 2.4) and CEN.PK (Figure 2.3, see also Figure 2.4) yeast strains. Panel A shows the growth, measured as optical density (OD660), of the wild type strain (open circles), of the HXT1 (closed circles) and HXT7 (open squares) overexpressing strains. Panels B and panels C show the glucose consumption and the ethanol production respectively. The results clearly show that the presence of an additional copy of a hexose transporter leads to a faster glucose consumption rate

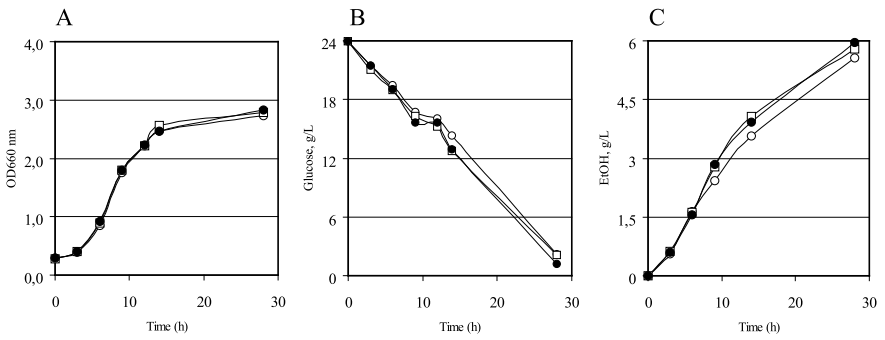


Figure 2.2: **Overexpression of HXT1 or HXT7 genes in the S. cerevisiae GRF18U strain.** Strains were flask-batch grown in minimal (YNB) medium, with glucose as a carbon source. (A) Growth was measured as optical density (OD 660 nm). (B) Residual glucose, g/L. (C) Ethanol produced, g/L. Data correspond to the mean values of three independent clones independently tested at least three times. Standard error is lower than 0.03%. Open circles: GRF18U-Control. Closed circles: GRF18U [HXT1] transformants. Open squares: GRF18U [HXT7] transformants.

and a faster ethanol production rate (panels B and C). It can be observed that similar glucose consumption rates have been observed from the different transformed strains, despite of the different biochemical features of the Hxt1 and Hxt7 transporters.

The two genes were initially tested because we could not *a priori* predict their positive or, eventually, negative effect. Indeed, similar effects were also obtained when glucose concentration was increased to 5% (Figure 2.4, only data obtained in the CEN.PK background overexpressing the HXT1 gene are shown). Biomass production is also increased, particularly in the CEN.PK yeast background; however the reason for such a behavior is not yet completely understood. It is also important to underline that during balanced exponential growth, the specific growth rates of the control and the transformed strains are equal (Figure 2.4). A very similar correlation (i.e., increased glucose consumption vs higher metabolite and higher biomass production) has been previously observed in our laboratory [12].
All the data are summarised in Table 2: the glucose consumption rate (at least in the first phases of the process) and the ethanol productivity and production were improved by overexpressing HXT1 or HXT7 in the two different yeast genetic backgrounds tested. It is worth to underline that when cells were grown in medium containing 5% glucose, also the nitrogen content was consequently increased (see Methods), determining the development of a higher biomass. This justifies the higher glucose consumption and ethanol production rates measured in these growth

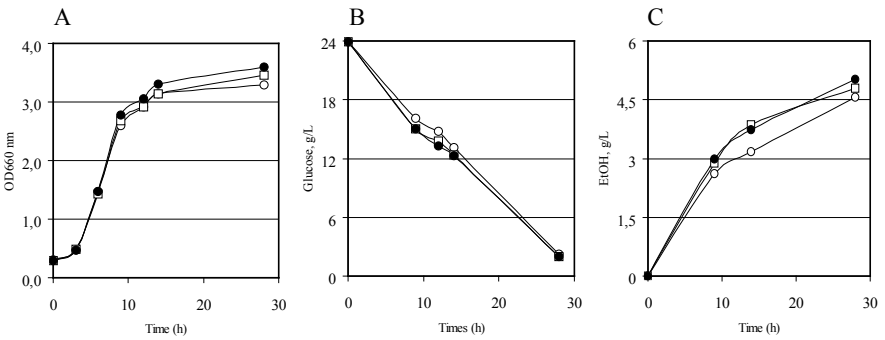


Figure 2.3: **Overexpression of HXT1 or HXT7 genes in the S. cerevisiae CEN.PK strain.** Strains were flask-batch grown in minimal (YNB) medium, with glucose as a carbon source. (A) Growth was measured as optical density (OD 660 nm). (B) Residual glucose, g/L. (C) Ethanol produced, g/L. Data correspond to the mean values of three independent clones independently tested at least three times. Standard error is lower than 0.03%. Open circles: CEN.PK-Control. Closed circles: CEN.PK [HXT1] transformants. Open squares: CEN.PK [HXT7] transformants.

conditions (Figure 2.4).

Finally, when transformants were grown in the above described media but under micro-anaerobic conditions, similar results have been obtained with respect to growth rate, substrate consumption and ethanol production (Table 2). Remarkably, in this case the improvement in the ethanol yield of the transformants was higher as the glucose consumption rate (Figure 2.4).

2.1.2 Effect of *HXT*1 overexpression in yeast engineered for lactic acid production

On the basis of the positive effect of the modification tested on yeast metabolism, the effect on a heterologous production has also been evaluated. Thus, the CEN.PK *S. cerevisiae* strain harbouring the integrative p022HXT1 expression vector was further transformed with the multicopy plasmid p212LDH bearing an engineered Lactobacillus plantarum LDH gene under the control of the glycolytic *Sc*TPI promoter (Figure 2.1). Very similar results were obtained in GRF18 background and overexpressing the HXT7 gene in both yeasts (data not shown). Independent transformants were shake flask cultured in YNB-minimal medium. Figure 2.5 shows the behaviour of the LDH (open circles) and of the HXT1-LDH (closed circles) overexpressing strains. Panels A and B report the cell density (as OD 660 nm) and the glucose consumption, while

Strains		Glc %	YNB %	aa mg/L	Agitation	μ_exp phase [1/h]	Rate Glc Consumption* [g/L/h]	Rate EtOH Production* [g/L/h]	Rate LA Production* [g/L/h]	Y_EtOH** [g EtOH/g Glc]	Y_LA** [g L.A./g Glc]
CEN.PK		2.39	1.34	50	YES	0.28 +/- 0.05	-0.77 +/- 0.08	0.23 +/- 0.09	-	0.19 +/- 0.11	-
CEN.PK	[*HXT1*]	2.39	1.34	50	YES	0.29 +/- 0.07	-0.83 +/- 0.08	0.27 +/- 0.04	-	0.21 +/- 0.05	-
CEN.PK	[*HXT7*]	2.39	1.34	50	YES	0.29 +/- 0.02	-0.82 +/- 0.08	0.28 +/- 0.03	-	0.20 +/- 0.03	-
CEN.PK		5.68	2.68	150	YES	0.33 +/- 0.05	-1.66 +/- 0.06	0.42 +/- 0.01	-	0.19 +/- 0.00	-
CEN.PK	[*HXT1*]	5.68	2.68	150	YES	0.33 +/- 0.03	-1.74 +/- 0.09	0.44 +/- 0.01	-	0.20 +/- 0.01	-
GRF18U		2.39	1.34	50	YES	0.25 +/- 0.05	-0.69 +/- 0.04	0.27 +/- 0.05	-	0.23 +/- 0.01	-
GRF18U	[*HXT1*]	2.39	1.34	50	YES	0.25 +/- 0.03	-0.79 +/- 0.08	0.32 +/- 0.06	-	0.25 +/- 0.08	-
GRF18U	[*HXT7*]	2.39	1.34	50	YES	0.25 +/- 0.04	-0.80 +/- 0.07	0.31 +/- 0.06	-	0.24 +/- 0.06	-
CEN.PK	[LDH]	2.04	1.34	50	YES	0.12 +/- 0.04	-0.38 +/- 0.07	0.09 +/- 0.08	0.08 +/- 0.09	0.17 +/- 0.01	0.21 +/- 0.01
CEN.PK	[*HXT1*] [LDH]	2.04	1.34	50	YES	0.14 +/- 0.08	-0.40 +/- 0.05	0.10 +/- 0.04	0.09 +/- 0.06	0.19 +/- 0.09	0.24 +/- 0.01
CEN.PK m850	[LDH]	9.14	1.70	-	YES	0.02 +/- 0.00	-1.21 +/- 0.00	-	0.77 +/- 0.00	-	0.67 +/- 0.00
CEN.PK m850	[*HXT1*] [LDH]	8.96	1.70	-	YES	0.03 +/- 0.09	-1.22 +/- 0.08	-	0.85 +/- 0.02	-	0.76 +/- 0.02
CEN.PK		2.13	1.34	150	NO	0.26 +/- 0.03	-1.22 +/- 0.03	0.41 +/- 0.08	-	0.40 +/- 0.03	-
CEN.PK	[*HXT1*]	2.13	1.34	150	NO	0.26 +/- 0.04	-1.30 +/- 0.02	0.45 +/- 0.02	-	0.45 +/- 0.08	-
CEN.PK		5.68	2.68	150	NO	0.23 +/- 0.01	-4.30 +/- 0.06	1.37 +/- 0.05	-	0.31 +/- 0.08	-
CEN.PK	[*HXT1*]	5.68	2.68	150	NO	0.23 +/- 0.04	-4.36 +/- 0.07	1.58 +/- 0.06	-	0.36 +/- 0.09	-

Figure 2.4: **Effect of the overexpression of HXT1 or HXT7 genes in the S. cerevisiae GRF18U or CEN.PK strains.** Table summarizes all the data obtained with wild type and engineered yeasts (see first column) grown in the indicated conditions (see columns 2-5). The specific growth, glucose consumption, ethanol and lactate production rates together with yield for ethanol and lactic acid production are given. For the described determinations, the respective standard error is indicated. Glc: glucose. aa: aminoacids. LA: lactic acid. * determined in exponential phase. ** determined at the timing of the highest metabolite (ethanol or lactic acid) production.

panels C and D the ethanol and the lactate accumulation during growth on defined YNB-2% glucose based medium.

Also in these transformants the expression of an additional copy of *HXT*1 lead to an increase in glucose consumption (Figure 2.5, panel B). Interestingly, both the ethanol and lactic acid productivities and titers are improved (Figure 2.5, panels C and D).

2.1.3 Effect of *HXT1* overexpression in homolactic yeasts

Considering the improved product titers and productivities obtained, we tested the lactic acid production in the engineered host strain CEN.PK m850 [LDH] (Figure 2.1), *ad hoc* constructed for being a low-pH homolactic producing yeast [10]. Said strain does not produce ethanol because it is totally devoided of pyruvate decarboxylase (Pdc) activity, it bears the L. plantarum LDH on a multicopy yeast expression plasmid and finally it has been selected, with an inverse metabolic engineering approach, for its acid tolerance. Figure 2.6 shows the behavior of the CEN.PK m850 [LDH] strain and of the same strain expressing an additional copy of the HXT1 transporter cultivated with 90 g/L of glucose. Independent transformants were tested for glucose consumption and lactic acid production.

In this case also, the strain expressing an additional HXT1 copy consumed glucose faster than the relative control even if both strains did not consume all of the carbon source present in the medium. This improved glucose consumption rate lead to produce a considerable additional amount of lactic acid (about 15% more) or the same amount in a shorter period of time (about 1.4 times faster, Figure 2.4). Data obtained suggested that the increase in glycolytic flux typical of the HXT strain was redirect, in the growth condition tested, towards the production of the lactic acid. Thus no improvement in biomass production was observed.
This last experiment shows the successful application of what was previously shown in laboratory strains (Figure 2.5) also in a strain already developed and optimised for industrial productions.

2.2 Discussion

The hexose transporter gene family in S. cerevisiae contains the sugar transporter genes *HXT*1 to *HXT*7, *GAL*2 and the glucose sensor genes

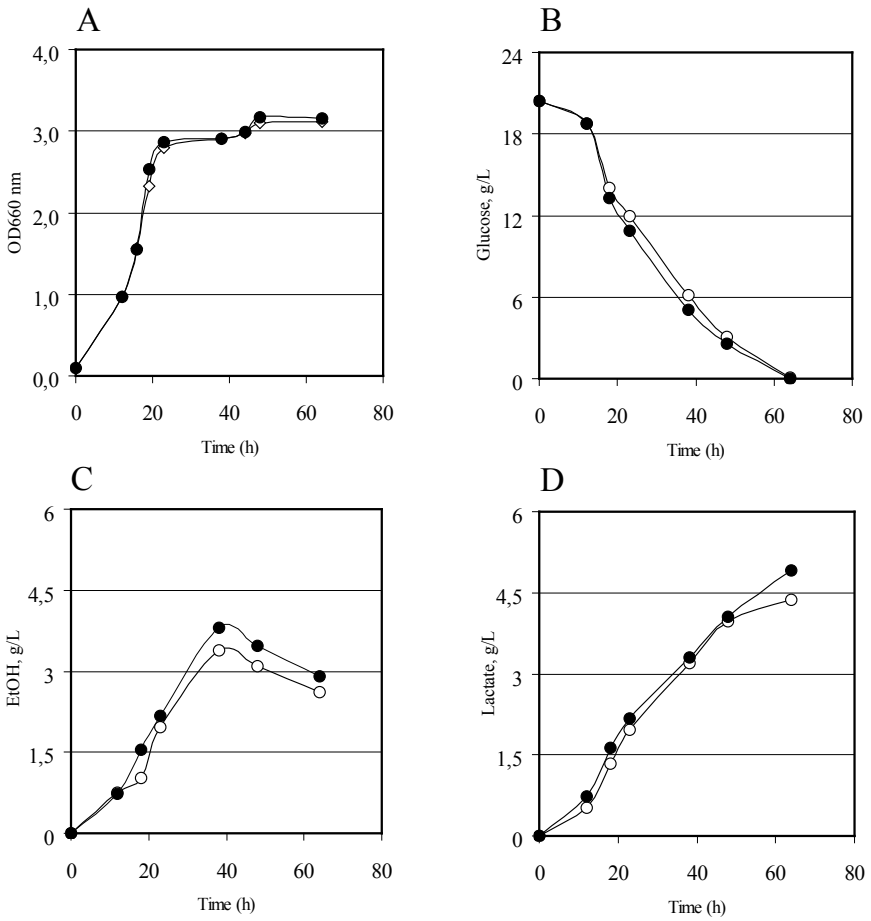


Figure 2.5: **CEN.PK S. cerevisiae strain overexpressing the *HXT*1 gene or the *HXT*1 and the *Lp*LDH gene.** Strains were flask-batch grown in minimal (YNB) medium, with glucose as a carbon source. (A) Growth was measured as optical density (OD 660 nm). (B) Residual glucose, g/L. (C) Ethanol produced, g/L. (D) Lactate produced, g/L. The data correspond to the mean values of three independent clones independently tested at least three times. Standard error is lower than 0.03%. Open circles: CEN.PK [LDH]-Control. Closed circles: CEN.PK [HXT1] [LDH] transformants.

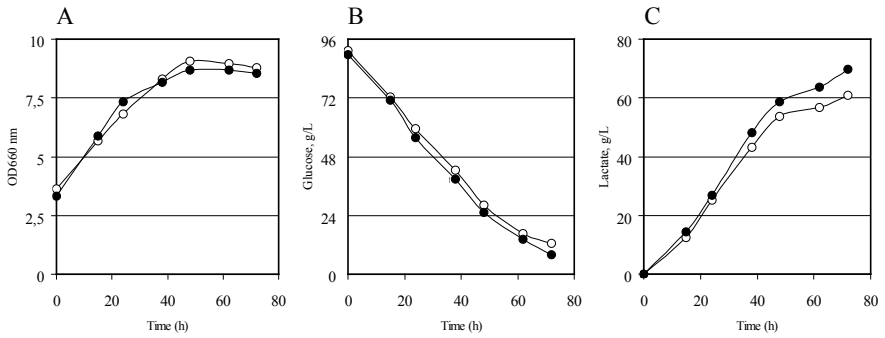


Figure 2.6: **Lactic acid production in the CEN.PK m850 [LDH] strain overexpressing the *HXT*1 gene.** (A) Growth was measured as optical density (OD 660 nm). (B) Residual glucose, g/L. (C) Lactic acid produced, g/L. The data correspond to the mean values of three independent clones independently tested at least three times. Standard error is lower than 0.03%. Open circles: CEN.PK m850 [LDH]-Control. Closed circles: CEN.PK m850 [HXT1] [LDH] transformants.

SNF3 and *RGT2* [31-33]. *HXT1* and *HXT3* genes have already been overexpressed in yeasts. More in detail, the effect of the overexpression of *HXT1* gene has been tested in a *S. cerevisiae* strain [28] during growth on complex-rich based media. A significant increase on the ethanol productivity (g/L/h) was observed. Also the ethanol yield, expressed as gram of ethanol produced per gram of substrate consumed, showed a significant (3%) improvement. This is in line with our findings. However, Gutirrez-Lomel *et al.* [28] observed no significant effects on the final ethanol concentration. On the other hand, while Gutirrez-Lomel et al. [28] examined strains producing 40-45 g/L of ethanol, we used physiological conditions leading to the accumulation of 4-6 g/L. Therefore, it could be speculated that a saturation limit could be reached when the strains are grown in the presence of a huge amount of glucose. A minor difference is that Gutirrez-Lomel *et al.* [28] did not observe any improvement in the biomass production. Once more, it should be underlined that the transformed strains have been grown under very different conditions (rich-complex or defined-minimal media, respectively). Guillaume *et al.* [52] have demonstrated that the pattern of fructose utilization during wine fermentation can be altered in yeasts harbouring a mutated HXT3 allele. More in details the authors found that the glycolytic flux could be increased by the overexpression of a mutated version of the transporter gene. Data demonstrate that the Hxt3 hexose transporter plays a key role in determining the glucose/fructose utilization ratio during wine fermentation. All these findings are in line with the data shown in this thesis as well as with the ones reported by Elbing

et al. [53]. Following a very elegant approach, the authors built a series of strains having different rates of ethanol production, linearly correlating with the maximal specific glucose consumption rates attained during exponential growth on glucose. However, the same authors concluded that the hexose transporter has no or very low control over glycolytic flux in the wild type cells growing in the presence of high glucose concentrations.
In conclution, even if the metabolically engineered S. cerevisiae yeast strains are among the most prominent recombinant hosts usable for the industrial production of lactic acid [54], [55], [56], [30], the overexpression of a hexose transporter has never been conceived to improve the productivity of this organic acid. In the recent past, aimed at improving the lactic acid production by metabolically engineered yeasts, we showed that the redirection of the pathway towards the lactate production can be strongly modulated by the genetic background of the host cell, by the source of the heterologous LDH enzyme, by improving its biochemical properties as well as by modulating (even if to very low extent) the export of lactate in the culture media [12]. In this work, we have modulated the lactic acid productivity by improving the efficiency of the first step of the pathway - the glucose uptake - leading to the accumulation of lactic acid from glucose.
Finally, it should be underlined that a variety of organic acids attract more and more attention as new building block materials for the chemical industry [54]. If produced by environmentally benign fermentation strategies, they can provide a sound alternative to petroleum derived, and therefore limited, building block materials. It can be anticipated that the production of these organic acids could be similarly improved by the overexpression of additional copies of one or more hexose transporters [29].

2.3 Methods

2.3.1 Yeast strains, transformation, media and cultivation

The *S. cerevisiae* strains used in this study derive from the following strains: GRF18U (*MATα, ura3; leu2-3,112; his3-11,15;* cir+) [57], CEN.PK strains 102-5B (*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2*) - Dr. P. Kotter, Institute of Microbiology, Johann Wolfgang Goethe-University, Frankfurt, Germany) [58], and CEN.PK m850 [10], [11]. Yeast transformations were performed basically according to the LiAc/ PEG/ ss-DNA protocol

[59]. The control strain is, for each background, the corresponding yeast strain transformed with the empty plasmid(s). Independent transformants and the respective control strains (at least three for each transformation) were cultivated in shake flasks in minimal synthetic medium (1.34% or 2,68% [w/v] YNB medium [catalogue no. 919- 15 Difco Laboratories, Detroit, Mich.] with 2% or 5% [w/v] glucose and 50 mg/L or 150 mg/L of appropriate amino acid(s), respectively). All strains were grown in shake flasks at 30℃. For aerobic growth, flasks were agitated at 160 r.p.m. and the ratio of flask volume/medium was of 5/1. For microaerobic condition, flasks were sealed and the ratio of flask volume/medium was of 10/6. Independent transformants derived from the strain CEN.PK m850 [LDH] were cultivated as previously described [11]. Briefly, growth kinetics were performed at 28℃in 250-mL quadruple baffled shake flasks in minimal medium containing 4.5 g/L $CaCO_3$, 1.7 g/L YNB without amino acids and without $(NH_4)_2SO_4$, 1 g/ L urea, 5 g/L ethanol, and with glucose 9% (w/v) as a carbon source. Cell growth was monitored by measuring the optical density at 660 nm at regular time intervals.

2.3.2 Gene amplification and plasmids construction

The *S. cerevisiae HXT1* [60] and *HXT7* [25] genes were PCR amplified using as a template the genomic DNA extracted from GRF18U strain by standard methods [54]. Pwo DNA polymerase (Roche catalogue no. 11 644 955 001) was used on a GeneAmp PCR System 9700 (PE Applied Biosystem, Inc.). Standard conditions used were 0.2 mM primers, 1.5 U of Pwo and 3 μL of genomic DNA. The program used for amplification of genes was as follows: after 5 min at 94℃, 30 cycles (each cycle consisting of 15 s at 94℃, 30 s at 57.5℃and 1 min 30 s at 72℃) were carried out, followed by 7 min at 72℃. Oligonucleotides pairs for HXT1 were as follows: $HXT1_{fw}$ (5-AAA ATC ATG AAT TCA ACT CCC GAT CTA-3) and $HXT1_{rev}$ (5-AGC TTG TTT AGT TTA TTT CCT GCTG AAA-3). Because of the high sequence homology between the coding sequence of the S. cerevisiae *HXT6* and *HXT7* genes [47], the latter was amplified in two steps. In the first step the oligos named 5HXT7 (5-A AAA ATG TCA CAA GAC GCT GCT ATT GCA-3) and 3HXT7exit (5-ATA TAT TAA AAA CGT ATT TAC TTT TCA AGT-3) were used, the second designed on an external region in respect to the gene that resulted different from the corresponding region of the *HXT6* gene. The single amplified band was secondarily used as a template for the two oligos 5HXT7 and 3HXT7 (5-AGT GTC GAC AAA TAA TTT GGT GCT GAA CAT-3), obtaining the sole open reading frame of the desired gene. The amplified fragments were sub-cloned into the Escherichia coli vector pSTBlue obtaining, respectively, the plasmids pSTBlueHXT1 and pSTBlueHXT7. The inserts were sequenced and resulted identical to the deposited S. cerevisiae corresponding sequences (*HXT1*, GeneID: 856494 and *HXT7*, GeneID: 851943). These coding sequences were used for the construction of the integrative expression plasmids p022HXT1 and p022HXT7, respectively, utilizing the basic *S. cerevisiae* integrative expression plasmid pYX022 (R&D Systems, Inc., Wiesbaden, D). For the construc-

tion of the plasmid p022HXT1, the recipient vector was *EcoR*I cut, blunted and dephosphorylated, while the insert was *Mlu*I blunt/*Pml*I excised from the pSTBlueHXT1 plasmid. For the construction of the plasmid p022HXT7, the recipient and the pSTBlueHXT7 vectors were *EcoR*I cut. For the construction of the plasmid named p212LDH, the coding sequence of *L. plantarum* LDH was *EcoR*I excised from previously described pSTplLDH [12] and sub-cloned into the S. cerevisiae expression vector pYX212 (multicopy, *URA3* auxotrophic marker R&D Systems, Inc., Wiesbaden, D) EcoRI opened and dephosphorylated. For the construction of the integrative plasmid p022KMX4, harboring an auxotrophic marker used only as a target gene and a dominant marker used for the selection of the transformants, the backbone of the plasmid pYX022 was used. pYX022 was *Kpn*I cut, blunt-ended and dephosphorylated and ligated with the KanR cassette derived from *Sph*I/*Sac*I blunt ending, from the plasmid pFA6KanMX4 [13]. p022KMX4 was EcoRI cut and dephosphorylated or EcoRI cut, bluntended and dephosphorylated and ligated with the *HXT1* or *HXT7* sequences cut as described above, resulting in the plasmids p022KMX4HXT1 or p022KMX4HXT7, respectively. A complete list of the transformed strains is given in Figure 2.1. DNA manipulation, transformation and cultivation of *E. coli* (Novablue, Novagen) were performed following standard protocols [62]. All the restriction and modification enzymes utilised are from NEB (New England Biolabs, UK) or from Roche Diagnostics.

2.3.3 Metabolite determination

Residual glucose and ethanol/lactic acid produced were determined with enzymatic kits from Megazyme, the glucose assay kit (K-GLUHKR), the Ethanol kit (KETOH) and L-lactic acid kit (K-LATE), respectively, according to the manufacturers instructions.

Chapter 3

Model-driven characterization of intracellular state of *Saccharomyces cerevisiae* strains improved in glucose uptake

The goal of this study was to investigate the differences previously described between HXT overepressing strain and the relative control linking the changes in the metabolic extracellular profile to systematic intracellular flux perturbations. Metabolites secreted and/or released by a cell reflect its internal state, and its composition and rate values varies in response to genetic or experimental perturbations due to change in intracellular pathway activities involved in the production and utilization of extracellular metabolites. In this study a constraint-based sampling approach on a genome-scale network of yeast metabolism was performed to systematically determine how the advantages showed by engineered strain were linked to changes in intracellular metabolic flux states.

"Model-driven characterization of the intracellular state of *Saccharomyces cerevisiae* strains improved in glucose uptake"
Manuscript in preparation

3.1 Results

3.1.1 Predicting optimal phenotypes

We utilized wet data described in the previous chapter to infer intracellular states. In particular we considered growth rate (GR), glucose consumption (GUR) and ethanol (ESR) release/secretion rate measured for wild-type and *Sc*HXT1 overexpressing strains in aerobic and semianaerobic conditions with 2% or 5% glucose as carbon source.
We first computed the maximum GR and ESR allowed by the measured GUR for each dataset. Thus, the model was constrained only by experimental GUR and the maximum growth and ES rates were calculated by using flux balance analysis (FBA) approach.

The experimental rates were lower than the calculated fluxes (Figure 3.1)

Name	Control Strain		ScHXT1 Strain	
-	In silico Rate	Experimental Rate	In silico Rate	Experimental Rate
GLC2% - AEROBIOSIS Dataset1				
experimental GUR	*-6.526*		*-6.612*	
Growth Rate [hr^{-1}]	0.279	0.282	0.291	0.290
EtOH Secretion Rate [mmol g DW^{-1} hr^{-1}]	8.863	7.584	8.802	8.389
GLC5% - AEROBIOSIS Dataset2				
experimental GUR	*-10.473*		*-10.626*	
Growth Rate [hr^{-1}]	0.326	0.330	0.327	0.330
EtOH Secretion Rate [mmol g DW^{-1} hr^{-1}]	17.100	10.264	17.426	10.472
GLC2% - MICROAEROPHILIA Dataset3				
experimental GUR	*-10.417*		*-10.700*	
Growth Rate [hr^{-1}]	0.255	0.257	0.257	0.260
EtOH Secretion Rate [mmol g DW^{-1} hr^{-1}]	17.930	14.913	18.467	14.417
GLC5% - MICROAEROPHILIA Dataset4				
experimental GUR	*-31.622*		*-32.956*	
Growth Rate [hr^{-1}]	0.233	0.235	0.232	0.232
EtOH Secretion Rate [mmol g DW^{-1} hr^{-1}]	60.594	39.605	63.272	46.979

Figure 3.1: **Maximal growth rate and ESR allowed by experimental GUR-** Comparison of *in silico* calculated and experimentally measured growth rate and ethanol secretion rate in all the conditions of oxygenation and glucose availability considered. Each dataset is related to the two yeast strains of interest: the ScHXT1 strain overexpresses an additional copy of the *HXT1* gene and the control strain is its control transformed with the empty plasmid

which represented the highest flux values provided by the particular GUR values used as constrain during the *in silico* computation. The differences observed could be due to the experimental set up used to obtain those datasets, which seemed to provide a non-optimal growth environment. This result suggested that it could be possible to further increase the ESR working on the process conditions because the yeast metabolism potentially supported an higher ethanol productivity. Considering the importance of the ethanol as biofuel, the possibility to improve the process productivity by modulating the growth condition

represents an important indication.
This sub-optimal phenotype must be taken into account during the following analysis because the intracellular states reflected these sub-optimal phenotypes. In order to determine further constrains to use in the subsequent calculation, we constrained the model using the different experimental datasets. The minimal oxygen (OUR), leucine and uracil uptake rates that can support these constrains were calculated using FBA approach (see Methods). The model was further constrained by these flux values. For each constrain, we assumed an error of ± 10% to set the lower and the upper bounds.
The Random Sampling Approach was used to calculate the secretion profiles in an *unbiased* manner (see Methods), in each condition of oxygenation and glucose availability for the two yeast strains of interest.

mmol/g DW/ hr		GLC 2% - Aerobiosis Control Strain	GLC 2% - Aerobiosis ScHXT1	GLC 5% - Aerobiosis Control Strain	GLC 5% - Aerobiosis ScHXT1
Oxygen Uptake Rate	**OUR**	**-4.80219**	**-5.05503**	**-3.76814**	**-3.71244**
Glucose Uptake Rate	**GUR**	-7.17	-7.26	-11.50	-11.66
Glycerol Secretion Rate	**Glyc SR**	0.02	0.02	0.03	0.03
Acetate Secretion Rate	**Ac SR**	0.36	0.19	0.64	0.85
EthanolSecretion Rate	**EtOH SR**	6.98	7.64	10.02	10.11
Growth Rate	**GR**	0.25	0.27	0.30	0.30
mmol/g DW/ hr		**GLC 2% - Microaerophilia Control Strain**	**GLC 2% - Microaerophilia ScHXT1**	**GLC 5% - Microaerophilia Control Strain**	**GLC 5% - Microaerophilia ScHXT1**
Oxygen Uptake Rate	**OUR**	**-1.38596**	**-1.54393**	**-1.20746**	**-1.33658**
Glucose Uptake Rate	**GUR**	-12.53	-11.75	-34.67	-36.12
Glycerol Secretion Rate	**Glyc SR**	0.04	0.03	0.20	0.19
Acetate Secretion Rate	**Ac SR**	0.19	0.20	0.47	0.37
EthanolSecretion Rate	**EtOH SR**	13.72	13.25	36.42	43.18
Growth Rate	**GR**	0.23	0.23	0.21	0.21

Figure 3.2: **Secretion profiles calculated by Flux Balance Analysis -** Secretion profiles calculated constraining the model with experimental data obtained in different conditions of oxygenation and glucose availability. In particular we considered the secretion rates related to acetate, glycerol and the growth rate which can be supported by the wet fluxes. Each secretion profile is related to the two yeast strains of interest: the ScHXT1 strain overexpresses an additional copy of the *HXT1* gene and the control strain is its control transformed with the empty plasmid

The results obtained (Figure 3.2) showed that, as expected, OUR decreased with the increase of glucose availability in both conditions of oxygenation. This suggested that growth in shake flask limits the oxygen availability and this was reflected in the way by which cells metabolized glucose; in particular the OUR decreased with the increase of glucose concentration in the medium and this correlates with the increase in the ESR. The results obtained by these computations indicated also that our microaerophilic growth conditions did not correspond to fully anaerobic conditions. Thus, the lowest OURs that support microaerophilic constrains were higher than zero (constraining the model setting OUR = 0 resulted in no solution). Furthermore, the lower OUR (glc5 % -no oxygen) corresponded to the higher GUR and this trend confirms data shown by Jouhten *et al.* [63]. Interestingly, the transformed strain utilized the oxygen faster than the relative control in all the tested conditions. This was probably due to the higher GUR typical of this strain.
In silico analysis predicted a glycerol production in all the tested conditions

(Figure 3.2). Glycerol participates in the maintenance of the redox balance when oxygen availability is limited [63] thus, its production could be explained by the sub-optimal ethanol production due to experimental set up but also by as biomass-dependent by-product. Considered the lower glycerol secretion rate of the ScHXT1 strain than the control, it is possible to speculate again that a further improvement of the ethanol productivity could be reached by modulating the growth condition.

3.1.2 The intracellular states: from phenotypes to intracellular behavior

In order to deeply understand the differences observed in the glucose metabolism between the two yeast strains considered, we inferred the intracellular flux distributions from the measured flux values (see Methods).

Perturbation Analysis
First of all, to highlight metabolic regions that were systematically affected by Sc$HXT1$ overexpression, reaction scores and reporter metabolite methods were used [36]. The Z reaction scores were calculated from sampling analysis to quantify flux changes between the engineered and wild-type strains, with $Z_{reaction} > 1.96$ corresponding to a two-tailed ρ-values < 0.05 and considered to be significantly perturbed [see Additional File A].
The results suggested that the number of metabolites and reactions affected by the modification of interest increased with GUR. Thus, the differences in the metabolism of the two strains tested seemed to correlate with the glucose availability. Here we analyze the first 28 most perturbed metabolites [Additional File A]. The selection was based on the Z metabolite scores which allowed to quantify the differences in flux samples between two conditions (wild-type and transformed strains). Similarly to $Z_{reaction}$, $Z_{metabolite} > 1.64$ corresponded to a two-tailed ρ-value lower than 0.05, representing a statistical significant perturbation.
In aerobic condition the major differences between control and engineered strains were associated with the biomass metabolism and the oxidative phosphorylation pathway, which was still involved in the biomass formation. In microareophilic condition glycolysis was the main perturbed pathway that causes also the perturbation of pyruvate-linked reactions and ethanol production. Also in this condition, the biomass biosynthesis seemed to be affected by the Sc$HXT1$ overexpression but in a less significant way. Based on these observation we deeper investigated the differences between *Sc*HXT1 and wild-type strains analyzing the fluxes through the pathways involved in the glucose metabolism.

Glycolysis
In all the conditions/strains considered the glycolysis trend could be divided into two parts: upper (which includes the previous reactions to aldolase) and

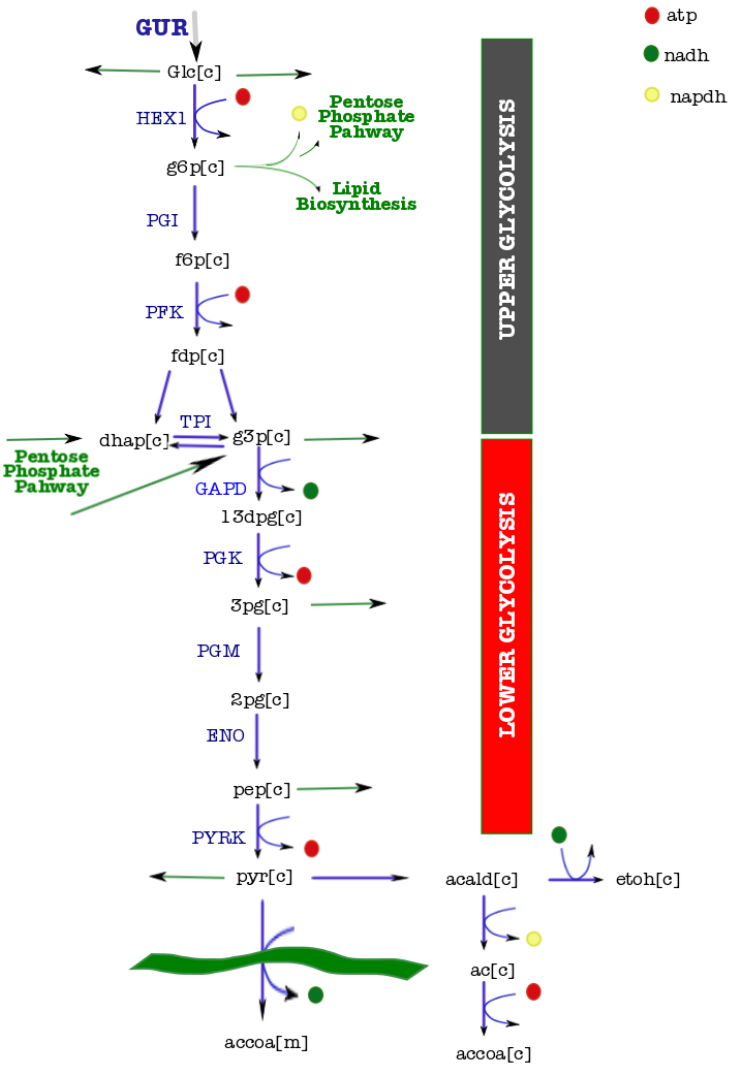


Figure 3.3: **Glycolytic Pathway -** Glc: glucose; g6p: glucose-6-phosphate; f6p: fructose-6-phosphate; fbp: fructose-1,6-bisphosphate; dhap: dihydroxyacetone phosphate; g3p: glyceraldheyde phosphate; 13dpg: 1,3-diphosphoglycerate; 3pg: 3-phosphoglycerate; 2pg: 2-phosphoglycerate; pep: phosphoenolpyruvate; pyr: pyruvate; acald: acetaldheyde; etoh: ethanol; ac: acetate; accoa: acetylCoA; HEX1: hexokinase; PGI: phosphoglucoisomerase; PFK: phosphofructokinase; TPI: triosephosphateisomerase; GAPD: glyceraldheyde-3-phosphate dehydrogenase; PGK: phosphoglucokinase; PGM: phosphoglucomutase; ENO: enolase; PYRK: pyruvate kinase

lower (which includes the reactions from glyceraldheyde 3 phosphate (g3p) to pyruvate (pyr)) glycolysis (Figure 3.3). Despite glucose was mainly metabolized by glycolysis, a fraction of the GUR was dispersed at glucose 6 phosphate (g6p) node; most of this flux re-joined into the glycolysis at triosophosphate isomerase (TPI) reaction level. The fluxes through all upper glycolytic reactions were lower in the Sc*HXT*1 strain than in the control (Figure 3.4). Only in glc2% - Microaerophilia (Figure 3.4), the Sc*HXT*1 upper glycolytic flux was higher than in the control, but in this case the reactions involved were not perturbed ($Z_{reaction}$ lower than 1.96). The lower glycolysis included ATP- and NADH-genic reactions (Figure 3.3). The engineered strain showed a higher flux values through these reactions than the control in all the conditions tested (Figure 3.4). Moreover these fluxes were *ca.* 2 fold higher than GUR, thus it is possible to highlight a positive relationship between GUR and lower glycolytic flux. Interestingly, the flux through pyruvate kinase (PYK) seems to be not affected by the Sc*HXT*1 overexpression, since the slight increase in its flux in the engineered strain was not statistically significant.

Summarizing, these data indicated that in all the conditions/strains tested the upper glycolytic flux was lower than GUR because most of the carbon was dispersed at g6p node. This flux went back to lower glycolysis through TPI reaction. In all the conditions considered the lower glycolytic flux was higher in the engineered strain than in the control. This could result in some differences in the redox and, more in general, in the energetic metabolism that could be associated with the *Sc*HXT1 phenotype.

Pentose Phosphate Pathway (PPP)
Despite the differences in the flux values (Figure 3.4), the PPP was significantly perturbed by the *Sc*HXT1 overexpression only in the presence of 5% glucose. For this reason we deeper analyzed how the carbon flux was distributed through this pathway only in this growth condition.
In aerobiosis, only two reactions involved in the conversion of sedoheptulose 7-phosphate (s7p) into dihydroxyacetone phosphate (dhap), were perturbed while in microaerophilia both oxidative and non-oxidative PPP were perturbed by our modification. In particular, while the oxidative flux was lower in the engineered strain, it was higher in the non-oxidative part. All together these findings suggested a possible relationship between glycolytic, PPP fluxes and OUR, indicating that the differences between the 2 strains were highlighted by changing the enviromental conditions. For example the NADPH biosynthesis seemed not to be affected by the Sc*HXT*1 overexpression when the glucose available was 20 g/L, independently of OUR. On the contrary, in the presence of 5% glucose the transformant showed a different behaviour depending on oxygen availability. In aerobiosis the flux was higher in the transformant than in the control while in the anaerobiosis was lower, consistently with a role of the OUR and more in general of growth condition, as determinants of glycolysis-PPP flux split.

	rxnNames	rxns	Aerobiosi Glc 2% Control Strain mmol/g DW/ hr	Aerobiosi Glc 2% ScHXT1 Strain mmol/g DW/ hr	Aerobiosis Glc 5% Control Strain mmol/g DW/ hr	Aerobiosis Glc 5% ScHXT1 Strain mmol/g DW/ hr	Microaerophilia Glc 2% Control Strain mmol/g DW/ hr	Microaerophilia Glc 2% ScHXT1 Strain mmol/g DW/ hr	Microaerophilia Glc 5% Control Strain mmol/g DW/ hr	Microaerophilia Glc 5% ScHXT1 Strain mmol/g DW/ hr
	Glucose Uptake Rate (mmol/g DW/hr)	**GUR**	**-7.165**	**-7.258**	**-11.500**	**-11.664**	**-12.533**	**-11.745**	**-34.667**	**-36.122**
Glycolysis	hexokinase (D-glucose:ATP)	**HEX1**	5.686	3.722	6.207	8.014	*6.025*	*7.257*	*10.178*	*14.850*
	phosphofructokinase	**PFK**	*4.354*	*4.083*	7.852	5.484	*4.493*	*5.564*	16.404	13.599
	fructose-bisphosphate aldolase	**FBA**	*4.310*	*4.037*	7.784	5.419	*4.413*	*5.494*	15.995	13.182
	triose-phosphate isomerase	**TPI**	5.980	6.031	10.053	10.197	*11.022*	*10.310*	31.301	33.261
	glyceraldehyde-3-phosphate dehydrogenase	**GAPD**	12.261	12.367	20.461	20.765	22.626	21.148	64.259	67.609
	phosphoglycerate kinase	**PGK**	-12.261	-12.367	-20.461	-20.765	-22.626	-21.148	-64.259	-67.609
	phosphoglycerate mutase	**PGM**	-12.093	-12.186	-20.192	-20.513	-22.344	-20.858	-63.391	-66.876
	enolase	**ENO**	12.093	12.186	20.192	20.513	22.344	20.858	63.391	66.876
	pyruvate kinase	**PYK**	*0.177*	*0.190*	*0.270*	*0.272*	*0.316*	*0.260*	*1.800*	*1.671*
PPP	glucose 6-phosphate dehydrogenase	**G6PDH2**	*1.012*	*1.051*	*1.191*	*1.250*	*1.591*	*1.459*	3.762	2.224
	6-phosphogluconolactonase	**PGL**	*1.019*	*1.058*	*1.201*	*1.261*	*1.600*	*1.468*	3.789	2.257
	phosphogluconate dehydrogenase	**GND**	*1.019*	*1.058*	*1.201*	*1.261*	*1.600*	*1.468*	*3.789*	*2.257*
	ribulose 5-phosphate 3-epimerase	**RPE**	*0.547*	*0.567*	*0.614*	*0.653*	*0.891*	*0.812*	1.940	0.878
	ribose-5-phosphate isomerase	**RPI**	*-0.472*	*-0.490*	*-0.586*	*-0.607*	*-0.708*	*-0.655*	*-1.849*	*-1.379*
	phosphopentomutase	**PPM**	*-0.068*	*-0.082*	*-0.115*	*-0.148*	*-0.139*	*-0.117*	*-0.510*	*-0.598*
	transketolase	**TKT1**	*0.339*	*0.354*	*0.402*	*0.423*	*0.533*	*0.485*	1.296	0.748
	transaldolase	**TALA**	*-1.396*	*-1.702*	-1.966	-4.452	*-6.254*	*-4.487*	-14.826	-20.106
	transketolase	**TKT2**	*0.207*	*0.213*	*0.212*	*0.230*	*0.358*	*0.327*	*0.644*	*0.129*
	Sedoheptulose 1,7-bisphosphate D-glyceraldehyde-3-phosphate-lyase	**FBA3**	*1.735*	*2.056*	2.368	4.875	*6.787*	*4.972*	16.122	20.854
Pyruvate Branch	pyruvate dehydrogenase	**PDHm**	1.646	1.795	1.657	1.611	0.721	0.788	1.666	1.222
	pyruvate carboxylase	**PC**	0.551	0.331	0.929	1.115	0.104	0.095	0.541	0.529
	aldehyde dehydrogenase (acetaldehyde, NADP)	**ALDD2y**	0.428	0.422	0.656	0.650	0.622	0.516	2.085	2.354
TCA cycle	acetyl-CoA synthetase	**ACS**					0.715	0.613	*3.001*	3.079
	citrate synthase	**CSm**	*1.496*	*1.641*	*1.439*	*1.410*	*0.775*	*0.735*	*2.125*	2.245
	Isocitrate dehydrogenase (NAD+)	**ICDHxm**	*0.073*	*0.115*	*0.071*	*0.075*	*0.041*	*0.036*	0.130	0.158
	oxoglutarate dehydrogenase (lipoamide)	**AKGDam**	*0.672*	*0.773*	*0.295*	*0.269*	*0.079*	*0.063*	*0.275*	0.229
	oxoglutarate dehydrogenase (dihydrolipoamide S-succinyltransferase)	**AKGDhm**	*0.672*	*0.773*	*0.295*	*0.269*	*0.079*	*0.063*	*0.275*	0.229

Figure 3.4: **Flux values through the main pathways involved in glucose metabolism** - Glycolysis, Pentose Phosphate Pathway (PPP), Pyruvate branched point and TCA cycle flux distributions; the flux values indicated corresponded to the mean of the samples resulting from sampling analysis. Each column indicates the flux values calculated in all the conditions of oxygenation and glucose availability of interest

Tricarboxylic Acid Cycle (TCA)
Interestingly, the fluxes through TCA cycle were not affected by the overexpression of Sc*HXT*1. Despite the experimental data used to constrain the model were obtained in the presence of oxygen, pyruvate was mainly decarboxylated into acetaldheyde by pyruvate decarboxylase (PYRDC) (Figure 3.4), confirming the respiro-fermentative metabolism of the cultivated cells. Furthermore, the TCA operated as a branched pathway leading to αketoglutarate as main product. This might be the reason why this pathway was not affected by the Sc*HXT*1 overexpression.

Oxidative Phosphorylation
Not all the reactions involved in this pathway were perturbed by the Sc*HXT1* overexpression. In all the conditions tested, the engineered strain showed a higher flux through the oxidative phosphorylation than the control, except in aerobiosis with glucose 5% (Figure 3.4). Thus, the *Sc*HXT1 strain seemed to deeper ri-oxidize NADH by this pathway than the control. Moreover, the NADH dehydrogenases flux, which increased with the reduction of OUR, was perturbed only in some conditions (Figure 3.5) (glc5% cytosol, glc2% and 5% mithocondrial - Microaerophilia). Interestingly, there were some differences between transformed and control strains in the compartment where NADH was mainly produced before its re-oxidation by respiratory chain. In the control cells NADH was mainly produced in the cytosol during aerobic growth while it derived from mitochondria in anaerobiosis. On the contrary, in the Sc*HXT*1 strain the NADH was mainly produced in the cytosol in all the tested conditions. All together these data indicated that there were many differences in the redox metabolism between the two yeast strains. In particular the cytosolic NADH producing fluxes were higher in the Sc*HXT*1 cells than in the control indicating that in the transformed strain the redox metabolism was more "active" in the cytoplasm than in the control cells.
Finally, the flux through ATP synthase was in all conditions tested higher in the engineered strain than in the control (Figure 3.5), except in the aerobiosis with glucose 2% where this flux was not affected by the modification of interest. It is possible to speculate that this reflects a different energetic needs of the engineered strain.

3.2 Discussion

This study showed that genome-scale metabolic networks can be used to integrate and analyze different experimental datasets by systematically identifying altered intracellular pathways related to measured changes in the phenotype. We identified statistically significant metabolic regions that were altered as a results of *Sc*HXT1 overexpression. First of all we considered the uptake and production rates obtained by *in silico* analysis. Constraining the model with experimantal GUR, it predicted the production of ethanol but also of glyc-

rxns Formula	rxnNames	rxns	Aerobiosi Glc 2%		Aerobiosis Glc 5%	
			Control Strain	ScHXT1 Strain	Control Strain	ScHXT1 Strain
			mmol/g DW/ hr	mmol/g DW/ hr	mmol/g DW/ hr	mmol/g DW/ hr
	O2 exchange	**EX_o2(e)**	**-4.802**	**-5.055**	**-3.768**	**-3.736**
Transport, Mitochondrial	O2 transport (diffusion)	O2tm	4.723	4.969	3.659	3.599
adp[m] + (3) h[c] + pi[m] --> atp[m] + (2) h[m] + h2o[m]	ATP synthase, mitochondrial	ATPS3m	12.488	13.121	*11.133*	*11.064*
(4) focytc[m] + (6) h[m] + o2[m] —> (4) ficytc[m] + (6) h[c] + (2) h2o[m]	**cytochrome c oxidase, mitochondrial**	**CYOOm**	**4.723**	**4.969**	**3.659**	**3.599**
2) ficytc[m] + (1.5) h[m] + q6h2[m] --> (2) focytc[m] + (1.5) h[c] + q6[m]	ubiquinol-6 cytochrome c reductase	CYOR_u6m	9.426	9.919	7.294	7.175
for[c] + h[c] + q6[m] --> co2[c] + q6h2[m]	formate dehydrogenase, cytosolic/ mitochondrial	FDNG	*0.017*	*0.014*	*0.018*	*0.023*
h[c] + nadh[c] + q6[m] --> nad[c] + q6h2[m]	**NADH dehydrogenase, cytosolic/ mitochondrial**	**NADH2-u6cm**	***2.474***	***2.176***	**3.918**	**1.891**
[m] : h + nadh + q6 --> nad + q6h2	**NADH dehydrogenase, mitochondrial**	**NADH2-u6m**	***1.569***	***2.072***	***1.449***	***1.632***
h2o + ppi --> h + (2) pi	inorganic diphosphatase	PPA	*2.112*	*2.168*	*2.878*	*2.914*
h2o + ppi --> h + (2) pi	inorganic diphosphatase	PPAm	*0.145*	*0.138*	*0.219*	*0.213*

rxns Formula	rxnNames	rxns	Microaerophilia Glc 2%		Microaerophilia Glc 5%	
			Control Strain	ScHXT1 Strain	Control Strain	ScHXT1 Strain
			mmol/g DW/ hr	mmol/g DW/ hr	mmol/g DW/ hr	mmol/g DW/ hr
	O2 exchange	**EX_o2(e)**	**-1.386**	**-1.544**	**-1.207**	**-1.337**
Transport, Mitochondrial	O2 transport (diffusion)	O2tm	1.268	1.434	0.824	0.891
adp[m] + (3) h[c] + pi[m] --> atp[m] + (2) h[m] + h2o[m]	ATP synthase, mitochondrial	ATPS3m	6.468	6.693	10.294	12.643
(4) focytc[m] + (6) h[m] + o2[m] —> (4) ficytc[m] + (6) h[c] + (2) h2o[m]	**cytochrome c oxidase, mitochondrial**	**CYOOm**	**1.268**	**1.434**	**0.824**	**0.891**
2) ficytc[m] + (1.5) h[m] + q6h2[m] --> (2) focytc[m] + (1.5) h[c] + q6[m]	ubiquinol-6 cytochrome c reductase	CYOR_u6m	2.463	2.810	1.341	1.493
for[c] + h[c] + q6[m] --> co2[c] + q6h2[m]	formate dehydrogenase, cytosolic/ mitochondrial	FDNG	*0.009*	*0.014*	*0.078*	*0.086*
h[c] + nadh[c] + q6[m] --> nad[c] + q6h2[m]	**NADH dehydrogenase, cytosolic/ mitochondrial**	**NADH2-u6cm**	**1.030**	**2.050**	***1.564***	***1.597***
[m] : h + nadh + q6 --> nad + q6h2	**NADH dehydrogenase, mitochondrial**	**NADH2-u6m**	**2.294**	**0.853**	**3.978**	**1.559**
h2o + ppi --> h + (2) pi	inorganic diphosphatase	PPA	2.927	2.675	*10.622*	*10.985*
h2o + ppi --> h + (2) pi	inorganic diphosphatase	PPAm	*0.319*	*0.234*	1.488	2.028

Figure 3.5: **Flux values through Respiratory Chain Reactions in each condition tested in the engineered and control strains** - flux values indicated for each reaction corresponded to the mean of samples derived from sampling analysis. Each column indicates the flux values calculated in all the conditions of oxygenation and glucose availability of interest for the two yeast strains considered. focytc: Ferrocytochrome C; ficytc: Ferricytochrome C; q6h2: Ubiquinol-6; q6: Ubiquinone-6; for: Formate; ppi: Diphosphate

erol and acetate. Jouhten *et al* [63] showed that CEN.PK cells cultevated in glucose-limited chemostat with different concentrations of oxygen produced acetate in any condition and glycerol only in fully anaerobic condition. These differences could be justified considering the different experimental set up used: shake flask provided a sub-optimal growth condition, leading to a sub-optimal growth and production rates, but also a glycerol release. Despite these differences, growth rate, glucose and ethanol fluxes followed the same trends as those shown by Jouhten *et al* [63]. The ethanol secretion rates increased with OUR reduction while glucose uptake rates increase with OUR increasing. FBA indicated also that microareophilic experimental measurements did not support a fully anaerobiosis constraints. The sub-optimal phenotype due to the experimental set up suggested by the *in silico* computation, represents a potential target to further improve the ethanol productivity in the context of biofuels production. Our data indicated that the yeast metabolism supported higher ethanol secretion rate than the experimentally determined flux. Thus, it is possible to speculate that this optimality could be reached by working on the process conditions.
Z scores analysis revealed a perturbation of the central carbon metabolism, the biomass biosynthesis and the oxidative pathways in all the growth conditions tested. The environment seemed to play a key role determining the significance of these perturbations: while in aerobiosis the *Sc*HXT1 overexpression mainly affected biomass biosynthesis, in anaerobiosis the redox metabolism was mostly influenced. This is consistent with the yeast metabolic response to the presence of oxygen and glucose availability, which are our environmental variables. Finally, our data suggested a positive correlation between growth rate and the re-oxidisation of assimilatory NADH by respiratory chain, confirming what was previously observed by Franzen *et al* [65]. Moreover the flux through ATP synthase decreased with decreasing of OUR as observed by [67]. In particular the ATP fraction derived from respiratory chain in cells cultivated in shake flask was lower than in chemostat and this could be related with the sub-optimal growth condition provided by shake flask.
It is clear that perturbation induced by our manipulation has widespread effects beyond just altering the glucose metabolism. Our findings indicated that the oxidative metabolism play an important role in determining the engineered strain features described in the previous chapter. Further analysis will be performed to identify the cause of these advantages.

3.3 Methods

3.3.1 Constraining the iMM904 network

The *S. cerevisiae* genome-scale metabolic network reconstruction by Mo *et al.* (iMM904, [66]) was used to further characterize the previously observed differences between *Sc*HXT1 over-expressing strain and the relative reference. The model was constrained by the uptake or secretion rates derived from the exper-

imental phenotype data previously described [68], assuming an error of ± 10% to set the lower and the upper bounds. These data were obtained using auxotrophic strains for uracil and L-leucine. These auxotrophies were simulated by deleting the appropriate genes from the model and supplementing the *in silico* strain with the appropriate - not limiting amounts of L-leucine and uracil. Furthermore, the experimental set up did not provide any information about the oxygen uptake rate (OUR, mmol g DW-1 hr-1) which is a critical parameter in the cell physiology. For this reason OUR, but also uracil and L-leucine uptake rates, were calculated by flux balance analysis using the experimental data constrained model. In particular, the three desired reactions were considered as objective function and the linear programming (LP) problem was solved minimizing these objectives assuming that cells growing in shake flask consumed the least possible amount of oxygen, leucine and uracil. This approach allowed to determine the minimum uptake rates that satisfy the experimental constrains. The rate values obtained were used as further constrains during the subsequent analysis. Ammonium, phosphate and sulphate were also considered as medium components and they were assumed to be non-limiting.

3.3.2 Random Sampling of Solution Space

A sampling-based network approach and statistical methods were used to link the extracellular profile changes to intracellular flux perturbation in an unbiased manner. The inferred perturbations in intracellular reaction fluxes were further analyzed using report metabolite and reaction approaches [66] in order to identify dominant metabolic features that were perturbed. In particular we used artificial centering hit-and-run (ACHR) Monte Carlo sampling to uniformly sample the metabolic flux solution space defined by the experimental constrains described above. Reactions and their participating metabolites found to be involved in intracellular loop were discarded from further analysis as these reactions can have an arbitrary flux value. For each sample which was generated the ACHR sampler was run for 5 million steps and a flux distribution was stored every 5000 steps [66]. The samples obtained with no experimental data were used as control samples to filter report metabolites/reactions whose scores were significantly high due to only random differences between sampling runs.

Standardized scoring of flux differences between transformed and control conditions

A *Zscore based approach* was used to quantify differences in flux samples between engineered and control strains in the different environmental conditions considered as described in [66]. All the *in silico* analysis were perfomed using the MATLAB (The MathWorks Inc., Natick, MA, USA) and the COBRA Toolbox [69] software packages with glpk solvers.

Chapter 4

Construction of Butanol-producing yeast through the expression of *Clostridium* pathway

The decreasing total reserve of petroleum and the growing concerns about supply security and climate changes have intensified interest in producing alternative fuels from renewable sources. Recently, efforts have been made to develop new processes for producing advanced biofuels, either chemically or fermentatively. Ideally, biofuels obtained by the second way would require minimal energy to separate them from fermentation broths, being non-toxic to the host microrganism and being efficiently produced from a variety of feedstocks. Compared to ethanol, n-butanol is more hydrophobic, has a higher energy density, can be transported through existing pipeline infrastructures and can be mixed with gasoline at any ratio. Thus, n-butanol is a substantially better biofuel than ethanol. n-Butanol can be naturally produced by a variety of Clostridial species. Despite the advances in biotechnology and the increased petroleum costs, Clostridia are not ideal because of the relative lack of genetic tools needed to manipulate their metabolism, their slow growth, their intolerance to n-butanol and oxygen and their by-products production. Thus, there is an increasing interest in producing n-butanol in a more suitable industrial organism. We chose S. cerevisiae as a host for n-butanol production because it is a genetically tractable, well characterized organism and it has been previously manipulated to produce other heterologous metabolites. The work here presented relates to the construction of yeasts able to produce n-butanol by the expression of six enzymatic activities from the ABE (Acetone Butanol Ethanol) Clostridium pathway.

4.1 Results

4.1.1 Construction of butanologenic *S. cerevisiae* strains

During this project we evaluated the expression of the *Clostrium* pathway in two different yeast backgrounds. The strains CEN.PK [70] and BY4741 [71] were both transformed with the plasmids described in Table 4.1. Initially, the

Table 4.1: **List of engineered strains used in this study**

Strains	Plasmids
First Strain Set	
CEN.PK control	pYX012 pYX022 pYX042 pZ3 pZ5
CEN.PK producer	pYX012[*Ca*THL][*Ca*BCD] pYX022[*Ca*HBD] pYX042[*Ca*ADHE1] pZ3[*Ca*ETFA][*Ca*ETFB] pZ5[*Ca*CRT]
BY4741 control	pYX012 pYX022 pYX042 pZ3 pZ5
BY4741 producer	pYX012[*Ca*THL][*Ca*BCD] pYX022[*Ca*HBD] pYX042[*Ca*ADHE1] pZ3[*Ca*ETFA][*Ca*ETFB] pZ5[*Ca*CRT]
Second Strain Set	
CEN.PK control*	pYX212 pYX022 pYX042 pZ3 pZ5
CEN.PK producer*	pYX212[*Sc*CCR] pYX022[*Ca*HBD] pYX042[*Ca*ADHE1] pZ3[*Sc*ERG10] pZ5[*Ca*CRT]
BY4741 control*	pYX212 pYX022 pYX042 pZ3 pZ5
BY4741 producer*	pYX012[*Ca*THL] pYX022[*Ca*HBD] pYX042[*Ca*ADHE1] pZ3[*Sc*ERG10] pZ5[*Ca*CRT]

effect of the single expression of each *Clostridium* gene was analyzed growing the yeast transformed with each gene individually in minimal synthetic medium (YNB) with glucose as carbon source. Data obtained indicated that the single expression of all these genes did not affect the yeast growth (Data not shown).

4.1.2 Enzyme assays for n-butanol synthetic enzymes expressed in *S. cerevisiae*

Cell extracts obtained from transformed CEN.PK (which expressed individually each gene encoded for the three enzymes of interest) and control CEN.PK were analyzed for ThiL, HBD and CRT enzyme activities (Figure 4.2). These activities were indirectly measured spectrophotometrically and the results showed a higher enzyme activity (mU/mg protein) in cell extracts obtained from producer than from control strain (Figure 4.1). ThiL activity was also measured in the control strain, confirming the presence in *S. cerevisiae* of a endogenous thiolase able to catalyze the condensation of two acetyl-CoA molecules to acetoacetyl-CoA. The activities measured suggested the *in vitro* functionality of the first three steps of the heterologous pathaway that should lead to the formation of crotonyl-CoA (Figure 4.1).

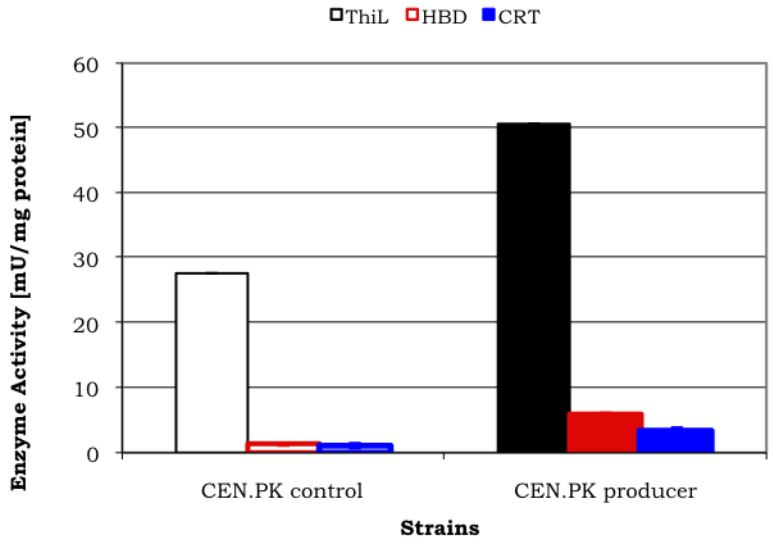


Figure 4.1: **Functional Analysis of the thiolase (ThL), 3-hydroxybutyryl-CoA dehydrogenase (hbd) and crotonase (crt) in *S. cerevisiae*** - Enzymatic Functionalities were determined by spectrophotometric method. Two yeast strains were considered: CEN.PK individually transformed with the genes encoded for the enzyme of interest (black: thiolase, red: 3-hydroxybutyryl-CoA dehydrogenase and blue: crotonase) and the CEN.PK control strain that was transformed with the empty plasmid. Each column of the histograms represented the enzyme activity (mU/mg protein) determined for each strain of interest. Data represent the mean values calculated from three indipendent experiments. One units is the amount of enzyme that converts 1 μmol of substrate to the product per min

4.1.3 n-Butanol production by butanolgenic *S.cerevisiae* strains

The reactions and the corresponding enzymes for n-butanol production are outlined in Figure 4.2. As enzymes with the same catalytic function from different organisms can have different catalytic activities, ability to be expressed, solubility, etc., we tested different candidates for the first and the fourth steps (enzymes in red in Figure 4.2).
The first set of strains, CEN.PK/ BY4741 producers, were engineered with all enzymes from *Clostridium acetobutylicum* (Figure 4.2). The second set of strains, CEN.PK/ BY4741 producer*, were constructed to evaluate the effects of the expression of different isoenzymes on n-butanol production. In particular, instead of the overexpression of the heterologous *Clostridium* gene, we overexpressed the endogenous *S. cerevisiae* thiolase (Erg10). Furthermore, we expressed an alternative butyril-CoA dehydrogenase (ccr) from *Streptomyces collinus*, which does not need accessor subunits such as the *Clostridium* enzyme and which was previously shown to be able to catalyze a critical step of the pathway of interest [44].
The n-butanol production was evaluated growing each strain (Table 4.1) in

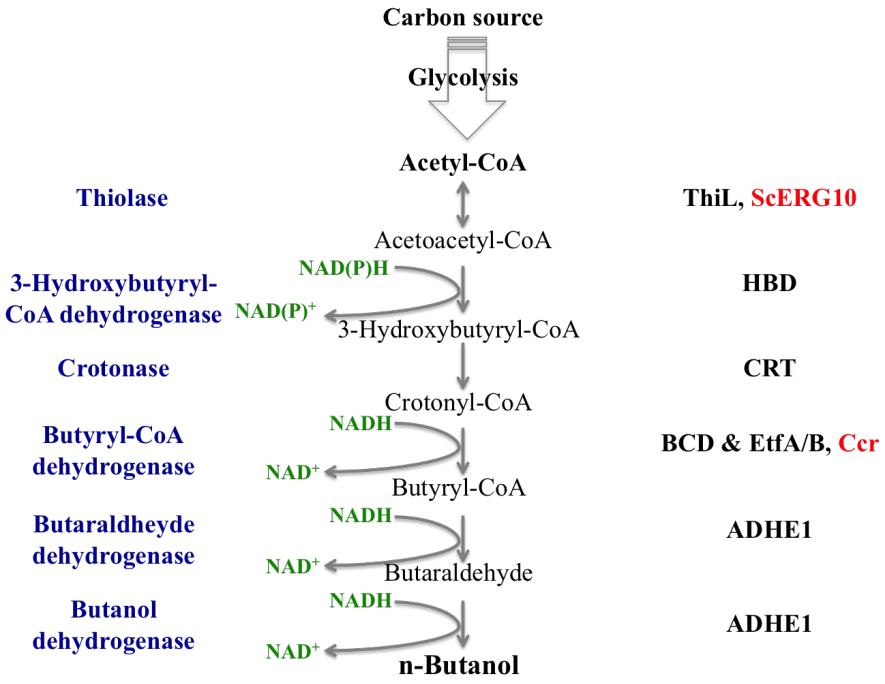


Figure 4.2: **The n-butanol biosynthetic pathway -** The enzyme in black are from *Clostridium acetobutylicum*. The enzymes in red are from other microorganisms: *ERG10*, *S. cerevisiae*; Ccr, *Streptomyces collinus*. Each candidate was evaluated in the context of the pathway for n-butanol production

minimal (YNB) medium with glucose as carbon source. Considering the crucial role of cofactors such as coenzymeA and NADH/NAD+ in the desired production, the two biosynthetic precursors (panthothenic and nicotinic acid respectively) were additionally supplied. The n-butanol production starts from acetyl-CoA which is linked to cell growth and CoA availability. For this reason BY4741* strains were also grown in minimal (YNB) medium with galactose as carbon source in order to avoid the catabolite repression.
For each strain and condition, cell growth, glucose consumption, ethanol, glycerol and n-butanol production were measured every 24h. Unfortunately, it was not possible to detect n-butanol in any conditions/ strains tested. Despite the apparent failure, we further investigated the extracellular metabolic profile of each strain. In particular, we analyzed the ethanol and glycerol productions as indicators of the redox state of the cells since these two products were involved in the NADH-rioxidation. Table 4.2 shows the growth and glucose consumption rates, ethanol and glycerol yields determined in the first 24 hours (which correspond to the exponential growth phase) in minimal (YNB) medium. Each engineered strain seemed to produce lower ethanol and glycerol amounts than the control, despite the growth rates seemed to be not affected by the expression of the *Clostridium* pathway. Interestingly, this was more evident during growth on galactose as carbon source (6.43 g/L/g DW and 7.83 g/L/g DW respectively). In this condition, BY4741 producer* accumulated about 0.821 times lower ethanol than the relative control. Furthermore, adding coenzyme A and NADH/NAD+ biosynthetic precursors, the ethanol yield decreased in both yeast background tested, while the growth rate increased (data not shown) indicating a possible role of these cofactors in the respiro-fermentative equilibrium. Despite n-butanol was not detected, the expression of the heterologous pathway seemed to affect the redox metabolism in both backgrounds tested. In particular, data obtained suggested a possible competition for the NADH between the alcoholic fermentation and the *Clostridium* activities.
Based on these experimental observations, we tried to *in silico* investigate the n-butanol production in order to identify the best medium conditions that lead to produce the desired alcohol. We firstly added the *Clostriodium* pathway in the iMM904 model [66], assuming that hbd used NADH rather than NADPH. Glucose and oxygen consumption uptake rates in each simulation were set to 10 mmol/ gDW/ hr and 1mmol/ gDW/ hr respectively, while only the upper bound of ESR was set to 13 mmol/gDW/hr. Based on yeast extract composition in each computation we added a nutrient to the *in silico* medium and the flux distribution was calculated by FBA approach using the biomass as objective. We evaluated both the individual or the simultaneous addiction of each nutrient analyzing the glycerol and ethanol secretion rates assuming that these two fluxes reflected the redox state of the cells.
Once identified the nutrient that lead to increase ethanol and glycerol fluxes, we used these new knowledges to further constrain the network. Finally, we optimized the butanol flux setting different growth rate values but, unfortunately, no production was predicted. It is clear that further computation will be performed in order to investigate the relationship between growth rate, medium composition, redox state and n-butanol production.

Table 4.2: **Growth rate, ethanol and glycerol yields -** Cells were grown in minimal (YNB) medium with glucose as carbon source. Growth rate (μ), ethanol (Y_{etoh}) and glycerol ($Y_{glycerol}$) yileds were calculated based on data obtained in the first 24 hours. Data correspond to the mean of two independent clones

Strains	μ [1/h]	$\mathbf{Y}_{etoh}$ [g/g]	$\mathbf{Y}_{glycerol}$ [g/g]
BY4741 control	0.094	0.26	0.11
BY4741 producer	0.094	0.14	0.07
CEN.PK control	0.089	0.45	0.12
CEN.PK producer	0.09	0.31	0.1
BY4741 control*	0.08	0.48	0.24
BY4741 producer*	0.08	0.46	0.25
CEN.PK control*	0.081	0.51	0.3
CEN.PK producer*	0.061	0.49	0.16

4.2 Discussion

The main technology platform for designing biofuel-producing organisms is metabolic engineering which allows to import exogenous genes and pathways that lead to express a desired biochemical function when the host cell does not possess it. Unfortunately, it is not always clear whether heterologous pathways will operate in the host like in the naturally producing cell. Thus, the same enzymes will lead to obtain different productions in different organisms, as is shown in Table 1.1. It is evident that the expression of the same genes in different host, in particular *E.coli* and *S.cerevisiae*, allows to produce a deeply different amount of the desired product (from 13.9 mg/L to the highest value of 1.2 g/L in *E.coli* and the sole reported production in the optimized *S. cerevisiae* equal to 2.5 mg/L [44], Table 1.1). These differences could be explained biochemically both on the basis of enzymes evolution and of metabolic network connectivity. Enzymes have evolved over time to respond to environmental needs and specific metabolic functions of the organisms that express them. Thus, individual reactions or pathways that are transferred in other organisms performed differently than in the natural host. Moreover, n-butanol production starts from acetyl-CoA which represents a central intermediate in yeast metabolism. In particular, some of the acetylCoA pool is trapped in the mitochondria, preventing it from being utilized in the cytosolic butanol pathway. In bacterial host this can not happen. This fact could also explain our results. The different extracellular metabolite profile of the engineered and wild-type strains suggested that the heterologous pathway somehow affected the yeast metabolic network, but not leading to a detectable n-butanol production. Further optimizations will be performed for example, by creating a pyruvate dehydrogenase bypass; pyruvate decarboxylase, acetaldehyde dehydrogenase and acetylCoA synthase [74] will be overexpressed to increase the cytosolic acetylCoA pool or the cytosolic overexpression of the pyruvate dehydrogenase will lead to obtain/increase n-butanol production.

The use of bioinformatic tools could also help to face the problem in a not easily predictable way. In particular, it is possible to speculate that the acetyl-CoA availability is associated with biomass growth, so strain design could be done by coupling cell growth and n-butanol production. A large-scale computational study of growth-coupled n-butanol production under several different substrate conditions, utilizing both constrain-based approach (COBRA) [69] and OptFlux algorithm [75] (which allows to consider (and optimize) in the same time, two different objective functions) is currently ongoing.

4.3 Methods

4.3.1 Strains and Media

C. acetobutylicum DSM 792 was available in our laboratory. *S. cerevisiae* strain CEN.PK 102-5B (*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2* - Dr, P. Kotter, Istitute of Microbiology, Johan Wolfgang Goethe-University, Frankfurt, Germany) [70] and BY4741 (*MATa his3-1 leu2 met15 ura3*) [71] were used as parent of all yeast strains.
The engineered strains obtained (Table 4.1) were grown in shake-flask at 30°Cin minimal synthetic medium (1.34% w/v YNB Biolife without amino acids) with 2% w/v of glucose as carbon source supplemented with G418 and NAT and methionine in the case of BY4741. BY4741* strain set was also grown in the presence of 2% w/v galactose as carbon source.

4.3.2 Plasmids construction

Desired genes were PCR amplified using as a template the genomic DNA extracted from *C. acetobutilicum* (DSM 792) and from *S. cerevisiae* CEN.PK for the *ERG10* amplification. Primers are listed in Table 4.3.

Pwo DNA polymerase was used on a GeneAmp PCR System 9700 (PE Applied Biosystem, Inc.). Standard condition used were 0.2 mM primers, 1.5 U of Pwo and 1.5 μL of genomic DNA. The program used for the amplification of genes was shown in Figure 4.3.

PCR products were subcloned into pSTBlue-1 with the perfect blunt cloning kit from Novagen, Inc. and checked by sequencing analysis. The coding sequences were subcloned into the *S. cerevisiae* expression vectors as described: ThiL, hbd, crt and etf B sequences were *EcoR*I subcloned into pYX012, pYX022 (RD Systems Inc., Wiesbaden, D), pZ_5 [20] and pZ_3 [72] respectively. Crt, etfA and etfB sequences were also cloned *EcoR*I into pYX042 (RD Systems Inc., Wiesbaden, D), pYX042 and pYX012 respectivily. adhEI was also cloned *EcoR*I into pYX022. For the construction of the double expression vector, *b*TPI

Oligo Names	Oligo details
$thiL_{fw}$	AGGAGGAGTAAAACATGAGAGATGT
$thiL_{rev}$	AACTATTTAGTCTCTTTCAACTACGAGA
hbd_{fw}	AGGGAGGTCTGTTTAATGAAAAAGG
hbd_{rev}	TGTAAACTTATTTTGAATAATCGTAGAAACCTTT
$etfA_{fw}$	AGTTAGGAGGGATTTTTCAATGAATAAAG
$etfA_{rev}$	TCTTTTTATTTATCTTAATTATTAGCAGCTTTAACT
$etfB_{fw}$	AGGAGGTTAAGAGGATGAATATAGTT
$etfB_{rev}$	TCCCTCCTAACTTAAATATAGTGTTCT
crt_{fw}	AGGATTAGTCATGGAACTAAACAATGT
crt_{rev}	TTACCTCCTATCTATTTTTGAAGCCT
$bcdt_{fw}$	AGATAGGAGGTAAGTTTATATGGATTTTAATT
bcd_{rev}	ACTATATTCATCCTCTTAACCTCCTTA
$adhEI_{fw}$	AGTGTATATTTATGAAAGTCACAACAGTAAA
$adhEI_{rev}$	TAAAAAGTAGTTGAAATATGAAGGTTTAAGGT
$erg10t_{fw}$	CAGATTACGTACGTACTCAAAATGTCTCAGAACGTTT
$erg10_{rev}$	GAGAAAATACGTAGAACGTAA
	TCATATCTTTTCAATGACAATAGA

Table 4.3: **Oligonucleotides:** Oligonucleotides pairs used for the amplification of the *C. acetobutylicum* ABE genes and *Erg10* yeast gene

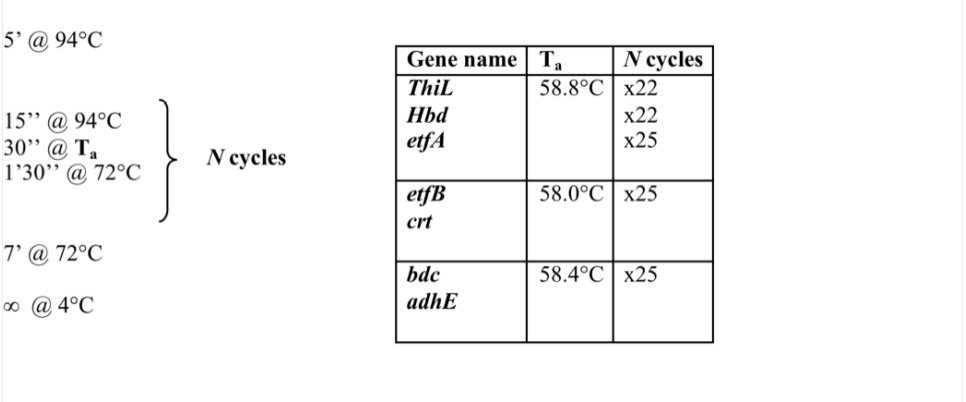


Figure 4.3: **PCR conditions used for the amplification of the *C. acetobutylicum* genes**

sequence [72] was obtained from pSTBlue[bTPI] (avaible in our laboratory) and subcloned into pYX012[ThiL] *Kpn*I cut. Bcd was *SnaB*I/Bluted/*Pml*I excised from pSTBlue[bcd] and subcloned into pYX012[ThiL] bTPI *Xba*I cut. Another double expression vector was obtained during this work pZ3[etfA/B]; [TPI-etfA-polyA] cassette was *FsP*I/ *Fse*I exiced from pYX042[etfA] and subcloned into pZ_3[etfB] opened *Fse*I and blunted. Furthermore *Erg10* sequence was obtained cutting *SanB*I from pSTBlue[erg10] and cloned into pZ_3 opened *EcoR*I and blunted. The coding sequence of ccr was optimized according to *S.cerevisiae* codon usage and synthesized by Eurofins MWG operon. ccr sequence was then *EcoR*I cut from pSTBluescriptII[ccr] and cloned into pYX012 *EcoR*I cut. ccr was also similarly cloned into the multi-copy plasmid pYX212.

4.3.3 Yeast transformation and strain construction

Transformation of all *S. cerevisiae* strains was performed using lithium acetate method [76]. The presence of all the desired sequences in the final strains were controlled by PCR using genomic DNA extracted from each strain as template.

4.3.4 Yeast cultivation

For each condition, cell growth was measured as optical density at 660nm. To measure n-butanol production, shake flask containing 150 mL of minimal synthetic medium (2% glucose or galactose with appropriate amino acid omissions as described above) were inoculated at 0.05 OD with the strain of interest. In order to achieve a "semi" anaerobic condition, the flasks were not agitated. Samples were collected at 24, 72, 120, and 144 h and analyzed for metabolites as discussed below.

4.3.5 Enzymatic assay

S. cerevisiae strains of interest were grown in shake flask in 220 mL of minimal medium (2.68% [w/v] YNB medium [catalogue no. 919- 15 Difco Laboratories, Detroit, Mich.]) with 5% of Glucose as carbon source, supplemented with necessary aminoacid and/or antibiotics to keep the selective pressure, and incubated at 30℃under aerobic condition until the exponential growth phase was reached. The cell extracts for THiL, HBD, and CRT enzyme assays were prepared as follows. The cells were collected, resuspended in the sonication buffer (100-mM Tris(hydroxymethyl) aminomethane hydrochloride or TrisHCl buffer and 2-mM 1,4-dithiothreitol; pH 7.5) before adding glass beads, and the mixture was homogenized at 4℃. Cellular debris were removed by centrifugation at 14000rpm, 4℃for 10 min, and the supernatant was used as cell extract. THiL activity was determined using acetoacetyl-CoA and CoA as substrates, and the decrease in acetoacetyl-CoA concentration was measured at 303 nm. The cell

extract was added to the solution containing 100-mM TrisHCl (pH 8.0), 10-mM MgCl2, 1 mM 1,4-dithiothreitol, 50 μM acetoacetyl-CoA, and 0.2 mM CoA to start the enzymatic reaction. Decrease in the absorbance was monitored in the sample solution and control solution, from which CoA was omitted, and the slope values were determined. The enzymatic activity was determined by calculating the difference in the slope values of the sample and control, and using the molar extinction coefficient of 14 M-1cm-1. HBD activity was measured at 345 nm by monitoring the decrease in NADH concentration resulting from β-hydroxybutyryl-CoA formation from acetoacetyl-CoA. The cell extract was added to the mixture containing 100-mM MOPS buffer (pH 7.0), 1 mM 1,4-dithiothreitol, 0.1 mM acetoacetyl-CoA, and 0.15 mM NADH, and the enzyme activity was calculated as above with the control, from which acetoacetyl-CoA was omitted, using the molar extinction coefficient of 6.220 M-1cm-1. CRT activity was measured by monitoring the decrease in crotonyl-CoA concentration 263 nm, as a consequence β-hydroxybutyryl-CoA formation from crotonyl-CoA [73]. The reaction mixture contained cell extract was prepared as described before, (100 mM TrisHCl buffer (pH 7.6) and 50 μM of crotonyl-CoA was added). The enzyme activity was determined as described above using the molar extinction coefficient of 6.700 M-1cm-1.

4.3.6 Metabolite detection

For metabolite analysis, cultures were sampled (2 mL) at 24, 72, 120, and 144 h. Cells were separated by centrifugation and the supernatants were stored at -20℃for the subsequent analytical quantifications. Glucose consumption, ethanol, n-butanol and glycerol production were measured by high performance liquid chromatography (HPLC, Aminex HPX-87H Column, 300 x 7.8 mm, Bio-Rad).

Chapter 5

Evaluation of yeast conversion capability for biofuels production

As described in the previous chapter, global energy and environmental problems have stimulated increased efforts towards biofuels synthesis from renewable resources. Compared to the traditional biofuel (i.e. ethanol), higher alcohols offer advantages as gasoline substitutes because of their higher energy density and lower hygroscopicity. One of the main issues to be solved is that none of the C4 and C5 alcohols have been produced from a renewable source with a sufficient yield to be considered as a gasoline substitute. It was reported that microorganisms and mammalian cells do not produce higher alcohols such as isobutanol, 2-methyl-1-butanol or 3-methyl- 1-butanol at industrially relevant quantities, although small amounts have been identified as by-products.

Despite other user friendly hosts organisms such as E. coli are readily applicable, we took advantage of the previously described metabolic capability of S.cerevisiae, in particular the multiplicity of its enzymatic activities involved in the aminoacid metabolism (in particular the branched chain aminoacid Leucine, Isoleucine and Valine), and the broad substrate range of the last steps in the αketo acid degradation [14], [77].

*αketo acids are intermediates in aminoacid metabolism derived from a deamination reaction. In yeast these compounds seem to be converted into alcohols by at least two different routes. In one case the carboxylation of the αketo acid is followed by the NADH-linked reduction of the resulting aldehyde (*Enrlich Pathway*), while in the other the αketoacid is oxidatively decarboxylated to the corresponding acyl-CoA derivates and then converted into the alcohols. Unfortunately, the enzymes involved in these pathways are not clearly identified yet. Some evidences described the roles of the pyruvate decarboxylases in the second route mentioned [77], while an* in vitro

characterization of a branched-chain αketo acid dehydrogenase was performed by Sinclair et al. *[78].*

Here, we initially explored the strain-dependent metabolic versatility of S. cerevisiae in order to select the best aminoacidic substrates for the n-butanol and other fusel alcohols production. After that, we briefly analyzed the pathway(s) involved in these conversions and based on the new knowledge derived from this study, a strategy aimed at re-directing the flux towards the desired product was implemented in S. cerevisiae.

5.1 Results

5.1.1 Analysis of wild-type *S. cerevisiae* metabolic versatility

At the beginning of this study we could not predict the great versatility shown by *S. cerevisiae*. Thus we tested different compounds as substrate for n-butanol production.
n-butanol can be obtained from a non-conventional aminoacid, the L-Norvaline, which is transamminated leading to the correspondent αketoacid, the αketovaleric acid, that is then metabolized to the correspondent alcohol. The wine strains are able to produce a wide range of alcohols and esters which are involved in the wine fragrance and organoleptic features. n-Butanol is one of these compounds produced during the wine production process. In particular, Lilly *et al.* reported a concentration lower than 1 mg/L after alcoholic fermentation of different grape types [15]. Based on this observation, we first evaluated the wine yeast ability to recognize and convert the αketovaleric acid and L-Norvaline into the desired alcohol. The strains (Table 5.1) were grown

Table 5.1: List of the strains used in this work

Strains	Source
Wild Vinary Strains	
VIN13 .	Wine yeast strain from Stellenbosh University (Sud Africa)
SAV	**Fermol Sauvignon** - wine yeast selected by Pascal Biotech industry and kindly granted by AEB spa.
AP	**Fermol Arome Plus** wine yeast kindly granted by AEB spa.
BL	**Fermol Blanc** - wine yeast selected by Pascal Biotech industry and kindly granted by AEB spa.
Lab Strains	
CEN.PK	[70]

in minimal medium in the presence or not of αketovaleric acid or L-Norvaline (see Methods) and n-butanol production was monitored by sampling the culture at different times. Surprisingly, despite their chemical nature, both these compounds were recognized by yeast cells, internalized and converted into the alcohol of interest (Figure 5.1). Figure 5.1 shows the growth and the n-butanol obtained from 5mM L-Norvaline (upper panels) or 5mM αketovalerate (lower panels) of the four wine strains tested. Cell growth seemed not to be affected by the presence of these two substrates, which were converted into n-butanol in a strain-dependent manner. Moreover, the n-butanol production seemed to be correlated on the substrate used, consistently with the number of step involved in the conversion.
After that we tested also the laboratory strains. The CEN.PK wild-type strain

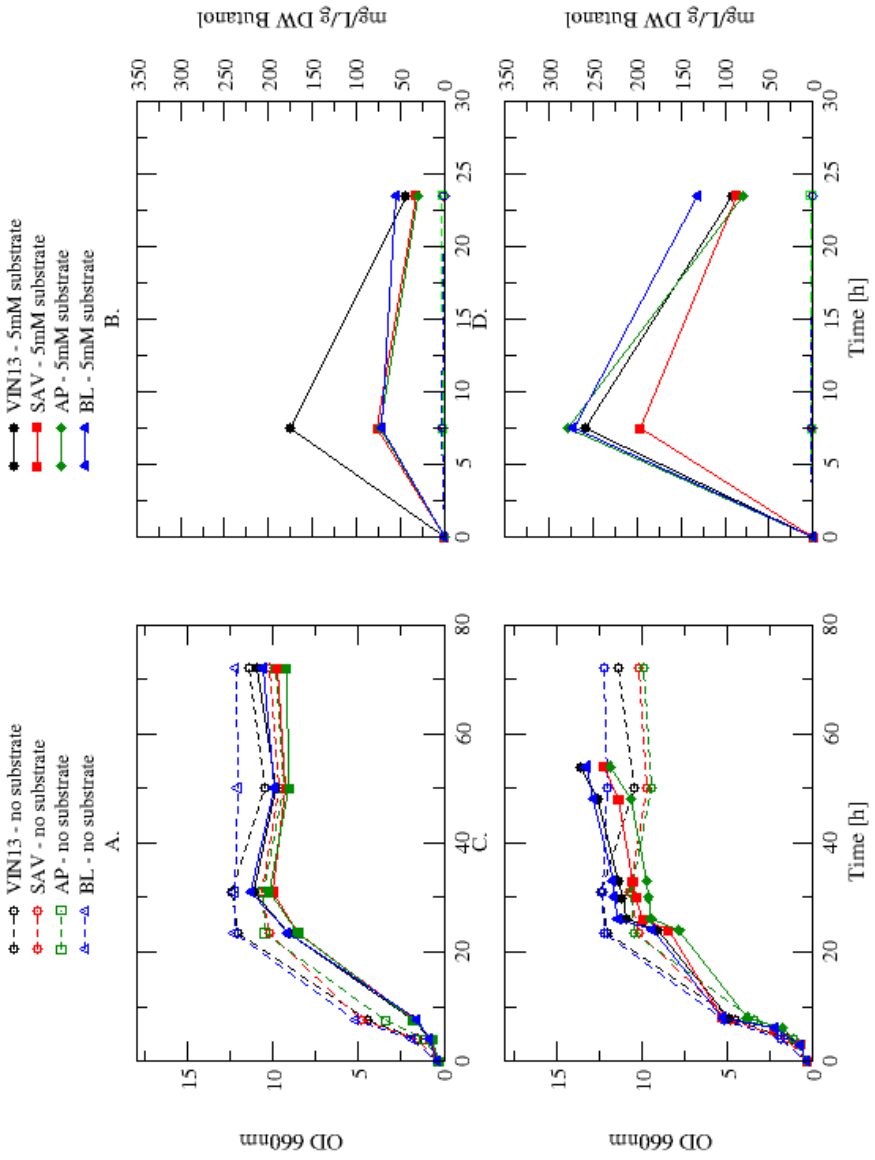


Figure 5.1: **Wine yeast strains growth and n-butanol production from L-Norvaline or αketovaleric Acid -** Four different wine strains (see Methods, Table 5.1) were flask-batch grown in minimal (YNB) medium, with glucose as carbon source, in the presence or not of 5mM of L-Norvaline (upper panels) and 5mM αketovaleric acid (lower panels). (A/C) Growth was measured as optical density (OD_{660nm}). (B/D) n-butanol production is normilized by biomass (mg/L/g DW). Data correspond to the mean of two different experiments. Standard error is lower than 0.03%. The yeast growth ability is not affected by the presence of the two precursors tested in the medium, which were converted in different amounts of n-butanol in 8 hours: about 80 mg/L from L-Norvaline and about 300 mg/L from αketovalerate

(Table 5.1) was grown in the same conditions previously described. Figure 5.2 shows the growth and the n-butanol production derived from 5mM of L-Norvaline (black line) or 5mM αketovalerate (red line). Also this lab strain was able to produce n-butanol from the two precursors tested even if in a less efficient manner than the wine strains, suggesting the loss of some metabolic versatility typical of the wild yeasts.
Considering that the highest (and sole) n-butanol production obtained in *S. cerevisiae* by the *Clostridium* pathway expression was 2.5 mg/L from 20 g/L galactose in 72 hours, our results represented a good starting point. Indeed, in 8 hours, the wine yeasts produced about 80 mg/L of n-butanol from 0.585 g/L (5mM) L-Norvaline and 300 mg/L from 0.580 mg/L (5mM) αketovalerate. In a less efficient way the lab strains accumulated from the same amounts of L-Norvaline and αketovalerate about 18 mg/L and 220 mg/L of n-butanol respectively, in 8 hours.
The presence of αketovalerate in yeast as intermediate of glycine metabolism

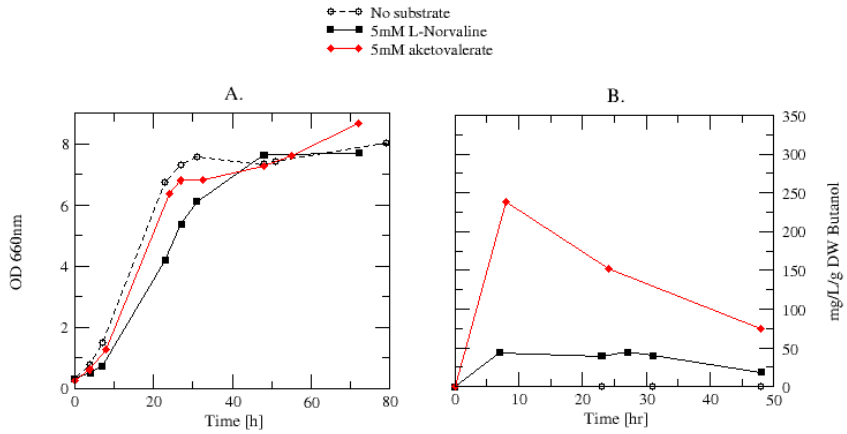


Figure 5.2: **Laboratory yeast growth and n-butanol production from L-Norvaline or αketovaleric Acid -** The CEN.PK lab strain was flask-batch grown in minimal (YNB) medium, with glucose as carbon source, in the presence or not of 5mM of L-Norvaline (upper panels) and 5mM αketovaleric acid (lower panels). (A/C) Growth was measured as optical density (OD_{660nm}). (B/D) n-Butanol production is normilized by biomass (mg/L/g DW). Data correspond to the mean of two different experiments. Standard error is lower than 0.03%. The yeast growth ability did not affected by the presence of the two precursors tested in the medium, which were converted in different amounts of n-butanol in 8 hours: about 18 mg/L from L-Norvaline and about 220 mg/L from αketovalerate

was detected through 13C-isotope-labeling analysis [79]. In particular the authors found this αketoacid growing cells in the presence of two different carbon sources, glucose and galactose, with glycine as the sole or main nitrogen source. They speculated that αketovalerate produced from glycine was further metabolized by an unknown activity leading to αketoisovalerate. This compound is the main precursor of valine and isobutanol, which is the correspondent fusel alcohol (Figure 5.3, [79]). In order to reproduce this intracellular scenario,

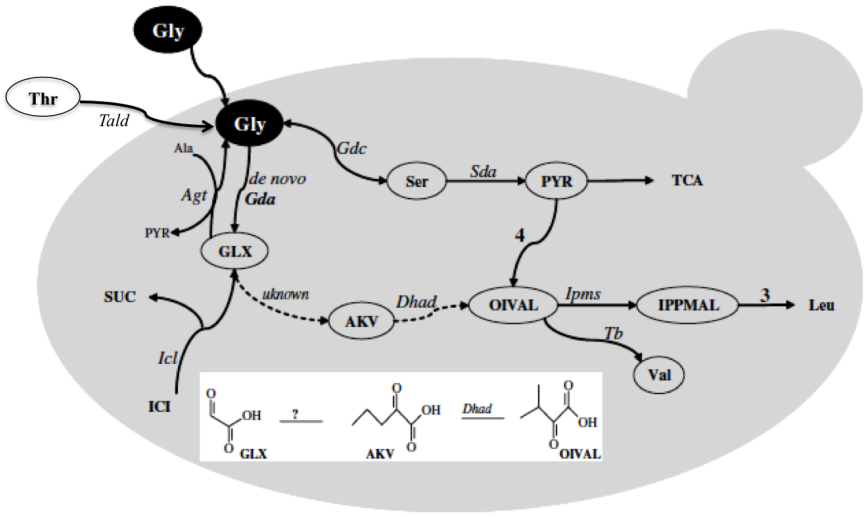


Figure 5.3: **Glycine metabolism in *Saccharomyces cerevisiae* -** Villas-Boas *et al.* proved that there are at least two pathways for glycine catabolism in *S. cerevisiae*: (1) via Gdc and (2) via a *de novo* described Gda. They postulated that αketovalerate was synthesized from glyoxylate by an unknown reaction/enzyme with its subsequent conversion to αketoisovalerate by Dhad. Gdc: glycine decarboxylase multienzyme complex; Sda: serine deaminase; Agt: alanine: glyoxylate aminotransferase; Gda: glycine deaminase; Dhad: dihydroxy acid dehydratase; Ipms: isopropylmalate synthase; Icl: isocitrate lyase; Tb: transaminase; Tald: threonine aldolase. B. Full arrows indicate confirmed pathways and dashed arrows indicate speculative pathways. The numbers specify the number of reaction steps in the pathway.

CEN.PK (Table 5.1) was cultivated in similar growth conditions in the presence of two different carbon sources, glucose and galactose, with glycine or with both glycine and ammonium as nitrogen source and n-butanol accumulation was monitored at different times. Panel A (Figure 5.4) shows the growth, measured as optical density (OD_{660nm}) of the lab strain in the glucose (black line) and galactose (red line) media with glycine as sole (dotted line) or main (solid line) nitrogen source. Panel B shows the glycine consumption and panel C and D shows the n-butanol and isobutanol production respectively, normalized by biomass (mg/L/g DW). It can be observed that the lab strain was able to grow in media with glycine as the sole nitrogen source (Figure 5.4, Panel A). Furthermore, this aminoacid represented a potential substrate for n-butanol production only if representing the sole nitrogen source (with glucose as car-

bon source). In this growth condition, glycine was used for the production of both n-butanol and isobutanol (Figure 5.4, Panel C and D). In the presence of galactose, cells seemed to metabolize glycine producing isobutanol as the main fusel alcohol. It is possible to speculate that the unknown enzyme involved in the conversion of αketovalerate into αketoisovalerate, could be partially repressed by glucose, leading to an accumulation of αketovalerate, which could be decarboxylated in an oxidative way to n-butanol.
Once identified the best condition for n-butanol production from glycine, we analyzed the pathway involved in more detail. In order to point out a possible relationship between glycine initial concentration and n-butanol production, wild-type lab strain was grown in the presence of different concentrations of this aminoacid as the sole nitrogen source, with glucose as carbon source. Growth was measured as optical density ((Figure 5.5, left panel) and n-butanol and isobutanol production were evaluated (Figure 5.5, right panel, black and red line respectively). The results obtained indicated that the growth rate seemed to be slightly affected by the increase in glycine initial concentration (Figure 5.5, left panel). Otherwise both n-butanol and isobutanol production increase only by increasing the glycine amount from 5 to 10 g/L. A further increase in the glycine initial concentration did not lead to a further increase in the alcohol productions (Figure 5.5, right panel). These findings represents a first evidence of the relationship between glycine supply and higher alcohol productions. Clearly, further experiments will be needed to deeper understand this relation and the competition between n-butanol and isobutanol accumulation.
Despite the data obtained indicated that glycine was converted into n-butanol by lab strain, other aminoacids (i.e. L-threonine and L-serine) which represented possible precursors for glycine in yeast (Figure 5.3), were not converted into the desired alcohol if they were supplied as the sole nitrogen source (data not shown).
The results shown indicated a strong a-specificity of the enzymatic activities involved in the degradation of the branched-chain aminoacids. A wide range of enzymes were involved in this part of the yeast metabolism and many of these are not annotated yet [77], [80], [81], [82]). Interestingly, the deletion of genes encoding for pyruvate decarboxylase (PDC) activities, which played an important but not essential role in the branched-chain aminoacid catabolism [77], did not lead to a complete loss in the ability to convert αketovalerate and glycine into n-butanol. Cells deleted in the *PDC* genes produced the alcohol of interest from these two substrates even with a reduced yield (data not shown), confirming the PDC role previously described by Schure *et al.* [77].
The substrates uptake but also the conversion of the αketoacid into the desired alcohol clearly represent two critical steps in order to direct the flux toward the production of interest. Dickinson and Norte [82] assumed that one or more branched-chain aminoacids are deaminated prior to further degradation of the carbon skeletons. It is possible to speculate that this could lead to a transient accumulation of the αketoacid intermediate which could be used in an undesired manner. Based on these observations we designed two different metabolic engineering strategies: first the constitutive expression of an additional copy of the gene encoding for the general aminoacid permease *GAP1* from *S. cere-*

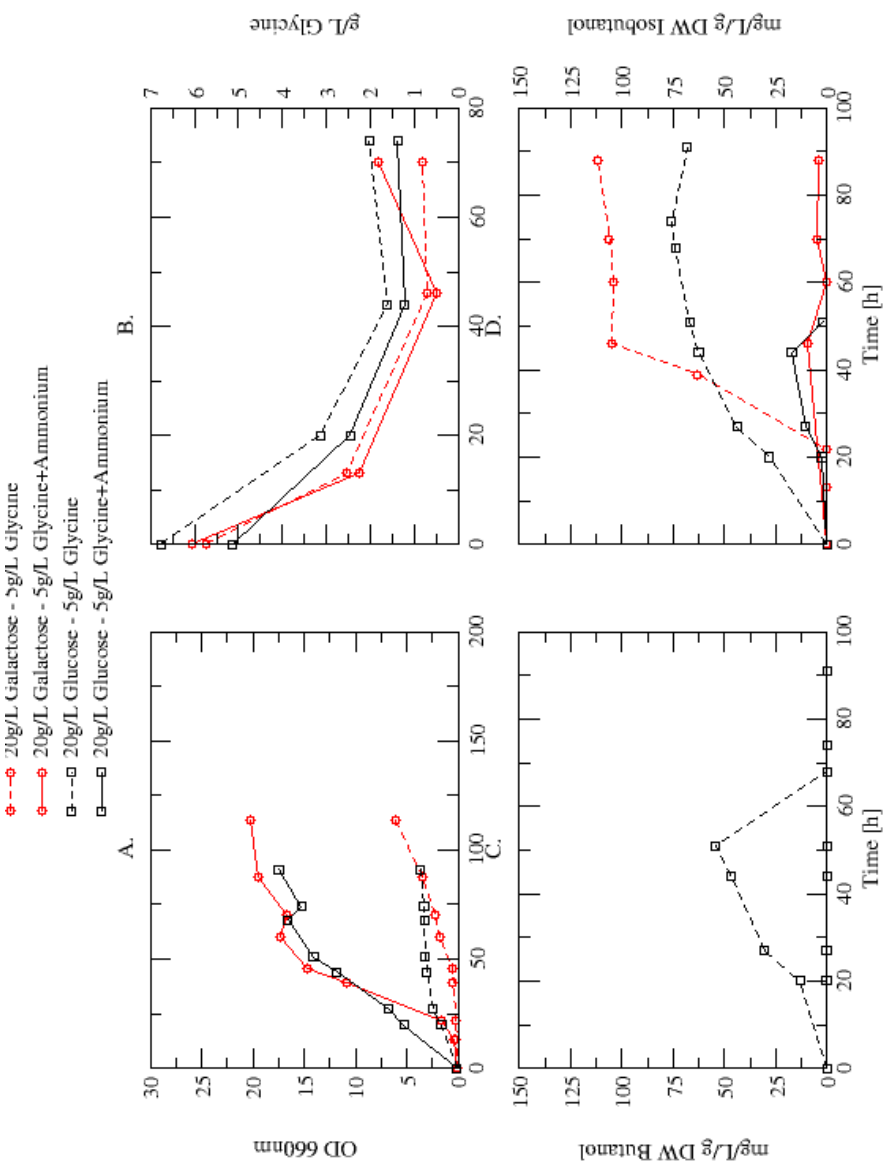


Figure 5.4: **Laboratory yeast growth, n-butanol and isobutanol production from Glycine -** The CEN.PK lab strain (Table 5.1) was flask-batch grown in minimal medium, with glucose or galactose as carbon source and glycine as the sole or main nitrogen font.(A) Growth was measured as optical density (OD_{660nm}). (B) Glycine consumption was measured at different times. (C/D) n-Butanol and Isobutanol production is normilized by the amount of biomass that produced it (mg/L/g DW), was determined. Data correspond to the mean of at least two different experiments. Standard error is lower than 0.03%.

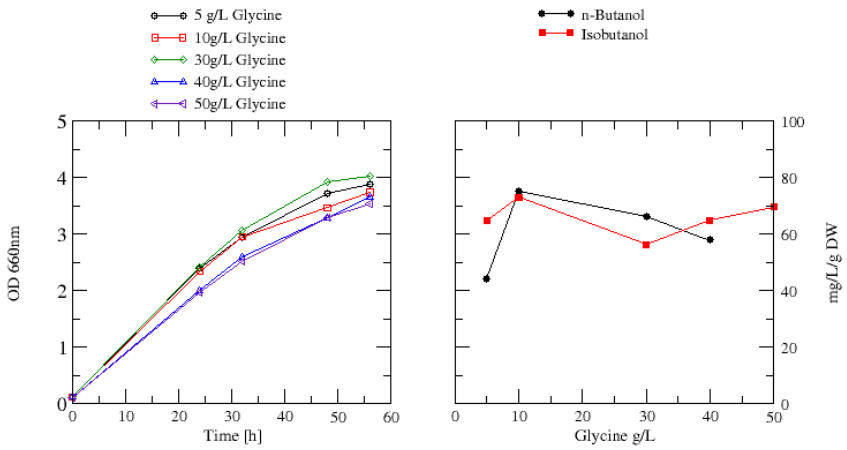


Figure 5.5: **Relationship between the n-butanol production and the initial concentration of glycine supplied -** The CEN.PK lab strain (Table 5.1) was flask-batch grown in minimal medium, with glucose as carbon source and different concnetration of glycine as sole nitrogen font.(A) Growth was measured as optical density (OD_{660nm}). (B) n-Butanol and Isobutanol production, normalized by biomass (mg/L/g DW), were detected. Standard error is lower than 0.03%.

visiae, second, the heterologous expression of an high affinity αketovalerate dehydrogenase was evaluated. The effects of the single or coupled expression of biochemical functions on n-butanol production from the previously selected compounds were analyzed.

5.1.2 Optimization of wild-type yeast bioconversion performance

Functional Analysis of the chosen enzymatic activities

The four genes coding for the αketoacid dehydrogenase complex from *Pseudomonas aeruginosa* (PaOxoDH) were cloned into yeast expression vectos (see Methods), under the control of the yeast constitutive promoter *Sc*TPI. This complex was described to have the same catalytic mechanism of pyruvate (PDH) and αketoglutarate dehydrogenase [85], [78]. It was formed by two catalytic subunits which directly interacted with the substrate, and two accessory subunits that were involved in the interaction with the cofactors. *In vitro* studies indicated a high affinity for the αketoacids and in particular for the αketovalerate [85], [78]. The functional analysis of the cloned genes was performed by an *in vitro* activity assay on protein extracts, following spectrophotometric production of an analogous of the NAD+ (see Methods). Considering the similar catalytic mechanism, we assumed a possible interaction between the catalytic subunits of the PaOxoDH and the accessory subsunits of the endogenous PDH. For this reason we also evaluated the PaOxoDH *in vitro* activity derived from the expression of the sole two genes coding for the two catalytic subunits (PaOxoDH+2, Table 5.2). The assay was performed as previously

Table 5.2: List of the strains obtained in this work

Strains	Plasmid
	Lab Strains
CEN.PK	pYX212 - pYX022 - pYX242 - pZ_3 - pZ_5
CEN.PK PaOxoDH+2	pYX212[*PaBDKA2*] - pYX022 - pYX242[*PaBDKA1*] - pZ_3 - pZ_5
CEN.PK PaOxoDH	pYX212[*PaBDKA2*] - pYX022 - pYX242[*PaBDKA1*] - pZ_3[*PaBDKB*] - pZ_5[*PaLPDV*]
CEN.PK Gap1	pYX212 - pYX022[*ScGAP1*] - pYX242 - pZ_3 - pZ_5
CEN.PK PaOxoDH-Gap1	pYX212[*PaBDKA2*] - pYX022[*ScGAP1*] - pYX242[*PaBDKA1*] - pZ_3[*PaBDKB*] - pZ_5[*PaLPDV*]

described [78]. The reaction was started with addition of αketovaleric acid (30 μmol) to the cell extracts obtained from the different yeast cultures directly into cuvette. The formation of reduced 3-acetylpyridine adenine dinucleotide (a NAD+-analog) was spectrophotometrically measured at 366 nm.
Figure 5.6 shows the NAD+-analouge formation rate measured for the control

(dotted black line), wine (dotted red line), PaOxoDH+2 (grey column) and PaOxoDH+4 (black column) strains (Table 5.2). Interestingly, the expression

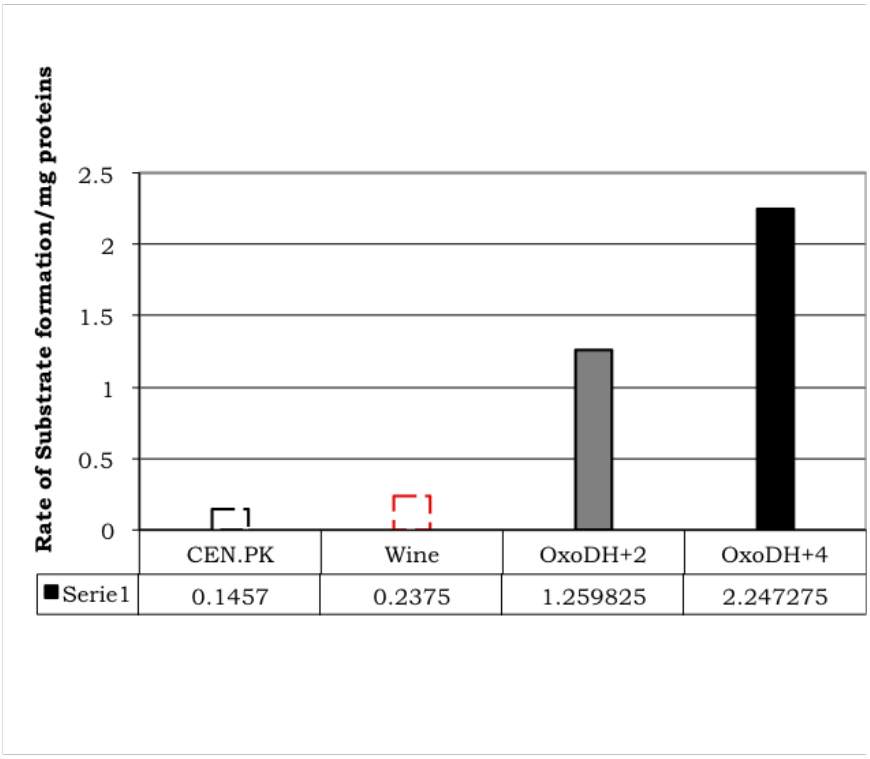


Figure 5.6: **Enzymatic activity of the PaOxoDH -** The CEN.PK, CEN.PK PaOxoDH and PaOxoDH+2 strains (Table 5.2) and the relative control were grown in minimal (YNB) medium with glucose as carbon source while wine strain was cultivated in rich medium with glycerol as carbon source*. The cells were harvested and the proteins were extracted. The assay was performed as described by [78].The reaction was started with addiction of cell extract and αketovaleric acid (30 μmol) as substrate directly to cuvette. The formation of reduced 3-acetylpyridine adenine dinucleotide (a NAD+-analog) was followed at 366 nm. The protein concentrations in cell extracts were estimated by the Lowry method. Bovine serum albumin (fatty acid free; Sigma Chemical Co.) was used as a standard. Data represent the mean values calculated from triplicate experiments (the values indicate the NAD+-analog formation rate normalized to mg protein [μmol min-1 mg protein-1] because its extinction coefficient is not reported). * Sinclair *(*et al.) [78] described the induction of the endogenous dehydrogenase in this growth medium.

of all the genes but also of only the catalytic genes allowed to strongly increase the NAD+-production rate (Figure 5.6). These results indicated that the complexes worked *in vitro* but they also showed a possible interaction between the heterologous catalytic subunits and the endogenous accessory parts. Figure 5.6 indicates also an (even very low) activity in the control extract. This could be due to a-specific residual activities which were able to use NADH-analog, but also it could confirm the presence, in yeast, of an endogenous αketoacid dehydrogenase complex [77], [80], [81].

To improve the substrate uptake, we constitutively expressed an additional copy of the yeast general aminoacid permease *GAP1*. Gap1 is an integral membrane protein strongly transcriptionally, transductionally and post-transductionally regulated [83]. It was able to transport all of the naturally occuring L-amino acids found in proteins and related compouds, such as ornithine and citrulline, and several D-aminoacids and toxic aminoacid analogs [84]. Despite many of them also have their specific transporter, no specific transporter is described for glycine. Thus Gap1 represented the only transporter able to internalize it [84]. The *GAP1* coding sequence was cloned into an integrative plasmid under the control of the constitutive ScTPI promoter and the functional analysis was performed by growing transformed cells in the presence of glycine as the sole nitrogen source.

Data obtained indicated an increase in the glycine consumption rate in both the strain expressing Gap1 individually and coupled with PaOxoDH (data not shown).

Analysis of the effects of the *PaOxoDH* and *GAP1* expression on n-butanol production

The genes coding for the dehydrogenase and the transporter of interest were expressed in yeast cells both individually and coupled. The strains obtained (Table 5.2) were grown in different minimal mediums supplied by the three selected compounds: αketovaleric acid, L-norvaline and glycine. For each condition tested, n-butanol and isobutanol productions were measured at different times.

- **Optimized yeast strains performance in the presence of αketovaleric acid**
  The PaOxoDH engineered strain and its relative control (Table 5.2) were grown in minimal (YNB) medium supplied with different concentration of αketovaleric acid (from 0 to 25 mM). Our evidences shown that the αketovalerate in the medium was completely consumed by the cells (data not shown); despite this, the medium with 25mM of this compound was set to pH 3, in order to favour the free internalization of the undissociated acid ($pK_a[\alpha\text{ketovalerate}] = 4.87$). Figure 5.7 shows the n-butanol production as a function of the amount of αketovalerate supplied. The control strain seemed to reach a *plateau* corresponding to an initial concentration of 10mM of substrate; funther increase in the αketovalerate amount did not lead to an increase in the n-butanol production. The engineered strain did not reach this *plateau*, indicating that a higher fraction of the internalized αketovalerate was used as substrate for the production of interest and confirming what was previously assumed: the limitation determined by the reaction(s) converting the αketoacid into alcohol.

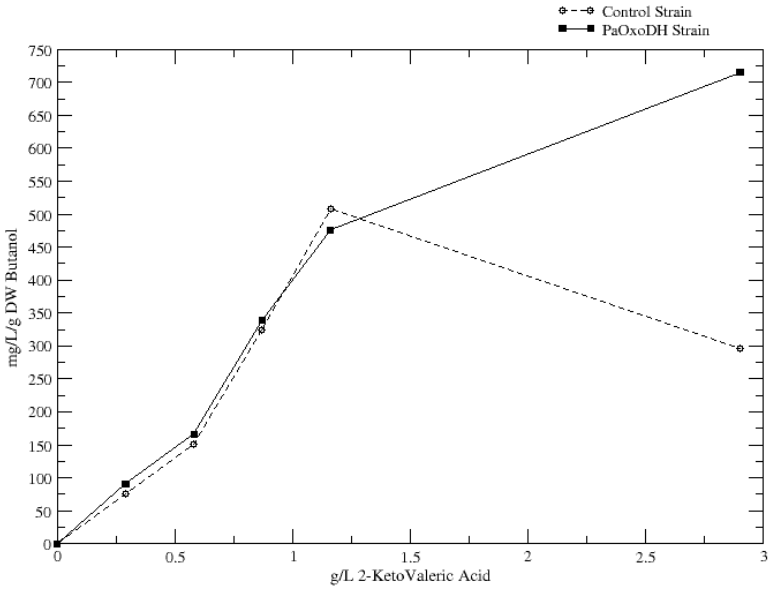


Figure 5.7: **n-Butanol production as a function of the initial amount of αketovaleric acid -** The CEN.PK PaOxoDH and the relative control strains were grown in the minimal (YNB) medium with different concentration of αketovalerate. Growth was measured as otical density (OD_{660nm} and n-butanol production was measured at different times. The plot shows the maximal n-butanol production obtained from each concentration of the substrate tested. To compare data obtained in the different conditions, the production was normalized by biomass (mg/L/g DW). Data represent the mean values calculated from two experiments (except the values correspondent to 25mM αketovalerate that represent a preliminary data)

- **Optimized yeast strains performance in the presence of glycine and L-norvaline**
  The engineered strains expressing PaOXODH and *GAP1* individually and coupled and the relative control (Table 5.2) were grown in the presence of two different carbon sources, glucose and galactose, with glycine as the sole or main nitrogen font as previously described. The n-butanol production was evaluated at different times. Table 5.3 shows the highest n-butanol/isobutanol production obtained from L-norvaline and glycine.

Table 5.3: **n-butanol produced from L-Norvaline in engineered strains -** The yeast strains described in Table 5.1 were grown in minimal medium supplied with L-norvaline 5mM or glycine as substrate for the n-butanol production. Growth was measured as optical density (OD_{660nm}) and n-butanol production was evaluated at different times. Data shown represent the highest n-butanol/isobutanol production obtained from each precursor. The production was normalized by the biomass (mg/L/g DW).

Substrate	**L-Norvaline 5mM**	**Glycine 5g/L**	**Glycine 5g/L**
Strains	**n-Butanol mg/L/g DW**	**n-Butanol mg/L/g DW**	**Isobutanol mg/L/g DW**
CEN.PK	40.01	54.59	75.89
CEN.PK PaOxoDH	43.00	n.d.	77.29
CEN.PK Gap1	41.28	73.50	72.51
CEN.PK PaOxoDH-Gap1	46.63	60.18	74.02

All the strains tested produced n-butanol only in the presence of glucose as carbon source and glycine as sole nitrogen source (Talbe 5.3). Interestingly, the modification performed affected also the isobutanol production when galactose was supplied as carbon source (data not shown). In this conditions the expression of *PaOxoDH*, individually or coupled with *GAP1*, seemed to play a role in the conversion of glycine into isobutanol but not into n-butanol, confirming an affinity of the PaOxoDH acitvity also for the isobutanol precursor, the αketoisovalerate [78]. It is possible to speculate that in galactose the glucose repression did not occur and the enzyme(s) involved in the conversion of αketovalerate into valine (Figure 5.3) was not inhibited. Thus, in the engineered cells, the glycine was internalized more rapidly and converted into valine in a more efficient way than the control, leading to a higher production of the correspondent alcohol. This confirmed what was previously hypothesized for the wild-type strain.
Further, these strains were also grown in minimal (YNB) medium supplied or not with 5mM of L-Norvaline and the n-butanol production was measured at different times (Table 5.3). All the optimized strains tested have shown a higher n-butanol production than the control in the condition considered (Table 5.3), confirming again our hypothesis about the roles of the substrate uptake and the αketoacid metabolization as determining steps in the n-butanol production.

5.2 Discussion

The increase in the energetic needs with the decrease in the fossil resource availability lead to consider biomass as the petroleum of the future. The possibility to use these kind of waste materials to produce high social value compounds such as biofuels, is a great challenge. Data shown here represent a further evidence of the production of n-butanol and isobutanol, which are two potential oil substitutes for the transport industry, from different aminoacidic compounds. The pathway(s) and enzymatic activities involved in this conversion were not identified yet. The debate concerning the existence of the *Ehrlich pathway* in yeast was not solved yet, even if a lot of routes for the production of fusel alcohol from the correspondent aminoacid were described [77], [80], [81]. The branched chain aminoacids were firstly deaminated leading to the correspondent αketoacids, which were converted into the relative alcohol by the formation of both the aldehydic and acyl-CoA intermediates.
Our results support the existence of both these pathways, highlighting the presence of specific and a-specific enzymes involved in the degradation of this aminoacid family.
Considering the wide range of enzymes that seemed to be involved in the conversion of interest, and their broad substrate specificity, the possibility to direct the metabolic flux solely towards the desired product(s) seemed to be the winning strategy. The higher production typical of the engineered strains fits this context. The expression of an αketovalerate-specific activity re-directed the flux towards the production of the desired alcohol, indicating the possibility to solve the competition for the substrate usage increasing the enzymatic affinity. Moreover, the substrate uptake seemed to play an important role as previously described in this thesis. The expression of the a-specific permease *GAP1* increased the glycine uptake rate leading to an higher n-butanol (or isobutanol, in a way that depends on the carbon source) production. As previously described [14], the effects derived from the increase in the fusel alcohol precursor availability were strongly associated with the growth conditions considered and also with the yeast strain utilized. This fact seemed to depend on the high connection between the branched chain catabolism and other metabolic regions, such as fatty acids and phenolic alcohols biosynthesis [14]. The main consequence of this high connectivity is a poor correlation between the aminoacid medium composition and the composition of the resulting fusel oil [80]. Further experiments will be performed to identify these connections in order to develop the best strategy to re-direct the flux towards the desired alcohol.

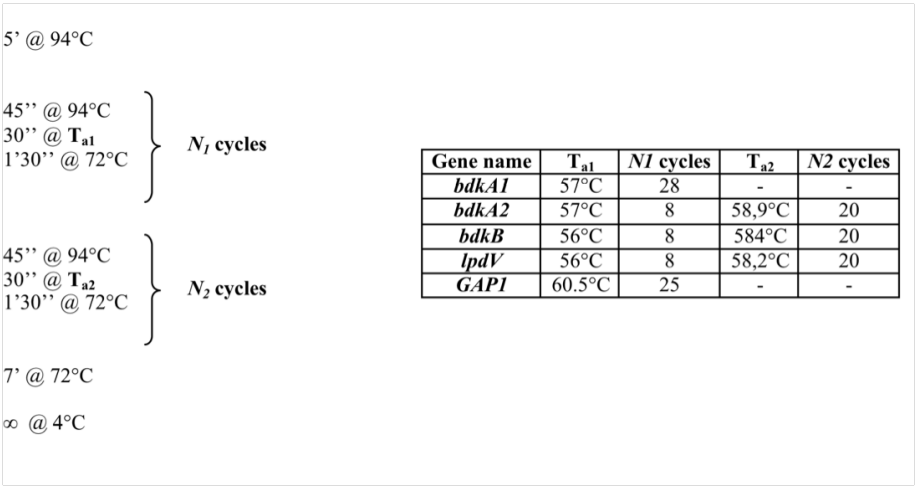
5.3 Methods

5.3.1 Strains, Media and Culture Conditions

The *S. cerevisiae* strains used in this study (Table 5.1) were derived from the CEN.PK strain 102-5B (*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2* - Dr, P. Kotter, Istitute of Microbiology, Johan Wolfgang Goethe-University, Frankfurt, Germany) [70]. Yeast transformations were performed according to the LiAc/PEG/ss-DNA protocol [76]. The control strain is the corresponding yeast strain transformed wih the empty plasmids. For each set of transformation, at least 2 independent clones were initially tested.
Yeast cultures were shake-flask grown in different minimal medium as will be described at 30°C. To evaluate the n-butanol production from αketovaleric acid or L-Norvaline, cells were grown in minimal synthetic medium (1.34% wv-1 YNB Biolife without amino acids) with 2% or 5% wv-1 of glucose as carbon source supplemented with G418 and NAT. αketovaleric acid was added at different concentrations as specifically indicated. The production from glycine was analyzed by growing the yeast strains in minimal medium [86] with 2% wv-1 of glucose or galactose as carbon source and glycine as sole or mainly nitrogen source. In this case a mixture of 5 g/L of glycine and ammonium sulfate was supplied.
For each condtion, samples were taken at different times for the determination of growth parameters, metabolite production, nutrient consumption and enzymatic activities.

5.3.2 Sequences amplification and cloning strategies

The coding sequences for the αketoacid dehydrogenase of interest were PCR amplified using as template the genomic DNA of *Pseudomonas aeruginosa* (catalogue ATCC 47085D-5). In a same way, the coding sequence of Gap1 was PCR amplified using CEN.PK genomic DNA as template. *Pwo* DNA polymerase was used on a GeneAmp PCR System 9700 (PE Applied Biosystem, Inc.). Standard conditions used were 0.2 mM primers, 1.5 U of Pwo and 4 μL of genomic DNA . The program and the oligos used for amplification of all these genes were as follow (Figure 5.8, Table 5.4). PCR products were cloned into pSTBlue-1 with the perfect blunt cloning kit from Novagen, Inc. and checked by sequencing analysis. The coding sequences were cloned into the *S. cerevisiae* expression vector as described in following: *bdkA1, bdkA2, bdkB* and *lpdV* sequences were subcloned *EcoR*I into pYX212, pYX242 (RD Systems, Inc., Wiesbaden, D.), pZ5 and pZ3 respectively, while *GAP1* sequence was *SnaB*I exceeded and cloned in *EcoR*I/bluneted pYX022 (RD Systems, Inc., Wiesbaden, D.).



Gene name	T_{a1}	*N1* cycles	T_{a2}	*N2* cycles
bdkA1	57°C	28	-	-
bdkA2	57°C	8	58,9°C	20
bdkB	56°C	8	584°C	20
lpdV	56°C	8	58,2°C	20
GAP1	60.5°C	25	-	-

Figure 5.8: **PCR conditions used for the amplification of the four genes of interest**

Oligo Names	Oligo details
$bdkA2_{fw}$	TCGGGGTatgAATGCCATGAAC
$bdkA2_{rev}$	CATTCAGACCTCCATCACACG
$bdkA1_{fw}$	AGGCCTGTCatgAGTGATTAC
$bdkA1_{rev}$	TTCATGGCATtcaTACCCCGA
$bkdB_{fw}$	GAGGTCTGAatgGGTACCCA
$bkdB_{rev}$	TCTGGCTCATCGCttaCTCCA
$lpdV_{fw}$	TTCCTGGAGTAAatgATGAGCCA
$lpdV_{rev}$	TCGCGGGAAtcaGATGTGCA
$ScGAP1_{fw}$	AAAATACGTAAATAAAAAATGAGTAATACTTCTTCGTAC
$ScGAP1_{rev}$	TTTTGTCGATGCATCGATTCATTAACACC

Table 5.4: **Oligonucleotides** Oligonucleotides pairs used for the amplification of genes coding for PaOxo DH and Gap1

5.3.3 Enzymatic assay

For the αketoacid dehydrogenase assay, the *S. cerevisiae* strains of interest were grown in shake flask in 220 mL of synthetic (YNB5X Glucose 5% Histidine (150mg/L) G418 NAT) liquid medium at 30℃, under aerobic condition until the exponential growth phase. The cell extracts were prepared as follows. The cells were collected by centrifugation at 4 000rpm, 4℃for 10 min and resuspended in a buffer B (200-mM Tris(hydroxymethyl) aminomethane hydrochloride or TrisHCl buffer pH 8.5 and 6mM 2-mercaptoethanol) and disrupted using glass beads. Cellular debris was removed by centrifugation at 14 000rpm, 4℃for 10 min, and the supernatant was used as a cell extract. αketoacid dehydrogenase activity was evaluated as described previously [78] by a continuous spectrophotometric assay. The assay comprised 0.6 mL of buffer B, cysteine-HCl (3 μmol), sodium azide (30 μmol), co-enzyme A (0.08 μmol), thiamine pyrophosphate (0.2 μmol), and the NAD+-analogue 3-acetylpyridine adenine dinucleotide (0.765 μmol) in a finel volume of 0.9 mL. The reaction was started with addiction of cell extract and αketovaleric acid (30 μmol) or αketoisovaleric acid (30 μmol) as substrate directly to cuvette. The formation of reduced 3-acetylpyridine adenine dinucleotide was followed at 366 nm.
The protein concentrations in cell extracts were estimated by the Lowry method. Bovine serum albumin (fatty acid free; Sigma Chemical Co.) was used as a standard.

5.3.4 Metabolite Determination

For each growth condition tested, samples were taken at different times. Cells were separated by centrifugation and the supernatants were stored at -20℃for the subsequent analytical quantifications. Glucose consumption, ethanol, n-butanol and isobutanol productions were measured by high performance liquid chromatography (HPLC, Aminex HPX-87H Column, 300 x 7.8 mm, Bio-Rad). Glycine consumption was evaluated by ninhydrin-based assay [87].

Chapter 6

Conclusions

Industrial biotechnology, also known as white biotechnology, is the application of modern biotechnology to the sustainable production of chemicals, materials, and fuels from renewable sources, using living cells and/or their enzymes. In particular metabolic engineering has enabled the introduction of metabolic pathways into host strains that do not naturally produce a desired compound or provides the technology to improve the natural host performance. Thus beneficial traits of certain platform host organism such as easy cultivation, substrate utilization, easy genetic manipulation, stress resistance etc., can be combined with the synthesis of desired metabolites.
A lot of interest has been generated in this field mainly because industrial biotechnology is often associated with reduced energy consumption, greenhouse gas emissions, and waste generation, and also might enable the paradigm shift from fossil fuel-based to bio-based production of value-added chemicals.
The fundamental force that drives the development and implementation of industrial biotechnology is the market economy, as biotechnology promises highly efficient processes at lower operating and capital expenditures. In addition, political and societal demands for sustainability and environment-friendly industrial production systems, coupled with the depletion of crude oil reserves, and a growing world demand for raw materials and energy, will continue to drive this trend forward [1].
Considering the combination of features that is required for industrial conversion, the ideal microrganism that consolidates all of the desirable industrial-prosessing traits does not exist or better, has not been identified or created yet. Both *E. coli* and *S. cerevisiae* have a long track record of successful industrial applications, but the latter was in this work chosen mainly because its fermentation properties. During this project, two different strategies were considered to convince our microrganism to efficiently produce different kinds of value-added compounds (lactic acid and biofules such as ethanol and n-butanol): one based on the transfer of an heterologous pathway and the other based on the exploration of a non-obvious ability hidden in the yeast metabolism.
First of all we demonstrated the role of glucose transport on endogenous and

heterologous production, overexpressing the *HXT1* and *HXT7* genes in different *S. cerevisiae* backgrounds. This modification improved glucose consumption rate leading to a significant increase in the ethanol but also in the lactic acid productivity (g/L/h). This unexpected result was further investigated through a mathematical approach. The intracellular flux distributions were inferred from the exchange rates (growth rate, glucose uptake and ethanol secretion rates) experimentally determined. The statistical analysis of the differences in these intracellular flux distributions between engineered strain and its control suggested that the perturbation induced by this modification has widespread effects beyond just altering the glucose metabolism. Also the oxidative metabolism seemed to determine the engineered strain features.
Once explored this unexpected relationship between substrate uptake rate and productivity, we tried to convince yeast to produce n-butanol by transferring the *Clostridium* pathway and investigating the opportunities offered by the yeast aminoacidic metabolism. No n-butanol was detected in the yeast which expressed the bacterial heterologous pathway but different aminoacidic sustrates were identified as potential precursor of the desired alcohol. Thus it is not possible to predict *a priori* the functionality of the heterologous pathway, especially when a lot of enzymatic activities were involved. On the other hand, the innate power of natural organism should not be underestimated. For this reason, we further exploited this "power" by improving the substrate uptake rate and by re-directing the flux toward the desired product. The overexpression of the gene encoding for the a-specific permease *GAP1* increased the glycine uptake rate leading to a higher biofuels accumulation (n-butanol or isobutanol in a way that depends on the carbon source). Moreover the expression of an αketovalerate specific dehydrogenase activity re-directed the flux toward the production of n-butanol increasing its production.
In summary, despite *S. cerevisiae* is widely recognized with *E. coli* as one of the better known microrganism, our data suggested that a lot about its metabolism still remains unknown and unexpected. Its fine regulation and the presence of bottlenecks hide many interesting and desirable properties from an industrial point of view. Furthermore, the consuetude to use conventional synthetic media did not allow to identify all the potential (and real) production capabilities of the microrganism of interest.
Our data showed the need to improve the substrate transport into the cell, whether glucose or other precursors, allowing the microrganism to express its full potential, converting in a more efficient way this substrate in the desired product(s). Moreover the tightly connection of the yeast metabolism and the wide substrate specificity of many enzymatic activities represented a fundamental starting point to produce non-natural compounds characterized by a chemical structure similar to naturally secreted metabolites. In this sense, the analysis both *in vivo* and *in silico* of the secretion/release profile in different medium and the study of the pathways/enzymatic activities involved in the conversion of interest could represent an alternative to the transfer of the entire metabolic pathway in order to improve titer, yield and productivity of the process. Thus, it is expected that the better quantification of the properties of various microrganisms through bioprospective methods [88] but also

through mathematical models will be used more extensively in the design of the metabolic engineering strategies and in particular the current efforts should focus more on the discovery of new biological functions than on the discovery of optimal new microrganisms.

Appendix A

Additional File

Most 28 perturbed metabolites

A.

Glc 2%

metNames	mets	Zmets Scores	nRxns linked
H2O	h2o[c]	11.46	174
5-Amino-6-(5'-phosphoribitylamino)uracil	5aprbu[c]	5.91	2
2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5'-phosphate	25dthpp[c]	5.33	2
H+	h[r]	5.14	5
4-(1-D-Ribitylamino)-5-aminouracil	4r5au[c]	4.56	3
Dolichol phosphate	dolp[r]	4.52	2
Mannan	mannan[r]	4.38	2
Dolichol phosphate	dolp[c]	4.12	2
Mannan	mannan[c]	3.57	2
Phosphate	pi[c]	3.39	85
Ammonium	nh4[c]	3.25	23
Dolichyl phosphate D-mannose	dolmanp[r]	3.01	2
6,7-Dimethyl-8-(1-D-ribityl)lumazine	dmlz[c]	2.96	2
D-Mannose 6-phosphate	man6p[c]	2.73	2
Nicotinamide adenine dinucleotide phosphate	nadp[c]	2.72	59
2,5-Diamino-6-hydroxy-4-(5'-phosphoribosylamino)-pyrimidine	25dhpp[c]	2.60	2
Riboflavin	ribflv[c]	2.58	2
D-Mannose 1-phosphate	man1p[c]	2.53	2
Nicotinamide adenine dinucleotide phosphate - reduced	nadph[c]	2.46	57
Diphosphate	ppi[c]	2.40	36
Formate	for[c]	2.35	9
GTP	gtp[c]	2.31	10
O2	o2[c]	2.29	22
GDP-D-mannose	gdpmann[c]	2.22	2
3,4-dihydroxy-2-butanone 4-phosphate	db4p[c]	2.07	2
Ergosterol	ergst[r]	1.80	2
Ergosterol	ergst[c]	1.79	2

B.

Glc 5%

metNames	mets	Zmets Scores	nRxns linked
2-Oxoglutarate	akg[e]	2.02E+13	2
L-Isoleucine	ile-L[m]	1.55E+13	5
dCDP	dcdp[c]	8.39E+12	2
Flavin adenine dinucleotide oxidized	fad[m]	6.42E+12	3
Flavin adenine dinucleotide reduced	fadh2[m]	6.42E+12	1
D-Glucose	glc-D[e]	3.84E+01	3
dTTP	dttp[c]	2.50E+01	2
dTDP	dtdp[c]	2.49E+01	2
D-Glucose	glc-D[c]	1.95E+01	1
UTP	utp[c]	1.91E+01	2
dUTP	dutp[c]	1.90E+01	5
Oxidized thioredoxin	trdox[c]	1.42E+01	4
Reduced thioredoxin	trdrd[c]	1.42E+01	1
3-Phospho-D-glyceroyl phosphate	13dpg[c]	1.20E+01	2
Glyceraldehyde 3-phosphate	g3p[c]	1.11E+01	2
3-Phospho-D-glycerate	3pg[c]	1.10E+01	2
Dihydroxyacetone phosphate	dhap[c]	1.08E+01	4
D-Erythrose 4-phosphate	e4p[c]	9.81E+00	2
O2	o2[e]	9.80E+00	2
O2	o2[c]	9.62E+00	2
H2O	h2o[c]	9.54E+00	2
4-(1-D-Ribitylamino)-5-aminouracil	4r5au[c]	8.43085	2
3,4-dihydroxy-2-butanone 4-phosphate	db4p[c]	8.37534	2
6,7-Dimethyl-8-(1-D-ribityl)lumazine	dmlz[c]	8.37351	9
Stearoyl-CoA (n-C18:0CoA)	stcoa[c]	7.79842	3
Sedoheptulose 1,7-bisphosphate	s17bp[c]	7.70568	2
dTMP	dtmp[c]	7.45586	2

B.

NO Oxygen

Glc 2%

metNames	mets	Zmets Scores	nRxns linked
L-1-Pyrroline-3-hydroxy-5-carboxylate	1p3h5c[m]	4216270000000000.00	1.00E+00
L-Isoleucine	ile-L[m]	1022660000000000.00	1.00E+00
dCTP	dctp[c]	434242000000000.00	1.00E+00
D-Glucose	glc-D[e]	75.72	2
D-Glucose	glc-D[c]	36.82	8
O2	o2[e]	23.00	2
3-Phospho-D-glyceroyl phosphate	13dpg[c]	22.62	2
Glyceraldehyde 3-phosphate	g3p[c]	17.62	7
O2	o2[c]	16.67	24
D-Glycerate 2-phosphate	2pg[c]	14.69	2
3-Phospho-D-glycerate	3pg[c]	13.88	3
Ferricytochrome c	ficytc[m]	12.66	5
Ferrocytochrome c	focytc[m]	12.66	5
O2	o2[m]	11.99	2
Phosphoenolpyruvate	pep[c]	11.54	6
H2O	h2o[m]	11.15	25
Ubiquinone-6	q6[m]	9.47	4
Ubiquinol-6	q6h2[m]	9.47	4
Dihydroxyacetone phosphate	dhap[c]	6.41	9
L-4-Hydroxyglutamate semialdehyde	4hglusa[m]	6.33	2
Ergosta-5,7,22,24,(28)-tetraen-3beta-ol	ergtetrol[c]	6.26	4
Oxidized thioredoxin	trdox[c]	6.04	11
Reduced thioredoxin	trdrd[c]	6.04	11
dTMP	dtmp[c]	5.66	5
Nicotinamide adenine dinucleotide	nad[c]	5.46	33
dTTP	dttp[e]	5.35	2
Ergosterol	ergst[c]	5.05	3

C.

NO Oxygen

Glc 5%

metNames	mets	Zmets Scores	nRxns linked
Acetate	ac[e]	22710500000000.00	1
D-Glucose	glc-D[e]	18.07	2
Ethanol	etoh[e]	11.64	2
CO2	co2[e]	8.64	2
isoamyl acetate	iamac[e]	7.42	2
Ergosta-5,7,22,24,(28)-tetraen-3beta-ol	ergtetrol[c]	6.08	4
3-Phospho-D-glyceroyl phosphate	13dpg[c]	6.00	2
O2	o2[c]	5.84	24
3-Phospho-D-glycerate	3pg[c]	5.60	3
D-Glucose	glc-D[c]	5.30	8
ergosta-5,7,24(28)-trienol	ergtrol[c]	5.12	3
Oxidized thioredoxin	trdox[c]	4.93	11
Reduced thioredoxin	trdrd[c]	4.93	11
GTP	gtp[c]	4.90	10
Pyruvate	pyr[m]	4.69	8
Glyceraldehyde 3-phosphate	g3p[c]	4.69	7
D-Glycerate 2-phosphate	2pg[c]	4.64	2
Succinate	succ[e]	4.57	2
H+	h[r]	4.46	5
O2	o2[e]	4.00	2
Ergosterol	ergst[c]	3.97	3
Dihydroxyacetone phosphate	dhap[c]	3.81	9
isoamyl acetate	iamac[c]	3.74	3
Ergosta-5,7,22,24,(28)-tetraen-3beta-ol	ergtetrol[r]	3.70	2
Ergosterol	ergst[r]	3.36	2
CO2	co2[m]	3.35	15
L-Malate	mal-L[m]	3.33	3

MOST PERTURBED REACTIONS

Control strain VS HXT1 AEROBIC Conditions
GLUCOSE 2%

subSystems	rxns	nZreac	Control Flux	hxt1 flux
Riboflavin Metabolism	DRTPPD	243.063	0.000251737	0.00026237
Transport, Endoplasmi	DOLPt2er	164.189	-0.205432	-0.21411
Transport, Endoplasmi	MANNANter	157.512	-0.205432	-0.21411
Glycoprotein Metaboli	DOLPMMer	109.759	0.205432	0.21411
Riboflavin Metabolism	GTPCII	109.222	0.000251737	0.00026237
Riboflavin Metabolism	RBFSb	108.709	0.000251737	0.00026237
Fructose and Mannose	PMANM	105.365	-0.205432	-0.21411
Riboflavin Metabolism	PMDPHT	98.9486	0.000251737	0.00026237
Phospholipid Biosynth	MI1PS	97.3087	0.00134768	0.00140461
Glycoprotein Metaboli	DOLPMTcer	90.1924	0.205432	0.21411
Riboflavin Metabolism	RBFSa	88.9267	0.000503474	0.000524739
Fructose and Mannose	MAN6PI	81.2256	-0.205432	-0.21411
Fructose and Mannose	MAN1PT	70.9932	0.205432	0.21411
Sterol Metabolism	C24STRer	70.6307	0.000177996	0.000185514
Riboflavin Metabolism	DROPPRy	70.5699	0.000251737	0.00026237
Transport, Endoplasmi	ERGSTter	70.367	0.000177996	0.000185514
	biomass_SC5_	**70.1761**	**0.25428**	**0.26502**
Riboflavin Metabolism	DB4PS	65.1688	0.000503474	0.000524739
Fatty Acid Biosynthes	DESAT18_2	64.7275	0.00128157	0.0013357
Transport, Endoplasmi	ERGTETROLt	57.4207	0.000177996	0.000185514
Transport, Mitochondr	O2tm	33.3591	4.72274	4.96901
	EX_o2(e)	30.0373	-4.80219	-5.05503
Transport, Extracellula	O2t	28.5684	4.80219	5.05503
Oxidative Phosphoryla	CYOR_u6m	24.4375	9.42621	9.91921
Oxidative Phosphoryla	CYOOm	20.5981	4.72274	4.96901
Transport, Extracellu	**GLCt1**	**16.2402**	**7.16509**	**7.25759**
Transport, Mitochondr	CO2tm	15.5823	-13.5801	-14.0411
	EX_etoh(e)	**15.0843**	**6.98049**	**7.64217**
Fatty Acid Biosynthes	FAS180	14.7222	0.00261851	0.00193194
Transport, Extracellu	**ETOHt**	**14.4117**	**-6.98049**	**-7.64217**
	EX_glc(e)	**13.515**	**-7.16509**	**-7.25759**
Transport, Extracellu	**LEUt2r**	**12.3572**	**0.0808094**	**0.0852448**
	EX_leu-L(e)	**11.3388**	**-0.0808094**	**-0.0852448**
Transport, Extracellu	**CO2t**	**10.137**	**-12.301**	**-13.2103**
Oxidative Phosphoryla	ATPS3m	8.93862	12.4877	13.1212
Nucleotide Salvage Pa	RNDR2	7.63773	0.00103623	0.00023432
Transport, Mitochondr	ATPtm-H	7.02689	12.8551	13.596
Sterol Metabolism	CHLSTI	6.99954	0.000131702	0.000141486

Control strain VS HXT1 AEROBIC Conditions
GLUCOSE 5%

subSystems	rxns	nZreac	Control Flux	hxt1 flux
Transport, Extr	GLCt1	47.2664	11.5	11.6641
Nucleotide Sal	NDPK4	44.1747	0.0011059	0.000331639
Nucleotide Sal	RNTR4	40.5446	0.000514661	0.00029709
	EX_glc(e)	34.0511	-11.5	-11.6641
Glycolysis/Glu	PGK	18.7961	-20.4605	-20.7651
Riboflavin Me	RBFSa	16.5192	0.000588606	0.000589775
	EX_o2(e)	15.4588	-3.76814	-3.71244
Glycolysis/Glu	FBA3	11.4712	2.36789	4.8748
Transport, Extr	DTTPt	9.5114	-0.0011059	-0.00033164
Nucleotide Sal	DTMPK	9.19681	0.0011059	0.000331639
Pentose Phospl	TALA	8.89509	-1.96612	-4.45201
Glycolysis/Glu	PGM	8.59609	-20.1924	-20.5132
Folate Metabol	DHFRi	8.20383	0.00322714	0.00221807
Glycolysis/Glu	GAPD	8.10688	20.4605	20.7651
	EX_ura(e)	8.09212	-0.0350356	-0.0340898
Oxidative Phos	CYOOm	8.02331	3.65852	3.59922
Glycolysis/Glu	PFK	7.71343	7.85217	5.48419
Purine and Pyr	TMDS	7.37475	0.00322714	0.00221807
Transport, Extr	URAt2	7.04435	0.0350356	0.0340898
Glycolysis/Glu	ENO	6.99939	20.1924	20.5132
Transport, Extr	O2t	6.88847	3.76814	3.71244
Glycolysis/Glu	FBA	6.75697	7.78414	5.41899
Complex Alcol	ALCD25xim	6.60032	0.0102027	0.0112803
Glycolysis/Glu	PFK_3	6.56657	2.36789	4.8748
Tyrosine, Tryp	TYRTAim	6.42384	0.030177	0.0267844
Glycerolipid M	G3PD1irm	6.2023	1.37919	2.85175
	EX_dttp(e)	6.1949	0.0011059	0.000331639
Transport, Mit	O2tm	6.17016	3.65852	3.59922
Tyrosine, Tryp	PPND2	6.14219	0.0269567	0.0363286
Sterol Metabol	C22STDSx	5.84233	2.51E-05	7.40E-05
Oxidative Phos	CYOR_u6m	5.81939	7.29448	7.17513
Nucleotide Sal	RNDR1	5.51064	0.00078184	0.000894439
Sterol Metabol	CHLSTI	5.3478	0.000178401	0.000129386
Fatty Acid Bio	ACACT1	5.05224	0.0160435	0.0113401
Fatty Acid Bio	FAS180COA	4.81045	0.00253293	0.00181999
Sterol Metabol	C5STDS	4.70073	2.97E-05	7.91E-05
Threonine and	AASAD2	4.63063	0.067819	0.0614589
Threonine and	AASAD1	4.55413	0.0350434	0.0395924

Nucleotide Salvage Pa	PUNP4	6.51684	0.000754508	0.000645364	Nucleotide Sal	DADK	4.4612	-0.00139147	-0.00150625
Transport, Extracellula	FUMt2r	6.26721	-0.120459	-0.0504367	Transport, End	ERGSTter	4.33185	0.000208093	0.000208506
	EX_co2(e)	**6.16298**	**12.301**	**13.2103**	Phospholipid E	AGAT_SC	4.32341	0.0289693	0.0296064
Nucleotide Salvage Pa	ADA	5.70578	0.0175276	0.025734	Fructose and M	MAN1PT	4.28203	0.240169	0.240646
Nucleotide Salvage Pa	NTD8	5.60207	0.000754508	0.000645364	Nucleotide Sal	NTD6	4.17719	0.000321283	0.000433929
Fatty Acid Biosynthes	FAS161	5.39784	0.00149313	0.00117116	Methionine Me	AHSERL2	4.13712	0.0229579	0.0172177
Transport, Extracellula	IAMOHt	5.28459	0.00418857	0.00535149	Fatty Acid Bio	FAS181	4.04532	0.00122834	0.00158705
	EX_h(e)	5.05672	3.52665	2.87053		EX_akg(e)	4.0205	0.0396489	0.0598937
Glycolysis/Gluconeog	GLUK	4.81684	1.62663	3.69132	Glycolysis/Glu	TPI	3.99052	10.0532	10.197
Pyruvate Metabolism	PYRDC	4.79869	7.74022	8.18351	Transport, Extr	CYSt2r	3.89732	-0.0132973	-0.00943523
Glycolysis/Gluconeog	TPI	4.74526	5.97993	6.03065	Glycoprotein M	DOLPMMer	3.79671	0.240169	0.240646
Valine, Leucine, and Is	LEUTA	4.72773	0.00544096	0.00669291	Valine, Leucine	ILETAm	3.78932	-159.511	-117.617
Cysteine Metabolism	CYSS	4.49973	0.00717141	0.0112191	Transport, End	MANNANter	3.77898	-0.240169	-0.240646
Fatty Acid Biosynthes	FAS181	4.46119	0.000730825	0.00165305	Purine and Pyr	CTPS2	3.77865	0.0525059	0.0473633
Valine, Leucine, and Is	DHAD1m	4.40347	0.380181	0.292551	Transport, End	ERGTETROL1	3.77485	0.000208093	0.000208506
	EX_iamoh(e)	4.33428	0.00418857	0.00535149		EX_3mbald(e)	3.74375	0.245136	0.105963
Sterol Metabolism	C22STDSx	4.33057	2.20E-05	1.47E-05		EX_h(e)	3.52726	9.45464	10.1298
Anaplerotic reactions	ME1m	4.32302	9.79526	10.209	Fatty Acid Bio	DESAT18_2	3.52529	0.00149827	0.00150125
Glycolysis/Gluconeog	GAPD	4.30161	12.2605	12.3666	Other Amino A	GTHP	3.50529	0.00970983	0.0168235
	EX_pyr(e)	4.03808	0.11654	0.053611	Purine and Pyr	PDE1	3.4756	0.0167536	0.031769
Purine and Pyrimidine	TMDPP	3.85003	0.000658339	0.000802972	Fatty Acid Bio	DESAT18	3.4705	0.00426532	0.00391752
Fatty Acid Biosynthes	FAS160COA	3.80279	0.00596513	0.00384152	**NAD Biosynth**	**NADK**	**3.4565**	**0.0619218**	**0.0728753**
Complex Alcohol Met	4MOPDC	3.78852	0.00544096	0.00669291	Nucleotide Sal	RNDR3	3.44257	0.000666666	0.00020932
Fatty Acid Biosynthes	DESAT18	3.78738	0.00396826	0.00324451	Purine and Pyr	CYTD	3.44223	0.0613947	0.062835
Glycolysis/Gluconeog	HEX1	3.72375	5.6857	3.72236	Phospholipid E	MI1PS	3.37619	0.00157556	0.00157869
	EX_epist(e)	3.70291	0.00165848	0.00204339	Riboflavin Me	DRTPPD	3.36936	0.000294303	0.000294888
Other Amino Acid Me	GTHO	3.69207	0.00669236	0.00852464	Glycerolipid M	G3PDm	3.34608	3.64641	5.53312
Glycolysis/Gluconeog	PGK	3.61498	-12.2605	-12.3666	Glycoprotein M	DOLPMTcer	3.33273	0.240169	0.240646
Sterol Metabolism	C5STDS	3.58448	4.63E-05	4.40E-05	Tyrosine, Tryp	PPND	3.30635	0.0308598	0.0221451
Purine and Pyrimidine	DURIPP	3.54819	-0.0017141	-0.00177441	Riboflavin Me	DROPPRy	3.27902	2.94E-04	2.95E-04
Sterol Metabolism	C4STMO1	3.52611	0.00377289	0.00428459	Oxidative Phos	NADH2-u6cm	3.26897	3.91814	1.89078
Transport, Mitochondr	ETOHtm	3.50888	-201.96	-131.652	Fructose and M	MAN6PI	3.25321	-0.240169	-0.240646
Sterol Metabolism	C4STMO2	3.50757	0.00377289	0.00428459	NAD Biosynth	NADPPPS	3.2005	0.0619218	0.0728753
Fatty Acid Biosynthes	DESAT16	3.4761	0.000927614	0.00135183	Sterol Metabol	HMGCOAS	3.15457	-0.0160435	-0.0113401
Valine, Leucine, and Is	ILETAm	3.47131	-88.5949	-218.918	Methionine Me	ADMDC	3.14648	0.0468247	0.0469875
Glycolysis/Gluconeog	PGM	3.4417	-12.0932	-12.1859	Riboflavin Me	GTPCII	3.13966	0.000294303	0.000294888
Glycerolipid Metaboli	G3PD1irm	3.3938	1.68463	2.50355	Transport, Extr	IAMOHt	3.10427	0.607999	0.777746
	EX_ura(e)	3.3393	-0.0303621	-0.0312997	Transport, Extr	3MBALDt	3.04949	0.245136	0.105963
x	PDHm	3.31239	1.64642	1.79545		biomass_SC5	3.03255	0.297276	0.297866
Valine, Leucine, and Is	ACLSm	3.28181	0.380181	0.292551	Complex Alcol	ALCD24yi	3.0306	0.0622118	0.254895
Transport, Mitochondr	CITtam	3.12724	-1.26293	-1.36383	Nucleotide Sal	NTD8	2.9464	0.000865264	0.000600738
Transport, Mitochondr	DHAPtm	3.12106	2.77975	2.05099	Purine and Pyr	ADNCYC	2.93951	0.0167536	0.031769
Valine, Leucine, and Is	KARA1im	3.11802	0.380181	0.292551	Fructose and M	PMANM	2.92712	-0.240169	-0.240646

Control strain VS HXT1 ANAEROBIC Conditions GLUCOSE 2%					Control strain VS HXT1 ANAEROBIC Conditions GLUCOSE 5%				
subSystems	**rxns**	**nZreac**	**Control Flux**	**hxt1 flux**	**subSystems**	**rxns**	**nZreac**	**Control Flux**	**hxt1 flux**
Transport, Extr	GLCt1	110.28	12.533	11.7452	Transport, Extr	GLCt1	296.19	34.6666	36.1215
	EX_glc(e)	64.2989	-12.533	-11.7452		EX_glc(e)	296.19	-34.6666	-36.1215
Glycolysis/Glu	GAPD	33.8846	22.6258	21.1481		EX_etoh(e)	168.874	36.4218	43.181
Transport, Extr	O2t	27.7598	1.38596	1.54393	Transport, Extr	ETOHt	168.874	-36.4218	-43.181
	EX_o2(e)	27.0926	-1.38596	-1.54393	Pyruvate Metal	PYRDC	109.108	39.2749	46.4164
Oxidative Phos	CYOOm	21.1818	1.26756	1.4335		EX_o2(e)	105.892	-1.20746	-1.33658
Glycolysis/Glu	PGK	20.0962	-22.6258	-21.1481	Transport, Extr	O2t	105.892	1.20746	1.33658
Glycolysis/Glu	ENO	19.7278	22.3435	20.8576	Transport, Extr	IAMACt	104.4	0.000781039	0.000289113
Glycolysis/Glu	PGM	16.2567	-22.3435	-20.8576		EX_iamac(e)	104.4	0.000781039	0.000289113
Glycolysis/Glu	TPI	15.6508	11.0215	10.3103	Glycolysis/Glu	PGK	103.949	-64.2585	-67.6091
Arginine and P	PHCHGSm	13.9286	-340.927	-206.539	Glycolysis/Glu	GAPD	103.949	64.2585	67.6091
Anaplerotic rea	PPCK	12.9811	-21.6764	-20.2805	Transport, Extr	CO2t	101.914	-36.2309	-45.4164
Oxidative Phos	CYOR_u6m	12.7877	2.46321	2.81021		EX_co2(e)	101.914	36.2309	45.4164
Transport, Mit	O2tm	8.66527	1.26756	1.4335	Glycolysis/Glu	PGM	100.09	-63.3911	-66.8761
	EX_dttp(e)	7.79127	0.000298178	0.00112781	Glycolysis/Glu	ENO	100.09	63.3911	66.8761
Nucleotide Sal	PUNP4	7.24418	0.000241856	0.00110487	Transport, Extr	ERGSTt	94.3924	6.23E-05	6.41E-05
Oxidative Phos	NADH2-u6m	7.22504	2.29442	0.85311		EX_ergst(e)	94.3924	-6.23E-05	-6.41E-05
Transport, Extr	DTTPt	6.96849	-0.00029818	-0.00112781	Transport, End	ERGSTter	92.9895	8.68E-05	8.34E-05
Nucleotide Sal	DTMPK	6.93081	0.000298178	0.00112781	Sterol Metabol	C24STRer	92.9895	8.68E-05	8.34E-05
Transport, End	ERGSTter	6.60365	9.84E-05	0.000100742	Transport, End	ERGTETROLt	92.9895	8.68E-05	8.34E-05
Oxidative Phos	NADH2-u6cm	6.55728	1.03009	2.05025	Sterol Metabol	C22STDS	90.2782	8.45E-06	1.47E-06
Purine and Pyr	TMDS	5.98426	0.00224422	0.00263944	Sterol Metabol	C5STDS	85.5644	2.68E-05	1.10E-05
Valine, Leucin	ILETAm	5.98247	-252.359	-123.247	Glycolysis/Glu	TPI	73.8915	31.3006	33.2605
Fructose and M	PMANM	5.98172	-0.187879	-0.189508	Sterol Metabol	C22STDSx	69.3727	1.83E-05	9.50E-06
	EX_etoh(e)	5.82706	13.7171	13.2491		EX_h(e)	68.0195	35.1037	28.0339
Riboflavin Met	GTPCII	5.57028	0.000230227	0.000232223	Nucleotide Sal	RNTR2	65.2894	0.000411638	0.000272862
Nucleotide Sal	NDPK4	5.48999	0.000298178	0.00112781	Transport, Extr	SUCCt2r	64.2098	-12.0658	-8.20605
Nucleotide Sal	NTD8	5.35334	0.000241856	0.00110487		EX_succ(e)	64.2098	12.0658	8.20605
Transport, End	ERGTETROLt	5.29124	9.84E-05	0.000100742	Anaplerotic rea	ME1m	63.295	40.7713	48.0759
Transport, Extr	ERGSTt	4.94722	6.44E-05	6.35E-05	Sterol Metabol	CHLSTI	59.2308	6.00E-05	7.24E-05
Glycoprotein M	DOLPMMer	4.90651	0.187879	0.189508	Transport, Mit	CO2tm	58.2938	-49.0154	-56.0073
	EX_h(e)	4.7658	10.5893	8.97661		EX_leu-L(e)	54.7958	-0.0678567	-0.067385
Sterol Metabol	C22STDS	4.76526	3.86E-07	2.27E-06	Transport, Extr	LEUt2r	54.7958	0.0678567	0.067385
Glycoprotein M	DOLPMTcer	4.68183	0.187879	0.189508	Oxidative Phos	ATPS3m	53.547	10.2937	12.6431
Transport, End	MANNANter	4.67493	-0.187879	-0.189508	Transport, Extr	DTTPt	51.9323	-0.0014664	-0.00065701
Riboflavin Met	RBFSa	4.58599	0.000460454	0.000464445	Nucleotide Sal	NDPK4	51.9323	0.0014664	0.000657005
Nucleotide Sal	RNTR3	4.5184	0.000338624	0.00010496		EX_dttp(e)	51.9323	0.0014664	0.000657005
Fatty Acid Bio	DESAT18_2	4.43016	0.00117207	0.00118222	Nucleotide Sal	DTMPK	51.9323	0.0014664	0.000657005

Transport, Mit	CO2tm	4.38305	-18.5959	-17.9912	Phospholipid E	MI1PS	48.9111	0.00112894	0.00111658
Phospholipid E	MI1PS	4.35076	0.00123253	0.00124321	Fatty Acid Bio	DESAT18_2	48.9111	0.00107356	0.00106181
Sterol Metabol	C24STRer	4.26464	9.84E-05	0.000100742	Riboflavin Met	GTPCII	48.9111	0.000210878	0.00020857
Sterol Metabol	C22STDSx	4.25574	1.07E-05	9.21E-06	Riboflavin Met	DB4PS	48.9111	0.000421757	0.000417139
	EX_nh4(e)	4.23474	-2.28261	-2.1341	Transport, End	DOLPt2er	48.9111	-0.172089	-0.170205
Riboflavin Met	DROPPRy	4.23354	0.000230227	0.000232223	Glycoprotein M	DOLPMMer	48.9111	0.172089	0.170205
Sterol Metabol	CHLSTI	4.16284	8.73E-05	8.93E-05		biomass_SC5_	48.9111	0.213008	0.210676
Riboflavin Met	DB4PS	4.1021	0.000460454	0.000464445	Transport, End	MANNANter	48.9111	-0.172089	-0.170205
Fructose and M	MAN6PI	4.00132	-0.187879	-0.189508	Glycoprotein M	DOLPMTcer	48.9111	0.172089	0.170205
Transport, End	DOLPt2er	3.93018	-0.187879	-0.189508	Fructose and M	MAN1PT	48.9111	0.172089	0.170205
Transport, Extr	ETOHt	3.85306	-13.7171	-13.2491	Fructose and M	PMANM	48.9111	-0.172089	-0.170205
Riboflavin Met	RBFSb	3.84426	0.000230227	0.000232223	Fructose and M	MAN6PI	48.9111	-0.172089	-0.170205
Purine and Pyr	DURIPP	3.81085	-0.00161105	-0.00211402	Riboflavin Met	DRTPPD	48.9111	0.000210878	0.00020857
	EX_ergst(e)	3.71925	-6.44E-05	-6.35E-05	Riboflavin Met	DROPPRy	48.9111	0.000210878	0.00020857
Fatty Acid Bio	FAS180COA	3.68472	0.00156738	0.00245433	Riboflavin Met	RBFSb	48.9111	0.000210878	0.00020857
Transport, Extr	H2Ot	3.68021	-9.36015	-8.813	Riboflavin Met	RBFSa	48.9111	0.000421757	0.000417139
Riboflavin Met	DRTPPD	3.54942	0.000230227	0.000232223	Riboflavin Met	PMDPHT	48.9111	0.000210878	0.00020857
Nucleotide Sal	RNDR1	3.50996	0.000643069	0.000700086	Pyruvate Metal	D-LACDm	48.6553	0.144754	0.0868819
Tyrosine, Tryp	CHORS	3.5003	0.17564	0.158669		EX_ura(e)	45.3888	-0.0252854	-0.0245389
Complex Alcol	ALCD25yi	3.49605	0.0112878	0.00357091	Transport, Extr	URAt2	45.3888	0.0252854	0.0245389
	EX_h2o(e)	3.48503	9.33731	8.7904	Nucleotide Sal	RNDR2	43.9748	0.00032475	0.000638742
	biomass_SC5_	3.43794	0.232553	0.234568	Oxidative Phos	NADH2-u6m	43.7905	3.97781	1.55932
Nucleotide Sal	RNDR2	3.41988	0.000261133	0.000821583	Anaplerotic rea	PPCK	43.5102	-60.2871	-63.968
Sterol Metabol	LNS14DM	3.38167	0.00250582	0.00272452		EX_thym(e)	40.0688	0.000260239	0.000581132
Fructose and M	MAN1PT	3.34517	0.187879	0.189508	Transport, Extr	THYMt3r	40.0688	0.000260239	0.000581132
Transport, Mit	ACtm	3.32922	-0.0468901	-0.102045	Purine and Pyr	TMDPP	40.0688	0.000260239	0.000581132
Transport, Extr	SUCCt2r	3.30637	-3.41082	-2.7554	Alternate Carb	D-LACDcm	40.0075	0.120208	0.0765287
	EX_succ(e)	3.30053	3.41082	2.7554	Oxidative Phos	CYOR_u6m	33.8027	1.34112	1.49301
	EX_pyr(e)	3.27861	0.124351	0.064995	Tyrosine, Tryp	DIAT	33.4016	0.0171647	0.0739125
Riboflavin Met	PMDPHT	3.2512	0.000230227	0.000232223	Pantothenate a	POLYAO2	33.4016	0.0171647	0.0739125
Fatty Acid Bio	FAS180	3.24828	0.00210579	0.00103822	Nucleotide Sal	RNDR1	32.1927	0.000544482	0.00021436
	EX_ttdca(e)	3.20E+00	0.00507679	0.00459956	Fatty Acid Bio	DESAT18	31.6679	0.00307995	0.0022186
Transport, Mit	ATPtm-H	3.06E+00	5.8702	6.27563	Alternate Carb	GLYOX	30.807	0.411422	0.318739
Arginine and P	4HGLSDm	3.06E+00	-340.927	-206.539	Alternate Carb	LGTHL	30.807	0.411422	0.318739
	EX_ura(e)	3.02E+00	-2.71E-02	-0.0277382	Fatty Acid Bio	FAS180	30.5844	0.00174026	0.00127441
Fatty Acid Bio	FAS161	3.01E+00	4.90E-04	0.00121482	Fatty Acid Bio	FAS181	30.5326	0.000856448	0.0016747
Fatty Acid Bio	DESAT16	2.91E+00	1.72E-03	0.00101827	Other	THIORDXi	28.7944	0.0632757	0.0190712
Glycine and Se	HSDyi	2.88E+00	1.65E-01	0.0957771	Fatty Acid Bio	FAS161	28.3275	0.00112326	0.000523153
Transport, Extr	URAt2	2.83E+00	2.71E-02	0.0277382	Other Amino A	GTHP	27.59	0.0152178	0.0427566
Pyruvate Metal	PYRDC	2.77E+00	1.48E+01	14.1798	Other Amino A	GTHO	27.59	0.0152178	0.0427566
	EX_iamac(e)	2.74881	0.000816287	0.000437845	Nucleotide Sal	RNTR1	27.5587	0.000517809	0.000728466
Nucleotide Sal	RNTR2	2.74214	0.000538848	0.000846254	Methionine Me	SHSL4r	27.2695	-314.513	-177.27
Transport, Extr	2MBALDt	2.67858	0.0265364	0.0220788	Alternate Carb	MGSA	27.1298	0.572081	0.539887

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MICROBIAL CELL FACTORIES

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Effect of *HXT*1 and *HXT7* hexose transporter overexpression on wild-type and lactic acid producing *Saccharomyces cerevisiae* cells

Giorgia Rossi[1], Michael Sauer[2,3], Danilo Porro[1*], Paola Branduardi[1]

Abstract

Background: Since about three decades, *Saccharomyces cerevisiae* can be engineered to efficiently produce proteins and metabolites. Even recognizing that in baker's yeast one determining step for the glucose consumption rate is the sugar uptake, this fact has never been conceived to improve the metabolite(s) productivity.
In this work we compared the ethanol and/or the lactic acid production from wild type and metabolically engineered*S. cerevisiae* cells expressing an additional copy of one hexose transporter.

Results: Different *S. cerevisiae* strains (wild type and metabolically engineered for lactic acid production) were transformed with the *HXT*1 or the *HXT7* gene encoding for hexose transporters.
Data obtained suggest that the overexpression of an Hxt transporter may lead to an increase in glucose uptake that could result in an increased ethanol and/or lactic acid productivities. As a consequence of the increased productivity and of the reduced process timing, a higher production was measured.

Conclusion: Metabolic pathway manipulation for improving the properties and the productivity of microorganisms is a well established concept. A high production relies on a multi-factorial system. We showed that by modulating the first step of the pathway leading to lactic acid accumulation an improvement of about 15% in lactic acid production can be obtained in a yeast strain already developed for industrial application.

Background

Natural *Saccharomyces cerevisiae* cells have long been utilized as very efficient biocatalysts, thanks to their native enzymatic capabilities. Ethanol, single cell proteins, flavours and fragrances are among the most traditional examples.

Since about three decades budding yeast can also be engineered and has been used to efficiently produce simple as well as complex molecules. Prominent examples are proteins with pharmaceutical applications, industrial enzymes, organic acids, new bio-fuels, biopolymers, vitamins and steroids, in a single fermentation step [1-8].

Glucose, either derived from starch and/or cellulosic materials, is the main carbon and energy source today available.

An economically sustainable bioprocess leading to the production of a homologous or heterologous low molecular compound requires a high yield (grams of product obtained by gram of substrate), high production titer (g/L) and high productivity (g/L/h) values.

It has been shown that high yields and high production titres can be obtained by recombinant redirection of the carbon flow towards the desired compound. In this respect, prominent examples are the production of lactic-, pyruvic- and malic- acid, glycerol and resveratrol [9-13]. Theoretically, high productivities could be obtained by increasing the carbon consumption rates itself (*i.e.*, essentially the glycolytic flux rate). It should be also underlined that an increased productivity, and therefore a reduction of the process duration, is not only implying a reduction in terms of costs. Cell factory viability (and therefore production) has also to be taken into account: very often the production process involves a stressful environment, leading to cell death during

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fermentation, as in the case of ethanol production, gradually reducing cell viability and thereby biocatalyst concentration [14].

Glucose transport, hexose phosphorylation, phosphofructokinase and pyruvate kinase activities have all been proposed to play central roles in the control of glycolysis flux rates [15-20].

Individual or simultaneous overproduction of glycolytic enzymes resulted either in no increases in glycolytic flux or in only incremental increases [21-25]. Furthermore, attempts to correlate glycolytic flux with enzyme levels under different physiological conditions have generally failed [26-28]. This is likely because the control of glucose catabolism is distributed over several different metabolic controls; in this context, glucose transport has been suggested to be one of the most important players [29,30].

Glucose transport in *S. cerevisiae* relies on a multi-factorial uptake system. More in details, the uptake of glucose by *S. cerevisiae* is controlled by multiple hexose transporters (Hxts) [31]. At least 20 *HXT* genes encoding these transporters have been identified [31,32]. Many and different studies were done to determine the respective biochemical features of these transporters (affinity and capacity), as well as to construct strains deleted in one or more *HXT* genes and to construct chimera proteins combining affinity and capacity of different transporters [33-35]. Remarkably, Otterstedt *et al.* [33] showed that a simple manipulation of the glucose uptake can strongly alter the mode of metabolic control.

Essentially, the various hexose transporters differ considerably in substrate specificity and affinity. Hxt1 and Hxt3 are low-affinity transporters (Km for glucose, ~50 to 100 mM), Hxt4 is a moderately low-affinity transporter, and Hxt2, Hxt6, and Hxt7 are high-affinity transporters (Km for glucose, ~1 to 4 mM) [36,37]. Hxt5 has been shown to be a transporter with intermediate to high affinity [38,39]. Both high- and low-affinity carriers have been shown to have a higher affinity for glucose than for fructose [37]. Analyses of the effect of *HXT* gene inactivation have shown that the hexose carriers Hxt1 to Hxt7 are the main transporters [37]. In this respect, it has been already shown that the ethanol (and CO_2) productivity and yield (grams of ethanol produced per gram of glucose consumed) can be improved by overexpression of *HXT*1 transporter in *S. cerevisiae* [40-43].

S. cerevisiae has long been studied for the production of organic acids like lactic, ascorbic, pyruvic and malic [[4,8,11] and [44]]. Indeed, yeast can grow and survive at low pH values, avoiding the accumulation of the respective salts [4,11].

Here we show improved lactic acid productivities, induced by an increase of the glucose consumption rate. Hxt1 and Hxt7 have been selected for this study. In spite of their different biochemical properties, the overexpression of *HXT*1 or *HXT*7 genes does lead to very similar results in the tested conditions. Moreover, we demonstrate that the increase of the glucose consumption rate has a positive effect not only in respect to microbial productivity and metabolite production, but also on biomass accumulation. Said phenomenon is more or less evident in respect to the yeast background.

Results

Effect of *HXT1* and *HXT7* overexpression in naturally ethanol producing yeasts

First the effect of the overexpression of the two different hexose transporters in two different yeast genetic backgrounds was studied. The strains GRF18U (the model yeast strain used in our laboratory) [45] and CEN.PK (a generally accepted reference yeast strain) [46] were both transformed with the integrative plasmids p022HXT1 or p022HXT7, respectively carrying the *HXT*1 [47] and *HXT*7 [31] genes under the control of the glycolytic *Sc*TPI promoter (Table 1: for each transformation, at least three independent transformants were analysed in three independent experiments). *HXT*1 and *HXT*7 encode for the two hexose transporters having the lowest and the highest affinity for glucose, respectively.

Initially, a functional analysis of the cloned *HXT*1 and *HXT*7 genes was performed by examining the effect of their expression in a *hxt1-7* deleted strain, RE700 [48]. While the control strain grows very poorly on glucose minimal medium, the transformed ones partially resumed the natural ability (data not shown). Then the natural abilities of the control and of the *HXT*1 or *HXT*7 transformed yeasts to produce ethanol were compared. Figure 1 and Figure 2 show the shake-flask batch growth kinetics in defined YNB-2% glucose medium of transformed GRF18U (Figure 1, see also Table 2) and CEN.PK (Figure 2, see also Table 2) yeast strains. Panel (A) shows the growth, measured as optical density (OD_{660}), of the wild type strain (open circles), of the *HXT*1 (closed circles) and *HXT*7 (open squares) overexpressing strains. Panels (B) and panels (C) show the glucose consumption and the ethanol production, respectively. The results clearly show that the presence of an additional copy of a hexose transporter leads to a faster glucose consumption rate and a faster ethanol production rate (panels B and C). It can be observed that similar glucose consumption rates have been observed from the different transformed strains, despite of the different biochemical features of the Hxt1 and Hxt7 transporters. A very low glucose concentration should have been tested to evidence the effect of the overexpression of the *HXT*7 transporter (which has the highest affinity for glucose): however, at that

Table 1 Transformed strains used in this study

Strains	Genotype	Plasmid*
GRF18U	*MATa, ura3, his3, leu2*	**p022** (*Sc*TPI; -; *HIS3*)
GRF18U [*HXT1*]	*MATa, ura3, his3, leu2*	**p022HXT1** (*Sc*TPI; *HXT1*; *HIS3*)
GRF18U [*HXT7*]	*MATa, ura3, his3, leu2*	**p022HXT7** (*Sc*TPI; *HXT7*; *HIS3*)
CEN.PK	*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2*	**p022** (*Sc*TPI; -; *HIS3*)
CEN.PK [*HXT1*]	*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2*	**p022HXT1** (*Sc*TPI; *HXT1*; *HIS3*)
CEN.PK [*HXT7*]	*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2*	**p022HXT7** (*Sc*TPI; *HXT7*; *HIS3*)
CEN.PK [LDH]	*MATa, ura3-52, his3-11, TRP1, MAL2-8c, SUC2*	**p022** (*Sc*TPI; -; *HIS3*) **p212LDH** (*Sc*TPI; LDH; *LEU2*)
CEN.PK [*HXT1*] [LDH]	*MATa, ura3-52, his3-11, TRP1, MAL2-8c, SUC2*	**p022HXT1** (*Sc*TPI; *HXT1*; *HIS3*) **p212LDH** (*Sc*TPI; LDH; *LEU2*)
CEN.PK [*HXT7*] [LDH]	*MATa, ura3-52, his3-11, TRP1, MAL2-8c, SUC2*	**p022HXT7** (*Sc*TPI; *HXT7*; *HIS3*) **p212LDH** (*Sc*TPI; LDH; *LEU2*)
CEN.PKm850 [LDH][50]	*MATa, pdc1::loxP, pdc5::loxP, pdc6::loxP, ura3-52*	**YEpLpLDH**[50] (*Sc*TPI; LDH; *URA3*) **p022KMX4** (*Sc*TPI; -; $G418^R$)
CEN.PKm850 [*HXT1*] [LDH]	*MATa pdc1::loxP pdc5::loxP pdc6::loxP ura3-52*	**YEpLpLDH**[50] (*Sc*TPI; LDH; *URA3*) **p022KMX4HXT1** (*Sc*TPI; *HXT1*; $G418^R$)
CEN.PKm850 [*HXT7*] [LDH]	*MATa pdc1::loxP pdc5::loxP pdc6::loxP ura3-52*	**YEpLpLDH**[50] (*Sc*TPI; LDH; *URA3*) **p022KMX4HXT7** (*Sc*TPI; *HXT7*; $G418^R$)

* Plasmid name. In brackets are reported the promoter, the harboured gene and the selection marker, respectively.

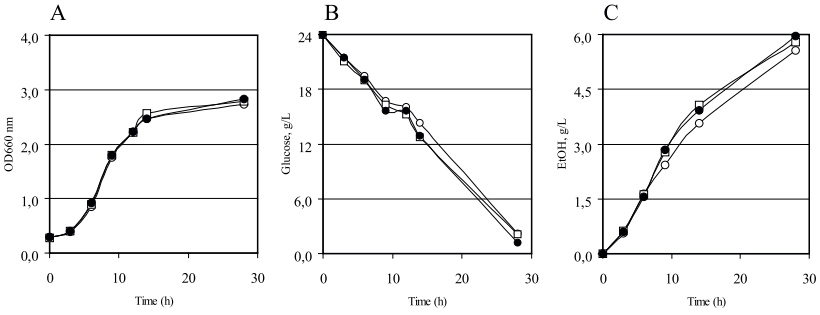


Figure 1 Overexpression of *HXT*1 or *HXT7* genes in the *S. cerevisiae* GRF18U strain. Strains were flask-batch grown in minimal (YNB) medium, with glucose as a carbon source. (A) Growth was measured as optical density (OD 660 nm). (B) Residual glucose, g/L. (C) Ethanol produced, g/L. Data correspond to the mean values of three independent clones independently tested at least three times. Standard error is lower than 0.03%. Open circles: GRF18U-Control. Closed circles: GRF18U [*HXT1*] transformants. Open squares: GRF18U [*HXT7*] transformants.

concentration the fermentation product formation would have also been very low. The two genes were initially tested because we could not *a priori* predict their positive or, eventually, negative effect.

Indeed, similar effects were also obtained when glucose concentration was increased to 5% (Table 2, only data obtained in the CEN.PK background overexpressing the *HXT*1 gene are shown). Biomass production is also increased, particularly in the CEN.PK yeast background. We do not have any explanation for that.

It is also important to underline that during balanced exponential growth, the specific growth rates of the control and the transformed strains are equal (Table 2). A very similar correlation (*i.e.*, increased glucose consumption *vs* higher metabolite and higher biomass production) has been previously observed in our laboratory [49]. All the data are summarised in Table 2: the glucose consumption rate (at least in the first phases of the process) and the ethanol productivity and production were improved by overexpressing *HXT*1 or *HXT*7 in the two different yeast genetic backgrounds tested. It needs to be underlined that when cells were grown in medium containing 5% glucose, also the nitrogen content was consequently incremented (see Methods), determining

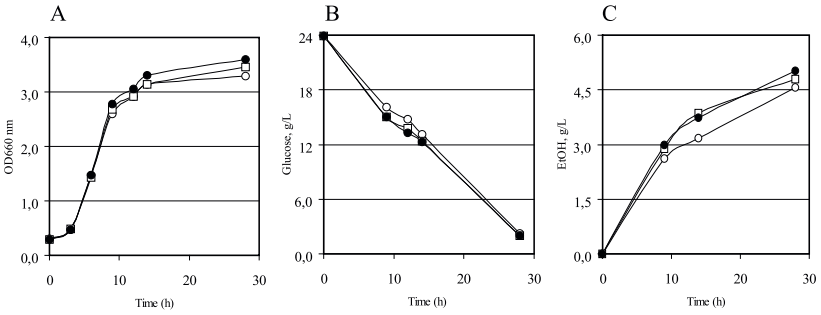


Figure 2 Overexpression of *HXT*1 or *HXT7* genes in the *S. cerevisiae* CEN.PK strain. Strains were flask-batch grown in minimal (YNB) medium, with glucose as a carbon source. (A) Growth was measured as optical density (OD 660 nm). (B) Residual glucose, g/L. (C) Ethanol produced, g/L. Data correspond to the mean values of three independent clones independently tested at least three times. Standard error is lower than 0.03%. Open circles: CEN.PK-Control. Closed circles: CEN.PK [*HXT*1] transformants. Open squares: CEN.PK [*HXT7*] transformants.

the development of a higher biomass. This justifies the higher glucose consumption and ethanol production rates measured in these growth conditions (Table 2).

Finally, when transformants were grown in the above described media but under micro-anaerobic conditions, similar results have been obtained in respect to growth rate and to substrate consumption and ethanol production (Table 2). Remarkably, in this case the improvement in the ethanol yield of the transformants was higher as the glucose consumption rate (Table 2).

Effect of *HXT1* overexpression in yeast engineered for lactic acid production

The CEN.PK *S. cerevisiae* strain harbouring the integrative p022HXT1 expression vector was further transformed with the multicopy plasmid p212LDH bearing an engineered *Lactobacillus plantarum* LDH gene [49] under the control of the glycolytic *Sc*TPI promoter (Table 1). We can briefly comment that very similar results were obtained in GRF18 background and overexpressing the *HXT*7 gene in both yeasts (data not shown).

Independent transformants were shake flask cultured in YNB-minimal medium. Figure 3 shows the behaviours of the LDH (open circles) and of the *HXT*1-LDH (closed circles) overexpressing strains. Panels (A) and (B) report the cell density (as OD 660 nm) and the glucose consumption, while panels (C) and (D) the ethanol and the lactate accumulation during growth on defined YNB-2% glucose based medium. It is important to underline that while the transformed CEN.PK strains shown in Figure 2 require uracil and leucine for growth, the CEN.PK transformed strains shown in Figure 3 require only leucine. It is reasonable to assume that because of that a slightly different amount on biomass accumulation was observed (compare Figure 2 and Figure 3).

It was observed also in these transformants that an additional copy of *HXT*1 lead to an increase in glucose consumption (Figure 3, panel B). Remarkably, both the ethanol and the lactic acid productivities and titres are improved (Figure 3, panels C and D).

Effect of *HXT1* overexpression in homolactic yeasts

Considering the improved product titres and productivities obtained, we tested the lactic acid production in the engineered host strain CEN.PK m850 [LDH] (Table 1), *ad hoc* constructed for being a low-pH homolactic producing yeast [50]. Said strain does not produce ethanol because it is totally devoid of pyruvate decarboxylase (Pdc) activity, it bears the *L. plantarum* LDH on a multicopy yeast expression plasmid and finally it has been selected, with an inverse metabolic engineering approach, for its acid tolerance. Figure 4 shows the behavior of the CEN.PK m850 [LDH] strain and of the same strain expressing an additional copy of the *HXT*1 transporter cultivated with 90 g/L of glucose. Independent transformants were tested for glucose consumption and lactic acid production. Under these growth/production conditions both strains did not consume all the glucose present in the medium. The control strain produced approximately 60 g/L of lactic acid at very low pH (after 70 h from the inoculum the external pH value was lower than 3.0). In the strain expressing an additional *HXT*1 copy no improvement in biomass production was observed, while the measured glucose consumption was faster; as a consequence, it could

Table 2 Effect of the overexpression of *HXT*1 or *HXT7* genes in the *S. cerevisiae* GRF18U or CEN.PK strains

Strains		Glc %	YNB %	aa mg/L	Agitation	μ_exp phase [1/h]	Rate Glc Consumption* [g/L/h]	Rate EtOH Production* [g/L/h]	Rate LA Production* [g/L/h]	Y_EtOH** [g EtOH/g Glc]	Y_LA** [g L.A./g Glc]
CEN.PK		2.39	1.34	50	YES	0.28 +/- 0.05	-0.77 +/- 0.08	0.23 +/- 0.09	-	0.19 +/- 0.11	-
CEN.PK	[*HXT1*]	2.39	1.34	50	YES	0.29 +/- 0.07	-0.83 +/- 0.08	0.27 +/- 0.04	-	0.21 +/- 0.05	-
CEN.PK	[*HXT7*]	2.39	1.34	50	YES	0.29 +/- 0.02	-0.82 +/- 0.08	0.28 +/- 0.03	-	0.20 +/- 0.03	-
CEN.PK		5.68	2.68	150	YES	0.33 +/- 0.05	-1.66 +/- 0.06	0.42 +/- 0.01	-	0.19 +/- 0.00	-
CEN.PK	[*HXT1*]	5.68	2.68	150	YES	0.33 +/- 0.03	-1.74 +/- 0.09	0.44 +/- 0.01	-	0.20 +/- 0.01	-
GRF18U		2.39	1.34	50	YES	0.25 +/- 0.05	-0.69 +/- 0.04	0.27 +/- 0.05	-	0.23 +/- 0.01	-
GRF18U	[*HXT1*]	2.39	1.34	50	YES	0.25 +/- 0.03	-0.79 +/- 0.08	0.32 +/- 0.06	-	0.25 +/- 0.08	-
GRF18U	[*HXT7*]	2.39	1.34	50	YES	0.25 +/- 0.04	-0.80 +/- 0.07	0.31 +/- 0.06	-	0.24 +/- 0.06	-
CEN.PK	[LDH]	2.04	1.34	50	YES	0.12 +/- 0.04	-0.38 +/- 0.07	0.09 +/- 0.08	0.08 +/- 0.09	0.17 +/- 0.01	0.21 +/- 0.01
CEN.PK	[*HXT1*] [LDH]	2.04	1.34	50	YES	0.14 +/- 0.08	-0.40 +/- 0.05	0.10 +/- 0.04	0.09 +/- 0.06	0.19 +/- 0.09	0.24 +/- 0.01
CEN.PK m850	[LDH]	9.14	1.70	-	YES	0.02 +/- 0.00	-1.21 +/- 0.00	-	0.77 +/- 0.00	-	0.67 +/- 0.00
CEN.PK m850	[*HXT1*] [LDH]	8.96	1.70	-	YES	0.03 +/- 0.09	-1.22 +/- 0.08	-	0.85 +/- 0.02	-	0.76 +/- 0.02
CEN.PK		2.13	1.34	150	NO	0.26 +/- 0.03	-1.22 +/- 0.03	0.41 +/- 0.08	-	0.40 +/- 0.03	-
CEN.PK	[*HXT1*]	2.13	1.34	150	NO	0.26 +/- 0.04	-1.30 +/- 0.02	0.45 +/- 0.02	-	0.45 +/- 0.08	-
CEN.PK		5.68	2.68	150	NO	0.23 +/- 0.01	-4.30 +/- 0.06	1.37 +/- 0.05	-	0.31 +/- 0.08	-
CEN.PK	[*HXT1*]	5.68	2.68	150	NO	0.23 +/- 0.04	-4.36 +/- 0.07	1.58 +/- 0.06	-	0.36 +/- 0.09	-

Table summarizes all the data obtained with wild type and engineered yeasts (see first column) grown in the indicated conditions (see columns 2-5). The specific growth, glucose consumption, ethanol and lactate production rates together with yield for ethanol and lactic acid production are given. For the described determinations, the respective standard error is indicated. Glc: glucose. aa: aminoacids. LA: lactic acid. * determined in exponential phase. ** determined at the timing of the highest metabolite (ethanol or lactic acid) production.

produce a considerable additional amount of lactic acid (about 15% more), or the same amount in a shorter period of time (about 1,4 times faster) (see also Table 2). In line with the previously described kinetics, similar results have been obtained overexpressing the *HXT*7 gene in the CEN.PK m850 background (data not shown).

This last experiment shows the successful application of what was previously shown in laboratory strains (Figure 3) also in a strain already developed and optimised for industrial productions.

In Table 2 the data related to the lactic acid producing m850 strain are also summarised and included.

Discussion

The hexose transporter gene family in *S. cerevisiae* contains the sugar transporter genes *HXT1* to *HXT17, GAL2* and the glucose sensor genes *SNF3* and *RGT2* [31-33]. *HXT*1 and *HXT*3 genes have already been overexpressed in yeasts. More in detail, the effect of the overexpression of *HXT1* gene has been tested in a *S. cerevisiae* strain [42] during growth on complex-rich based media. A significant increase on the ethanol productivity (g/L/h) was observed. Also the ethanol yield, expressed as gram of ethanol produced per gram of substrate consumed, showed a significant (3%) improvement. This is in line with our findings. However, Gutiérrez-Lomelì *et al.*[42] observed no significant effects on final ethanol concentration. On the other hand, while Gutiérrez-Lomelì *et al.*[42] examined strains producing 40-45 g/L of ethanol, we used physiological conditions leading to the accumulation of 4-6 g/L. Therefore, it could be speculated that a saturation limit could be reached when the strains are grown in the presence of a huge amount of glucose.

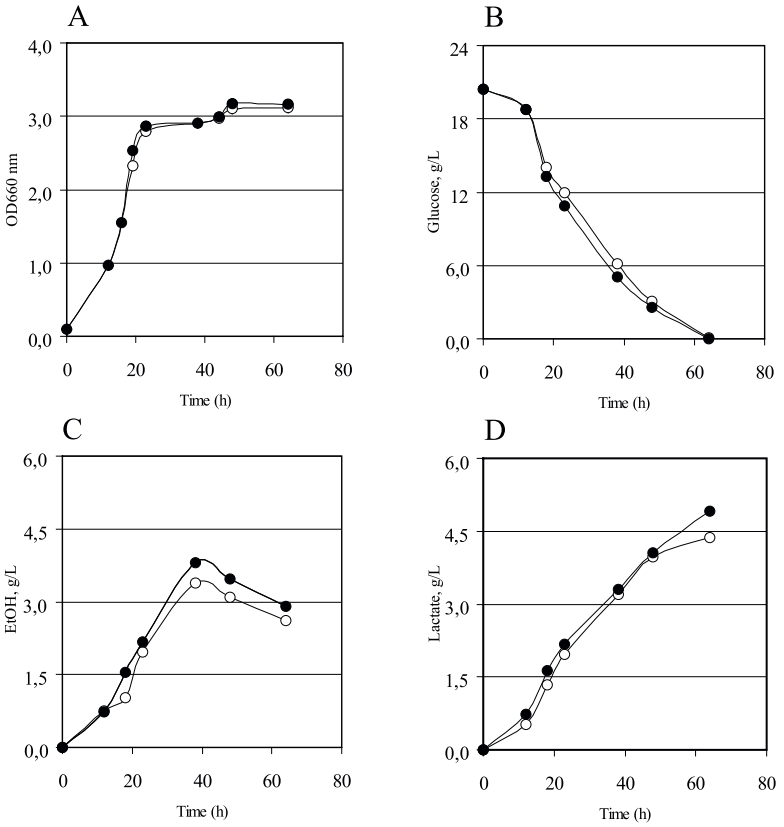


Figure 3 CEN.PK *S. cerevisiae* strain overexpressing the *HXT*1 gene or the *HXT*1 and the *Lp*LDH gene. Strains were flask-batch grown in minimal (YNB) medium, with glucose as a carbon source. (A) Growth was measured as optical density (OD 660 nm). (B) Residual glucose, g/L. (C) Ethanol produced, g/L. (D) Lactate produced, g/L. The data correspond to the mean values of three independent clones independently tested at least three times. Standard error is lower than 0.03%. Open circles: CEN.PK [LDH]-Control. Closed circles: CEN.PK [*HXT*1] [LDH] transformants.

A minor difference is that Gutiérrez-Lomelì *et al.*[42] did not observe any improvement in the biomass production. Once more, it should be underlined that the transformed strains have been grown under very different conditions (rich-complex or defined-minimal media, respectively).

Guillaume *et al.*[51] have demonstrated that the pattern of fructose utilization during wine fermentation can be altered in yeasts harbouring a mutated *HXT3* allele. More in details the authors found that the glycolytic flux could be increased by the overexpression of a mutated version of the transporter gene. Data demonstrate that the Hxt3 hexose transporter plays a key role in determining the glucose/fructose utilization ratio during wine fermentation. All these findings are in line with the data shown in this paper as well as with the

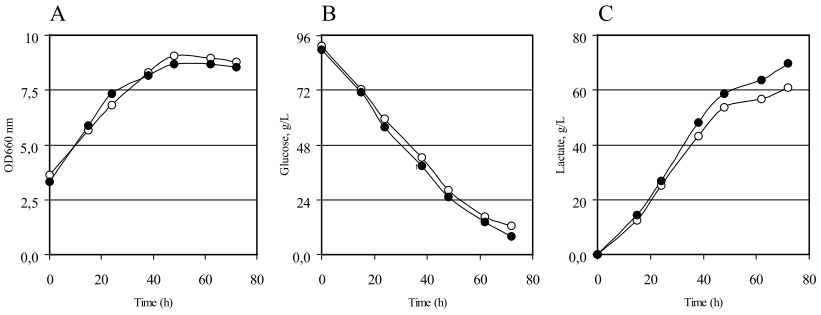


Figure 4 Lactic acid production in the CEN.PK m850 [LDH] strain overexpressing the *HXT1* gene. (A) Growth was measured as optical density (OD 660 nm). (B) Residual glucose, g/L. (C) Lactic acid produced, g/L. The data correspond to the mean values of three independent clones independently tested at least three times. Standard error is lower than 0.03%. Open circles: CEN.PK m850 [LDH]-Control. Closed circles: CEN.PK m850 [*HXT1*] [LDH] transformants.

ones reported by Elbing *et al.*[15]. Following a very elegant approach, the authors built a series of strains having different rates of ethanol production, linearly correlating with the maximal specific glucose consumption rates attained during exponential growth on glucose. However, the same authors concluded that the hexose transporter has no or very low control over glycolytic flux in the wild type cells growing in the presence of high glucose concentrations.

Concluding, even if the metabolically engineered *S. cerevisiae* yeast strains are among the most prominent recombinant hosts usable for the industrial production of lactic acid [4,8,12,44], the overexpression of a hexose transporter has never been conceived to improve the productivity of this organic acid. In the recent past, aimed to improve the lactic acid production by metabolically engineered yeasts, we showed that the redirection of the pathway towards the lactate production can be strongly modulated by the genetic background of the host cell, by the source of the heterologous LDH enzyme, by improving its biochemical properties as well as by modulating (even if to very low extent) the export of lactate in the culture media [49]. In this article, we modulate the lactic acid productivity by improving the efficiency of the first step of the pathway - the glucose uptake - leading to the accumulation of lactic acid from glucose.

Finally, it should be underlined that a variety of organic acids draw more and more attention as new building block materials for the chemical industry [4]. If produced by environmentally benign fermentation strategies, they can provide a sound alternative to petroleum derived, and therefore limited, building block materials.

It can be anticipated that the production of these organic acids could be similarly improved by the overexpression of additional copies of one or more hexose transporters [43].

Methods

Yeast strains, transformation, media and cultivation

The *S. cerevisiae* strains used in this study derive from the following strains: GRF18U (*MATα, ura3; leu2-3,112; his3-11,15*; cir+) [45], CEN.PK strains 102-5B (*MATa, ura3-52, his3-11, leu2-3/112, TRP1, MAL2-8c, SUC2* - Dr. P. Kötter, Institute of Microbiology, Johann Wolfgang Goethe-University, Frankfurt, Germany) [46], and CEN.PK m850 [50,52]. Yeast transformations were performed basically according to the LiAc/PEG/ss-DNA protocol [53]. The control strain is, for each background, the corresponding yeast strain transformed with the empty plasmid(s).

Independent transformants and the respective control strains (at least three for each transformation) were cultivated in shake flasks in minimal synthetic medium (1.34% or 2,68% [w/v] YNB medium [catalogue no. 919-15 Difco Laboratories, Detroit, Mich.] with 2% or 5% [w/v] glucose and 50 mg/L or 150 mg/L of appropriate amino acid(s), respectively).

All strains were grown in shake flasks at 30°C. For aerobic growth, flasks were agitated at 160 r.p.m. and the ratio of flask volume/medium was of 5/1. For microaerobic condition, flasks were sealed and the ratio of flask volume/medium was of 10/6.

Independent transformants derived from the strain CEN.PK m850 [LDH] were cultivated as previously described [52]. Briefly, growth kinetics were performed

at 28°C in 250-mL quadruple baffled shake flasks in minimal medium containing 4.5 g/L $CaCO_3$, 1.7 g/L YNB without amino acids and without $(NH_4)_2SO_4$, 1 g/L urea, 5 g/L ethanol, and with glucose 9% (w/v) as a carbon source. Cell growth was monitored by measuring the optical density at 660 nm at regular time intervals.

Gene amplification and plasmids construction

The *S. cerevisiae HXT*1 [47] and *HXT*7 [31] genes were PCR amplified using as a template the genomic DNA extracted from GRF18U strain by standard methods [54]. Pwo DNA polymerase (Roche catalogue no. 11 644 955 001) was used on a GeneAmp PCR System 9700 (PE Applied Biosystem, Inc.). Standard conditions used were 0.2 mM primers, 1.5 U of Pwo and 3 μL of genomic DNA. The program used for amplification of genes was as follows: after 5 min at 94°C, 30 cycles (each cycle consisting of 15 s at 94°C, 30 s at 57.5°C and 1 min 30 s at 72°C) were carried out, followed by 7 min at 72°C. Oligonucleotides pairs for *HXT*1 were as follows: HXT1_fw (5'-AAA ATC ATG AAT TCA ACT CCC GAT CTA-3') and HXT1_rev (5'-AGC TTG TTT AGT TTA TTT CCT GCTG AAA-3'). Because of the high sequence homology between the coding sequence of the *S. cerevisiae HXT*6 and *HXT*7 genes [47], the latter was amplified in two steps. In the first step the oligos named 5'HXT7 (5'-A AAA ATG TCA CAA GAC GCT GCT ATT GCA-3') and 3'HXT7exit (5'-ATA TAT TAA AAA CGT ATT TAC TTT TCA AGT-3') were used, the second designed on an external region in respect to the gene that resulted different from the corresponding region of the *HXT*6 gene. The single amplified band was secondarily used as a template for the two oligos 5'HXT7 and 3'HXT7 (5'-AGT GTC GAC AAA TAA TTT GGT GCT GAA CAT-3'), obtaining the sole open reading frame of the desired gene.

The amplified fragments were sub-cloned into the *Escherichia coli* vector pSTBlue obtaining, respectively, the plasmids pSTBlueHXT1 and pSTBlueHXT7. The inserts were sequenced and resulted identical to the deposited *S. cerevisiae* corresponding sequences (*HXT*1, GeneID: 856494 and *HXT*7, GeneID: 851943). These coding sequences were used for the construction of the integrative expression plasmids p022HXT1 and p022HXT7, respectively, utilizing the basic *S. cerevisiae* integrative expression plasmid pYX022 (R&D Systems, Inc., Wiesbaden, D). For the construction of the plasmid p022HXT1, the recipient vector was *Eco*RI cut, blunted and dephosphorylated, while the insert was *Mlu*I blunt/*Pml*I excised from the pSTBlueHXT1 plasmid. For the construction of the plasmid p022HXT7, the recipient and the pSTBlueHXT7 vectors were *Eco*RI cut. For the construction of the plasmid named p212LDH, the coding sequence of *L. plantarum* LDH was *Eco*RI excised from previously described pSTplLDH [49] and sub-cloned into the *S. cerevisiae* expression vector pYX212 (multicopy, *URA*3 auxotrophic marker R&D Systems, Inc., Wiesbaden, D) *Eco*RI opened and dephosphorylated. For the construction of the integrative plasmid p022KMX4, harboring an auxotrophic marker used only as a target gene and a dominant marker used for the selection of the transformants, the backbone of the plasmid pYX022 was used. pYX022 was *Kpn*I cut, blunt-ended and dephosphorylated and ligated with the Kan^R cassette derived from *Sph*I/*Sac*I blunt ending, from the plasmid pFA6KanMX4 [55]. p022KMX4 was *Eco*RI cut and dephosphorylated or *Eco*RI cut, blunt-ended and dephosphorylated and ligated with the *HXT*1 or *HXT*7 sequences cut as described above, resulting in the plasmids p022KMX4HXT1 or p022KMX4HXT7, respectively. A complete list of the transformed strains is given in Table 1.

DNA manipulation, transformation and cultivation of *E. coli* (Novablue, Novagen) were performed following standard protocols [54]. All the restriction and modification enzymes utilised are from NEB (New England Biolabs, UK) or from Roche Diagnostics.

Metabolite determination

Residual glucose and ethanol/lactic acid produced were determined with enzymatic kits from Megazyme, the glucose assay kit (K-GLUHKR), the Ethanol kit (K-ETOH) and L-lactic acid kit (K-LATE), respectively, according to the manufacturer's instructions.

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Authors' contributions

GR participated in the design of the study and planned and carried out the experimental work including plasmid and strain construction, yeast cultivation and data analysis. MS participated in the design of the study and in data interpretation. DP and PB designed the whole study, performed data interpretation and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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THE END