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PROCESS AND METABOLIC ENGINEERING FOR THE PRODUCTION OF VITAMIN B_9 IN YEASTS AS EXAMPLE OF INDUSTRIAL SYMBIOSIS AND CIRCULAR ECONOMY

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Ho sempre il tuo Bel belè nei pensieri, spero di renderti ancor più fiera di me.

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SUMMARY

Lignocellulose is the major structural component of woody and non-woody plants, representing a major potential source of renewable organic matter. Lignocellulose is primarily composed by two carbohydrate polymers, cellulose and hemicellulose and by lignin (an aromatic polymer). These complex polymers on the one hand often constitute a residual biomass of agro-food production chain, but at the same time they contain different sugar monomers and phenolic precursors, harbouring an enormous biotechnological value, since they can potentially be converted into different value-added products.

In a scenario where the world population is increasing together with the generation of waste and pollution at the expenses of planet resources and human wellbeing, this project aims at proposing an example of circular bioeconomy and industrial symbiosis. More in detail, the project starts from the quali-quantitative evaluation of residual agricultural biomasses to the valorization of a subset of interest for our territory into folates, exploiting yeasts as microbial cell factory.

Folate (Vitamin B_9) is a water-soluble B vitamin with important roles in nucleic acid synthesis, repair and methylation, produced only by green plants and some microorganisms: for these reasons it represents an essential nutritional component for humans. Vitamin B_9 commercially available is chemically synthetized as folic acid, suboptimal in terms of bioactivity for humans; the production of natural folates by microbial fermentation is therefore becoming a sustainable and desirable alternative for human supplementation. During the project the ENEA methodology for the analysis of resource flows and for the creation of possible synergies between the various companies present in the Lombardy region was acquired and applied. Thanks to this work it was possible to identify the main waste biomasses produced in the area in the agro-food sector and unfermented grape marcs was then selected

for further studies in laboratory, and compared with previously utilised residual biomasses, deriving from sugar process of production.

The non-conventional yeast *Scheffersomyces stipitis* was exploited as natural but never investigated host for the production of vitamin B_9. The growth was optimized and folate production was assessed first in shake flasks and then in bioreactor in formulated media mimicking lignocellulose hydrolysates. The maximum folate production was 3.7 ± 0.07 mg/L, which to date is the highest reported when considering wild type microorganisms.

Moreover, folate production was evaluated in shake flasks starting from three different residual biomasses: sugar beet molasses (SBM), sugar beet pulp (SBP) and unfermented grape marcs (UGM). *S. stipitis* was able to metabolize these biomasses, reaching folate titers of 188.2 ± 24.86 µg/L, 130.6 ± 1.34 µg/L and 101.9 ± 6.62 µg/L respectively.

In parallel, the yeast *Saccharomyces cerevisiae*, amenable for genetic manipulation, was engineered into the anabolic pathway of folate production to acquire novel knowledge on possible targets for unlocking bottlenecks of production. Eight different genes were manipulated for the first time in the same genetic background and exploiting different engineering strategies. This was pivotal for testing the best strain in bioreactor and in bringing folate production and productivity up to 620.0 ± 12.30 µg/L and 41.33 μg_{fol}/Lh respectively, among the highest in the literature.

Overall, these results provide solid evidence of possible up-cycling microbial-based processes of lignocellulosic biomasses that characterize specific territory. The value in terms of circularity of the resources, minimization of management costs of wastes and generation of values in the logic of industrial symbiosis was demonstrated, matching the initial scope of the PhD project.

The work lays the foundation for techno-economic analysis and life cycle assessment that could be used at industrial level for considering the industrial scaling up of these results.

SOMMARIO

La lignocellulosa è il principale componente strutturale delle piante legnose e non, e rappresenta una delle principali fonti potenziali di materia organica rinnovabile. La lignocellulosa è composta principalmente da due polimeri di carboidrati, cellulosa ed emicellulosa e dalla lignina (un polimero aromatico). Questi polimeri complessi da un lato costituiscono spesso una biomassa residua della filiera agroalimentare, ma allo stesso tempo contengono differenti monomeri zuccherini e precursori fenolici, aventi un enorme valore biotecnologico, poiché potenzialmente possono essere convertiti in differenti prodotti ad elevato valore aggiunto.

In uno scenario in cui la popolazione mondiale è in aumento insieme alla generazione di rifiuti ed inquinamento a scapito delle risorse del pianeta e del benessere umano, questo progetto mira a proporre un esempio di bioeconomia circolare e simbiosi industriale. Più in dettaglio, il progetto parte dalla valutazione quali-quantitativa delle biomasse agricole residue fino alla valorizzazione di un sottoinsieme di interesse per il nostro territorio in folati, sfruttando i lieviti come *cell factory* microbiche.

Il folato (Vitamina B_9) è una vitamina B idrosolubile con ruoli importanti nella sintesi, riparazione e metilazione degli acidi nucleici, prodotta solo dalle piante verdi e da alcuni microrganismi: per questi motivi rappresenta una componente nutritiva essenziale per l'uomo. La vitamina B_9 disponibile in commercio è sintetizzata chimicamente come acido folico, non ottimale in termini di bioattività

per l'uomo; la produzione di folati naturali mediante fermentazione microbica sta quindi diventando un'alternativa sostenibile e desiderabile per l'integrazione umana. Nel corso del progetto è stata acquisita ed applicata la metodologia ENEA per l'analisi dei flussi di risorse e per la creazione di possibili sinergie tra le varie aziende presenti nella regione Lombardia. Grazie a questo lavoro è stato possibile identificare le principali biomasse di scarto prodotte nell'area nel settore agroalimentare e le vinacce non fermentate sono state quindi selezionate per ulteriori studi in laboratorio, e confrontate con biomasse residue precedentemente utilizzate, derivanti dal processo di produzione dello zucchero.

Il lievito non convenzionale *Scheffersomyces stipitis* è stato sfruttato come ospite naturale per la produzione di vitamina B_9, per la prima volta in questo lavoro. La crescita è stata ottimizzata e la produzione di folati è stata valutata prima in beuta e successivamente in bioreattore in terreni formulati che imitano gli idrolizzati di lignocellulosa. La produzione massima di folati è stata di 3,7 ± 0,07 mg/L, che ad oggi è la più alta riportata se si considerano i microrganismi di tipo selvatico.

Inoltre, è stata valutata la produzione di folati in beuta a partire da tre diverse biomasse residue: melassa di barbabietola da zucchero (SBM), polpa di barbabietola da zucchero (SBP) e vinacce non fermentate (UGM). *S. stipitis* è stato in grado di metabolizzare queste biomasse, raggiungendo titoli di folati rispettivamente di 188,2 ± 24,86 µg/L, 130,6 ± 1,34 µg/L e 101,9 ± 6,62 µg/L.

Parallelamente, il lievito *Saccharomyces cerevisiae*, suscettibile di manipolazione genetica, è stato ingegnerizzato nel percorso anabolico della produzione di folati per acquisire nuove conoscenze sui possibili bersagli per sbloccare i precursori che ne limitano la produzione. Otto geni diversi sono stati manipolati per la prima volta nello stesso background genetico e sfruttando diverse strategie ingegneristiche. Questo è stato fondamentale per testare il miglior ceppo nel bioreattore e per

portare la produzione e la produttività di folati rispettivamente a 620,0 ± 12,30 µg/L e 41,33 µgfol/Lh, tra i più alti in letteratura.

Nel complesso, questi risultati forniscono una solida evidenza di possibili processi di upcycling a base microbica di biomasse lignocellulosiche che caratterizzano un territorio specifico. È stato dimostrato il valore in termini di circolarità delle risorse, minimizzazione dei costi di gestione dei rifiuti e generazione di valori nella logica della simbiosi industriale, rispondente allo scopo iniziale del progetto di dottorato. Il lavoro getta le basi per l'analisi tecnico-economica e la valutazione del ciclo di vita che potrebbero essere utilizzate a livello industriale per considerare l'espansione industriale di questi risultati.

INTRODUCTION

From the European Green Deal to Biorefineries

The decisive decade has already begun.

19 out of 20 of the warmest years recorded occurred after 2000. Average global temperatures have risen considerably since the pre-industrial revolution and peaked between 2010 and 2019, the warmest decade recorded up to now. The current global average temperature is between 0.94 and 1.03 °C higher than at the end of the nineteenth century and scientists believe that a two-degree Celsius increase from pre-industrial levels could have dangerous consequences on both the climate and the environment.

Most of the scientific evidence available shows that this anomaly is due to the increase in greenhouse gas (GHG) emissions produced by human activities.

The European Union is third in the world for greenhouse gas emissions after China and the United States, followed by India, Russia and Japan.

According to the data for 2019, the energy sector was responsible for 77.1% of total emissions, followed by the agricultural sector (10.55%), industry (9.10%) and the waste sector (3.32%) (Figure 1).

(https://www.europarl.europa.eu/news/it/headlines/society/20180703STO07129/le-soluzioni-dell-ue-per-contrastare-i-cambiamenti-climatici).

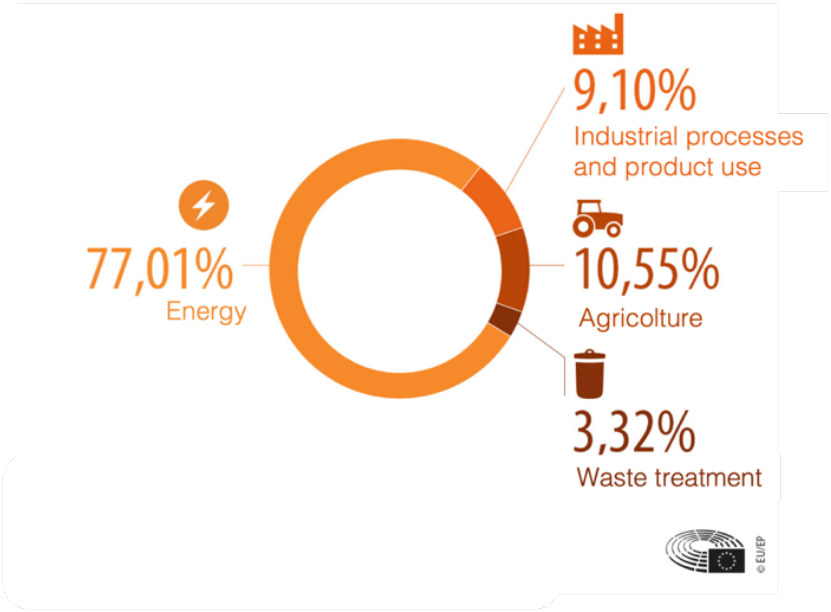

Figure 1: Greenhouse gas emissions in the EU in 2019 by sector. Adapted from: European Environment Agency (EEA)

Moreover, it is also important to consider the Earth Overshoot Day (EOV), calculated as the moment of the year when humans have already utilized the total amount of resources produced by the planet in one year. Earth Overshoot Day is computed by dividing the planet's biocapacity (the amount of ecological resources Earth is able to generate that year), by humanity's Ecological Footprint (humanity's demand for that year), and multiplying by 365, the number of days in a year. If the date is closer to the beginning of the year, it means that the resources of the following year will already be consumed, thus impoverishing the earth from resources for future generations. As you can see from the graph in Figure 2 this is already happening, as if in '71 the EOV was in December, this year (2022) we have already passed it (Day 2020).

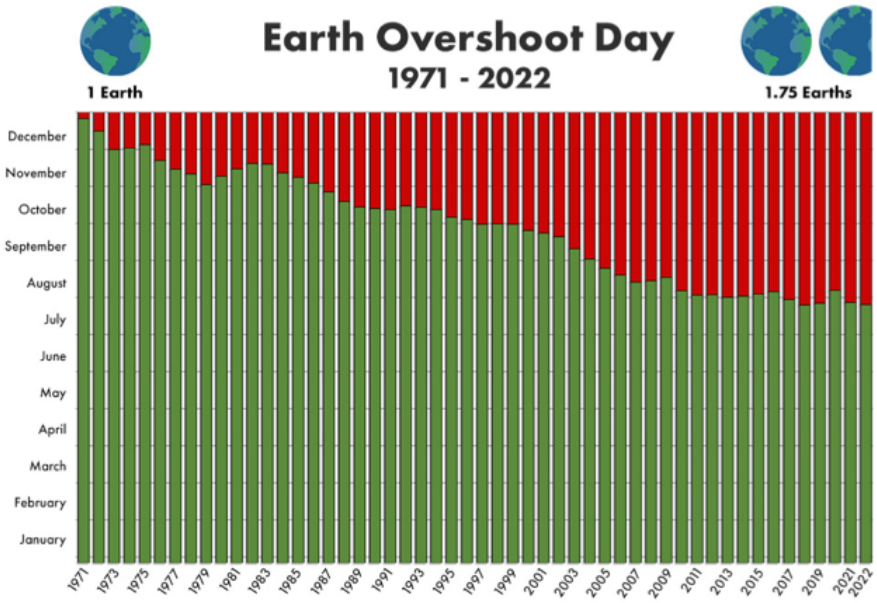

Figure 2: Past Earth Overshoot Day. From: Day, E.O., Earth overshoot day.

Nowadays, the world is living in a crucial period to answer the emergencies that have been threatening climate and biodiversity. This decade is, therefore, decisive to fulfill the commitments made under the Paris Agreement, in the interests of the health, well-being and prosperity of all.

In 2021, the EU made climate neutrality legally binding, by setting a net zero emissions target by 2050. This net zero emissions target is preserved in the climate law.

Moreover, the European Green Deal is the roadmap for the EU to become climate neutral by 2050. The EU has set an example by setting ambitious targets to reduce net emissions by at least 55% by 2030 compared to 1990 levels, these targets are embodied in the “Fit for 55” package presented by the Commission in July 2021.

The European Green Deal lays the foundations for the economy of tomorrow with reference strategies on biodiversity, circular economy, zero pollution, sustainable

and smart mobility, renovations, sustainable food, hydrogen, batteries, offshore renewable energy and much more.

Hence, a systemic transformation of the economy will be required to achieve the 2030 target. Complementary targeted policies at EU and national level will accelerate behavioral change, technological innovation and related applications. (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52021DC0550)

One of these policies is certainly the concept of circular economy that represents a radical paradigm shift, within which to develop new sustainable business models, able to increase the potential closure of production cycles and the efficient use of local resources (Korhonen, Honkasalo et al. 2018). According to a recent report from the World Bank, 3.4 billion tons of waste will be generated annually within the next 30 years and industrialized countries – that represent 16 percent of the world's population – are responsible for generating a third of the global waste (Ceccotti, Bruno et al. 2022).

Therefore, the concept of circular economy It is not just a question of eliminating or minimising the production of waste, but of radically changing the conflicting vision between economic and environmental interests, traditionally considered in antithesis, with a new and broader concept of well-being, which includes both.

In this doctoral thesis I would like to make clear how the concepts of bioeconomy and biorefinery can be pivotal for the objectives discussed above.

Bioeconomy is already a worldwide business that involves the exploitation of different types of biomass: agriculture, feed, pulp and paper, food and beverage, fisheries, and forestry are the most involved sectors within bioeconomy boundaries (Bracco, Calicioglu et al. 2018). Bioeconomy is a key protagonist in fostering environment-driven measures across countries, and, as announced by the European Commission, it "needs to have sustainability and circularity at its heart"(Strategy 2018).

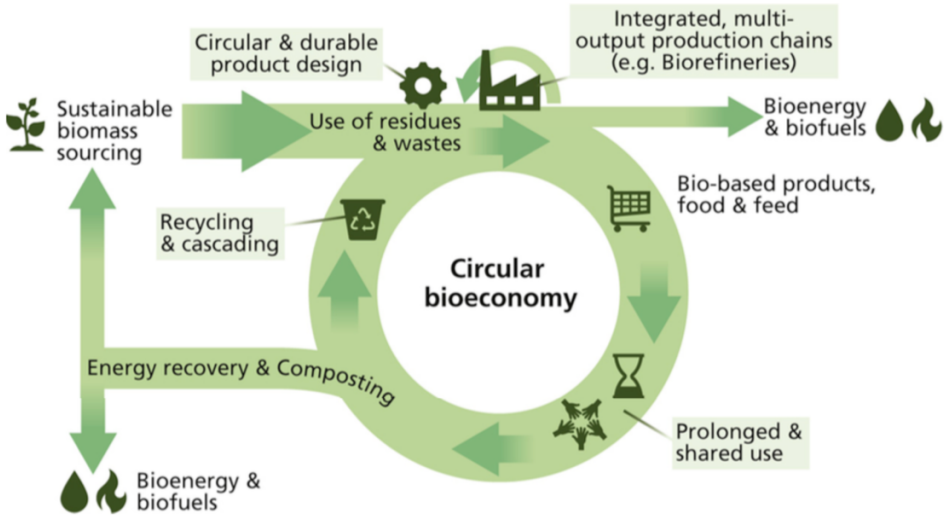

Figure 3: The circular bioeconomy and its elements, from Stegmann, Londo & Junginger (2020) (Stegmann, Londo et al. 2020).

As part of the strategies and tools for closing resource cycles, there is an increasing interest in industrial symbiosis (IS). The term was borrowed from biology and refers to the involvement of traditionally separate entities in a collective approach to obtain competitive advantages, based on the physical exchange of materials, energy, water and by-products; the key points of the IS are the collaboration and the synergistic possibilities offered by the geographical proximity of the activities (Chertow 2000). Furthermore, more recent definitions also introduce the role of information exchange, know-how and opportunities for innovation, also considering the importance of social integration (Lombardi and Laybourn 2012, Mulrow, Derrible et al. 2017). The idea behind the SI is to produce products and services by reducing the use of energy and resources through cooperation, using the by-products of one activity as the input of another (Yeo, Masi et al. 2019, Neves, Godina et al. 2020). The creation of effective IS can lead to the development of urban and rural sites and the recovery and modernization of brownfields, can

promote the emergence of new jobs, and can encourage more sustainable development, reducing:

- greenhouse gas emissions;
- exploitation of resources;
- production of waste.

There are several drivers for the development of IS, from increasing profits and competitiveness, to social, environmental and regulatory reasons (Neves, Godina et al. 2020).

In this perspective, biorefineries are configured as one of the key technologies for the development of industrial symbiosis: biorefineries can be described as the sustainable processing of non-fossil feedstock (e.g. biomass and organic waste) in a broad spectrum of marketable products (food, feed, materials, molecules) and energy (fuels, electricity, heat), through the efficient use of resources (Katakojwala and Mohan 2021). The development of these systems, therefore, could make the development of IS further efficient and promote carbon neutrality, thanks to the possibility they offer of exploiting waste and biomass from agriculture, aquaculture and forestry, and given the need for these systems to be integrated into the territory and infrastructure (Bertacchi, Jayaprakash et al. 2022).

For these reasons, I believe that biorefineries and industrial symbiosis are novel technological and economic drivers that embody and reflect the principles of the European Green Deal and can therefore guide the transitions of years to come.

Residual biomasses and microbial cell factories

1.3 billion tons of lignocellulosic biomass (LCB) are produced globally every year, and only a small fraction is exploited for the production of biomolecules (Bertacchi, Jayaprakash et al. 2022).

LCB relates to second-generation biomass feedstocks and presents an alternative to first-generation biomass feedstocks that compete with food crops for land (Mahmud, Sonia et al. 2020). LCB is mainly composed of three polymers: 35-50% cellulose, 20-35% hemicellulose and 10-25% lignin together with small amounts of other components like acetyl groups, mineral and phenolic substituents (Figure 4) (Isikgor and Becer 2015).

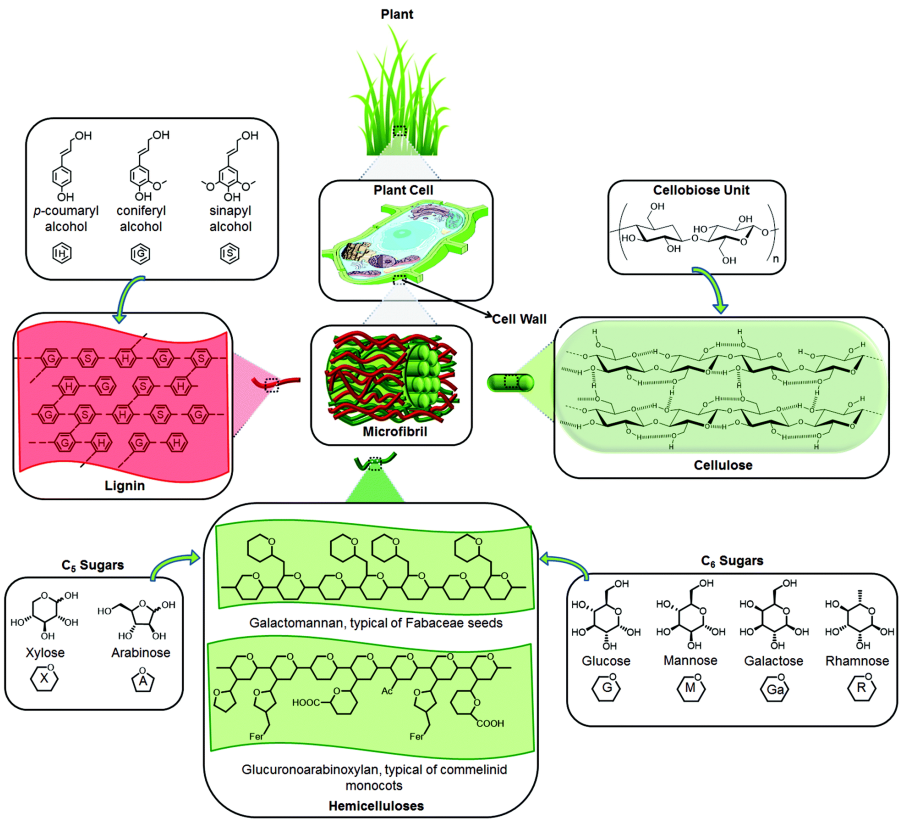

Figure 4: Composition of lignocellulosic biomass, from (Isikgor and Becer 2015).

Cellulose is a polymer of β-D-glucose units linked together by β (1-4) glycosidic bonds, organized in fibrils with a partly ordered and partly amorphous structure. Hemicellulose is a complex polymer of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acid sugars, the main components of which are xylans (in the case of hardwood trees) or glucomannans (in the case of conifers); hemicellulose is linked to cellulose fibrils through hydrogen bonds, while it binds lignin through covalent bonds (LCC bonds, Lignin-Carbohydrate Complex bonds). Lignin is an amorphous heteropolymer, composed of three main units, alcohol p-coumaryl, coniferyl alcohol and sinapyl alcohol; it gives structural support to the plant, makes it impermeable, and acts as a barrier against microbial attacks (Biswas, Teller et al. 2020, Bertacchi, Jayaprakash et al. 2022). These three polymers, cellulose, hemicellulose and lignin, are interlinked resulting in a complex and rigid three-dimensional structure that hinders its industrial utilization.

To release the sugars contained in the LCB and make them available to the microorganisms, it is necessary to carry out a pre-treatment to weaken the recalcitrant structure of the biomass. There are numerous types of pre-treatment, which can be used alone or combined such as: mechanical, thermal, acid, alkaline, oxidative, or enzymatic pre-treatment (ATW and Zeeman 2009). In this work, being limited by the unavailability of a steam explosion facility, we choose to pre-treat the biomass with an autoclaving process: the biomass is heated under the pressure of 1 Atm to temperatures around 120 °C. During this pre-treatment, part of the hemicellulose and lignin is solubilized and hydrolysed, despite in much less extent if compared to the steam explosion process; the acids released by the hydrolysis of the hemicellulose catalyse the further hydrolysis of the liberated oligomers (ATW and Zeeman 2009). To increase the quantity of released sugars, the autoclave treatment was followed by enzymatic hydrolysis. Generally, the enzymatic hydrolysis steps are preferred to chemical treatments (e.g. acid, alkaline, oxidative),

because the enzymes operate in conditions more compatible with microbial growth and allow to obtain less impacting wastewater (Katakojwala and Mohan 2021). Depending on the feedstock and pretreatment process, different hydrolysates can be obtained. The pretreatment is one of the most important step in the entire bioprocess as both the efficiency of the conversion and downstream processing steps depend on it (Bertacchi, Jayaprakash et al. 2022).

Among the diverse methods of biomass valorization, microbial fermentations offer many possibilities for obtaining the desired products. The diverse nature of the biomass feedstocks, together with the biodiversity of microorganisms, has the potential to lead to the production of a diverse array molecules of industrial interest (Branduardi 2021).

Industrial symbiosis (IS) can promote the development of this scenario: for example, by-products generated from a specific industrial process could be consider as an input substrate for another process. It is important to highlight that IS could help the realization of the concept of circular bioeconomy of industries already present in the territory without dedicating additional land for the generation of resources. In fact, Land Use and Land Cover Change (LUCC) is another hot topic that is being discussed today (Chang, Yue et al. 2018),

Microorganisms and their activities are vitally important to virtually all processes on Earth. Microorganisms matter because they affect every aspect of our lives – they are in us, on us and around us (Lund, De Biase et al. 2020).

These microbes play key roles in nutrient cycling, biodegradation/biodeterioration, climate change, food spoilage, the cause and control of disease, and biotechnology. Thanks to their versatility, microbes can be put to work in many ways: making life-saving drugs, the manufacture of biofuels, cleaning up pollution, and producing/processing food and drink (Pham, Yilma et al. 2019). Thanks to that, cells

can be compared to factories where everything happens but on a much smaller scale, and hence the term microbial *cell factory* was coined.

More recently, with advances in genomics, metabolic engineering, systems and synthetic biology, microorganisms have been used to produce biopharmaceuticals (hormones, enzymes, antibiotics, vitamins), polymers, monomers, valuable chemicals and other biological products (Adegboye, Ojuederie et al. 2021). Unlocking the potential of microorganisms in industrial processes, we can turn global problems (waste, pollution and resource scarcity) into solutions (products, materials, chemicals).

With a view to developing a sustainable industrial process, the PhD focused on the use of two yeasts, in particular *Saccharomyces cerevisiae* and *Scheffersomyces stipitis*. Generally speaking, yeasts, unlike bacteria, are acidophilic organisms: running the fermentations at low pH has the advantage of minimizing the growth of the most common bacterial contaminants. Furthermore, yeasts are generally robust organisms, are not subject to phage contamination, and show better tolerance to inhibitors, making them more suitable for large-scale fermentations (Curran, Leavitt et al. 2013, Zhu, Wang et al. 2021).

S. cerevisiae is undoubtedly the most studied eukaryotic microorganism in the laboratory, and the same time used at industrial level. Moreover, numerous molecular biology tools have been developed for its genetic manipulation. This microorganism possesses a cellular organization very similar to that of higher eukaryotic cells, and at the same time the typical advantages of unicellular organisms in terms of growth speed, ease of cultivation. In fact, it is able to grow quickly on simple medium, which makes studying in this system faster and cheaper. Furthermore, it exists both in the haploid and in the diploid state, with the obvious advantage of making possible to study the effect of both dominant and recessive mutations. Simple genetic engineering tools applicable to this yeast have been

developed, including transformation with high-efficiency single-copy or multi-copy plasmids, gene deletion methods, and simple molecular biology protocols (Branduardi, Smeraldi et al. 2008).

The possibility of carrying out genomic engineering in a relatively simple way, together with the status of a GRAS organism (Generally Recognized As Safe), make *S. cerevisiae* an excellent model organism, allowing to transfer the results obtained in less studied and known yeasts (Parapouli, Vasileiadis et al. 2020).

It is certainly one of the organisms that has undergone the greatest human domestication, as for centuries it has been the agent responsible for fermentation for the production of alcoholic beverages and for the leavening of sweet and savory pastries in bread making (Porro, Sauer et al. 2005).

S. stipitis is a Crabtree negative yeast, so it triggers the production of ethanol only in response to a decrease in oxygen, unlike *S. cerevisiae* (Silva, Mussatto et al. 2012, Shin, Kim et al. 2019). In a bioprocess, this feature is desirable, as it allows to avoid the formation of ethanol as a by-product, increasing, in this way, the theoretical production yield. Furthermore, *S. stipitis* has a wider preference of substrates if compared to *S. cerevisiae* among which: glucose, fructose, xylose, arabinose, mannose, cellobiose, and oligomers of mannans and xylans (Silva, Mussatto et al. 2012). Differently to *S. cerevisiae*, *S. stipitis* is able to metabolize pentose sugars (Jeffries and Van Vleet 2009), being generally considered to be the yeast capable of consuming xylose more efficiently: this aspect is of particular interest in the development of a biobased bioprocess, as xylose is the most abundant sugar released from LCB after glucose (Liang, He et al. 2014, Hilliard, Damiani et al. 2018). The main disadvantage of this yeast is the scarce availability of molecular biology tools: *S. stipitis* is resistant to most antibiotics, and belongs to the CUG clade (CUG encodes the amino acid serine, instead of leucine) (Jeffries and Van Vleet 2009). Furthermore, the ARS and CEN sequences of *S. cerevisiae* are not functional in *S.*

stipitis, and the transformation efficiency with integrative constructs is low because the repair of double strand breaks occurs through the Non-Homologous End Joining mechanism (NHEJ) (Cao, Gao et al. 2017, Cao, Gao et al. 2018). Despite this, there are in the literature attempts to apply the CRISPR / Cas9 technology based on the use of the auxotrophic marker *SsURA3* (Cao, Gao et al. 2017, Cao, Gao et al. 2018), and the use of resistance to zeocin as the dominant marker (Laplaza, Torres et al. 2006).

In my doctoral journey I studied these two yeasts to improve the consumption of the main sugars released by the pre-treatment of lignocellulosic biomass and consequently to obtain a better valorisation of different agricultural waste, investigating the production of Vitamin B_9.

Vitamin B_9

Vitamin B9 or folate is a general term that refers to a large group of molecules that share a general structure - as shown in Figure 5 - and differ in their oxidation state, in substitutions in the pteridine ring or in the number of glutamic acid residues in the molecule (Sybesma, Starrenburg et al. 2003). Folate is composed of three building blocks that derive from compounds synthesized in other central metabolic pathways. The pteridine ring (in red) derives from guanosine triphosphate (GTP), which in turn is produced in the biosynthesis pathway of purines. Glycolysis, the pentose phosphate pathway (PPP) and the shikimate pathway are necessary for the creation of 4-para-aminobenzoic acid (pABA, in blue). Glutamate (in green) is produced as an amino acid and can generate mono-glutamate or poly-glutamate forms of folate based on the amount of residues anchored to the chain (Levin, Giladi et al. 2004).

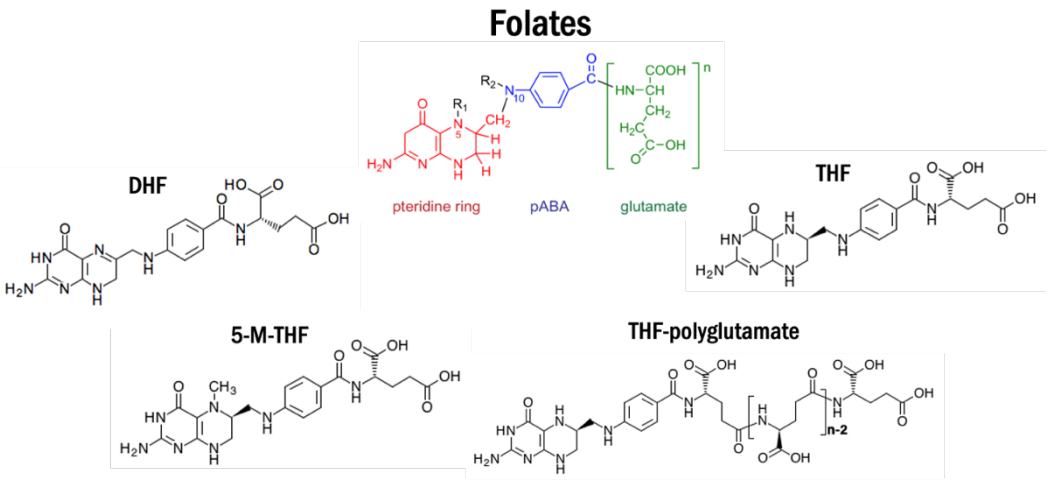

Figure 5: Top central: schematic representation of the general structure of a folate molecule, in which the three fundamental parts are distinct with different colours. The pteridine ring (red) deriving from GTP, the pABA (blue) produced from chorismate, and the glutamate (green). The vitamers of folate (black) differ for the state of oxidation (DHF, THF), for carbon substitutions (5-M-THF) and for the presence of several glutamates linked together in a poly-glutamate tail (THF-poly-glutamate). Images adapted from KEGG.

Only plants and some microorganisms have the pathway for the *de novo* biosynthesis of folate (Figure 6), which is very conserved within the various species despite the evolution that has distinguished them (Rossi, Amaretti et al. 2011). The biosynthesis of folate requires the synthesis of each of the three constituent parts, the pteridine ring, pABA and the residues of L-glutamic acid. The pteridine ring derives from the purine biosynthesis pathway, and the first step is the hydrolysis of GTP to 7,8-dihydroneopterin-3'-triphosphate (DNP-PPP) and formate, catalyzed by GTP cyclohydrolase I (EC 3.5.4.16) (encoded by the gene *FOL*2); through further four enzymatic steps, DNP-PPP is converted into (7,8-dihydropterin-6-yl) methyldiphosphate (DPM-PP), which together with pABA represents the substrate of dihydropteroate synthase (encoded by *FOL*1).

pABA is synthesized by the shikimate pathway, the precursors of which are phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), intermediates of glycolysis and of the pentose phosphate (PPP) pathway, respectively. The first reaction of the shikimate pathway is the condensation of PEP and E4P to DAHP (3-deoxy-D-arabinoheptulosonate-7-phosphate), catalyzed by DHAP synthase.

In *S. cerevisiae* there are two isoforms of DHAP synthase, encoded by *ARO*3 and *ARO*4 (Künzler, Paravicini et al. 1992); Aro3p and Aro4p are negative feedback inhibited by L-phenylalanine and L-tyrosine respectively (Zhu, Wang et al. 2021). The shikimate pathway, in fact, leads to the synthesis of aromatic amino acids (L-Phe, L-Tyr, L-Trp), which therefore exercise control upstream of the pathway. There are two mutated versions of these two genes (*ARO*3^{K222L} (Hassing, de Groot et al. 2019) and *ARO*4^{K229L} (Luttik, Vuralhan et al. 2008)) insensitive to feedback inhibition from L-Phe and L-Tyr. DHAP is a substrate of the pentafunctional enzyme Aro1p (encoded by *ARO*1), which catalyzes the conversion of DHAP into 5-enolpiruvil-shikimate-5-phosphate (EPSP) by a series of five different reactions. In *Escherichia coli* these steps are catalyzed by five distinct enzymes (*Aro*B, *Aro*D, *Aro*E, *Aro*L,

*Aro*A); the overexpression of single genes in *Saccharomyces cerevisiae* allowed to observe that the phosphorylation step of shikimate to shikimate-3-phosphate (catalyzed by *Aro*L) is the one with the greatest control of the flow from DHAP to EPSP (Rodriguez, Kildegaard et al. 2015, Hassing, de Groot et al. 2019). EPSP is converted to chorismate by the action of the chorismate synthase (encoded by *ARO*2). The chorismate is a central metabolite of the pathway, as on the one hand it can be converted to pABA through the ADC synthase (EC 2.6.1.85) and the ADC lyase (EC 4.1.3.38) (encoded respectively by *ABZ*1 and *ABZ*2), on the other hand it is also a precursor for the biosynthesis of aromatic amino acids and their derivatives.

Subsequently, DPM-PP and pABA are converted from Fol1p to dihydroxyteroate (H2pteroate), which is reduced to 7,8-dihydrofolate (DHF) by dihydrofolate synthase (EC 6.3.2.12) (encoded by *FOL*3), and then to 5,6,7,8-tetrahydrofolate (THF) from DHF reductase (EC 1.5.1.3) (encoded by *DFR*1). Finally, THF is a substrate of folylpolyglutamate synthase (*MET7*), which catalyzes the addition of L-glutamic acid residues for the generation of poly-glutamate vitamers.

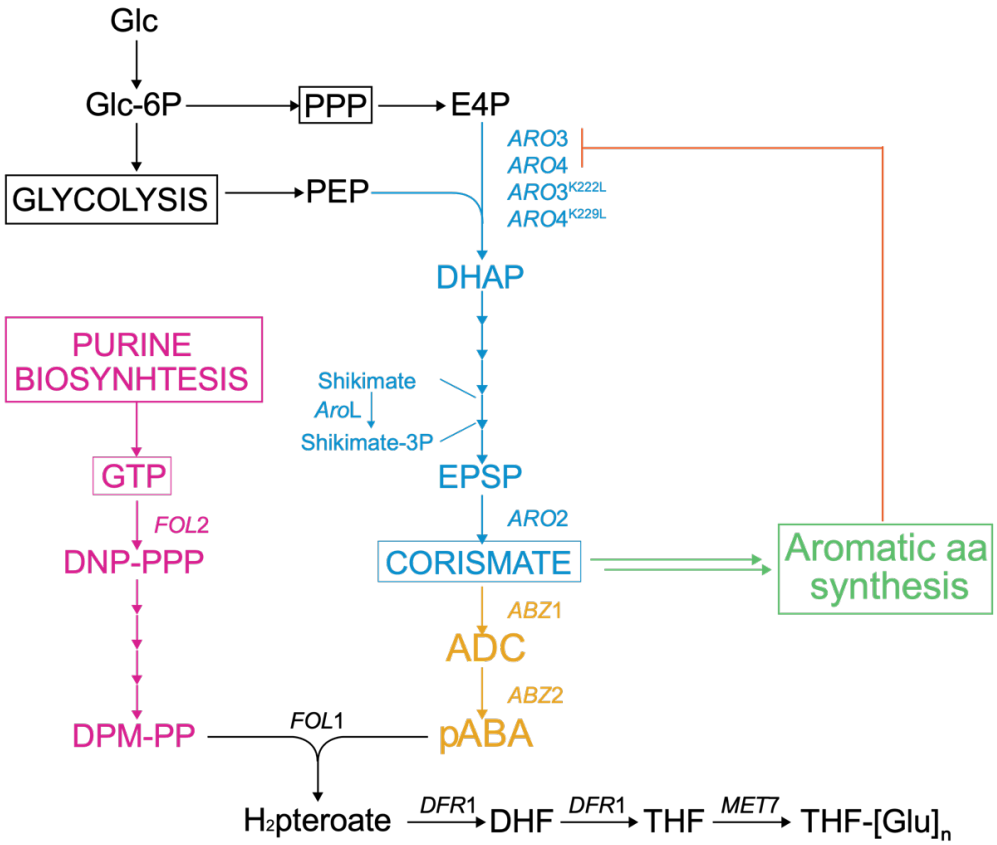

Figure 6: Tetrahydrofolate biosynthesis pathway in yeasts. The biosynthesis of THF requires the synthesis of the pteridine ring starting from GTP (pink), and of pABA, a derivative of the shikimate pathway (blue and yellow). Glc: glucose, Glc-6P: glucose-6-phosphate, PPP: Pentose phosphate pathway, E4P: erythrosium-4-phosphate, PEP: phosphoenolpyruvate, DHAP: 3-deoxy-D-arabinoheptulosonate-7-phosphate, EPSP: 5 -enolpyruvil-shikimate-5-phosphate, ADC: 4-amino-4-deoxy-chorismate, DNP-PPP: 7,8-dihydroneopterin-3'-triphosphate, DPM-PP: (7,8-dihydropterin-6-yl) methyldiphosphate, H2pteroate: dihydropteroate, DHF: 7,8-dihydrofolate, THF: 5,6,7,8-tetrahydrofolate, THF- [Glu] n: THF polyglutamate. ARO3 / ARO4: DHAP synthase, AroL: shikimate kinase (E. coli), ARO1: pentafunctional protein Aro1, ARO2: chorismate synthase, ABZ1: ADC synthase, ABZ2: ADC lyase, FOL2: GTP cyclohydrolase I, FOL1: dihydropteroate synthase, FOL1: dihydroptero synthase: dihydrofolate synthase, MET7: folylpolyglutamate synthase. Scheme adapted from (Cherry, Adler et al. 1998, Kanehisa and Goto 2000, Luttik, Vuralhan et al. 2008).

Folate are hydrophilic anionic molecules that are unable to diffuse across biological membranes: for this reason, in mammalian cells and tissues there are specialized transport systems for these vitamin cofactors. Absorption exploits several genetically and functionally distinct transporters, such as folate receptors, the organic anion transporter family, proton-coupled folate transporters (PCFT), and

reduced folate transporters that are ubiquitously expressed (Matherly, Hou et al. 2007). Each mechanism plays a unique role in mediating transport through epithelia and systemic tissues and contributes to folate homeostasis in humans (Zhao, Matherly et al. 2009). In our body, folate mono-glutamates and FA are absorbed in the intestine, at the level of duodenum and in the apical part of the jejunum, through the PCFT receptors present in the microvilli of the enterocytes. Since most of the folate ingested with the diet are in poly-glutamate forms, their conversion into folate mono-glutamates is necessary to be absorbed in the intestine, and this happens thanks to the enzymatic action of glutamate carboxypeptidase II (GCII) anchored in the intestinal villi. Once into intestinal cells, both natural mono-glutamate folate and FA are converted into 5-methyl tetrahydrofolate (5-MeTHF): in this form they can be transported by multidrug-resistance-associated proteins (MRP) towards the blood vessels and into the circulatory system (Blom and Smulders 2011) (Figure 7). To carry out the various biological functions, the molecules leave the bloodstream and inside the cell and mitochondria are reconverted into poly-glutamate forms through the action of folylpolyglutamate synthase (FPGS). This event is necessary for their retention in the various organs' tissues. Furthermore, folate-dependent enzymes have greater affinity for poly-glutamate forms which are therefore considered the biologically active vitamin cofactors in our organism (Akhtar, Orsomando et al. 2010).

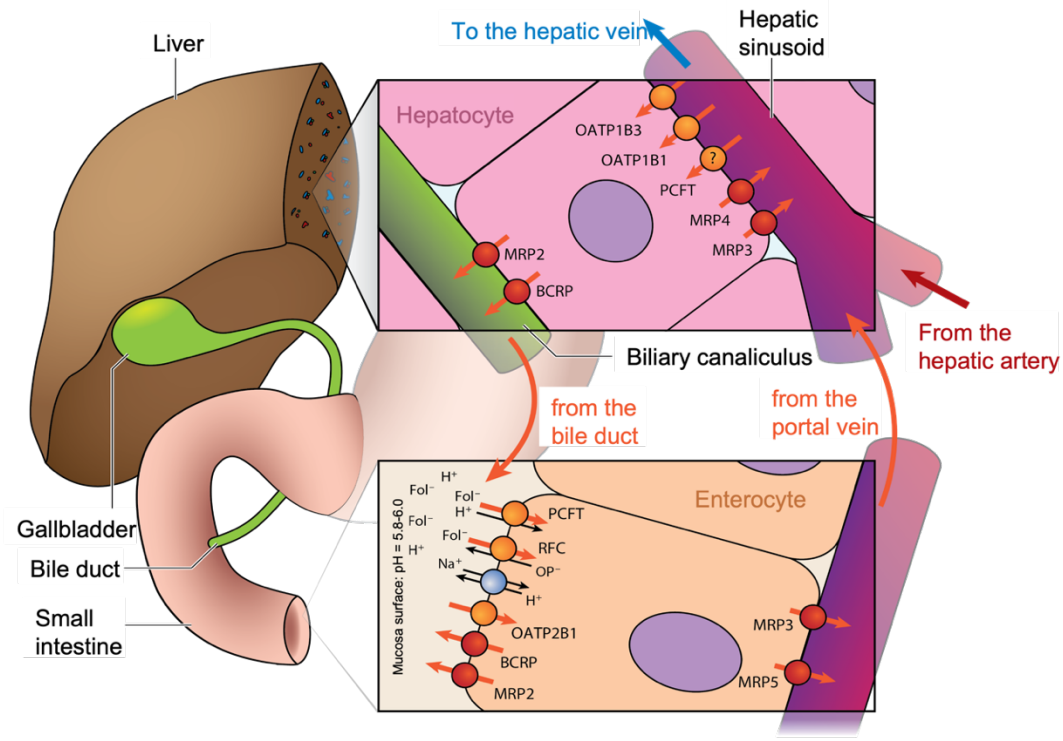

Figure 7: Intestinal absorption and enterohepatic circulation of folates. Folate is mainly absorbed in the duodenum and in the jejunum (small intestine), at the level of the apical membrane of the edge of the brush through the specific PCFT transporter; uptake takes place in an acidic environment (pH = 5.8-6.0), maintained by Na + / H + exchangers expressed on the apical membrane. There is a second folate specific transporter (RFC), expressed in all tissues and cell lines, which is responsible for delivering folate to cells from the systemic circulation. The PCFT transporter is specific for the monoglutamate forms of folate, therefore, the uptake of dietary folates (generally polyglutamates) requires a preliminary step of hydrolysis, carried out by the glutamate carboxypeptidase II. The OATP2B1 transporter is able to transport AF, 5-methylTHF and 5-formylTHF, but the activity is very poor; BCRP and MRP2 re-transport folate from enterocytes to the lumen of the intestine, but the influence on folate absorption is not clear. AF is reduced to DHF and THF by DHFR, and subsequently converted to 5-methylTHF in enterocytes. MRP3 and MRP5 are expressed in the basement membrane of enterocytes, which mediate the transport of 5-methylTHF and any non-metabolized AF in the hepatic portal vein, to reach the hepatic sinusoid. From here, folates reach the systemic circulation via the hepatic veins, or are transported to the hepatocytes at the level of the basolateral membrane, by the transporters OATP1B1 and OATP1B3; PCFT is expressed at the basolateral membrane, but the role is unclear. Folate is stored in hepatocytes in polyglutamate form or transported in the biliary system (hydrolysis to monoglutamate forms is carried out by the γ-glutamyl hydrolase enzyme). Image adapted from (Saini, Nile et al. 2016).

Folates are important cofactors in the C1 metabolism, as they act as donors and acceptors of monocarbon units, and for this reason they are involved in the synthesis reactions of purines and thymidylate, in the synthesis of the amino acids methionine (Met), serine (Ser), glycine (Gly), formylmethionine (fMet, for the synthesis of formyl-methionyl-tRNA in mitochondria), in the synthesis of pantothenate (vitamin B_5), and in the catabolism of histidine (His); moreover, folates play a key role in the methyl cycle, which supplies S-adenosylmethionine (SAM) to methyltransferases, capable of transferring methyl groups to a wide range of substrates (proteins, lipids, DNA and hormones) (Blancquaert, Storozhenko et al. 2010).

Hence folate is an essential molecule for cell division, for embryonic and fetal development and for the maintenance of cardiovascular and neurological functions (Strobbe and Van Der Straeten 2017). Human, on the other hand, is unable to synthesize folate which for this reason represents an essential food component (Walkey, Kitts et al. 2015). Thus, the U.S. Food and Drug Administration (FDA) and the European Union suggest a daily intake of 400µg and 240µg, respectively; in addition, a higher intake of folate is suggested for pregnant and lactating women (Sybesma, Starrenburg et al. 2003).

This vitamin can be taken through diet: it is present in vegetables (especially in dark green leafy ones), fruit, legumes, milk, meat, eggs and seafood (Jägerstad 2012) and nowadays fortified food and food supplements are available to increase the daily assimilation.

However, vitamin B_9 currently on the market is almost exclusively in the form of folic acid obtained by chemical synthesis, while large-scale biotechnological processes for the production of natural folate do not yet exist (Acevedo-Rocha, Gronenberg et al. 2019). Folic acid is not biologically active in itself; inside our body it is activated into tetrahydrofolate (THF) through biotransformation catalyzed by

dihydrofolate reductase (DFR, EC 1.5.1.3). Unfortunately, human DFR shows a low conversion rate of FA in the biologically active form, consequently the intake of synthetic vitamin B_9 can lead to its accumulation in the bloodstream (Bailey and Ayling 2009).

This accumulation creates problems for our organism, as it can mask a vitamin B_{12} deficiency, not solving the problems related to it (Choi, Yates et al. 2014). This problem can be solved by fortifying foods with natural folate or by creating biotechnological processes that exploit the ability of microbial cell factories to produce natural forms of vitamin B_9.

To better illustrate the work done, the thesis is divided into chapters that recapitulates the main objectives and results of the project.

In chapter 1 we deal with the concept of industrial symbiosis, taking advantage of working tables created by ENEA in which various companies of the agricultural and food and beverage sectors present in the area of interest have been invited. These companies compared and made available material outputs and inputs, which were afterwards analyzed by ENEA proprietary workflow to create a book of qualy-quantitative material fluxes and to identify possible synergies on the territory and thus favoring the creation of industrial symbiosis.

Chapter 2 and 3 focus on the valorization of a non-Saccharomyces yeast, *Scheffersomyces stipitis*, as a promising host for lignocellulosic biomass valorization in products of high-added value, as vitamins. In particular, in Chapter 2 the aim was to optimize growth and folate production in minimal defined media resembling a lignocellulosic hydrolysate in terms of sugar composition and content. Three different growth parameters were examined and improved: oxygenation, media composition, nitrogen supplementation.

The first two chapters were propaedeutic for developing the work illustrated in Chapter 3 in which the yeast *S. stipitis* was grown on hydrolysates deriving from three different biomasses from the agro-food sector, sugar beet pulp (SBP), sugar beet molasses (SBM) and unfermented grape mask (UGM). UGM was also selected thanks to the work carried out in the first chapter, as it is one of the most present residual resources in the Lombardy region. The idea of this chapter is therefore to exploit the concept of biorefinery, to create new technologies that make the closing of the life cycle of all products deriving from the agro-food sector potentially possible.

Chapter 4 is dedicated to engineering strategies intended to increase the production of vitamin B_9 in the yeast *Saccharomyces cerevisiae*, amenable for genetic manipulation. Eight different genes were manipulated for the first time in the same genetic background and exploiting different engineering strategies. This was pivotal for testing the best strain in bioreactor and in bringing folate production and productivity.

OBJECTIVES

The purpose of this PhD project is therefore to create, through bioprospection and metabolic engineering approaches, microbial cell factories suitable for the production of natural forms of vitamin B_9, which can then be used to enhance agri-food waste in the perspective of industrial symbiosis and circular economy.

In my vision this thesis is an example on how to close industrial cycles by the use of biotechnology and biorefineries, an important starting point for lowering environmental impact due to the creation of agricultural waste and the production of molecules by chemical synthesis.

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Chapter 1

Industrial symbiosis potential on specific agri-food and metallurgical value chains in Lombardy region

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LM contributed to the flows analysis reported in this article, bibliographic research and drafting of a part of the introduction and the results obtained

ABSTRACT

This paper focuses on the project activities carried out by ENEA related to Industrial Symbiosis (IS) as part of the Creiamo project, funded by CARIPLO Foundation, aimed at identifying and promoting new destinations and economic opportunities for by-products and waste deriving from the olive and wine sectors, under a circular economy perspective. Due to the pandemic, the ENEA's methodology for promoting and implementing IS has been adapted in order to perform from remote all the activities with the companies involved. An engagement campaign was carried out in the territory of Brescia with the support of several local associations. The IS – Operative Meeting (OM) with enterprises was held remotely on 19 February 2021. About 100 potential synergistic actions have been identified, mainly involving material resources. Following an initial processing of data, summary reports were prepared, one for each company. Significant resource flows were selected according to the quantities involved and to (their) economic value. As a consequence of this work, two operative handbooks have been drawn up for companies that are willing to transform synergies from theory to practice.

Keywords*:* eco-innovation, industrial symbiosis methodology, Lombardy, oil and wine residues, synergies.

BACKGROUND

By 2050, it is expected that the world will have a minimum of 9 billion habitants and our common desire is to ensure a comparatively high level of prosperity and welfare for all. This goal is not achievable using the present linear mode of economy, because the natural resources of the Earth are already being depleted. A profound shake-up is required and a whole re-thinking of the Western industrial system and economy needs to happen (Sacchi et al., 2021).

Circular economy aims to replace the linear economy concept into a new circular model, where the value of products and materials is maintained as long as possible (Korhonen et al., 2018). It is not just a question of eliminating or minimising the production of waste, but of radically changing the conflicting vision between economic and environmental interests, traditionally considered in antithesis, with a new and broader concept of well-being, which includes both. The circular economy represents a radical paradigm shift, within which to develop new sustainable business models, able to increase the potential closure of production cycles and the efficient use of local resources.

As part of the strategies and tools for closing resource cycles, there is an increasing interest in IS, which aims at transferring and sharing of resources (raw materials, water, waste, energy waste, services, skills, tools, etc.) between companies and / or other operators present in nearby areas (EU Committee of standardisation, 2018). This approach constitutes not only a potential competitiveness factor for industrial activities, but also an enrichment factor for the area, which sees all of its resources valued locally and not dispersed, delegated or given away to third parties. The European Commission has attributed a strategic role to IS for the efficient use of resources, clearly identified in various programming and funding documents (e.g., European Resource Efficiency Platform, COM (2011) 571, COM (2014) 398, COM (2015) 614). Interest in IS was also registered in Italy, with the development

of several projects dedicated to the closure of resource cycles and with the inclusion of IS in some regional planning tools (Simboli, 2014, 2015) (Notaricola, 2016).

ENEA is responsible for implementing and promoting IS using a validated methodology as result of decades of experience on this issue. In 2011 ENEA started the development and the implementation of an IS network model thanks to three projects in three Italian regions: the “Eco-Innovation Sicily” project (Cutaia et al., 2015; 2014a; Luciano et al., 2016); the "Green Project - Industrial Symbiosis" in Emilia-Romagna (Cutaia et al., 2016; 2014b); and the "Industrial Park of Rieti-Cittaducale" project in Lazio (La Monica, 2016).

The Creiamo project, financed by CARIPLO Foundation, has as its partner the University of Brescia in the role of coordinating the activities, the University of Milano Bicocca and ENEA, which dealt with aspects relating to the circular economy, the IS, and the promotion of intersectoral synergies in particular.

This article, as part of the aforementioned project, illustrates the activity carried out by ENEA to identify and promote new destinations and economic opportunities for by-products and waste deriving from olive and wine supply chains, under a circular economy perspective. By achieving this objective, the competitiveness of companies operating in the Lombardy Region, and in Brescia province specifically, will consequently be increased, also thanks to the creation of new business models (e.g., IS).

The project adopted a system eco-innovation strategy through the creation of a network of IS in Lombardy, through which enterprises will be able to achieve economic, environmental and social benefits.

Moreover, the project represents the first structured attempt to implement IS in the region and involved various productive sectors in addition to the one directly involved in the project (olive and wine supply chain). The agri-food industry, with a focus on the production of oil and wine have been studied in the project.

Waste resources coming from these sector, olive and grape marc, Olive Mill Wastewater (OMWW) were considered for the implementation of specific valorisation strategies.

The choice of synergies to be implemented was guided by the characteristics and quantities of the resources made available by the companies involved in the project.

The potential of circular economy in agri-food chain

The agri-food chain includes the primary sectors of agriculture, animal husbandry and fishing, the food industry that deals with the transformation of raw materials and the production of beverages, the industry for the enhancement of by-products and waste, the distribution and commercial phase.

In the Communication of the European Commission "Roadmap to a Resource Efficient Europe", the waste produced along the agri-food chain is mentioned as one of those on which to intervene primarily (European Commission, 2011).

The circular economy model aims to overcome the limits of the current system, increasing production performance and simultaneously generating an improvement in the quality of the soil, water and air. This is achieved by exploiting the reuse of agricultural residues, finding the right application for each type of residue, through a series of sequential processes. The companies must identify the actual potential of all substances that do not constitute the final product, identifying them as a source of income and not as a cost that must be incurred for their disposal. In this context, an important role is played by technological and process innovation, through the adoption of new technologies applicable to residues for their enhancement. The creation of cooperatives or districts of IS can further favour the reuse of resources in new processes. Therefore, the involvement of all stakeholders, such as research and development institutes, industry

associations and government bodies, as well as companies, is primary to create favourable conditions for the development of new business ideas.

Circularity in the olive oil and wine processing sectors

Lombardy constitutes the most important agricultural region in Italy. Overall, 13.5% of the value of agricultural and forestry production and 11.3% of agricultural added value are, in fact, produced in Lombardy. In absolute values, the Lombard agricultural sector involves 41,116 companies, with an extension of the Utilised Agricultural Area (UAA) equal to 958,378 ha, or 7.6% of the national share. Overall, there are 56,000 production facilities operating in the agri-food sector (agricultural production, related activities and food processing), which involve about 200,000 workers, of whom 143,000 are permanently employed, equal to 3% of the Lombard total (Data in ISMEA 2020).

In the European Union, the mills generate about 9.6 million tons / year of by-products that can be used in other areas. With a sales volume of 3 billion euros (ISTAT data in ISMEA 2018), the olive oil supply chain represents 2% of the total turnover of the agri-food industry. Italy ranks second among the olive oil producing countries in the world.

Olive pomace (exhausted pulp, stone and seeds) and OMWW are significant by-products in Lombardy with a high environmental impact when not properly treated. However, at the same time these by-products are also rich in high-value compounds, which can be used directly after extraction, or enhanced as ingredients for other industrial sectors: food industry, feed industry, the nutraceutical and cosmetic sectors.

Grapes represent one of the largest fruit productions globally, an amount of 60-70 million tons are produced every year; 60% of the grapes produced are used as

"pressed grapes", for the production of wine or grape juice (Muhlack et al. 2018), (Gómez-Brandón et al. 2019). In particular, the European Union is the largest wine producer, accounting for 65% of global production; in 2018 in Italy more than 8.6 tons of grapes were harvested, and in Lombardy alone in 2019 wine production reached 130 million litres (Chebbi et al. 2021). During the wine production process, the pressing of the grapes generates solid residues (pomace), consisting of stalks, skins, seeds and water, which represent about 25% of the grape mass; for the production of 6L of wine is estimated to produce about 1 kg of pomace leftover, for a total of 10.5-13.1 million tons of pomace per year (Muhlack et al. 2018), (Gómez-Brandón et al., 2019). Given the high quantities of pomace produced annually by wineries, the sector is under pressure to implement plans for an adequate and sustainable disposal of this biomass: the pomace, in fact, is characterised by high COD and BOD (Chemical / Biochemical Oxygen) values, which makes disposal an important and costly environmental problem (Campanella et al. 2017).

There are multiple possibilities for valorising residues and processing by-products of these two production chains, some of which are traditional and consolidated practices, others more innovative and under development. The current principal valorisation processes for olive pomace, OMWW and grape marc are shown in Table 1.

Table 1. Strengths and limitations of olive oil and wine processing chain residues valorising methods

Resource	***Technology***	***Strengths***	***Limitations***	***Reference***
Olive Pomace	Extraction of useful molecules	The valorisation of organic wastes through fast pyrolysis appears to be a highly promising option for decreasing pollutants and reducing consumption of natural resources.	Is influenced by several factors that can have a significant impact on the feasibility of this approach	(Difonzo et al. 2021)

	Pyrolysis	The valorisation of organic wastes through fast pyrolysis appears to be a highly promising option for decreasing pollutants and reducing consumption of natural resources	Developing a novel cost-effective and environment-friendly process	(Dorado et al. 2021)
	Bioconversion	Exhausted olive pomace (EOP) represents a potential candidate stream to be utilized in biotechnological processes	EOP composition includes significant amounts of extractives and pectin, which are both usually discarded and are not utilized in the valorisation process of the raw material	(Paz et al. 2020)
	Anaerobic Digestion			(Elalami et al. 2020)
	Bioremediation			(Flores-Céspedes et al. 2020)
	Animal feed	Replacing conventional feed with waste biomass produced by crop-industrial processes can be a good practice for the sustainability of crop-livestock systems and an interesting solution for their disposal	Maintainment of the products quality	(Chiofalo et al. 2020)
Olive Mill Wastewater (OMWW)	Purification through membranes	High content of hydroxytyrosol, tyrosol, oleuropein, verbascoside, vanillic acid, and luteolin isolated		(Tundis et al. 2020)
	Hydrothermal carbonization	Is considered as a promising technique for wastes conversion into carbon rich materials for various energetic, environmental and agricultural applications		(Azzaz et al. 2020)
	Animal feed	Using polyphenols from olive oil waste as feed supplements in animal diets can be a strategy to reduce adverse environmental effects of this by-product and		(Branciari et al. 2020)

		to enhance the quality of products of animal origin		
	Bioconversion	The process was successfully validated on an industrial scale without any pre-treatment, dilution and/or supplementation of the raw waste. Bio-detoxified OMW produced by this sustainable and low-cost process would be suitable for new non-chemical fertigation or soilless applications		(Ramires et al. 2020)
Grape marcs	Extraction of useful molecules	Natural extracts obtained from grape pomace are particularly interesting and the technology already exist	Seasonality of raw materials	*(*Brazinha et al. 2014*)*
	Pyrolysis	Process applied as pre-treatment step for grape marc within energy generation	A crucial first step in developing a novel cost-effective and environment-friendly process	(Marculescu and Ciuta 2013)
	Bioconversion	High by-product valorisation process	Technology improvement	(Campanella et al. 2017)
	Animal feed		The nutritive value of grape pomace varies depending on the proportion of seeds and pulp	(Guerra-Rivas et al. 2017)
	Bioremediation			(Chebbi et al. 2021)

MATERIALS AND METHODS

The methodology, developed by ENEA, is the result of consolidated experience on IS (Cutaia et al. 2015).

It is based on three basic pillars:

1) Language of symbiosis: a shared language that is expressed in the formats for collecting information (personal data and relating to input and output resources);

2) Communication with companies: reciprocal, collaborative and continuous throughout the whole symbiosis implementation process**;**

3) Knowledge and experience: this aspect concerns the knowledge base that allows ENEA to intercept, support and enhance collaboration between enterprises and the exchange of resources as a basic prerequisite for the implementation of IS paths (Fig. 1).

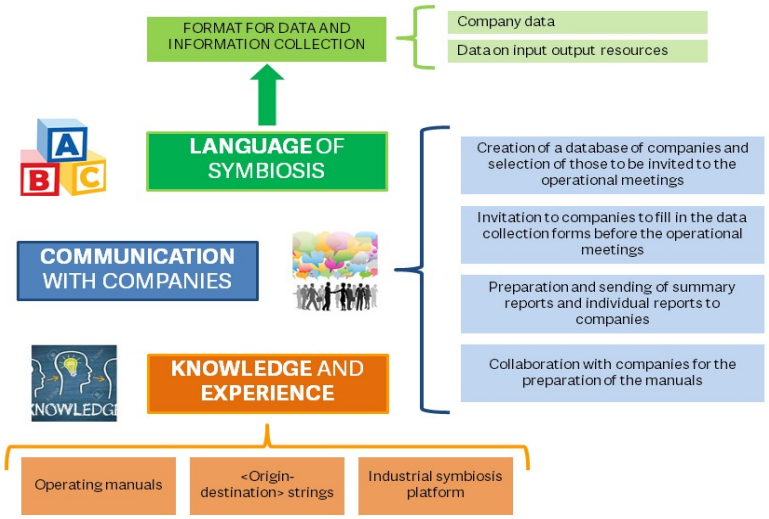

Fig. 1. The three pillars of the ENEA methodology

According to methodology, activities related to the implementation of IS followed these operational phases:

• Step 1_ Identification of stakeholders in the Lombardy region: preparation of company's database, selection of enterprises to be involved including business associations and local authorities.

• Step 2_Preparatory activity for the OM: Invitation of companies; registration; preliminary data collection about the resources.

• Step 3_OM with companies: sharing of resources, finding synergies, conducting the OM and facilitating the identification of matches.

• Step 4_Identification of significant resource flows and potential synergies: analysis of data relating to the OM resources, analysis of the synergies that emerged during the OM, summary reports elaboration, search for new synergies and identification of significant flows.

• Step 5_ Study of synergies in collaboration with companies: study of technical feasibility and environmental aspects through LCA, assessment of economic impacts, study of legislation and technical standards.

• Step 6_ Drafting of the operative handbooks.

For the Creiamo project, the ENEA methodology to support enterprises in the realisation of IS matches, has been adapted to be carried out in telematic mode, due to the pandemic situation.

Therefore, both OM and information exchanges with the enterprises and stakeholder involved took place remotely. At first, ENEA drew up a specific online form that companies filled out before the OM took place. These forms contain company personal information and shared or requested resources data (type, quantity, availability, characteristics, etc.). This anonymous database (names of the companies have been replaced with unique codes) was used as a base of information during the online operating session. Participants anonymously expressed their preferences on the use or willingness to share involved resources, allowing an initial match identification.

The IS OM was held on February 19, 2021, aimed at enterprises in the province of Brescia and neighbouring provinces.

RESULTS AND DISCUSSION

There were 22 firms participating in the OM and they belong to very different production sectors, as it can be seen from the graph (Fig. 2)

During the meeting 96 resources were shared, mostly material resources (about 89%). Of these, 24 were input resources (required by companies), and 72 output resources (offered for sharing) composed of waste, by-products or surpluses (Fig. 3).

Overall, 102 synergies were identified, 77 synergies on output resources and 25 on input resources. To these must be added the synergies identified by ENEA downstream of the workshops, which envisaged intermediate treatments of the resources made available (Fig. 4).

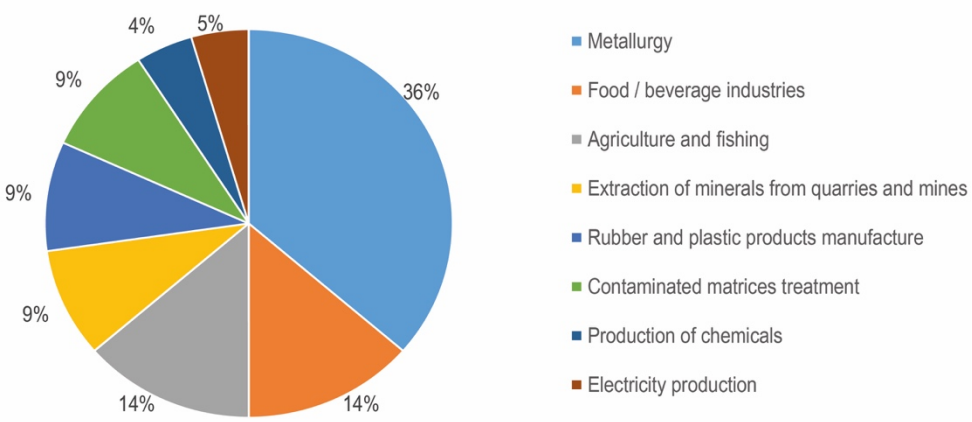

Fig. 2. Production sectors of the 22 companies that participated at the IS OM. (ENEA elaboration)

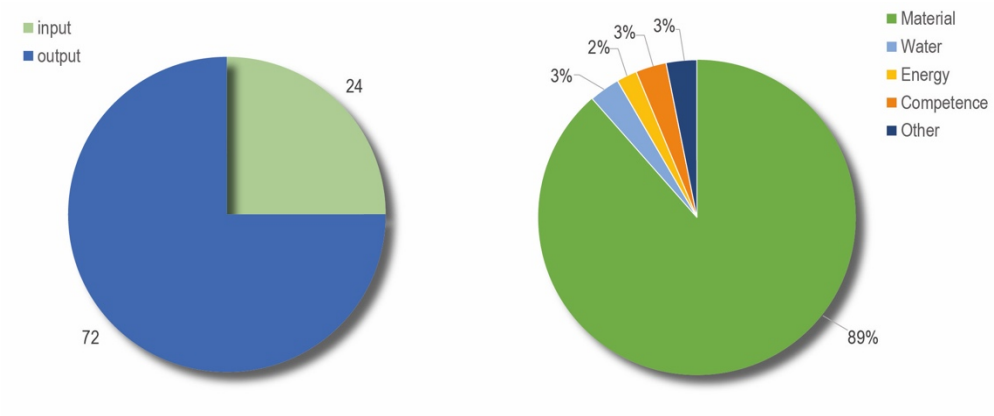

Fig. 3. Shared resources (ENEA elaboration)

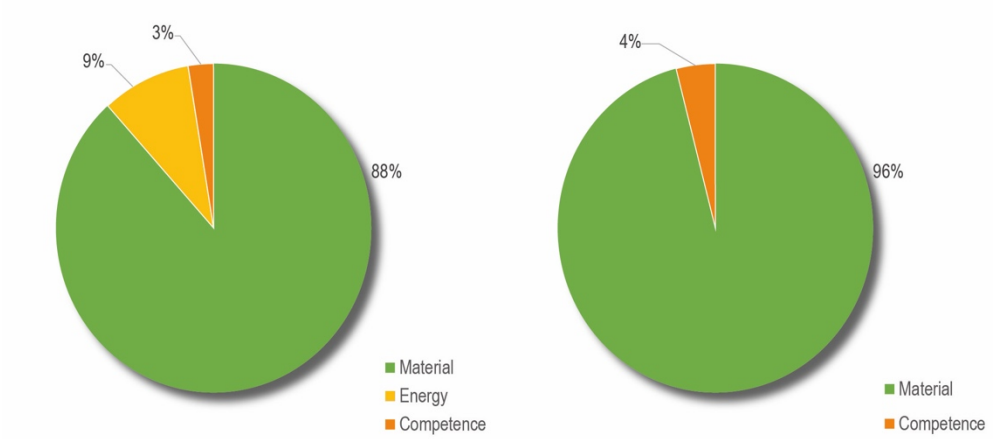

Fig. 4. Synergies emerged during the OM (ENEA elaboration)

Following the OM activity, thanks to the contribution of Confindustria Brescia, the resources database was integrated with information on companies that were unable to participate. The identification of new potential synergies and the drafting of a summary report for each company followed this phase. The document contains a summary of the shared and requested resources and a description of the potential synergies identified (Fig. 5).

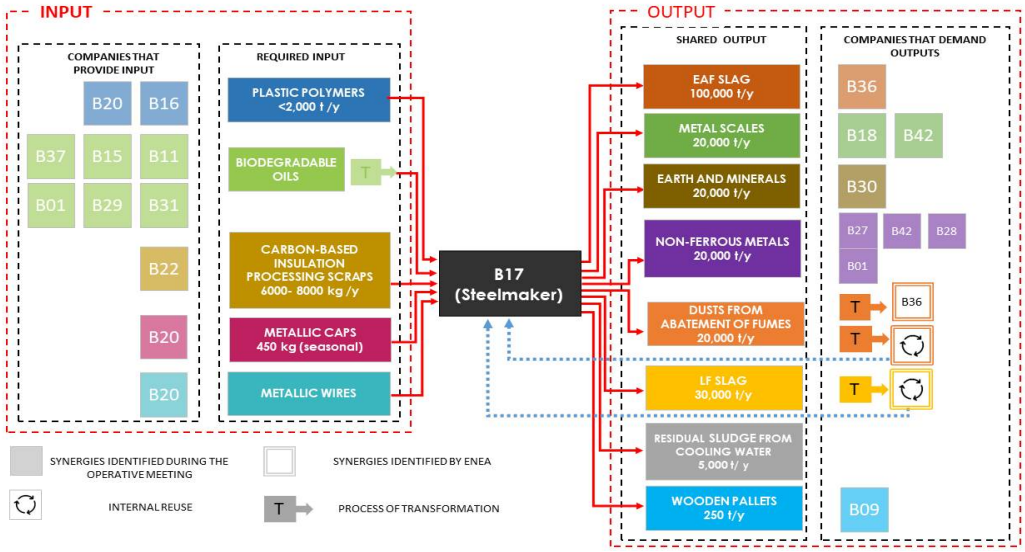

Fig. 5. Example of a summary scheme of the synergies identified as reported in the summary report

A further phase of investigation led to the identification of the most significant resource flows both from a quantitative and qualitative point of view. The valorisation solutions for these flows were studied in-depth in a technical handbook:

- Technical handbook on the synergies identified for organic resources, i.e., waste from olive oil and wine productive process, in particular olive and grape marc and OMWW. In this handbook, three flows of organic resources have been studied starting from waste from the companies involved:
  - Olive pomace, produced by three different farms with the possibility of being enhanced:
    - Through the extraction of compounds with higher added value such as polyphenols or antioxidants, in turn addressed to the fishing, cosmetic or nutraceutical industry.
    - For the production of bio-oils used for combustion.
    - For the production of natural bio-surfactants.

- OMWW, produced by three different farms with the possibility of being valorised:
  - Through the extraction of compounds with higher added value such as polyphenols or antioxidants, in turn addressed to the fishing, cosmetic or nutraceutical industry.
  - For the production of natural bio-surfactants.
- Grape marc, output classified as by-products from two different farms with the possibility of being valorised:
  - Through the extraction of compounds with higher added value such as polyphenols or antioxidants, in turn addressed to the fishing, cosmetic or nutraceutical industry.
  - For the production of bio-oils used for combustion.
  - For the production of natural bio-surfactants.

Scenario for the enhancement of wine and olive oil waste: Extraction of high added value molecules

Biomasses are renewable energy sources that are distinguished from other renewable sources (such as wind, solar, geothermal) as the available energy is stored in the chemical bonds present in the various molecules that compose it. This means that they are a source of energy but also of basic chemical compounds or high added value and biomaterials.

The extraction of chemical compounds with high added value from biomass already represents an important market in the pharmaceutical (chitosan used as a vehicle for drugs or for the production of sutures and synthetic leather), cosmetics (serums and creams enriched in chitosan and extracts polyphenols), nutraceutical (dyes

derived from biomass, texture modifiers and food supplements) and agricultural (pyrethrum-based insecticides); so the extraction processes can be defined as sustainable, it is important to use technologies with high energy efficiency and which are based on low environmental impact solvents obtained preferably from renewable sources (Herrero and Ibañez 2018).

Olive pomace, OMWW and grape marcs represent a very varied source of bioactive compounds that could have potential applications in various markets as mentioned before.

Real cases have been identified during the OM (Fig. 6). Among the companies involved, two of them (indicated with the code B03, B16) requested as input molecules with high added value such as antioxidants and polyphenols, intended for the fishing industry, to increase the quality of feed, or for the creation of plastics for food packaging, in order to increase the freshness and shelf life of packaged foods. Among the companies present none was able to directly share these types of molecules but five of them (B11, B15, B37, B29, B31) shared olive pomace, OMWW and grape marcs for a total of 1040 m^3/y, 1300 m^3/y e 100 tons/y respectively.

In literature there is much research aimed at extracting these products from pomace, OMWW and pomace as they are present at high titers (Table 1).

The extraction of these high added value molecules is now carried out on an industrial scale starting from edible plants and crops.

Therefore, a possible strategy to valorise these by-products could be the implementation of these extraction processes, which already exist, using the resources examined in this work as starting biomass.

Fig. 6 shows, more in detail, the case study developed by ENEA; the green and purple arrows highlight the distance of olive pomace, OMWW and grape marcs respectively from the companies that supply these outputs to the different

processing firms identified by ENEA in the Lombardy region. Furthermore, the grey arrows highlight the distances between the processing companies identified and the ones that require polyphenols and antioxidants as input.

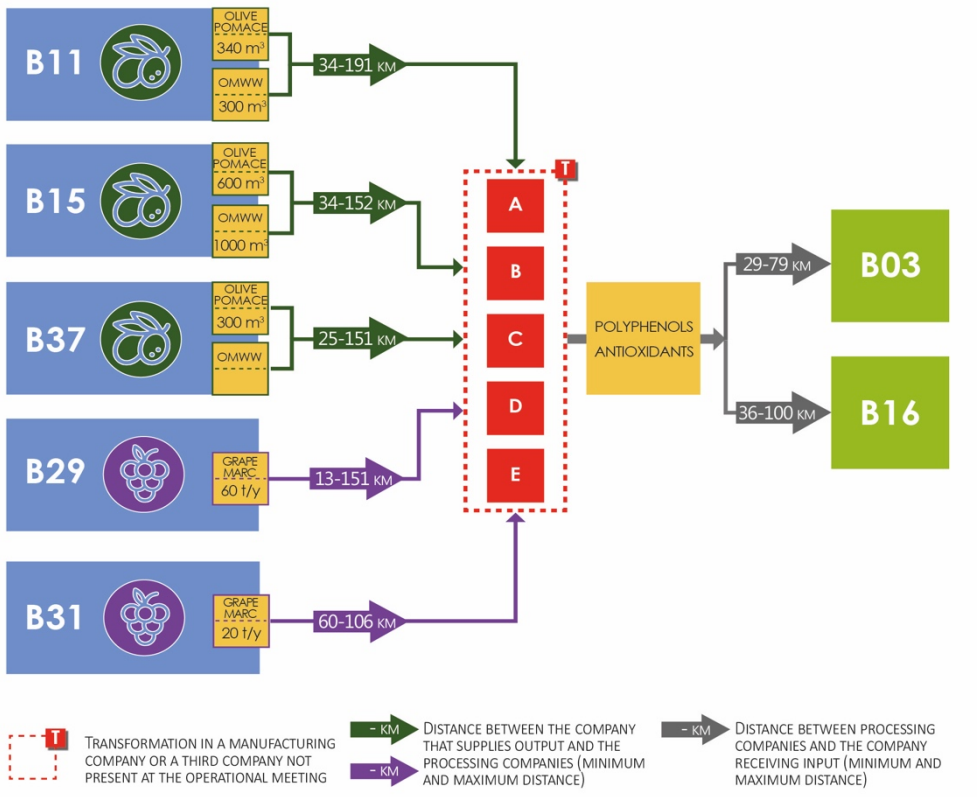

Fig. 6. Enhancement scenario of olive pomace, grape marcs and OMWW for the extraction of high added value molecules

In the arrows (Fig. 6) a range of distances is shown from the smallest to the largest between the various companies taken in consideration. These values can therefore undergo changes since the intermediate extraction process, which must be done on the resources, is carried out on site by the companies in question or by third-part companies. The distances between firms are one of the factors that has the greatest impact on economic costs but also on the environmental impact. For this reason, one of the foundations of IS is the closeness between companies and for this reason in this work the distances between them have been analysed in detail.

In general, it has been observed that the distances between the companies that provide resources (B11, B15, B37, B29, B31) and those that receive them for treatment (A, B, C, D, E) lie in a range of distance that goes from 13 to 191 km, while between the "T" companies and those that use the final resource (B03, B16) they are found in a range that goes from 29 to 100 km. It is possible to observe in detail the specific ranges for each company in the synergy diagram (Fig. 6)

CONCLUSIONS

The IS activities carried out in the Creiamo project have aroused interest among the companies who actively participated in synergies implementation, confirming environmental, social and economic advantages deriving from resources exchange between enterprises. In fact, the development paths outlined, such as the extraction of high added value molecules from oil and wine production waste and the use of black slag as an artificial inert from steel mill waste, can be taken as a pilot case for the development of analogous synergies in territories where there is a productive fabric that produces the same kind of resources. The feedback effects mainly materialize in economic and environmental advantages, as in the case of the production of antioxidants and polyphenols, where the use of agro-industrial by products determines a reduction in production costs, an increase in value chains and a closure of production cycles with the relative reduction of environmental impacts.

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Chapter 2

First report on Vitamin B_9 production including quantitative analysis of its vitamers in the yeast *Scheffersomyces stipitis*

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ABSTRACT

Background: the demand for naturally derived products is continuously growing. Nutraceuticals such as pre- and post-biotics, antioxidants and vitamins are prominent examples in this scenario, but many of them are mainly produced by chemical synthesis. The global folate market is expected to register a CAGR of 5.3% from 2019 to 2024 and reach USD 1.02 billion by the end of 2024. Vitamin B_9, commonly known as folate, is an essential micronutrient for humans. Acting as a cofactor in one-carbon transfer reactions, it is involved in many biochemical pathways, among which the synthesis of nucleotides and amino acids. In addition to plants, many microorganisms can naturally produce it, and this can pave the way for establishing production processes. In this work, we explored the use of *Scheffersomyces stipitis* for the production of natural vitamin B_9 by microbial fermentation as a sustainable alternative to chemical synthesis.

Results: Glucose and xylose are the main sugars released during the pretreatment and hydrolysis processes of several residual lignocellulosic biomass (such as corn stover, wheat straw or bagasse). We optimized the growth conditions in minimal medium formulated with these sugars and investigated the key role of oxygenation and nitrogen source on folate production. Vitamin B_9 production was assessed first in shake flasks and then in bioreactor, obtaining a folate production up to 3.7 ± 0.07 mg/L, which to date is the highest found in literature when considering wild type microorganisms. Moreover, the production of folates was almost entirely shifted towards reduced vitamers, which are those metabolically active for humans.

Conclusions: for the first time the non-*Saccharomyces* yeast *S. stipitis* was used to produce folate. The results confirm its potential as a microbial cell factory for folate production, which can be also improved both by genetic engineering strategies and by fine-tuning the fermentation conditions and nutrient requirements.

Keywords

Nutraceuticals, Biorefinery, 5MTHF, THF, *Scheffersomyces stipitis*

1. BACKGROUND

Stephen DeFelici introduced the term “nutraceuticals” to categorize a wide range of molecules with a claimed medical or health benefit (Hugenholtz and Smid 2002). Over the past few years, a large number of new nutraceuticals has been launched on the food and pharmaceutical market, among which vitamins represent an important category. In a scenario of environmental sustainability, biotechnological production of vitamins is starting to replace the chemical synthesis (Wang, Liu et al. 2021), with interesting examples including vitamins B_2, B_{12}, C, and K (Pappenberger and Hohmann 2013, Fang, Kang et al. 2017, Averianova, Balabanova et al. 2020, Kang, Baek et al. 2022). However, a biotechnological process for the large-scale production of vitamin B_9 (or folic acid, FA) has not been implemented yet, and the market still relies on its chemical production, which requires unsustainable, petroleum-based reagents (Revuelta, Serrano-Amatriain et al. 2018).

Folate is the term encompassing the different natural forms of the water-soluble vitamin B_9. All the vitamers share a common structure consisting in a pteridine ring linked to a molecule of para-aminobenzoic acid (pABA) by a methylene bridge, and one or more glutamyl residues. Humans depend on an adequate and constant intake of this essential nutrition component, as it is a central cofactor in many metabolic reactions required for biosynthetic and cellular processes, such as methylation reactions and the synthesis of DNA, RNA and proteins (Bailey and Caudill 2012). Indeed, the National Institute of Health (NIH) suggests a Recommended Dietary Allowance (RDA) of 400 µg dietary folate equivalents (DFE)

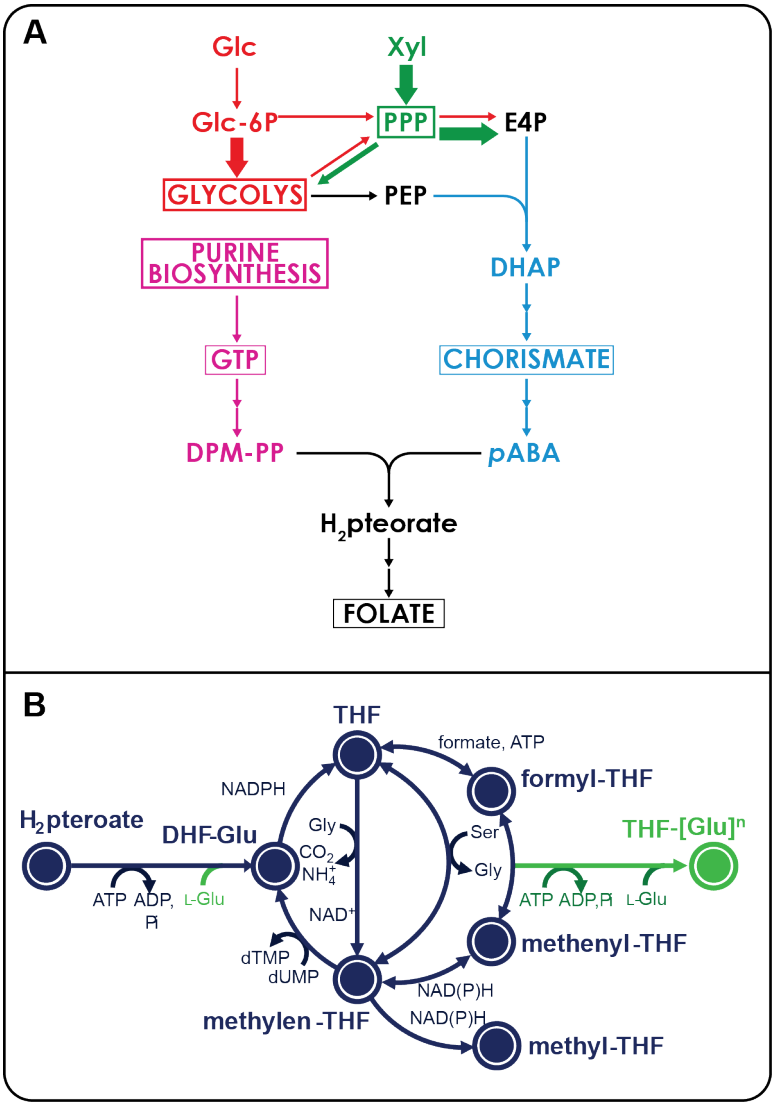
for adults; the European Union (EU) suggests an RDA of 250 µg DFR. A higher intake (600–1000 µg DFE) is advised for pregnant women (Jägerstad 2012).

Nowadays, the most widely used vitamer for the formulation of food supplements is FA; however, this form is not bioactive, since humans require the activity of dihydrofolate reductase (DHFR) in the enterocytes (Ohrvik and Witthoft 2011) to reduce FA to one of its active forms (e.g., tetrahydrofolate (THF) and 5-methyl-THF (5MTHF)). Nevertheless, the low reaction rate of DHFR (Revuelta, Serrano-Amatriain et al. 2018) limits the absorption of FA, which might lead to its accumulation in the bloodstream. This could mask a vitamin B_{12} deficiency and increase the risk of developing prostate and colorectal cancer (Choi, Yates et al. 2014, Saini, Nile et al. 2016). Food supplements enriched with natural forms of folate – which contain the bioactive vitamers – could prevent this issue. Only plants and a few microorganisms possess the entire pathway for the de novo biosynthesis of these bioactive molecules, therefore the development of more environmentally friendly processes using microorganisms to produce natural folate is becoming crucial (Revuelta, Serrano-Amatriain et al. 2018).

Many studies have focused on the use of lactic acid bacteria – such as *Lactococcus (Lc.) lactis* and *Streptococcus thermophilus* – as potential producers, since they are commonly used in fermented dairy goods and thus can be applied to fortify such products (LeBlanc, de Giori et al. 2007). However, lactic acid bacteria are well known for their limitations and challenges on an industrial scale, among which the requirement of complex nutritional media for normal growth and a still difficult optimization and control of the metabolic activities (Saeed A and Salam A 2013). Yeasts, on the other hand, are in general more robust than bacteria and less subject to contaminations, and thus are generally preferred for large-scale fermentations (Curran, Leavitt et al. 2013, Zhu, Wang et al. 2021). Conveniently, different *Saccharomyces cerevisiae* strains and other yeasts like *Metschnikowia lochheadii*,

Debaryomyces melissophilus and *Debaryomyces vanrijii* have shown to be promising for folate synthesis, with production ranging from 40 to 140 µg/g of cell dry weight (Hjortmo, Patring et al. 2005).

In this work, *Scheffersomyces stipitis* was selected as a new potential yeast platform for vitamin B_9 production. *S. stipitis* is a Crabtree negative yeast (Su, Willis et al. 2015), which means the onset of ethanol production is not correlated to the sugar concentration: this aspect is interesting from an industrial point of view, since the most commonly used yeast (i.e., *Saccharomyces cerevisiae*) requires more complex fed-batch fermentations to avoid ethanol production. Moreover, *S. stipitis* has the ability to grow on a wide panel of sugars and oligomers, containing both hexose and pentose sugars (Jeffries and Van Vleet 2009). The most interesting characteristic for this study is its high flux through the pentose phosphate pathway (Shin, Kim et al. 2019), which is relevant for the production of Vitamin B_9, since there is a higher availability of erythrose 4-phosphate (E4P), a precursor of one of the moieties of folate (Scheme 1).



Scheme 1: The figure shows the de-novo folate biosynthesis pathway in S. stipitis. (A) The two main building blocks of folate derives from GTP (highlighted in purple) and pABA (highlighted in blue). GTP is produced in the purine biosynthesis pathway. pABA derives from chorismate, the last metabolite produced in the shikimate pathway which has E4P and PEP as precursors (highlighted in black). The first intermediate is produced in the PPP pathway (highlighted in green), while the second metabolite is an important molecule mostly produced through glycolysis (highlighted in red). The arrow thickness suggests a different flux through the pathways depending on the carbon source. (B) Representation of the pathway in which the different vitamers of vitamin B9 are produced.

Glc = glucose; Xyl = xylose; Glc-6P = glucose 6-phosphate; PPP = pentose phosphate pathway; E4P = erythrose 4-phospate; PEP = phosphoenolpyruvate; DHAP = 3-deoxy-D-arabino-heptulosonate-7-phosphate; DPM-PP = (7,8-dihydropterin-6-yl) methyl diphosphate; pABA = para-aminobenzoic acid; H2-pteroate = dihydropteroate; THF = tetrahydrofolate; DHF = dihydrofolate; Gly = glycine; Ser = serine; Glu = glutamate.

In this work, we first optimized the growth of *S. stipitis* on minimal Verduyn medium formulated with 20 g/L of glucose or xylose as carbon and energy source. We assessed the key role of salts, oxygen and nitrogen source to support the growth and allow the complete consumption of the carbon source. Furthermore, we assessed *S. stipitis* growth on optimized Verduyn synthetic medium formulated with a mixture of glucose and xylose, considering the potential to use this strain for the valorization of residual lignocellulosic biomasses. We obtained a robust folate production of 3.72 ± 0.07 mg/L (192.7 ± 46.9 µg/gCDW), both in baffled flasks and bioreactor, which to the best of our knowledge is the highest found in literature when considering wild type microorganisms.

2. RESULTS

2.1 Growth optimization on glucose or xylose as carbon source

S. stipitis was grown on Verduyn minimal medium, using 20 g/L glucose or xylose as carbon source. Under these conditions, only a small consumption of the carbon source was observed (around 4 g/L), both in presence of glucose or xylose (Figure 1, A e B); the result is not entirely unexpected, as Verduyn is a medium optimized for *Saccharomyces cerevisiae*, and it is likely that *S. stipitis* exhibits different demands in terms of nutrients needed for growth.

In line with this hypothesis, by doubling the salts present in the medium (Verduyn-S), a complete consumption of glucose was observed (Figure S1A). Considering the rather slow consumption of the carbon source and the low biomass yield (Figure S1A) it was hypothesized that growth might be limited by the availability of O2, as reported by Silva, et al. 2012 (Silva, Mussatto et al. 2012).

Increasing the medium oxygenation by changing the ratio between media and flask volume, cells reached higher OD and biomass yield, despite an incomplete consumption of the carbon source and EtOH production (Figure S1B). To minimize that, the kinetics were repeated in baffled flasks that allow a greater oxygenation than the traditional ones (Li, Xia et al. 2013). Moreover, all the nutrients present in Verduyn-S medium have been doubled (Verduyn-S2), except for the carbon source, and with this formulation *S. stipitis* was able to consume all the glucose present in the medium, reaching 30.5 OD with a substrate consumption rate of 1.73 g/Lh (Figure 1C). The biomass yield, however, was lower than in the previous condition (1.36 OD/g), due to the production of EtOH, which is around 1 g/L, probably due to the high amount of biomass; once again, oxygen appears to be the most difficult parameter to control in shake flask fermentations.

Likewise, growth on xylose 20 g/L was investigated. The information obtained from the growth optimization of *S. stipitis* in Verduyn with glucose was used to optimize its growth on xylose. The kinetics were performed in baffled flasks using Verduyn-S2 medium; in these conditions, the strain exhibited a better growth, compared with the initial tested condition (Figure 1B), in spite of an incomplete xylose consumption (Figure S2).

To obtain the complete consumption of the carbon source, we tested the possibility to repeat the kinetics but changing the nitrogen source from ammonium sulfate to urea, as this formulation is often used for *S. stipitis* (Agbogbo, Coward-Kelly et al. 2006, Silva, Mussatto et al. 2012). To keep the same amount of nitrogen, 9.2 g/L urea were added to the medium (Verduyn-S2U). As shown in Figure 1D, urea allowed the complete consumption of 20 g/L xylose with a consumption rate of 2.40 g/Lh, and the biomass reached a final OD of 20.9. However, given the higher growth rate, a production of ethanol (3.59 g/L) was observed, which could explain the lower

biomass yield (0.91 OD/g). As observed previously, oxygen appears as the limiting factor for the growth in flasks.

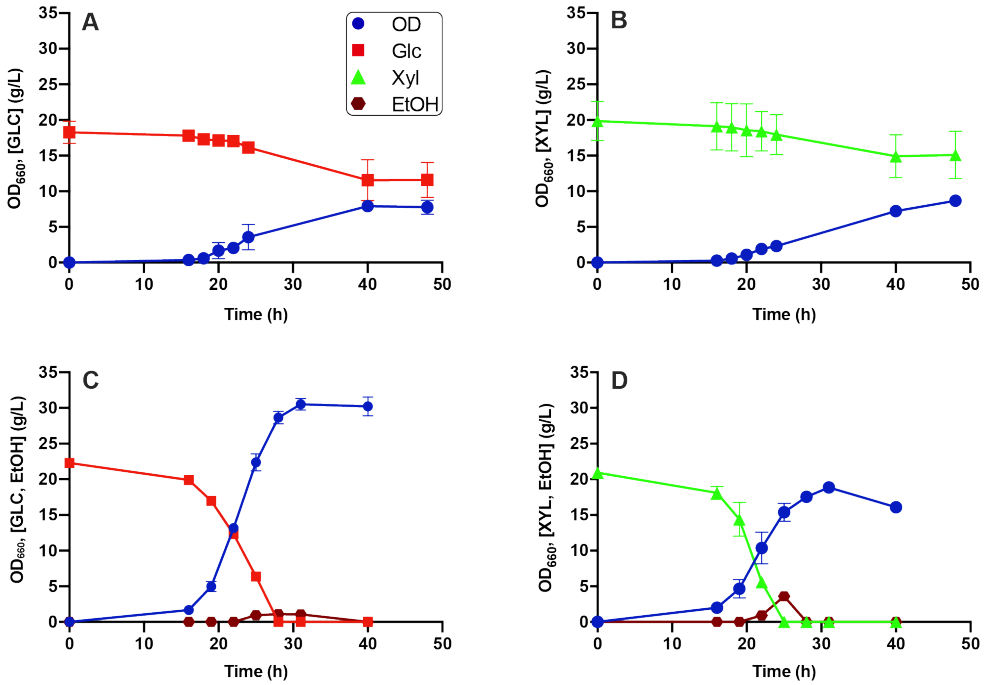


Figure 1: S. stipitis fermentation profiles in the presence of glucose or xylose. The graphs show growth in: (A) Verduyn 20 g/l glucose. (B) Verduyn 20 g/L xylose. (C Verduyn-S2 (20 g/l glucose) in baffled flasks. (D) Verduyn-S2U (20 g/l xylose) in baffled flask. Values are the mean ± standard deviation of three independent experiments.

2.2 Growth optimization on media formulated with glucose and xylose as carbon sources

In literature there are several studies on the composition of different lignocellulosic residual biomasses, see as examples (Grohmann and Bothast 1994, Van Maris, Abbott et al. 2006). As reported, in corn stover, wheat straw or bagasse and others, the glucose concentration is often double with respect to xylose. With the final aim to use *S. stipitis* to valorize different residual biomasses for vitamin B_9 production, we opted to simulate that sugar composition, formulating a minimal medium that contains glucose and xylose in a 2:1 ratio. In preliminary studies (Figure S3, A and B), *S. stipitis* showed a behavior similar to what we observed when grown on glucose or xylose alone. Since previous results showed that Verduyn-S2 medium is required to allow the consumption of 20 g/L of glucose, and since the use of urea as an alternative nitrogen source allows the complete consumption of 20 g/L of xylose, the kinetics were repeated in baffled flasks using Verduyn-S2U. We could confirm that this medium is able to support the complete consumption of the carbon sources. We also observed that xylose consumption starts only when the concentration of glucose present in the medium goes below 10 g/L. Indeed, as reported in literature, *S. stipitis* has two transport systems for sugars; one at high and one at low affinity, which operate simultaneously. Glucose inhibits xylose transport through the high-affinity system but competes with xylose for the low-affinity transport (Kilian and Van Uden 1988). Considering the data obtained with single sugars, it might be argued that the preference for glucose when present at high concentration is explained by the difference in the biomass accumulation (30.5 OD on glucose and 20.9 OD on xylose, see again Figure 1 panels C and D). This means that *S. stipitis*, although it would seem to consume glucose more slowly, prefers this sugar because it allows a faster growth and a higher biomass yield. In Verduyn-S2U

with 20 g/L of glucose and 10 g/L of xylose cell density reached 29.8 OD, with a yield of 0.92 OD/g (Figure 2). This low yield can be justified by the production of about 3.8 g/L of EtOH, once again due to insufficient oxygenation.

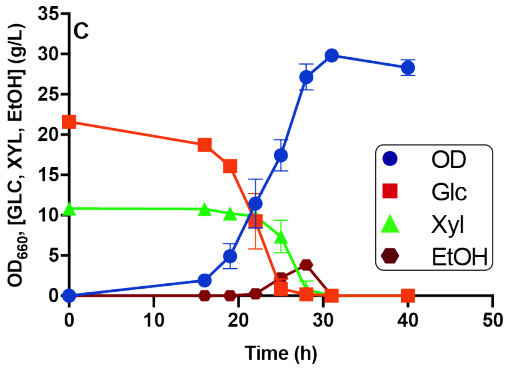


Figure 2: S. stipitis fermentation profile in the presence of glucose and xylose. The graph shows yeast growth on Verduyn-S2U Glc 20 g/L, Xyl 10 g/L in baffled flasks. Values are the mean ± standard deviation of three independent experiments.

2.3 Folate production in different growth conditions

The overall production of folate was evaluated in the most promising growth conditions, which are Verduyn-S2 glucose 20 g/L, Verduyn-S2U xylose 20 g/L, and Verduyn-S2U glucose 20 g/L and xylose 10 g/L, all in baffled flasks (see again Fig 1C, 1D, 2C). Folate concentration was measured at different time points (25, 28 and 31 hours), corresponding to Exponential Phase (EP), Late Exponential Phase (LEP) and Early Stationary Phase (ESP) of growth, respectively. We observed that the overall folate production changes in different growth conditions, the best medium being the one with xylose and urea (1D, 2C).

Figure 3A shows total folate concentration in the different media. For all the conditions tested, the highest production was obtained in the ESP (31h); in particular, production reached 0.9 ± 0.12 mg/L on glucose, 2.7 ± 0.10 mg/L on xylose

and 3.7 ± 0.07 mg/L on glucose and xylose. Due to the higher sugar concentration, growth on the mixed medium resulted in the highest folate production. Indeed, folate production was 4.5 and 1.4 times higher than in the media containing only glucose and only xylose, respectively.

Figure 3B shows the amount of the different vitamers produced by *S. stipitis* in the conditions tested in this study. After 31 hours of fermentation, in conditions 1D and 2C *S. stipitis* was able to produce 1.9 ± 0.05 and 2.7 ± 0.04 mg/L of THF, 0.6 ± 0.01 mg/L and 0.97 ± 0.08 mg/L of 5MTHF, and 0.2 ± 0.05 mg/L and 0.1 ± 0.02 mg/L of 5FTHF, respectively, while FA was not identified in any sample analyzed.

Since the total carbon concentrations provided in the different runs are different, to compare results it is important to calculate fermentation parameters, and here we used the following: product yield (YP), growth rate (μmax) and duplication time (TD). On glucose as sole carbon source the cells express the fastest μmax (0.34 h-1), which is also reflected in the shorter TD (2.0 h), but the strain has the lowest YP = 6·10-5 gP/gS. In Verduyn-S2U with xylose as carbon source the situation is reversed, as the strain presents the lowest growth rate (0.28 h-1) and a longer duplication time (2.5 h), but the highest YP = 12.8·10-5 gP/gS. In the medium with mixed sugars, we registered an intermediate performance, with a growth rate of 0.30 h-1 and duplication time of 2.3 h, but with a YP = 11.5·10-5 gP/gS, which is close to the performance reached with xylose as sole carbon source.

Considering these observations, the mixed medium with both glucose and xylose was selected for the scale up in bioreactor, in order to obtain more precise data about growth and folate production by S. stipitis, and in order to assess the possible production performances of this process.

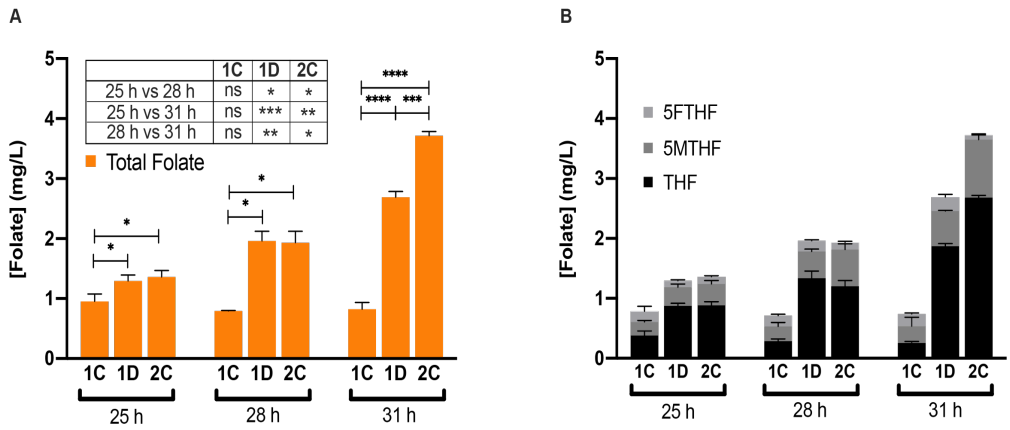


Figure 3: S. stipitis folate production and characterization over time in different growth conditions. A) Total folate production (orange bars). B) Characterization of the different folate vitamers produced. (1C) Kinetics on Verduyn-S2, Glc 20 g/L m/f = 1:5 in baffled flask; (1D) Kinetics on Verduyn-S2U, Xyl 20 g/L, m/f = 1:5 in baffled flasks; (2C) Kinetics on Verduyn-S2U, Glc 20 g/L, Xyl 10 g/L m/f = 1:5 in baffled flasks. Values are the mean ± standard deviation of three independent experiments. A two-way ANOVA was performed to analyze the effect of medium composition and growth phase on folate production.

2.4 Growth and folate production in bioreactor

To test the robustness of the process in a larger volume while assuring a full aerobic condition and to acquire data for quantitative analysis, we moved to 2L stirred tank bioreactors, growing the cells in the same medium tested in baffled flasks Verduyn-S2U (20 g/L glucose, 10 g/L xylose); results are shown in Figure 4A. Thanks to the possibility of monitoring and maintaining the desired settings for pH and dissolved oxygen, we observed the highest cell density of this study (40 OD), with no EtOH production; moreover, *S. stipitis* reached the stationary phase at 24-27 h, showing a reduced fermentation time, with a growth rate of 0.27 h-1.

Consistently with the results obtained in shake flasks, the peak for folate production was obtained in the ESP (27h), reaching a concentration of 3.4 ± 0.99 mg/L. The peak production corresponds to 192.7 ± 46.9 µg·gCDW-1, which is reflected in a specific yield of 5.6 ± 1.4 µg·gCDW-1·gS-1. Figure 4B shows the production of the

different vitamers. At 27 h (peak production), THF is the main form, with a concentration of 2.6 ± 0.9 mg/L, corresponding to 76% of the total production. 5MTHF is the second most abundant form, reaching a concentration of 0.6 ± 0.04 mg/L (18% of total production). Only a small production of 5FTHF was observed (in a range between 0.04 ± 0.049 and 0.05 ± 0.012 mg/L).

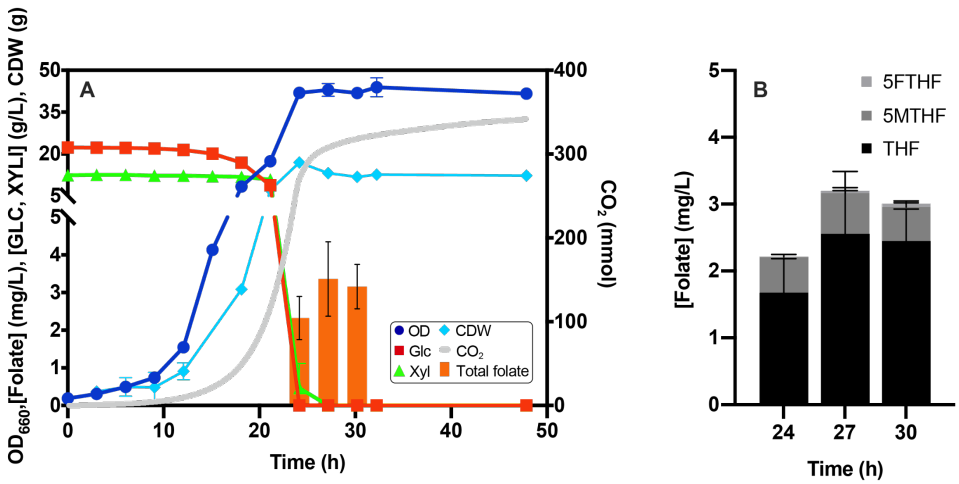


Figure 4: Fermentation of S. stipitis in bioreactor on a mixed medium. A) Fermentation profile and folate productions. B) Characterization of the different folate vitamers produced. Values are the mean ± standard deviation of independent experiments.

3. DISCUSSIONS

3.1 Growth optimization in shake flasks

In this work we investigated the ability of *S. stipitis* to produce folate, considering various advantages that this yeast can offer. Numerous non-Saccharomyces yeasts are known to have evolved in ecological niches different from those of provenance of *S. cerevisiae*: some niches are characteristic for the quality of the substrates, or for the presence of substances usually inhibiting growth, and consequently led to the selection of yeasts with interesting characteristics for growth on complex substrates and to produce new molecules (Yamakawa, Kastell et al. 2020), ideal for developing a bioprocess.

Indeed, *S. stipitis* can metabolize different sugars and a wide range of oligomers; it is one of the yeasts with the best ability to ferment xylose to ethanol (Jeffries and Van Vleet 2009), in combination with a virtual zero production of by-products in anaerobiosis, such as acetate, acetoin, 2,3-butanediol, pentitols and glycerol (Liang, He et al. 2014, Su, Willis et al. 2015). Unlike *S. cerevisiae, S. stipitis* is a negative Crabtree yeast and it has been shown in several studies (Silva, Mussatto et al. 2012, Su, Willis et al. 2015, Hilliard, Damiani et al. 2018, Shin, Kim et al. 2019) that the production of ethanol is triggered when the availability of oxygen becomes limiting, and not by high glucose concentration. This feature can be advantageous in a bioprocess because it allows to limit the production of ethanol as a carbon sink to maintain the redox balance in aerobic conditions. Another advantage of *S. stipitis* is an increased carbon flux through the Pentose Phosphate Pathway (PPP), which is common in several hemiascomycetes yeasts (Blank, Lehmbeck et al. 2005). Indeed, a profiling analysis of metabolic fluxes in *S. stipitis* showed that 41% of glucose-6-phosphate (G6P) is oxidized through PPP, while in *S. cerevisiae* the flux is only equal to half of that value (Papini, Nookaew et al. 2012); moreover, the percentage of

phosphoenolpyruvate (PEP) produced through at least one transketolase (i.e. through the PPP) is around 60% for *S. stipitis*, while for *S. cerevisiae* it is basically zero, as PEP is virtually synthesized only through glycolysis (Fiaux, Çakar et al. 2003). This characteristic is very interesting for the synthesis of folate – and more generally for the wide range of compounds that derive from the shikimate pathway – because the low availability of E4P is generally a limiting factor for this type of productions in *S. cerevisiae*; a greater flux through the PPP should be reflected in a greater availability of intermediates and, consequently, in a greater flux through the shikimate pathway. As reported by Gao and colleagues, *S. stipitis* was engineered for shikimate production in aerobic conditions, and the authors commented that in non-limiting oxygen conditions PEP and E4P precursors might be more abundant than in oxygen-limited conditions (Gao, Cao et al. 2017). Since one of the two main building blocks of folate has pABA as precursor, and since most studies focus on anaerobic fermentation, we decided to optimize the growth on minimal Verduyn medium to sustain the aerobic state, which in turn could promote folate production.

The goal in the first part of the work was to optimize the chemical-physical parameters to maximize growth and production, while at the same time deepening the physiological and metabolic aspects; in fact, defining the composition of the fermentation medium is an important step to increase productivity in bioconversion processes (Silva, Mussatto et al. 2012).

Table S2 summarizes the results obtained in shake flask fermentations. Growth optimization on glucose mainly required additional nutrients to sustain a high biomass production, combined with an increased oxygenation to avoid the production of ethanol as a carbon sink.

The fermentations on xylose under full aerobiosis, however, allowed us to observe an unusual behavior: growth speed and xylose consumption were about three

times smaller when compared with the same conditions using glucose as carbon source. The use of urea as an alternative nitrogen source to ammonium sulfate was essential to allow fast growth and the complete consumption of xylose. This behavior is still not clear to us, however the complete consumption of xylose with urea as a carbon source could be explained by the different energetics of the metabolism when urea is the nitrogen source (Figure 5). Ammonia (NH_3) has a pKa of 9.25, so the concentration of ammonium (NH_4^+) remains virtually the same for pHs between 3-7 (Cueto-Rojas, Milne et al. 2017), indicating that most of the nitrogen supplied as $(NH_4)_2SO_4$ is present as an ammonium ion. In *S. cerevisiae*, ammonium uptake occurs by facilitated diffusion thanks to an ammonium permease, encoded by *MEP*2 and *MEP*1. The accumulation of intracellular NH_4^+ is favored by the negative membrane potential, but the maintenance of homeostasis requires the pumping of a proton to the outside by the H^+-ATPase Pma1, with a net consumption of 1 ATP for every ammonium ion introduced (Cueto-Rojas, Milne et al. 2017). Ammonia can permeate the cell membrane by diffusion, but in *S. cerevisiae* the balance is shifted towards the export (Cueto-Rojas, Milne et al. 2017). Therefore, the uptake of 1 mole of ammonium requires 1 mole of ATP and causes the acidification of the growth medium. The same genes coding for the ammonium permease (*MEP*2, *MEP*1) and for the H^+-ATPase Pma1 (*PMA*1) are present in *S. stipitis* as well (Kanehisa and Goto 2000), so it is possible to hypothesize that the ammonium uptake mechanism is the same. On the other hand, in *S. stipitis* urea uptake happens through a urea permease, encoded by the *DUR*3 and *DUR*4 genes (Jeffries and Van Vleet 2009) and the transport occurs via a simport with an H^+ ion (Kanehisa and Goto 2000). Since budding yeasts lack the urease enzyme, urea amidolysis (encoded by *DUR*1 and *DUR*2) is required for the metabolism of urea (Sibirny 2019). This bifunctional enzyme first catalyzes an ATP-dependent carboxylation to produce allophanate (urea-1-carboxylate), and subsequently

catalyzes its decarboxylation to 2 ammonium ions and CO_2 (Sibirny 2019). It is interesting to note the elegant stoichiometry of urea uptake and its subsequent transformation reactions (Figure 5) (Caspi, Billington et al. 2018): the proton imported from the permease and the two protons generated by the carboxylation are all used in the decarboxylation step, with a neutral balance of protons. We then hypothesize that the uptake of urea does not require to be coupled to an active transport of protons. Therefore, the uptake of 1 mole of urea (which produces 2 moles of NH_4^+) requires only 1 mole of ATP, half of the energy required for ammonium uptake; moreover, the uptake of urea causes an increase – rather than a decrease – of the medium pH, which might cause less stress to the cells when compared to the growth at a low pH. It is therefore possible to hypothesize that the complete consumption of the carbon source is due to a greater availability of energy. This behavior could also be enhanced by the probable different gene expression with the two nitrogen sources (da Cruz, Cilli et al. 2002). Moreover, the shift in specificity of xylose reductase (XR) towards NADPH, may play a role in cofactor imbalance in the presence of high concentrations of xylose and oxygen (see Figure S4 and related comments for further details).

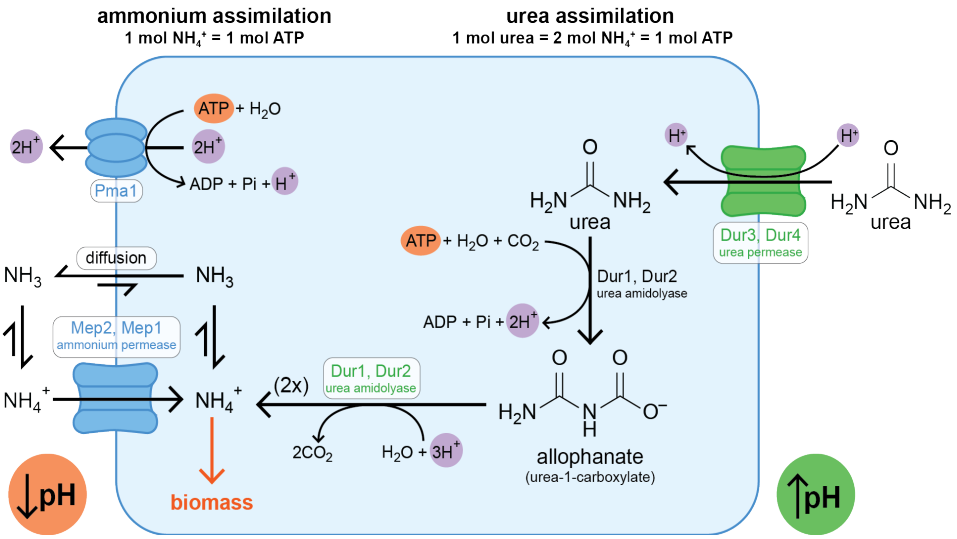


Figure 5: Proposed putative pathway for ammonium and urea uptake in S. stipitis. Ammonium (left) is internalized in the cell by facilitated diffusion via ammonium permease; to maintain the potential difference across the plasma membrane the H+-ATPase Pma1 expels a proton, causing acidification of the growth medium. Urea (right) is internalized through a symport with an H+ ion (causing an increase in the pH of the growth medium), and it is converted into ammonium via urea amidolyase.

3.2 Folate production

In this work the aerobic growth of *S. stipitis* in Verduyn-based medium formulated with different carbon and nitrogen sources was studied. Folate production was evaluated in the optimized conditions at different time points by using a HPLC-UV method to measure four different folate vitamers (THF, 5MTHF, 5FTHF and FA), as this is relevant for assessing their bioactivity. In addition, the occurrence of the target analytes quantified in different growth conditions were qualitatively confirmed by exploiting a HRMS system (High Resolution Mass Spectrometry) (see Par. 5.5.4). Table S3 and Figure S5 report the MS and MSMS spectra of the analytes identified in yeast medium.

Hjortmo and co-workers (Hjortmo, Patring et al. 2008) reported that in *S. cerevisiae* the folate production peak is reached at the early stationary phase. Indeed, we

observed a similar behavior with *S. stipitis*, reaching peak production at the ESP, right after the depletion of the carbon source and the rapid consumption of ethanol. To the best of our knowledge, this is the first study investigating *S. stipitis* ability to produce folate. Furthermore, it is also the first study in which the different folate vitamers produced by *S. stipitis* were characterized, showing that this yeast mainly produces vitamers in their reduced forms, such as THF, 5MTHF, and 5FTHF even though yeast growth is pursued in aerobiosis. Figure 3B shows that medium composition and growth phase significantly affects the level of the different forms produced. While on glucose *S. stipitis* produced more balanced amounts of the various forms, in the media containing xylose and urea the production is biased towards THF.

This behavior was confirmed during bioreactor fermentation. Indeed, we observed a comparable folate production with respect to the same growth medium in shake flasks (2C), suggesting that this process is robust and scalable. More interestingly, the bioreactor setup provided better growth conditions which in turn allowed a shorter fermentation time and an improvement in the productivity, probably due to the lack of ethanol production.

Table 1 shows the highest folate productions found in literature, obtained with different microorganisms, ranging from bacteria, to yeasts, to filamentous fungi. The productions obtained in the present study with *S. stipitis* on minimal medium are among the highest reported in literature, generally being around 10 times higher than the ones obtained with other microorganisms. The only exception is an engineered strain of the filamentous fungi *Ashbya gossypii* (Serrano-Amatriain, Ledesma-Amaro et al. 2016), which reaches a production of 6.60 mg/L of folate, exceeding approximately 2 times our results. However, in addition to being engineered, A. gossypii requires an adenine auxotrophy to sustain such a high

production, combined with the supplementation of pABA, with consequences on economics of the possible process.

Table 1: Folate productions of different organisms reported in literature. Reported productions of this study represent the sum of single vitamers titles.

Microorganism	Medium	[Sugars] (g/L)	Production	Ref.
S. stipitis	Verduyn-S2	Glc 20	0.95 ± 0.12 mg/L	This study
	Verduyn-S2U	Xyl 20	2.69 ± 0.10 mg/L	This study
	Verduyn-S2U	Glc 20; Xyl 10	3.72 ± 0.07 mg/L	This study
(bioreactor)	Verduyn-S2U	Glc 20; Xyl 10	3.36 ± 0.99 mg/L (192.7 ± 46.9 $\mu g/g_{CDW}$)	This study
S. cerevisiae	YPD	Glc 20	0.25 mg/L	(Hjortmo, Patring et al. 2008)
	Verduyn 2X	Glc 20	0.36 mg/L	(Hjortmo, Patring et al. 2008)
Yarrowia lipolytica	YPD	Glc 20	0.3 ± 0.1 $\mu g/g_{CDW}$	(Jach, Sajnaga et al. 2021)
Metschnikowia lochheadii	CBS modified	Glc 20	90 $\mu g/g_{CDW}$	(Hjortmo, Patring et al. 2005)
Escherichia coli	M9	Glc 4	0.27 mg/L	(Zhu, Koepsel et al. 2003)
Streptococcus thermophilus	M17	Lac 5	0.20 mg/L	(Sybesma, Starrenburg et al. 2003)
Lactococcus lactis	M17	Lac 5	0.29 mg/L	(Sybesma, Starrenburg et al. 2003)
A. gossypii	MA2 + *p*ABA	Glc 10	6.60 mg/L	(Serrano-Amatriain, Ledesma-Amaro et al. 2016)

4. CONCLUSIONS

To the best of our knowledge, this is the first report exploiting the yeast *S. stipitis* for the production of folate. We formulated media considering the main sugars present in lignocellulosic biomasses as carbon sources. The different optimizations in shake flasks allowed the selection of the best condition (Verduyn-S2U medium with 20 g/L of glucose and 10 g/L of xylose, Y_P = $11.5 \cdot 10^{-5}$ g_P/g_S and growth rate 0.30 h^{-1}) to be repeated and studied in detail in bioreactor. The production obtained in bioreactor in the present study with *S. stipitis* on minimal medium (3.36 ± 0.99 mg/L, or 192.7 ± 46.9 $\mu g/g_{CDW}$) is the highest reported in literature comparing wild type microorganisms.

Furthermore, the production of folate was found to be predominantly shifted towards vitamers in their reduced forms (2.6 ± 0.9 mg/L of THF, 0.6 ± 0.04 mg/L of 5MTHF and 0.04 ± 0.049 mg/L of 5FTHF), making *S. stipitis* a very promising organism from a nutraceuticals market since the majority of folate available in food supplements tend to be in the oxidized forms, thus requiring an endogenous reduction step to be metabolically active. The obtained results demonstrate the potential of *S. stipitis* as a microbial cell factory for natural folate production, which can reasonably be further improved by genetic engineering strategies and/or by fine tuning the fermentation conditions and nutrient requirements.

5. MATERIALS AND METHODS

5.1 Strain and medium compositions

The haploid, Crabtree negative yeast *S. stipitis* (culture collection CBS 6054) was used in the experiments. The strain was maintained in 20% (v/v) glycerol at -80 °C (Master Cell Banks, MCB) after growth in YPD medium composed of (per liter): yeast extract 10 g, tryptone 20 g and glucose 20 g.

Defined synthetic media Verduyn (Verduyn, Postma et al. 1992) were composed of (per liter): $(NH4)_2SO_4$ 5 g; KH_2PO_4 3 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; trace elements 1X (EDTA 30 mg; $ZnSO_4 \cdot 7H_2O$ 9 mg; $CoCl_2 \cdot 6H_2O$ 0.6 mg; $MnCl_2 \cdot 4H_2O$ 2 mg; $CuSO_4 \cdot 5H_2O$ 0.6 mg; $CaCl_2 \cdot 2H_2O$ 9 mg; $FeSO_4 \cdot 7H_2O$ 6 mg; $Na_2MoO_4 \cdot 2H_2O$ 0.8 mg; H_3BO_3 2 mg; KI 0.2 mg); vitamins 1X (D-biotin 0.10 mg; calcium D-pantothenate 2 mg; nicotinic acid 2 mg; myo-inositol 50 mg; thiamine hydrochloride 2 mg; pyridoxal hydrochloride 2 mg; *para*-aminobenzoic acid 0.4 mg. Depending on the experiment, the carbon sources used were (per liter): glucose 20 g; xylose 10 g or 20 g; or glucose 20 g and xylose 10 g. The pH of the media was adjusted to 5.5.

Growth optimization required as first to doubling the concentrations of some salts as follows: $(NH4)_2SO_4$, 10 g; KH_2PO_4, 6 g; $MgSO_4 \cdot 7H_2O$, 1 g, obtaining what we called "Verduyn-S" medium. Furthermore, the final growth and production runs have been obtained in Verduyn-S2, where in addition to salts also trace elements and vitamins present in the Verduyn-S have been doubled; finally, when indicated ammonium sulfate has been replaced with 9.2 g/L of urea (Verduyn-S2U). Table S1 summarizes all the media compositions used in this study.

Yeast extract was purchased from Biolife Italia S.r.l., Milan, Italy. All other reagents were purchased from Sigma- Aldrich Co., St Louis, MO, USA.

5.2 Growth conditions in shake flasks

Seed cultures from YPD plates were initially grown overnight in glass tubes in 2 mL YPD; cells were then inoculated for the intermediate inoculum (starting OD 0.2) in 50 mL glass tubes containing 5 mL of the same culture medium used for the subsequent run and grown for 8 h: this step is important to accustom the cells to the final culture conditions. Cells were then inoculated in 250 mL (baffled) shake flasks (starting OD 0.001), filled with 50 mL of the minimal medium under investigation. All growths were performed in a rotary shaker at 160 rpm and 30 °C.

5.3 Growth conditions in batch bioreactors

For batch fermentations, 2 L stirred tank bioreactors (BIOSTAT® A plus, Sartorius Stedim Biotech GmbH, Goettingen, Germany) equipped with Visiferm DO ECS 225 for pO2 measurement and Easyferm Plus K8 200 for pH measurement (both from Hamilton Bonaduz AG, Bonaduz, Switzerland) were used at a working volume of 1 L. The temperature was kept constant at 30 °C and pH was set to 5.5, maintained by automatic addition of 5M KOH and 5M HCl. The stirring rate was set to 300 rpm in cascade to maintain the oxygen concentration, which was set to 30% of saturation, to guarantee a completely aerobic condition to the cell culture. Filtered air (pore size 0.2 µm) was continuously sparged through the reactor at a flow rate of 1 vvm. Foam formation was controlled by the addition of an emulsifier agent (Triton 100X, Fisher reagents) and a silicon agent (Polydimetylsiloxane, Sigma-Aldrich). MCB was restreaked in YPD plates. A single colony was taken and grown in YPD liquid medium overnight. From this culture, individual aliquots of 1.3 mL were created and kept in 20% (v/v) glycerol at -80 °C (Working Cell Banks, WCB).
WCB were defrozen and inoculated directly in 50 mL glass tubes containing 5 mL of YPD and incubated for 8 h. Cells were then inoculated in 500 mL shake flasks in 100 mL of minimal medium for the intermediate cultures (starting OD 0.04) and grown

overnight. For the inoculum, cells were harvested, washed twice with physiological solution (0.9% NaCl), and used to inoculate the bioreactor (starting OD 0.25). Samples were collected every three hours until 30 h and then at the end of the fermentations, stopped at 48 h.

5.4 Biomass and metabolites quantification

S. stipitis growth was followed by measuring the optical density at 660nm (OD660) (UV-1800; Shimadzu, Kyoto, Japan). For bioreactor experiments cellular dry weight (CDW) was estimated by a correlation with OD660, using the equation: CDW = 0.3998OD + 0.1307.

HPLC analyses were performed to quantify the amount of glucose, xylose, ethanol. Prior to analysis, all samples obtained in Par 5.3 were centrifuged (14000 rpm, 10', 4°C) and filtered with 0.2 µm PTFE filters (AISIMÔ CORPORATION CO., LTD). For the analysis of glucose, xylose, and ethanol, a Rezex ROA-Organic Acid H+ column (00H-0138-KO) 300 x 7.8 mm, 8 µm (Phenomenex, USA) coupled with a precolumn Micro-Guard Cation H+ refill cartridges 30 x 4.6 mm, 8 um (Biorad, USA) was injected with 20 µL of sample. The mobile phase was H_2SO_4 0.01 M pumped isocratically at a flow of 0.5 mL/min for 40 min. Column temperature was kept at 40 °C. Separated components were detected by a refractive index detector (RID) and peaks were identified by comparison with known reference standards dissolved in Ultrapure H2O (18 MΩ) obtained by using a Milli-Q purification system (Millipore, Bedford, USA). Calibration curves were prepared in a range between 20 and 0.625 g/L.

5.5 Total folate determination and quantification

5.5.0 Reagents

All reagents were purchased from Merck, Germany, apart from Ultrapure H_2O (18 MΩ) obtained by a Milli-Q purification system (Millipore, Bedford, USA), THF purchased by Schircks Laboratories, Switzerland, 5MTHF and 5FTHF obtained from US Pharmacopeia, USA. MS grade solvents were obtained from Romil SpA, Italy.

5.5.1 Solutions preparation

- Potassium phosphate buffer 1M (Buffer P) was prepared by adjusting the pH of a solution of KH_2PO_4 1M to 6.4 by adding H_2KPO_4 1M; ascorbic acid was then added to a final concentration of 10% (w/v); the solution was filter-sterilized and stored at 4 °C.

- Purification of rat serum for polyglutamyl-folate deconjugation was adapted from Patring, et al. 2005 (Patring, Jastrebova et al. 2005). Briefly, 1 mL of activated charcoal is added to 10 mL of rat serum and allowed to react for 1h at 4 °C with constant stirring; the solution was then filtered, divided into 2 mL aliquots, and stored at -20 °C.

5.5.2 Sample preparation

3 mL or 10 mL of culture suspension were collected from shake flasks or bioreactor, respectively. To avoid folate degradation in samples before and during the analysis, 10% of Buffer P 1M were added to each sample and N2 was sparged to replace the oxygen present in the medium; samples were stored at -80 °C and thawed prior to use.

The measurement of the total folate produced requires the release of intracellular folate directly into the culture supernatant (in which extracellular folates are

already present). The protocol was adapted from Patring, et al. 2005 (Patring, Jastrebova et al. 2005); intracellular folates were extracted by heating at 100°C for 15'; after centrifugation (4 °C, 14000 rpm, 10 min) 1 mL of the supernatant was treated with 50 µL of rat serum at 37°C for 3 h. After filtration the sample was used for the analysis. The deconjugation step with rat serum is required to obtain all the present folates in the mono-glutamate forms: this allowed the further quantitative analysis at HPLC with the direct standards correlation.

5.5.3 Solid Phase Extraction

Solid Phase Extraction (SPE) was performed by using IsoluteTM SAX cartridge (Biotage, Sweden) with strong anion-exchange sorbent 500mg/3mL. For elution under reduced pressure, a Sigma vacuum manifold was used. The clean-up procedure was as follows: the SPE cartridge was activated and conditioned by sequential elution of 5 mL of MeOH and H2O and phosphate buffer 0.1 M (Na_2HPO_4) containing 1% ascorbic acid w/v (pH 7) without allowing the column to run dry. Then, samples obtained in Par 5.5.2 were equilibrated to pH 7 by a few drops of NaOH 5 M and then loaded and passed through the cartridge adjusting the vacuum, to keep a constant flow rate of 2-3 drops per second. When all the sample was eluted, the cartridge was washed with 5mL of phosphate buffer 0.1 M (pH 7) and completely dried. Analytes were eluted into a glass vial with 5 mL of 0.1 M sodium acetate containing 10% (w/v) sodium chloride and 1% (w/v) ascorbic acid. Prior to HPLC analysis, all samples were filtered through a 0.5 µm Millipore filter (Bedford, MA, USA).

5.5.4 LC-MS separation and qualitative analysis

The identification of folic acid vitamers in the samples was carried out using a Waters ACQUITY UPLC system coupled with a Waters Xevo G2-XS QTof Mass Spectrometer (Waters Corp., Milford, MA, USA). All analytes were separated on a UPLC system equipped with a Zorbax SB-C18 column (100 mm × 2.1 mm, 3.5 µm). The mobile phases were both MS grade H2O (A) and MeOH (B), both containing 0.1% formic acid (HCOOH), with gradient elution as follows: 0–2.0 min, 5–10% B; 2.0–17.0 min, 10–35% B; 17.0–18.0 min, 35%–95%. After each run the column was washed for 5 mins (95% B) and then equilibrated for further 5 mins at the initial conditions (5% B) before the next sample injection. Elution was performed at a flow rate of 0.5 mL/min, and the injection volume was 5 µL. The column temperature was set at 30°C. The Xevo G2-XS QTof Mass Spectrometer, equipped with an ESI source, was used in negative ionization mode to acquire full-scan MS and the spectra were recorded in the range of m/z 100–1000. The source parameters were as follows: electrospray capillary voltage 2.5 kV, source temperature 150°C, and desolvation temperature 500°C. The cone and desolvation gas flows were 10 and 1000 L/h, respectively. A scan time of 0.5 s was employed. The cone voltage was set to 60 V, and ramping collision energies ranged from 6 to 30 V to produce abundant ions before detection at the ToF. The mass spectrometer was calibrated with 0.5 M sodium formate and leucine-enkephalin (100 pg/µl) was used as LockMass (m/z 554.2615, 2 kV ionization voltage), which was infused simultaneously with the flow of column at 10 µl/min and acquired for 1 s each 10 s. The base peak chromatograms (BPI) were acquired at low (6) and high (30) energy from which the peaks identification was performed. Folate vitamers identity was confirmed by exploiting analytical standards as reference. Standard solutions were prepared by dissolving 5MTHF, 5FTHF, THF and FA in H2O (pH = 9) containing 1%

(w/v) sodium ascorbate. The MassLynx software (version 4.2) was used for instrument control, data acquisition and data processing.

5.5.5 Quantitative analysis by HPLC-UV

To determine the amount of folic acid vitamers in samples a 1260 Infinity HPLC system (Agilent Technologies, USA) coupled to a UV detector was exploited. The analytes were separated by a Zorbax SB-C18 column (4.6 x 250 mm, 5 µm) coupled with a precolumn (4.6 x 12.5 mm, 5 µm) both purchased from Agilent Technologies, USA. The mobile phases were: aqueous TBS (Tetrabutylammonium sulfate) 5 mM, K_2HPO_4 3 mM, KH_2PO_4 3 mM pH 7.5 (A) and MeOH (B), with gradient elution as follows: 0-11.0 min, 10-55% B; 11-13 min 55-60%, 13-15 min 55-95% B, 15-18 min 95% B and 18-19 min 95-10% B, 2 and 8 min of equilibration (10% B) was performed before the next sample injection. Elution was performed at a flow rate of 1 mL/min and the injection volume was 100 µL. The column temperature was set at 30°C. UV spectra were acquired in the range of 190 – 600 nm and two wavelengths, 280 and 310 nm, were employed for the detection of target analytes. A calibration curve for each analyte was made in a range between 0.1 and 10 µg/mL and regression coefficients were used only if associated with a R2 > 0.98.

5.6. Fermentation analysis

Specific growth rate (μ_{max}) was calculated mathematically by an equation obtained from plotting values of OD vs. time on Excel.

Folate yields on consumed sugars (here Y_p) were calculated using equation (1):

(1) $Y_P = F_p/\Delta_{sug} \cdot 100$

Where F_p is the amount of folate produced and Δ_{sug} the amount of sugar consumed.

Duplication time (T_d) was calculated using equation (2):

(2) $T_d = \ln 2 / \mu_{max}$

Consumption rate (C_r) was calculated using equation (3):

(3) $C_r = \Delta_{sug}/\Delta t$

Where Δt is the time interval corresponding to the exponential phase of the growth kinetics.

5.7. Statistical analysis

A two-way ANOVA was performed to analyze the effect of medium composition and growth phase on folate production followed by a post hoc Tukey-Kramer test for multiple comparisons. P-values are represented as follows: P>0.05, ns; P≤0.05, *; P≤0.01, **; P≤0.001, ***; P≤0.0001, ****.

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SUPPLEMENTARY FILES

Table S1. *Composition of the different synthetic media used in this study.*

	Verduyn	Verduyn-S	Verduyn-S2	Verduyn-S2U
$(NH_4)_2SO_4$	5	10	20	-
Urea	-	-	-	9,2
KH_2PO_4	3	6	12	12
$MgSO_4 \cdot 7H_2O$	0,5	1	2	2
Trace elements	1X	1X	2X	2X
Vitamins	1X	1X	2X	2X

Trace elements 1X (per liter): EDTA, 30 mg; ZnSO4·7H2O, 9 mg; CoCl2·6H2O, 0.6 mg; MnCl2·4H2O, 2 mg; CuSO4·5H2O, 0.6 mg; CaCl2·2H2O, 9 mg; FeSO4·7H2O, 6 mg; Na2MoO4·2H2O, 0.8 mg; H3BO3, 2 mg; KI, 0.2 mg. Vitamins 1X (per liter): (D-biotin, 0.10 mg; calcium D-pantothenate, 2 mg; nicotinic acid, 2 mg; myo-inositol, 50 mg; thiamine hydrochloride, 2 mg; pyridoxal hydrochloride, 2 mg; para-aminobenzoic acid, 0.4 mg. Values are expressed in g/L.

Table S2. *Growth conditions, biomass yields and folate production on minimal Verduyn medium.*

	Condition	Initial C/N (mol/mol)	Oxygenation	Sugars consumption (g/L)	OD	Y_X (OD/g)	Total folate (mg/L)	EtOH production (g/L)
1A	Glc 20 g/L (Verduyn)	8,8	m/f = 2:5	20	7,91	0,43	-	
S1A	Glc 20 g/L (Verduyn-S)	4,4	m/f = 1:5	18	22,23	1,24	-	3.2 ± 0.23
S1B	Glc 20 g/L (Verduyn-S)	4,4	baffled	10	21,27	2,05	-	
1C	Glc 20 g/L (Verduyn-S2)	2,2	baffled	20	30,5	1,36	0.8 ± 0.11	1.1 ± 0.01
1B	Xyl 20 g/L (Verduyn)	8,8	baffled	10	12,64	1,29	-	
S2	Xyl 20 g/L (Verduyn-S2)	2,2	baffled	15	23,04	1,51	-	
1D	Xyl 20 g/L (Verduyn-S2U)	2,7	baffled	20	19,02	0,91	2.7 ± 0.10	3.6 ± 0.12
S3A	Glc 20 g/L + Xyl 10 g/L (Verduyn)	13,2	m/f = 2:5	20 (Glc)	7,86	0,41	-	
S3B	Glc 20 g/L + Xyl 10 g/L (Verduyn-S2)	3,3	m/f = 1:5	10 (Glc)	20,08	1,56	-	
3	Glc 20 g/L + Xyl 10 g/L (Verduyn-S2U)	3,8	baffled	20 (Glc) + 10 (Xyl)	29,83	0,92	3.7 ± 0.07	3.8 ± 0.39

Table S3. *LC/MS based qualitative determination of folic acid vitamers in S. stipitis samples in negative ion current.*

Vitamer	Retention time	Formula	$[M-H]^-$	MS/MS fragments	Reference
THF	3,78	$C_{19}H_{23}N_7O_6$	444,44	357, 315, 272, 228, 128	STD
5MTHF	4,74	$C_{20}H_{25}N_7O_6$	458,46	371, 329, 286, 242, 128	STD
5FTHF	8,38	$C_{20}H_{23}N_7O_7$	472,44	357, 315, 271, 228, 128	STD

Figure S1: The figure shows the fermentation profile on (A) Verduyn-S 20g/L glucose m/f 1:5 and (B) Verduyn-S 20g/L glucose in baffled flasks. Figure key: OD (full blue circles), glucose consumption (full orange squares), ethanol production (dark brown hexagons).

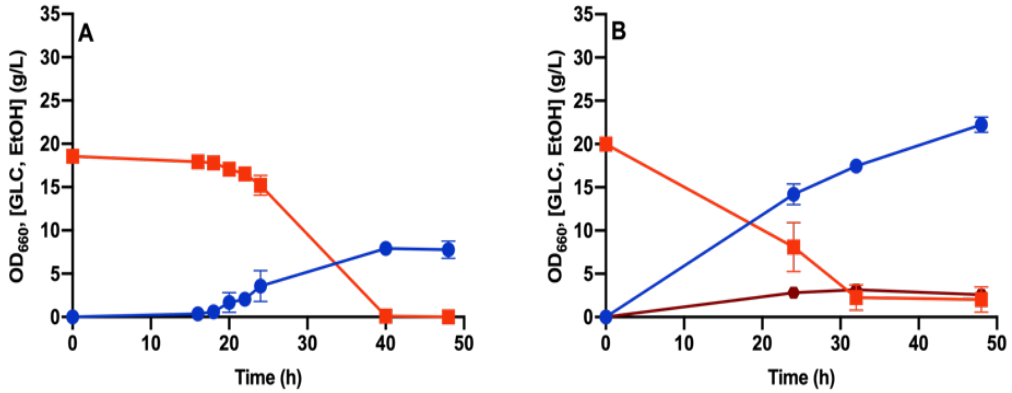


Figure S2: The figure shows the fermentation profile on (A) Verduyn-S2 20 g/L xylose in baffled flasks. Figure key: OD (full blue circles), xylose consumption (full green triangles).

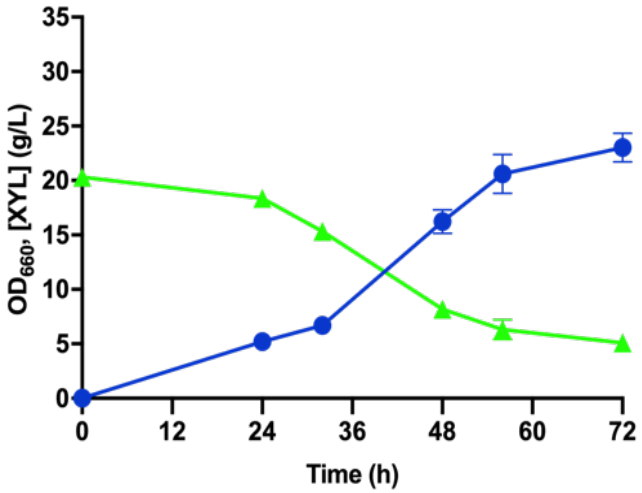


Figure S3: The figure shows the fermentation profile on (A) Verduyn 20g/L glucose + 10 g/L xylose in baffled flasks and (B) Verduyn-S2 20g/L glucose + 10 g/L xylose in baffled flasks. Figure key: OD (full blue circles), glucose consumption (full orange squares), xylose consumption (full green triangles).
In Verduyn medium, glucose was consumed within 50 h, the final OD reached was 7.9, with a biomass yield of 0.41 OD/g; xylose consumption was observed only after glucose depletion, due to glucose catabolite repression mechanism; however, S. stipitis was able to consume only 1.5 g/L of xylose (Figure S3, A). Accordingly, the growth profile and biomass yield are very similar to those observed on glucose alone (Figure 1A).
Given the positive effect of high oxygenation and the results obtained on individual sugars, the kinetics were repeated in baffled flasks (Figure S3, B). The expected result was the rapid consumption of 10 g/L of glucose, followed by a slowdown in growth, as observed previously. The observed behavior, however, is again unexpected: S. stipitis did consume 10 g/L of glucose, but it exhibited a much slower growth, while achieving a high biomass titer and yield (20.1 OD; 1.56 OD/g). This behavior is similar to the one observed in the presence of xylose 20 g/L alone (Figure 1B).

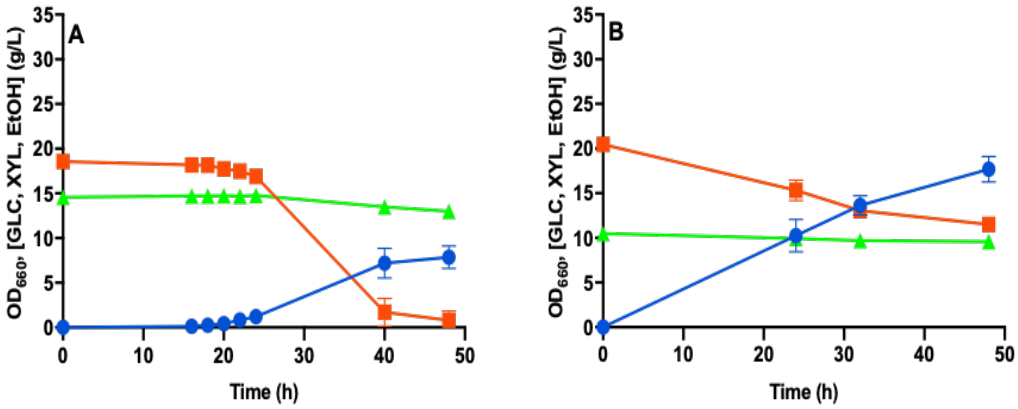


Figure S4: Cofactor imbalance for xylose assimilation under different oxygen conditions.
The different energetics of the uptake of ammonium sulfate and urea partially explain our observations regarding fermentation profiles under different oxygenation. It is necessary to consider that nitrogen metabolism is strictly regulated by the Nitrogen Catabolite Repression (da Cruz, Cilli et al. 2002) and that in S. cerevisiae it has been observed that the gene expression pattern is different when the available nitrogen source is ammonium or urea (Godard, Urrestarazu et al. 2007). This implies that the higher consumption of xylose is probably due to other, more complex regulatory phenomena as well.
Indeed, these observations are not sufficient to explain why this behavior occurs only in the presence of high concentrations of xylose, and not in the other conditions that were tested. While glucose requires two phosphorylation steps to be metabolized, xylose requires a reduction to xylitol (by xylose reductase, XR) and a subsequent oxidation to xylulose (by xylose dehydrogenase, XDH). The XR requires NAD(P)H as cofactor, while the XDH cofactor is only NAD+ dependent. Interestingly, the specificity of XR for NADH is greater when growth is limited by oxygen, as NADH cannot be readily re-oxidized on the electron transport chain; when oxygen uptake is sufficient, NADH is less available and causes a specificity shift of XR towards NADPH, with a consequent increase in flow through the PPP (Hilliard, Damiani et al. 2018, Shin, Kim et al. 2019). These observations could justify the different behavior observed in the presence of xylose or glucose, due to a possible imbalance of the redox cofactors in the presence of high concentrations of xylose and oxygen.

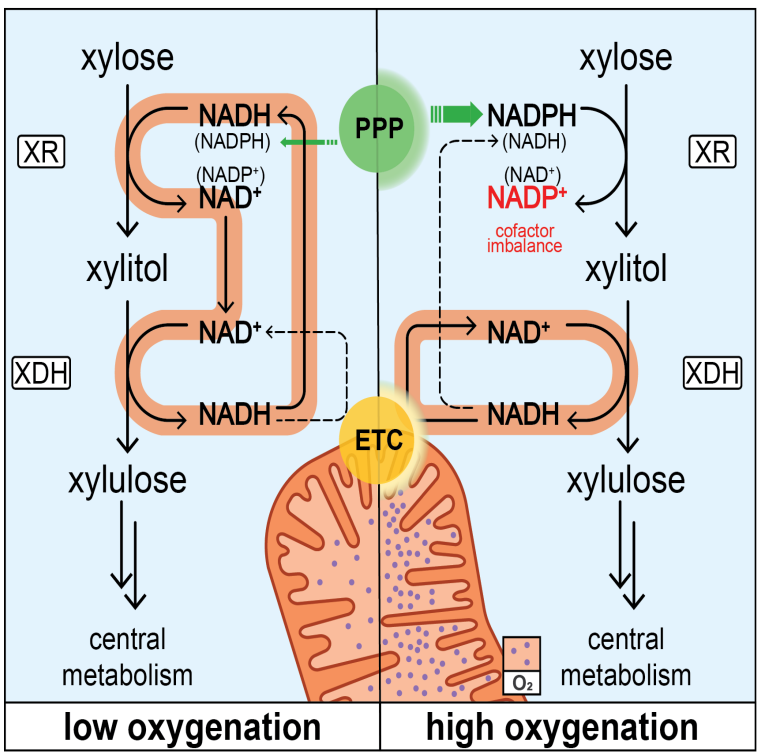
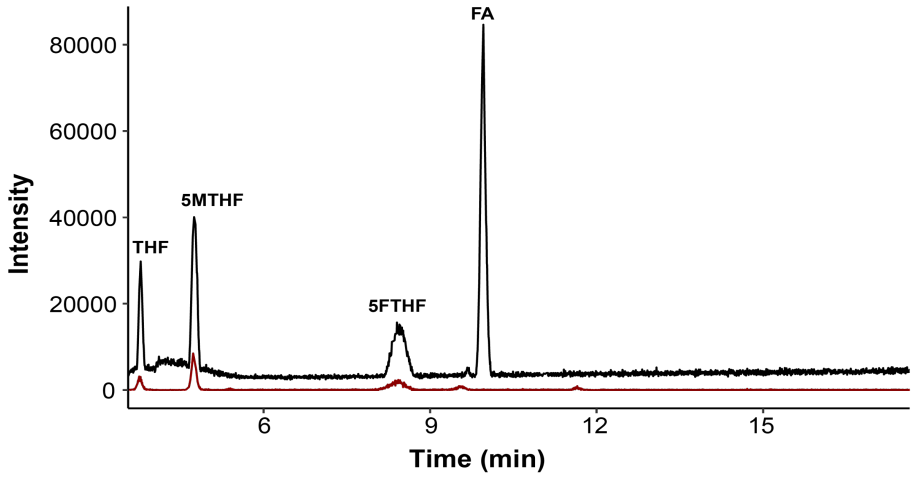


Figure S5: EIC (Extracted Ion Chromatogram) in negative ion current of a representative sample (in brown) showing the occurrence of the three reduced vitamers and the absence of folic acid compared with the reference standards (in black).



Chapter 3

Scheffersomyces stipitis ability to valorise different residual biomasses for Vitamin B_9 production

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ABSTRACT

Background

Sugar beet pulp (SBP), sugar beet molasses (SBM), and unfermented grape marcs (UGM) represent important waste in the agro-food sector. If suitably pretreated, hexose and pentose sugars can be released in high quantities and can subsequently be used by appropriate cell factories as growth media and for the production of (complex) biomolecules, accomplishing the growing demand for products obtained from sustainable resources. One example is vitamin B_9 or folate, a B-complex vitamin currently produced by chemical synthesis, almost exclusively in the oxidized form of folic acid (FA). It is therefore desirable to develop novel competitive strategies for replacing its current fossil-based production with a sustainable bio-based process.

Objectives and Results

In this study, we assessed the production of natural folate by the yeast *Scheffersomyces stipitis*, investigating SBM, SBP and UGM as potential growth media. Pretreatment of SBM and SBP had previously been optimized in our laboratory; thus, here we focused only on UGM pretreatment and hydrolysis strategies for the release of fermentable sugars. Then, we optimized the growth of *S. stipitis* on the three media formulated from those biomasses, working on inoculum pre-adaptation, oxygen availability and supplementation of necessary nutrients to support the microorganism. Folate production, measured with a microbiological assay, reached 188.2 ± 24.86 µg/L on SBM, 130.6 ± 1.34 µg/L on SBP and 101.9 ± 6.62 µg/L on UGM.

Conclusion

Here we demonstrate the flexibility of *S. stipitis* in utilizing different residual biomasses as growth media. Moreover, we assessed the production of folate from waste, and to the best of our knowledge, we obtained the highest production of folate from residual biomasses ever reported, providing the first indications for the future development of this microbial production process.

Keywords

Biorefinery, Vitamin B9, *Scheffersomyces stipitis*, Sugar Beet Pulp, Sugar Beet Molasses, Unfermented Grape Marcs

1. BACKGROUND

The continuous demand for nutritional security due to the fast world population increase is leading towards finding new solutions and technologies for waste valorization. “The state of food security and nutrition in the world 2019” – a document by the Food and Agriculture Organization of the UN – reported that globally about 820 million people are still starving and/or suffering from food insufficiency. The 2030 agenda for sustainable food supply and the Zero hunger challenge put forward the aim of producing “quality food from waste” (Organization 2019, Kapri, Singh et al. 2020). Moreover, the waste accumulation is a true problem for the ecosystem, since its high water and nutrients content promotes microbial decomposition leading to large methane and carbon dioxide emissions, causing problems for humans, animals and the environment (Helkar, Sahoo et al. 2016, Kumar, Yadav et al. 2017). However, agricultural waste is increasingly appointed as a valuable source of nutraceuticals (or food additives), which are compounds that can provide additional health benefits in a regular diet (Lasrado and Rai 2018). There are several examples in literature on the chemical extraction of diverse nutraceuticals from these residual biomasses, and in recent years environment-friendly protocols are more and more applied (Ghafoor, Sarker et al. 2022, Koraqi, Qazimi et al. 2022, Souza, da Silva Ramalhão et al. 2022), following the principle of green chemistry. Nonetheless, the yield is generally low, and with the addition of economic considerations on the bioprocess the problem of organic waste remains unsolved, unless alternative or complementary solutions are found. Among those, one of the most promising is to use these biomasses as feedstock in biorefineries. Lignocellulosic waste is composed of lignocellulose, a complex natural polymer consisting in a combination of lignin, hemicellulose and cellulose (Howard, Abotsi et al. 2003). The chemical structure of these three polymers makes them a substrate of enormous biotechnological value (Malherbe

and Cloete 2002), as they can be converted into products of interest through physical, chemical, and biological processes. In particular, the latter comprises the use of microorganisms, such as bacteria, fungi or algae, defined as microbial cell factories. It is important to underline that the food and beverage industry produces a large amount of residues – among which lignocellulosic residues are also abundant, and not all of them are currently valorized in up-cycling processes. However, prior to be used as feedstock for microbial-based biotransformation, the raw biomass generally requires a pretreatment step to open up the lignocellulosic structure to ease enzyme accessibility during hydrolysis, either in a separate enzymatic reaction (Separate Hydrolysis and Fermentation, SHF) or combining the two steps (Simultaneous Saccharification and Fermentation, SSF). Many pretreatment strategies are available, and they can be exploited as is or in combination (mechanical, thermal, acid, base, oxidative, enzymatic) (Hendriks and Zeeman 2009). Steam explosion is a common thermal pretreatment strategy, in which the biomass is heated under pressure at around 240°C and then rapidly de-pressurized and cooled, making the water inside the biomass explode, opening up the lignocellulosic compact structure and releasing sugars oligomers and organic acids; the latter step further catalyzes the hydrolysis of the released oligomers (Hendriks and Zeeman 2009). Since this pretreatment requires specific instruments, an autoclave pretreatment is a strategy commonly used in a research lab setting to mimic the effect of a steam explosion, despite being less effective, especially with lignin enriched biomasses. This pretreatment is generally followed by a hydrolysis step, preferably enzymatic, since enzymes operate at conditions compatible with microbial growth (Katakojwala and Mohan 2021) and produce cleaner waste when compared, for example, to chemical hydrolysis.

In this work, for the first time, Unfermented Grape Marc (UGM) and other two important agricultural residues, Sugar Beet Pulp (SBP) and Sugar Beet Molasses

(SBM), have been used for the production of vitamin B_9, exploiting the microbial cell factory *Scheffersomyces stipitis*.

S. stipitis is a Crabtree negative yeast, naturally able to consume both hexose and pentose sugars (Jeffries and Van Vleet 2009), making it an interesting cell factory for the valorization of diverse residual biomasses, such as those considered in this work. Nonetheless, the ability of this yeast to valorize residual biomasses in compounds of interest is almost unexplored, as it is mainly considered as potential cell factory for bioethanol production (Biazi, Santos et al. 2022, Campos, Ribeiro et al. 2022).

In this work, we initially tested different pre-treatment conditions to select the best on UGM for sugar monomers release: the results were compared to the theoretical maximum value previously determined by total acid hydrolysis. We selected a simple autoclave pretreatment since it allowed the release of almost all the available sugars. Pretreatment conditions for SBP were adapted from Martani and colleagues, while no pretreatment was necessary for SBM (Martani, Maestroni et al. 2020).

Prior to use, the obtained supernatants were appropriately diluted, and growth supplements were added when necessary to prepare them as growth media. We then optimized growth and sugar consumptions of *S. stipitis* using the three different biomasses. In particular, we focused on inoculum pre-adaptation, oxygenation and addition of key nutrients for supporting growth, based on the experience from our previous study on defined media (Mastella, Senatore et al. 2022).

Hence, folate production was assessed in shake flasks fermentations where yeasts were grown in the optimized media derived from biomasses. Folate amounts and yield obtained from the different biomasses were calculated with an indirect microbiological assay to determine productions and yields. The results show that *S.*

stipitis was able to produce comparable – if not competitive – amounts of folate, when compared to the data reported in literature with other cell factories. The highest production was achieved in SBM (188.2 ± 24.86 μg/L), followed by SBP (130.6 ± 1,34 μg/L), and by UGM (101.9 ± 6.62 μg/L); the highest yield was obtained on SBP (9.54 μg/g ± 0.17) as a growth substrate.

These results confirm the potential of *S. stipitis* as a cell factory for the production of folates from residual biomasses and provide a solid starting point for further optimization at bioreactor scale, where attention can be devoted at maximizing the production while fully exploiting all the nutritional elements present in the different residual biomasses.

2. MATERIALS AND METHODS

2.1. Chemicals and agricultural residual biomasses

All the chemicals used in this work, including glycerol ($C_3H_8O_3$), sodium hydroxide (NaOH), sulfuric acid (H_2SO^4), sodium chloride (NaCl), and sodium citrate, were purchased from Sigma-Aldrich Company.

Sugar beet pulp (SBP) and sugar beet molasses (SBM) were provided by Cooperativa Produttori Bieticoli (CoProB), Minerbio (BO, Italy); unfermented grape marc - obtained from the production of white wine - (UGM) was collected from local farmers in the Lombardy region within the framework of the national funded project CREIAMO (https://creiamo-circulareconomy.com/). SBM was stored at 4°C, while SBP and UGM were stored at -20°C in order to better preserve their chemical and biological properties.

2.2. Yeast strain and growth conditions

Scheffersomyces stipitis (Culture Collection, CBS 6054) was grown in 100 mL, 250 mL or in 250 mL baffled-shake flasks at 30°C and 160 rpm, using different medium: flask volume (m/f) ratios. Hydrolyzed SBP 3% and 5%, SBM 1:16 and UGM 5% were used as growth media (see next paragraph for details on the preparation). Cells were inoculated at an initial optical density (OD660) of 0.05 in SBP and UGM, while in SBM the inoculum was calculated to obtain an initial OD 0.0005, to be able to follow the exponential phase of growth. Growth was measured by monitoring the media absorbance at 660 nm (UV-1800; Shimadzu, Kyoto, Japan).

2.3 Folate detection

The extracellular or intracellular amounts of free folates (folate vitamers harboring at maximum three glutamyl units) produced by the yeast strain were determined indirectly by a microbiological assay, using *Lactobacillus rhamnosus* (NRRL culture collection, strain B-442) as the test microorganism. This bacterium is able to grow proportionally to the concentration of folic acid present in the medium: this allows to build a calibration curve that correlates the final OD reached by *L. rhamnosus* to the concentration of folates in the samples (Horne and Patterson 1988).

The microbiological assay was performed in 96-well microtiter plates, following a protocol adapted from Sybesma and colleagues (Sybesma, Starrenburg et al. 2003). The wells were filled by adding: 100 µL of twofold-concentrated Folic Acid Casei Medium (FACM, HiMedia, Mumbai, India), 100 µL of an unknown or reference sample in 0.1 M potassium phosphate buffer (pH 6.4) containing 1% (w/v) ascorbic acid (Sigma-Aldrich, St. Louis, USA), and 20 µL of *the L. rhamnosus* inoculum. The plates were incubated at 37°C and the turbidity was measured after 18 hours using a multiscan spectrophotometer set at 595 nm (VICTOR^{tm} X3, PerkinElmer). Control

wells were inoculated without *L. rhamnosus* to check the absorbance of the FACM medium, later subtracted from the absorbance of the samples.

The analysis of the total folate concentration (including forms with long chains of poly-glutamates) was performed as for free folate but after enzymatic deconjugation of the folate samples with rat serum (Sigma-Aldrich, St. Louis, USA) as source for γ-glutamyl hydrolase activity. The purified rat serum was added to the folate samples at the final concentration of 20% (v/v) (Sybesma, Starrenburg et al. 2003). After 3 hours of incubation at 37°C, the enzyme was inactivated by heating for 5 min at 100°C. Samples were cooled down and after centrifugation at 14000 rpm for 20 min at 4 °C, the supernatant was collected and used for the microbiological assay, as described above.

2.4. Raw materials and preparation treatments

SBM was diluted 1:4 (vv-1) with distilled water prior to autoclave sterilization. Pre-treatment step and enzymatic hydrolysis were required on SBP to release the sugars contained in the (hemi)cellulose fraction; a 10% stock was prepared as described by Martani and colleagues (Martani, Maestroni et al. 2020). A 10% stock of UGM was prepared as follows: 100 g of total solids (TS) of ground biomass were suspended in distilled water to a final volume of 1L; after sterilization in autoclave, the suspension was centrifuged in order to separate the solids from the liquid phase; the pH was adjusted to 5.5 with NaOH 15M and stored at 4°C. When necessary and indicated, $(NH_4)_2SO_4$ (10 g/L) or urea (4.6 g/L), and/or KH_2PO_4 (6 g/L) and $MgSO_4 \cdot /L_2O$ (1 g/L) were added to the media.

Sugar content of all three biomasses were analyzed by HPLC (as described below); nitrogen content (ammonia, urea, and primary amino nitrogen) in UGM 10% was

determined with the Urea/Ammonia Assay Kit (Rapid) (K-URAMR) and the Primary Amino Nitrogen Assay Kit (PANOPA), both purchased by Megazyme.

In this work we also focused on the study of UGM, starting from a characterization by acid hydrolysis, we then optimized the best conditions for its pre-treatment.

In order to identify the best condition in terms of time and released sugars, different enzymatic hydrolysis protocols were tested (no enzymes added, pH, temperature, and enzymatic cocktail), either directly in the supernatant, or in citrate buffer 50mM (sterile sodium citrate tribasic 14.7 g/L at pH 5.5); the different conditions tested are listed in Table 1. Prior to enzymatic hydrolysis, a 3% w/v solution was autoclaved in order to both sterilize and pretreat the biomass. Enzymatic hydrolyses were carried out by adding 100 µL/gTS of the enzymatic cocktails NS22119 or NS22201 (which are the same cocktails used for SBP), kindly provided by Novozymes (Novozymes A/S, Copenhagen, Denmark). Enzymes were used in large excess to avoid limiting conditions, and directly added in the supernatant. For the experiments in citrate buffer, the supernatant was pre-emptively substituted with an equal volume of citrate buffer. Sugar release was studied by taking 1 mL samples at 0, 3, 7 and 24 hours from the addition of the enzymatic cocktail; samples were then analyzed by HPLC.

The percentage of sugar released on total solids %(sug/TS) was calculated as the ratio between the concentration of the released sugars (g/L) and the concentration of TS (3 gTS/100 mL). The percentage of sugar released with respect to the total acid hydrolysis was evaluated with equation 2, where c_{enz} is the concentration of the sugar in the supernatant measured with the HPLC, $85mL$ is the volume of supernatant, and $\%_{sug}(g_{acid}/g_{TS}) \cdot 3g_{TS}$ is the quantity of sugar released by the total acid hydrolysis.

Eq. (2)

$$\%\left(\frac{g_{enz}}{g_{acid}}\right) = \frac{\frac{g_{enz}}{L} \cdot 85mL \cdot \left(\frac{1L}{1000mL}\right)}{\%_{sug}\left(\frac{g_{acid}}{g_{TS}}\right) \cdot 3g_{TS}} \cdot 100$$

Table 2: Tested enzymatic hydrolysis conditions (S, supernatant; B, citrate buffer). Three independent experiments were performed for each condition.

Conditions	Biomass	rpm	Temperature	pH	S/B	Cocktail
1a	Wet	130	30°C	6	S	-
1b	Wet	130	30°C	6	S	N S22119
2a	Wet	130	50°C	6	S	-
2b	Wet	130	50°C	6	S	N S22119
3a	Wet	130	30°C	5.5	B	-
3b	Wet	130	30°C	5.5	B	N S22119
4a	Wet	130	50°C	5.5	B	-
4b	Wet	130	50°C	5.5	B	N S22119
5a	Wet	130	30°C	6	S	-
5b	Wet	130	30°C	6	S	N S22201

In order to determine the initial concentration of raw materials being efficiently pretreated by autoclaving, different UGM solutions (3%, 5% and 10% w/v) were used; after treatment and centrifugation, the different supernatants were neutralized with NaOH 15M and a 1mL sample analyzed with the HPLC. Three independent experiments were performed.

2.5. Characterization of unfermented grape marc

UGM was stored at -20°C; before analysis and use, the frozen biomass was ground with a food processor, to homogenize this heterogeneous biomass as much as possible. To determine the amount of total solids (%TS), 2g of frozen ground UGM were lyophilized over night; %TS was calculated as the ratio of the weight after and before lyophilization. Data refers to three independent experiments performed.

Total amount of glucose (Glc), arabinose (Ara), and insoluble fraction were determined with a modified version of the National Renewable Energy Laboratory (NREL) protocol for the determination of structural carbohydrates and lignin in biomass (https://www.nrel.gov/docs/gen/fy13/42618.pdf), to perform a complete acid hydrolysis. Briefly, 300 mg of TS were weighted in 50 mL bottles; 3 mL of 72 % (v/v) H_2SO_4 were added, and the solution was incubated at 30°C for 1h, stirring every 10 minutes. The solution was diluted to 5.4 % (v/v) by quickly adding 37 mL of distilled water, mixed by inversion, and then autoclaved at 121 °C for 20 minutes. The solution was filtered with a Büchner funnel, using pre-weighted filter paper. The retained solids were dried in a microwave until constant weight. The filtered liquid was transferred to a beaker and neutralized with NaOH 15M until pH 5-6 was reached; the volume of the solution was measured, and the samples were analyzed by HPLC (as described below). The total amount of sugars present in the biomass was calculated with equation (1):

Eq. (1)

$$[sugar]\,(g) = c_{HPLC} \cdot V_{filtered}$$

where c_{HPLC} is the concentration measured by HPLC, and $V_{filtered}$ the volume of the solution. Three independent experiments were performed.

To determine the total amount of fructose (Fru), 1.5g of TS were added to 50mL of distilled water to obtain a 3% (w/v) solution, autoclaved at 121°C for 20 min; samples were allowed to cool and H2SO4 was added until pH 2 was reached. Acid hydrolysis was carried out in a water bath set at 100 °C, 100rpm; 1mL samples were taken after 1h and 3h, neutralized with NaOH 15 M and analyzed by HPLC (as described below). The volume of the solution was measured, and total amount of fructose in grams was calculated with Eq. (1). Three independent experiments were performed.

Presence of Glc and Fru was confirmed with the D-Fructose/D-Glucose Assay Kit (Megazyme).

2.6. HPLC analysis

Glucose, fructose, sucrose (Suc), arabinose, arabitol, acetic acid, galacturonic acid, lactic acid, and ethanol (EtOH) concentrations were determined by HPLC (Agilent 1100/1200, Agilent Technologies, Inc.) using a Rezex^{tm} ROA-Organic Acid H+ (8%) 300 x 7.8 mm column (Phenomenex, Torrance, CA, USA), at 40°C; H_2SO_4 0.005N was used as mobile phase, pumped at 0.5 mL/min; analysis time was set to 40 min. Separated components were detected by a refractive-index detector and peaks were identified by comparison with known standards. Prior to analysis, all samples were centrifuged (14000 rpm, 10', 4°C) and filtered with 0.20 µm PTFE filters (AISIMÔ CORPORATION CO., LTD).

2.7 Statistical analysis

All statistical analyses where P-values are indicated, were performed using a two-tails, unpaired, heteroscedastic Student's t-test.

3. RESULTS AND DISCUSSIONS

3.1 Growth and folate production in Sugar Beet Molasses (SBM)

SBM is a thick, dark brown liquid containing roughly 800 g/L sucrose and a minor quantity of fructose. Different dilutions (1:4, 1:8, 1:16, 1:20, 1:32 v/v) were tested in shake flasks (m/f ratio of 2:5) as growth medium, and the highest concentration that S. stipitis was able to withstand was the one of SBM 1:16; this dilution was thus selected for further investigations. In this condition we observed only a partial consumption of the carbon source and the production of small quantities of ethanol (data not shown), suggesting an insufficient aeration and the presence of one or more limiting nutrients, most probably in terms of the nitrogen source, of which SBM is poor (Martani, Maestroni et al. 2020).

To determine which nutrient(s) might be limiting, $(NH_4)_2SO_4$ (2, 5, or 10 g/L), or $MgSO_4$ (1 g/L) and KH_2PO_4 (6 g/L), or a combination of the three ($(NH_4)_2SO_4$ 10 g/L, $MgSO_4$ 1g/L, KH_2PO_4 6 g/L) were added to SBM 1:16. These nutrients were specifically chosen since they are the main components of Verduyn medium (Verduyn, Postma et al. 1992), which is considered as a reference for the preparation of minimal medium for the yeast *Saccharomyces cerevisiae*. The results are shown in Figure S1. Interestingly, the addition of $(NH_4)_2SO_4$ as nitrogen source had a negative effect on growth, leading to an OD660 lower than the one measured in the sole SBM 1:16; the addition of $MgSO_4$ and KH_2PO_4 (with or without $(NH_4)_2SO_4$) allowed to reach a biomass 2-3 times higher than in the control condition. Growth on SBM 1:16 + MgSO4 (1 g/L) and KH2PO4 (6 g/L) was further investigated in 250 mL shake flask (m/f ratio of 2:5) (Figure 1, A): the cells reached an OD_{660} of 26, consuming less than 30% of the available sugars, while showing two distinct growth phases ($\mu1 = 0.08 \pm 0.014h^{-1}$, $\mu2 = 0.02 \pm 0.003h^{-1}$) and the production of ethanol.

To improve growth and sugar consumption, the kinetic was repeated by changing three different parameters (separately): nitrogen availability, salts concentration and aeration. We tested the addition of a lower concentration of $(NH_4)_2SO_4$ (2 g/L) to the medium optimized for salt content, but also in this case we saw a negative effect on growth rate (Fig S2, A), confirming what seen before; similarly, doubling the amount of $MgSO_4$ (2 g/L) and KH_2PO_4 (12 g/L) did not show any differences with the previous fermentation (Fig S2, B). Differently, the reduction of the m/f ratio to 1:5 to improve aeration allowed to reach twice the amount biomass (Figure 1, B); however, the production of ethanol and the constant (not exponential) growth profile suggested that oxygen was still the limiting factor.

Thus, the growth kinetic was repeated in baffled flasks (m/f ratio of 1:5) on SBM 1:16 + $MgSO_4$ (1 g/L) and KH_2PO_4 (6 g/L): growth was indeed faster ($\mu = 0.42 \pm 0.022$ h-1) and more than 90% of the sucrose was consumed, ethanol was not detected and the culture reached a final OD660 of 70 (Figure 1, C).

Here it is interesting to discuss the assimilation of sucrose by *S. stipitis*, to better understand glucose, fructose, and sucrose consumptions on SBM. Sucrose assimilation is well described in *Saccharomyces cerevisiae*: its genome contains seven loci encoding invertase (*SUC*1, *SUC*2, *SUC*3, *SUC*4, *SUC*5, *SUC*7, and *SUC*8), but since the reference strains (S288C) only encodes *SUC*2, most studies have focused on that gene (Taussig and Carlson 1983). In *S. cerevisiae*, *SUC*2 exists in two distinct forms that are localized in the cytoplasm as well as secreted, thus allowing invertase activity both intra- and extracellularly. BLAST alignment of *ScSUC2* on *S. stipitis* available genomes (taxid 4924 and CBS 6054) returned no results, suggesting that *S. stipitis* genome does not encode for an invertase homologue of *SUC*2. Research with the keyword "sucrose" on *S. stipitis*' (CBS 6054) annotated genome on ncbi outputs four putative genes involved in sucrose metabolism: *SUC1.3*, *SUC1.4* and *SUC1.5* are identified as probable sucrose utilization proteins; *SNF8* (sucrose non-

fermenting) is suggested to be involved in glucose derepression. Local (Smith-Waterman algorithm) and global (Needleman-Wunsch algorithm) alignment of *SUC1.3*, *SUC1.4* and *SUC1.5* showed identity around 50%, and similarity around 60-70% (BLOSUM62 matrix); no N-terminal secretion sequence was identified, but *SUC1.3* is characterized by an additional 45 amino acids at the C-terminal. Alignment of *SUC1.3*, *SUC1.4* and *SUC1.5* with *ScSUC2* showed no identity, in accordance with the results obtained with Blast. These results suggest that *S. stipitis* genome could encode for at least three similar invertases, non-homologues of *ScSUC2*, responsible for the ability of this yeast to consume sucrose; the lack of ER signal sequence suggests that these proteins are intracellular. Alignment of *SsSNF8* on Blast with *S. cerevisiae*'s genome suggests that *SsSNF8* is homologue to *ScSNF8* (36% identity), which is reported to be involved in glucose derepression in *S. cerevisiae*, in particular in carbon catabolite repression of polymerase II transcription by glucose (yeastgenome.org).

Indeed, when grown on minimal medium with 20 g/L of sucrose as carbon source (data not shown), no release of glucose or fructose in the medium was observed. These results are consistent with the work of Kobayashi and colleagues (Kobayashi, Inokuma et al. 2021), where no significant accumulation of monosaccharides derived from sucrose hydrolysis was observed in the culture supernatants when *S. stipitis* was grown on YPS. These results point to the existence of an intracellular invertase. In a subsequent work, Kobayashi and colleagues (Kobayashi, Inokuma et al. 2022) investigated catabolite repression on sugars in sugarcane molasses, which has a relative high glucose and fructose content when compared to SBM, where they only represent 0.3% of the dry mass each. Their results showed that sucrose consumption was clearly suppressed by the presence of glucose and fructose. This effect could also explain what we observed on SBM, where however the lower monosaccharides concentration and the high biomass titers probably alleviate the

catabolite repression over sucrose. Moreover, it must be noted that precise HPLC quantification of small amounts of glucose and fructose in a complex matrix is challenging: the chromatograms were characterized by small shoulders corresponding to glucose and fructose peaks, but quantification was not possible (Supplementary Figure S3). These results, in contrast with growth on defined media and the annotated genome, suggest the presence of an extracellular invertase. Taken together, these observations suggest that *S. stipitis* encodes both an intracellular and an extracellular invertase, yet further studies are needed to better characterize this aspect and to identify the putative extracellular invertase(s).

Folate production was evaluated under this condition at the end of the exponential phase and the onset of the stationary phase (Figure 1, C, yellow bars). The highest production (188.2 ± 24.86 µg/L) was obtained at 64h, with a yield on total sugars of 2.33 µg/g.

The highest production on SBM found in literature is 150 µg/L, obtained with *S. cerevisiae* in bioreactor, with a yield on total sugars of 2.21 µg/g (Hjortmo, Patring et al. 2008): these findings are comparable to our results obtained in shake flasks, suggesting the true potential of *S. stipitis* as a folate producer, especially when considering a higher aeration and the controls available within a bioreactor.

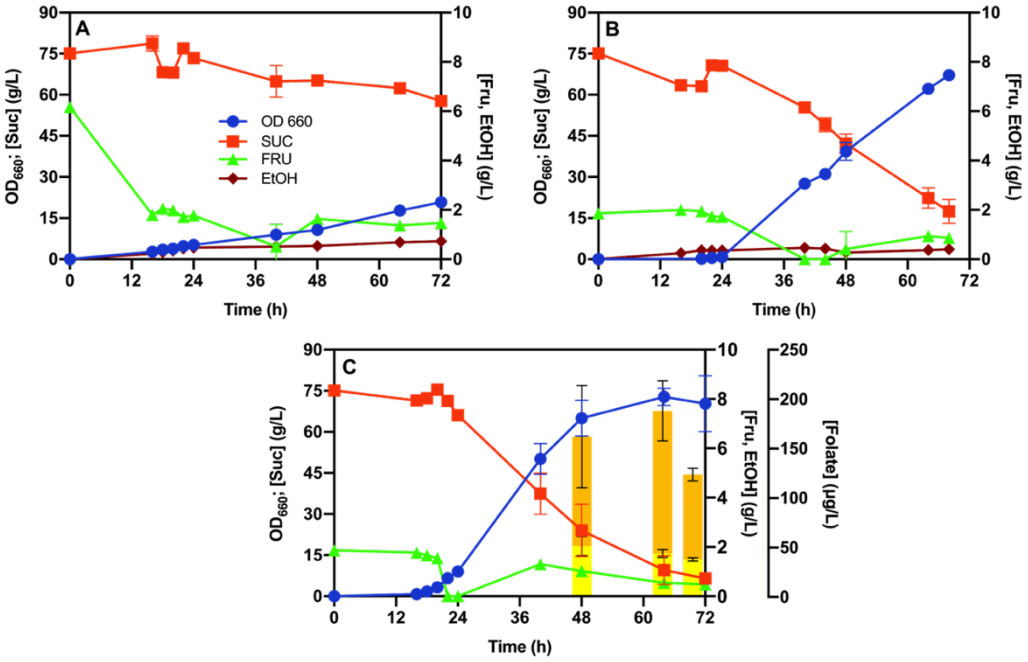


Figure 1. Growth and folate production profiles on SBM + MgSO4 (1 g/L) + KH2PO4 (6 g/L). (A) Growth and sugar consumption with a m/f ratio of 2:5. (B) Growth and sugar consumption with a m/f ratio of 1:5. (C) Growth, sugar consumption and folate production in baffled flasks (m/f = 1:5). The left y-axis shows OD660 (blue, circles) and sucrose concentration (dark orange, squares); the right y-axis shows fructose (green, triangles) and ethanol (brown, diamonds) concentration; the secondary y-axis shows the concentration in bars of free (yellow) and total (orange) folate. Values are mean ± standard deviation of three independent experiments.

3.2 Growth and folate production in Sugar Beet Pulp (SBP)

It is known that SBP pre-treatment and hydrolysis allows the release of glucose and small amounts of arabinose, while also producing growth inhibitors, such as acetic and lactic acid; the latter is probably due to an initial contamination of the biomass from bacteria, as reported by Kühnel and colleagues (Kühnel, Schols et al. 2011), but also galacturonic acid released from pectin as described by Perpelea and co-authors (Perpelea, Wijaya et al. 2022). Thus, the use of SBP requires to find a compromise between a sufficiently high concentration of sugars and *S. stipitis's* tolerance to inhibitors. Martani and colleagues have already characterized sugars

and acids released during the pre-treatment process of SBP (Martani, Maestroni et al. 2020).

Given these information, SBP toxicity was assessed in a preliminary study by growing *S. stipitis* in different concentrations of SBP (3%, 4%, 5%, 6% w/v); SBP 3% allowed growth, while the growth on SBP 4% and 5% was characterized by a long lag phase; growth was not observed for higher concentrations (data not shown).

Initially, SBP 3% was selected for further investigations, and growth was monitored in shake flasks (m/f ratio of 2:5): as shown in Figure 2, A, all the glucose is consumed, and arabinose starts being consumed at 40h, probably due to glucose catabolite repression.

Since sugars concentration in SBP 3% is low (roughly 7 g/L of glucose, the most abundant), before characterizing the growth at a higher aeration, *S. stipitis* was grown on SBP 5% (roughly 15 g/L Glc and 2g/L Ara) but testing a pre-adaptation phase. Pre-cultures were grown in SBP 2.5% and 5% and YPD was used as a control (Figure S3). The pre-adaptation on SBP 2.5% significantly reduced the duration of the lag phase and thus allowed efficient growth also on SBP 5% (Figure 2, B): biomass reached 11 OD at 88h, with still a minor but present residue of glucose, no consumption of arabinose and the production of 2 g/L of EtOH. Since *S. stipitis* is a Crabtree negative yeast (Jeffries and Van Vleet 2009), to improve the aeration and thus avoid the production of EtOH (as with SBM), the m/f ratio was lowered to 1:5 and baffled shake flasks were used. Indeed, this condition allowed to double the amount of biomass with a faster consumption of glucose, completely depleted already at 48h; arabinose was also completely consumed, and no ethanol production was observed (Figure 2, C). Folate production (yellow and orange bars) was evaluated at the end of the exponential phase and reached 130.6 ± 1.34 µg/L at 48h, with a yield on total sugars of 9.54 µg/g. These results are interesting, because even if the production is lower than on SBM, the yield is 4 times higher.

Furthermore, this residual biomass did not require any addition of specific salts or nutrients as in the case seen above. Here, only oxygenation and strain pre-adaptation were pivotal for improving the growth.

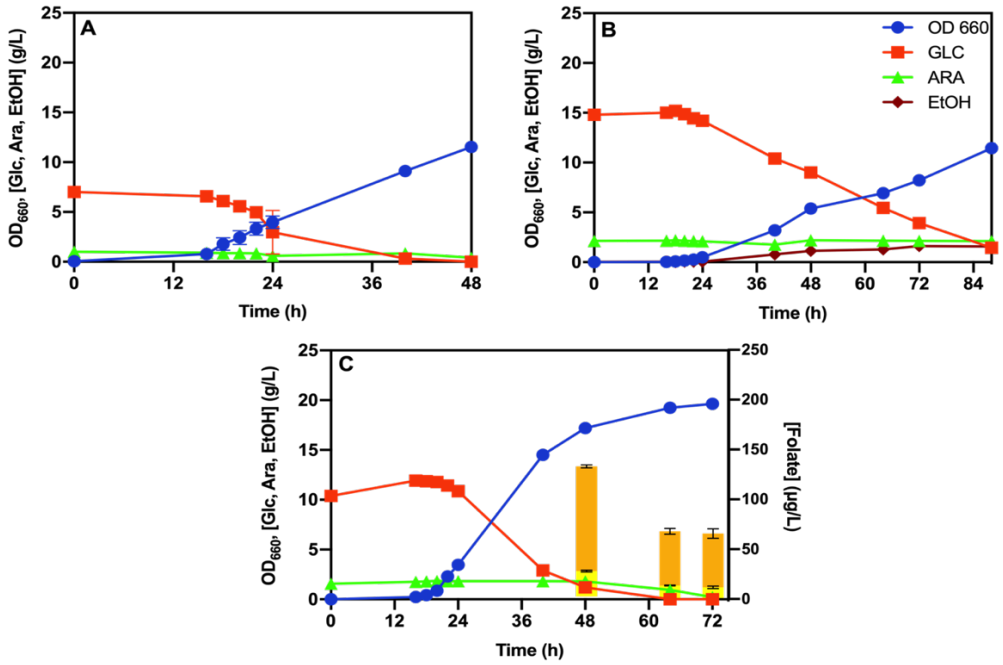


Figure 2. Growth and folate production profiles on SBP. (A) Growth and sugar consumption on SBP 3% with a m/f ratio of 2:5. (B) Growth and sugar consumption on SBP 5% with a m/f ratio of 2:5 with pre-inoculum adaptation in SBP 2.5%. (C) Growth and sugar consumption on SBP 5% with a m/f ratio of 1:5 in baffled flasks with pre-inoculum adaptation in SBP 2.5%. The left y-axis shows OD660 (blue, circles), glucose (dark orange, squares), arabinose (green, triangles) and ethanol (brown, diamonds) concentration. The right y-axis shows the concentration in bars of free (yellow) and total (orange) folate. Values are mean ± standard deviation of three independent experiments.

3.3 Growth and folate production in UGM

While SBM and SBP had been previously characterized in our laboratory on other non-Saccharomyces yeasts (Martani, Maestroni et al. 2020), UGM required a characterization study and the development of an efficient pre-treatment protocol for the subsequent use as growth medium. Since the starting biomass is wet, %TS was determined for each batch.

A complete acid hydrolysis was carried out to determine the total amount of sugars in the biomass. The NREL protocol (see Methods), however, did not allow a precise quantification of fructose, probably due to its degradation to 5-hydroxymethylfurfural, occurring at temperatures above 106°C and at a pH lower than 2 (Bower, Wickramasinghe et al. 2008). An alternative hydrolysis protocol which allowed sampling over time was developed, and the results obtained for the other sugars were in agreement with the NREL protocol. Our results show that more than 50% of the biomass is composed by sugars, namely Fru (26.56%), Glc (24.96%) and Ara (1.21%), expressed as percentage on TS (g/gTS).

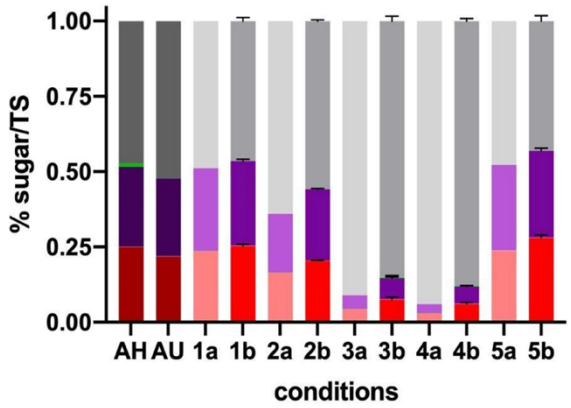
3.3.1 Development of a pre-treatment and hydrolysis protocol for UGM

To develop a protocol for releasing nutrients from UGM, we followed a strategy similar to the one we applied to SBP (Martani, Maestroni et al. 2020). The ground biomass is autoclaved to partially simulate a steam explosion, and then an enzymatic hydrolysis is carried out. Since many factors can affect hydrolysis efficiency (Bertacchi, Jayaprakash et al. 2022), different enzymatic hydrolysis conditions were tested (Table 1, par 2.6.2), using two different enzymatic cocktails: NS22119 contains several carbohydrases, to release both hexose and pentose

sugars, while NS22201 contains cellulases for the degradation of cellulose and other β-glucans. Hydrolysis efficiency was determined as percentage of released sugars with respect to the total acid hydrolysis, which was used as a baseline.

The histograms in Figure 3 show the percentage of sugar released on gTS for each condition tested, as well as the results obtained with the acid hydrolysis (AH) and the sole autoclave pre-treatment (AU). The autoclave pre-treatment process allowed to release amounts of glucose and fructose close to the results obtained with the complete acid hydrolysis, representing 21.8% and 25.8% g/gTS, respectively (AU in Figure 3). To further improve the quantity of sugars released, different conditions for enzymatic hydrolysis were tested; preliminary studies allowed us to determine the best agitation parameters and showed no difference when the starting biomass was wet or dry (data not shown). The hydrolyses were tested at 30°C and 50°C, either by adding the enzymatic cocktail NS22119 directly in the supernatant of the autoclaved biomass, or replacing it with citrate buffer (1b, 2b, 3b, 4b, Figure 3, condition listed in Table 1, par 2.6.2). As highlighted in Figure 3, enzymatic hydrolysis conducted in the supernatant allowed a recovery of around 75% of the total sugars present in the biomass, showing very little difference with the negative controls, regardless of the temperature or the enzymatic cocktail (conditions 1, 2 and 5, Table 1, par 2.6.2); hydrolyses in citrate buffer (conditions 3 and 4, Table 1, par 2.6.2) were far less efficient, and the same results were obtained by using the enzymatic cocktail NS22201 (5b, Figure 3), confirming that most of the sugars are released in the supernatant during the autoclaving step (condition AU). These results are similar to what Corbin and colleagues reported, who characterized the composition of Sauvignon Blanc white marc (Corbin, Hsieh et al. 2015): the main carbohydrates left after juicing are monosaccharides (Glc and Fru), oligosaccharides and water-soluble polysaccharides, which represent roughly 70% of the total carbohydrates and 38% of TS. In our case, the monosaccharides

released after the pre-treatment represent more than 50% of the TS, and thus probably more than 70% of the total carbohydrates. Moreover, Corbin and colleagues (Corbin, Hsieh et al. 2015) reported that enzymatic hydrolysis with cellobiases from *Aspergillus niger* did not improve sugar release, which is consistent with our results. Since no significant improvements were observed – apart from the release of very small quantities of arabinose (<1 g/L) with the enzymatic cocktail NS22119 – in the logic of the development of an industrial process, the sole autoclave pretreatment was selected, to cut down both process time and reagents cost.



Conditions	Biomass	rpm	Temperature	pH	S/B	Cocktail
1a	Wet	130	30°C	6	S	-
1b	Wet	130	30°C	6	S	N S22119
2a	Wet	130	50°C	6	S	-
2b	Wet	130	50°C	6	S	N S22119
3a	Wet	130	30°C	5.5	B	-
3b	Wet	130	30°C	5.5	B	N S22119
4a	Wet	130	50°C	5.5	B	-
4b	Wet	130	50°C	5.5	B	N S22119
5a	Wet	130	30°C	6	S	-
5b	Wet	130	30°C	6	S	N S22201

Figure 3: Pre-treatment results. The graph represents the percentage of released sugar on gTS for each condition tested (listed in Table 1, par. 2.6.2). AH, Acid Hydrolysis; AU, autoclave pre-treatment. a, negative control (no enzymatic cocktail); b, enzymatic cocktail added. The results are the mean ± standard deviation of three independent experiments.

To prepare the medium for fermentation, different total solid concentrations were tested (3%, 5%, 10%). The amount of sugars released is proportional to the TS concentration (Glc: R2 =0.9971; Fru: R2 = 0.9973), and no significant release of growth inhibitors, such as galacturonic acid, acetate, lactic acid, or formic acid was observed. UGM 10% was therefore selected as the stock medium, containing on average 19.1 g/L ± 2.14 g/L of Glc and 23.5 g/L ± 3.23 g/L of Fru. Ammonia, urea and PAN (Primary Amino Nitrogen) concentrations in UGM 10% were measured to

assess the availability of nitrogen: ammonia concentration was 32.2 ± 1.38 mg/L, urea was not detected, and the PAN value was 71.3 ± 1.94 mgN/L, for a total amount of nitrogen lower than 100 mgN/L. These quantities are one order of magnitude smaller than the concentrations of nitrogen generally used to cultivate *S. stipitis.*

3.3.2 Fermentation profile and folate detection in UGM

S. stipitis's fermentation profile was evaluated on different UGM dilutions (2%, 3%, 5%, 10% w/v); growth was observed in all conditions except for UGM 10%, which also caused problems when reading OD660 due to the turbidity of the medium (data not shown). Given the results obtained so far, growth kinetics on UGM were all carried out in shake flasks with an m/f ratio of 1:5, to allow sufficient aeration.
Growth profiles on UGM 3% (Figure 4, A) and UGM 5% (Figure 4, B) were further characterized, following the fermentation for 72 hours after the inoculum: glucose is depleted in both conditions, but only 1.5 g/L of fructose are consumed; moreover, we noticed that growth caused an increase in the pH of the medium (from 5.5 to 6.5 at the end of the fermentation). Since there were not significant differences in the two conditions UGM 5% was selected for further characterization, given its initial higher sugars concentration. Following the same strategy used for SBM, $MgSO_4$ (1 g/L) and KH_2PO_4 (6 g/L), or/and $(NH_4)_2SO_4$ (10 g/L) were added to UGM 5% to test for the presence of limiting nutrients (Figure S4). The addition of $MgSO_4$ and KH_2PO_4 did not significantly ameliorate the growth (compare with Figure 4B); the addition of $(NH_4)_2SO_4$ as nitrogen source – either alone or in combination with $MgSO_4$ and KH_2PO_4 – allowed to reach a higher biomass titer, with a faster and better consumption of sugars if compared with what observed on the sole UGM 5%; however, a very long lag phase (>72h) was observed, and the pH at the end of the fermentation was low (pH 2.5-3). This could be explained by the mechanism of

ammonium uptake, which requires pumping of a proton in the medium (Cueto-Rojas, Milne et al. 2017). These results suggest that nitrogen is a limiting factor, but that $(NH_4)_2SO_4$ is not the ideal source for S. stipitis on UGM 5%; moreover, addition of $MgSO_4$ and KH_2PO_4 in combination with $(NH_4)_2SO_4$ allowed to reach a higher biomass titer and the complete depletion of both glucose and fructose, suggesting that these two salts are also a limiting factor.

Given these results, urea was tested as an alternative nitrogen source to $(NH_4)_2SO_4$; urea was used at a concentration of 4.6 g/L, which provides the same amount of nitrogen as 10 g/L $(NH_4)_2SO_4$. Growth in UGM 5% + 4.6 g/L urea + 1 g/L $MgSO_4$ + 6 g/L KH_2PO_4 was assessed in shake flasks (Figure 4, C): the cells reached 17 OD, doubling the value obtained in UGM 5%, and comparable to the biomass reached in kinetics with $(NH_4)_2SO_4$ and salts. Glucose and fructose are both depleted and, coherently to the proposed mechanism of urea uptake (Mastella et al., 2022) an increase in pH at the end of the fermentation (from 5.5 to 7.5-8) is observed. Due to the faster growth, aeration becomes insufficient, as the production of 2.5 g/L ethanol is observed.

To avoid ethanol production, the growth was repeated in baffled flasks (m/f = 1:5) (Figure 4, D). The higher aeration did avoid ethanol production and allowed cells to prolong the primary exponential phase, reaching 25 OD. Folate production was evaluated at the end of the exponential phase, with a peak concentration at 40 h of 101.9 ± 6.62 µg/L, and a yield of 5.30 µg/g on total sugars.

Folate production on UGM was the lowest obtained in this study, however the yield was higher than in SBM.

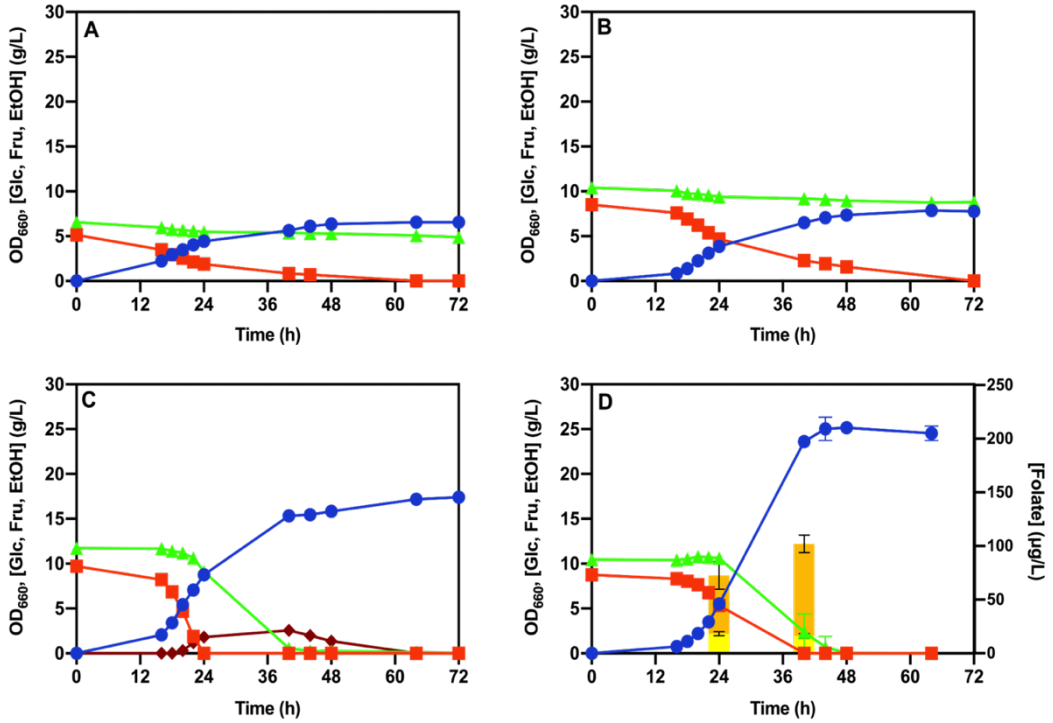


Figure 4. Growth and folate production profiles on UGM. (A) Growth and sugar consumption on UGM 3% with a m/f ratio of 1:5. (B) Growth and sugar consumption on UGM 5% with a m/f ratio of 1:5. (C) Growth and sugar consumption on UGM 5% + 4,6 g/L urea + 6 g/L KH_2PO_4 + 1 g/L $MgSO_4$ with a m/f ratio of 1:5. (D) Growth, sugar consumption and folate production on UGM 5% + 4,6 g/L urea + 6 g/L KH_2PO_4 + 1 g/L $MgSO_4$ in baffled flasks (m/f = 1:5) The left y-axis shows OD660 (blue, circles) and glucose concentration (dark orange, squares); the right y-axis shows fructose (green, triangles) and ethanol (brown, diamonds) concentration; the secondary y-axis shows the concentration in bars of free (yellow) and total (orange) folate. Values are mean ± standard deviation of three independent experiments.

4. CONCLUSIONS

S. stipitis was studied for the production of folate from different residual biomasses for the first time; while pre-treatment protocols were already available for SBM and SBP, UGM required a characterization step and the development of a pre-treatment and hydrolysis protocol. The NREL standard procedure was not efficient for fructose quantification, thus an alternative protocol was developed.

S. stipitis was able to grow on all the three different media, formulated on the residual biomasses. Interestingly, SBM and SBP did not need addition of nitrogen: these media contain enough YAN (Yeast Available Nitrogen) to sustain growth, even to high cell densities. This was not for UGM: the addition of $(NH_4)_2SO_4$ did improve growth, but caused an extremely long lag phase, which was solved by using urea instead.

The highest production of folate (188.2 ± 24.86 µg/L) was obtained on SBM, showing however the lowest yield; the best yield was obtained on SBP (9.54 ± 0.17 µg/g). *S. stipitis* proved to be a versatile and robust yeast able to grow on different residual biomass, consuming all the sugars in the media (both hexose and pentose) with almost no additional nutrient requirements, making the overall process less costly. In terms of folate production on SBM we were able to obtain a 33% higher titer than the best reported in literature (obtained with *S. cerevisiae* in bioreactor with SBM, (Hjortmo, Patring et al. 2008). To the best of our knowledge, this is the highest folate concentration obtained from residual biomasses ever reported. Future studies should focus on scaling-up the process in bioreactor, in order to better control and ameliorate the production, while acquiring the data for a techno-economic analysis that will provide insights on the stage of technological development of this microbial production process.

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SUPPLEMENTARY FILES

Figure S1

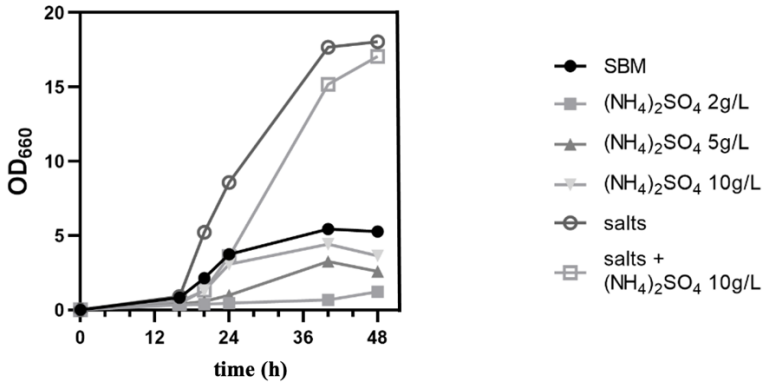

Figure S1. Effect of the addition of Verduyn nutrients in SBM-based medium on the growth of *S. stipitis*. The different conditions tested are listed in the figure key on the side (SBM = control condition without any modification; salts = 6 g/L KH_2PO_4 + 1 g/L $MgSO_4$).

Figure S2

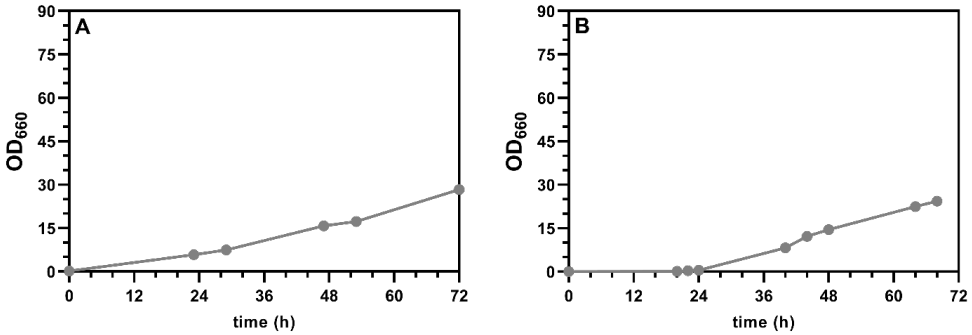

Figure S2. SBM optimization tests of SBM + 6 g/L KH_2PO_4 and 1 g/L $MgSO_4$ for the growth of *S. stipitis*. (A) Effect of the addition of 2 g/L $(NH_4)_2SO_4$. (B) Effect of the addition of double the amounts of $MgSO_4$ and KH_2PO_4 (final concentrations are 12 g/L KH_2PO_4 and 2 g/L $MgSO_4$).

Figure S3

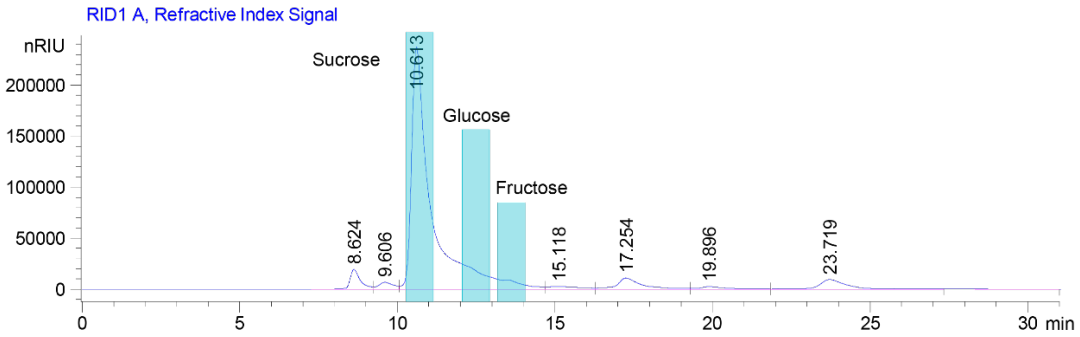

***Figure S3: Chromatograms of S. stipitis growth on Verduyn medium with 20 g/L of Sucrose**. Here we can observe small shoulders corresponding to glucose and fructose peaks, but quantification was not possible*

Figure S4

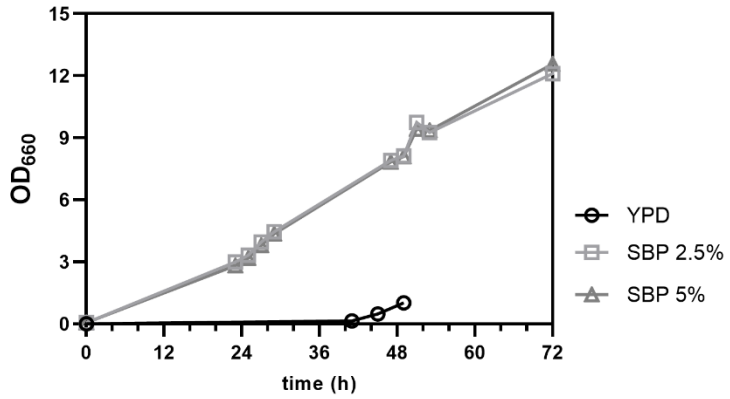

Figure S4. Growth of *S. stipitis* on SBP 5% with the pre-inoculums performed in YPD, SBP 2.5%, or SBP 5%.

Figure S5

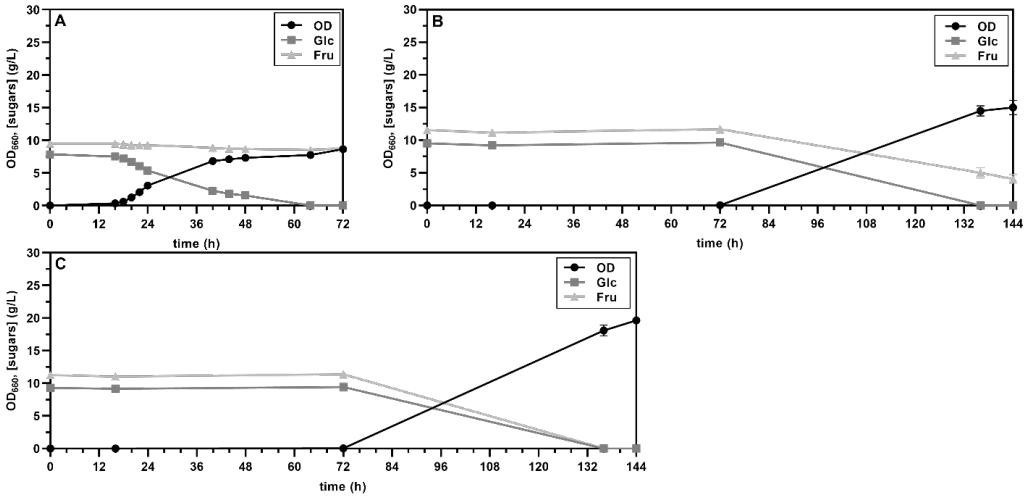

Figure S5. Effect of the addition of Verduyn main salts in UGM on the growth of S. stipitis. (A) Effect of the addition of 6 g/L KH_2PO_4 and 1 g/L $MgSO_4$. (B) Effect of the addition of 10 g/L $(NH_4)_2SO_4$. (C) Effect of the addition of 6 g/L KH_2PO_4, 1 g/L $MgSO_4$ and 10 g/L $(NH_4)_2SO_4$.

Chapter 4

Metabolic engineering approaches to increase folate production of Saccharomyces cerevisiae

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ABSTRACT

Folate vitamers (vitamin B_9) are humans' essential micronutrients that function as cofactors in one-carbon transfer reactions, mainly those involved in the synthesis of nucleotides and amino acids. All the vitamin B_9 commercially available are chemically synthetized in the form of folic acid, which does not maximize their biological effects. The production of natural folates by microbial fermentation represents therefore a sustainable and preferable alternative.

Previously, different *Saccharomyces cerevisiae* strains have been engineered and tested in different production media to increase the endogenous production of folate. However, the studies performed so far do not allow to systematically compare the different pathways manipulations, as they were performed in strains with different genetic backgrounds, or the vitamers were measured either as extracellular or intracellular products, not allowing to unveil the real potential of *S. cerevisiae*.

Here, we focused on maximizing folate production by engineering the production of the two main building blocks concurring to the synthesis of folate, 4-Aminobenzoic acid (pABA) and dihydropteridine.

In the first part of this work, we relied on classical engineering strategies, integrating the expression cassettes (all under the control of the constitutive *Sc*TPI promoter) in loci for auxotrophies complementation. The simultaneous overexpression of several combinations of these genes did not result in an increased production of folate in comparison to the benchmark strain, overexpressing only the first gene of the pteridine pathway, *FOL2* (strain F). Either the expression system or a metabolic bottleneck could justify the results.

To verify our first hypothesis, we took advantage of a new genetic engineering toolkit: in this setting, the same genes were overexpressed by the control of two different strong promoters and integrated in well characterized and highly

expressed genetic loci, originating the strain EAFS. Furthermore, for testing the second hypothesis, two exogenous genes were cloned and added in EAFS strain, to increase the availability of the upstream precursors of pABA, originating the EAFSXP strain. Both strains reached higher folate title, in comparison to the F and all previously tested strains, highlighting the importance of overexpression method. Nonetheless, no significant differences between EAFS and EAFSXP strains were observed.

Given the results obtain, the EAFS strain was further tested in bioreactor, reaching a folate production of 620.0 ± 12.30 μg/L, which is one of the highest titer ever reported in literature using *S. cerevisiae* as *cell factory*.

Keywords: Folates production, *Saccharomyces cerevisiae*, metabolic engineering, bioreactor fermentation

BACKGROUND

Folates are a group of water-soluble compounds belonging to the B vitamin family (B_9). They share a common structure consisting in a pteridine ring linked to a molecule of para-amino-benzoic acid (pABA) by a methylene bridge, and at least one glutamic acid molecule. The vitamers of folate differ for the state of oxidation (folic acid, di-hydrofolate, tetrahydrofolate), for carbon substitutions (i.e., methyl, formyl, methylene, or methenyl) and for the presence of a diverse number of glutamate units linked together in a poly-glutamate tail (Figure 1).

R_1	R_2	
H	H	THF
CH_3	H	5-methylTHF
CHO	H	5-formylTHF
H	CHO	10-formylTHF
H_2C—		5,10-methylenTHF
HC—		5,10-methenylTHF

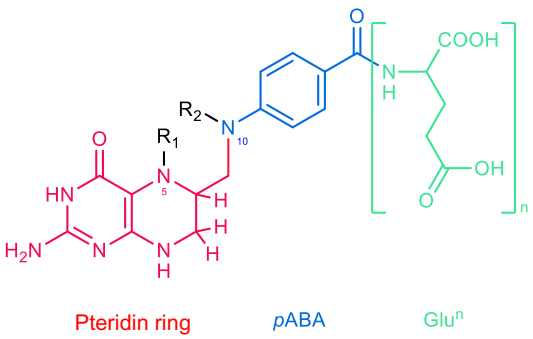

Figure 8: Chemical structure of the vitamers of tetrahydrofolate (THF). Tetrahydrofolate (THF) is composed of three main parts: a pteridine ring (pink), a pABA molecule (blue), and one or more L-glutamic acid residues (green). The different vitamers differ in the side chains R1 and R2 shown in the table alongside, in the oxidation state and also in the glutamation tail. Picture adapted from (Strobbe and Van Der Straeten 2017).

Folates act as donor of C1 units, thus playing an essential role in a wide range of human biochemical pathways such as: DNA synthesis and methylation (Crider, Yang et al. 2012); protein synthesis (McBreairty, Robinson et al. 2016); homocysteine metabolism (Blom and Smulders 2011); red blood cells formation (Koury and Ponka 2004); formation of embryonic tissues (Moyers and Bailey 2001). Due to the central role of folate, its deficiency leads to several physiological disorders including:

anemia (Strobbe and Van Der Straeten 2017); cardiovascular diseases; neural tube defects (TNDs) and colon cancer (Blom and Smulders 2011, Ferrari, Torrezan et al. 2019). As vitamin B_9 is biosynthesized de novo only by plants and some microorganisms, it is an essential component for animal nutrition, obtainable either by diet or by food supplementation. Folates are present in a variety of foods: green leafy vegetables, dark green vegetables (such as broccoli and Brussels sprouts), orange juice, beans, and other legumes (Jägerstad and Jastrebova 2013). Fruits, dairy products, poultry and eggs are also important sources of folates (USDA 2018). As prescribed by the National Institutes of Health (NIH), in the USA the recommended dietary allowance (RDA) for folates in adults is 400 μg dietary folate equivalents (DFE), whereas the folate RDA in the European Union is 240 μg DFE (Efsa 2014); a higher intake (600–1000 μg DFE) is advised for pregnant women (Strobbe and Van der Straeten 2017). As reviewed by (McLean, de Benoist et al. 2008) folate deficiency is a global health issue, therefore the two main strategies developed to increase folate content in foods are biofortification and food supplementation. Several examples of folate biofortification in plants (e.g. rice, tomato, wheat and beans) are reported in literature, (Saini, Nile et al. 2016), as well as in dairy products and fermented food by using folate-producing lactic acid bacteria (LABs) (Saubade, Hemery et al. 2017). However, there are some concerns regarding the use of bio-fortified food, as folate stability during food processing and preservation remains problematic; by contrast, folates food supplementation seems a good alternative overcome this limitation (Choi, Yates et al. 2014). Unfortunately, all the vitamin B_9 commercially available is chemically synthetized in the form of folic acid, that seems not to have the desired biological effects, as it can mask symptoms of vitamin B_{12} deficiency which is linked to an increase of prostate cancer risk, whereas natural folates do not display these collateral effects (Choi, Yates et al. 2014).

For these reasons, the production of natural folates by microbial fermentation appears desirable. Moreover, the current improvements on the development of microbial hosts able to exploit residual biomasses would make the overall process more sustainable compared to the chemical synthesis.

We focused our attention on *Saccharomyces cerevisiae*, which, in addition of being a microbial cell factory widely used in biotechnological processes, is also a natural producer of different folate vitamers (Hjortmo, Patring et al. 2005), and a microorganism certified as GRAS. Yeast's folate biosynthetic pathway is well characterized: it consists in the biosynthesis of the pteridine ring starting from GTP, common precursor of the riboflavin biosynthetic pathway, its condensation with para-amino benzoic acid (pABA), deriving from chorismate, and the addition of glutamate residue(s) (Figure 2).

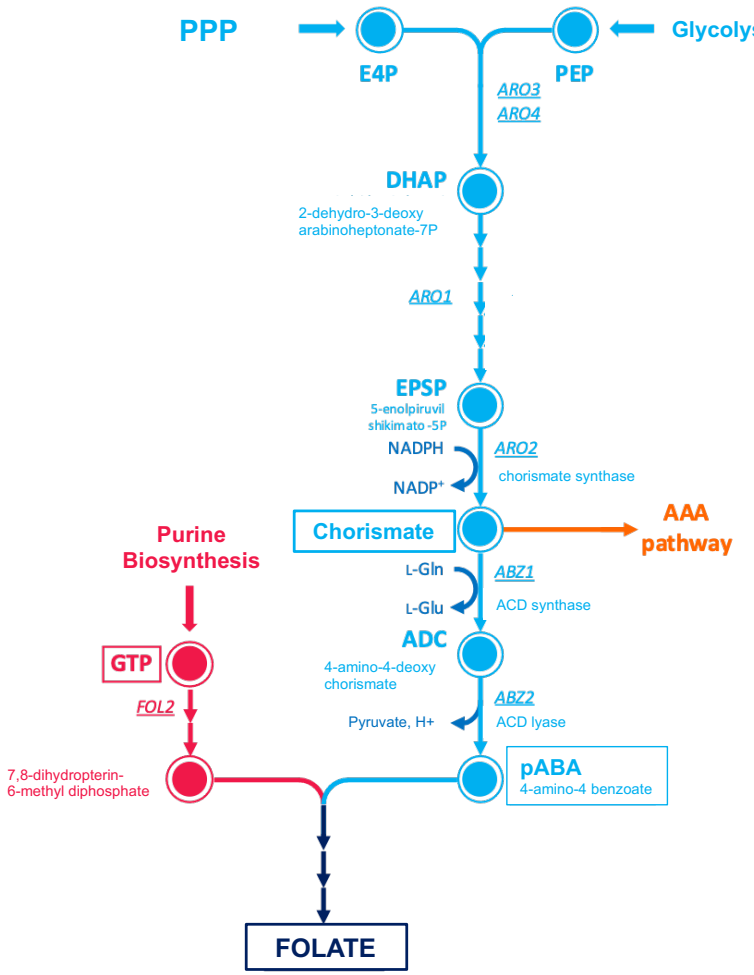

Figure 9: Pathway of biosynthesis of tetrahydrofolate in S. cerevisiae. The biosynthesis of THF requires the synthesis of the pteridine ring starting from GTP (red), and of pABA, a derivative of the shikimate pathway (blue). Glc: glucose, Glc-6P: glucose-6-phosphate, PPP: Pentose phosphate pathway, E4P: erythrosium-4-phosphate, PEP: phosphoenolpyruvate, DHAP: 3-deoxy-D-arabinoheptulosonate-7-phosphate, EPSP: 5 -enolpyruvil-shikimate-5-phosphate, ADC: 4-amino-4-deoxy-chorismate, DNP-PPP: 7,8-dihydroneopterin-3'-triphosphate, DPM-PP: (7,8-dihydropterin-6-yl) methyldiphosphate, H2pteroate: dihydropteroate, DHF: 7,8-dihydrofolate, THF: 5,6,7,8-tetrahydrofolate, THF- [Glu] n: THF polyglutamate. ARO3 / ARO4: DHAP synthase, ARO1: pentafunctional protein Aro1, ARO2: chorismate synthase, ABZ1: ADC synthase, ABZ2: ADC lyase, FOL2: GTP cyclohydrolase I, FOL1: dihydroptero synthase, FOL1: dihydroptero synthase: dihydrofolate synthase, MET7: folylpolyglutamate synthase.

Genetic modification approaches have been proposed to boost the flux of the main metabolites involved in the biosynthesis of folate towards its accumulation (Hjortmo, Patring et al. 2008, Gientka and Duszkiewicz-Reinhard 2010, Liu, Walkey et al. 2016) in *S. cerevisiae*. In these studies, genetic manipulations were performed

testing the strains in different growth condition, and the production of vitamin B9 was evaluated either at the intracellular or extracellular level (Table 1).

More in detail, in the study of Liu and co-authors (Liu, Walkey et al. 2016), the goal was to increase the amount of folate produced by the wine strain named M2 through the overexpression of single genes present in the folate biosynthesis pathway, or in a combination of double overexpressions. The strains created were used in wine production and the concentrations of folate were assayed by a microbiological assay (Table 1). The results show that the overexpression of the *FOL*2 gene leads to the greatest increase in folate. Differently, the study conducted by Hjortmo and co-authors (Hjortmo, Patring et al. 2008) aimed at finding the best growth conditions to obtain the highest level of intracellular folate using the wild type SQK2n strain background. The authors tested the strain in shake flask and then in bioreactor, on synthetic Verduyn or rich YPD medium and also on molasses. The best intracellular folate production was obtained on minimal medium.

From this report it is evident that a comparison is extremely difficult, making difficult to establish the real potential of *S. cerevisiae* in terms of folates production.

Table 3: Genetic manipulations and growth condition tested in literature for the production of folate

Background	Overexpressed gene(s)	Growth condition	Folate determination	Folate production	Reference
M2	-	Wine fermentation	Extracellular by microbiological assay	36.6 ± 7 µg/L	(Liu, Walkey et al. 2016)
M2	*FOL*2			122.9 ± 9.7 µg/L	
M2	*ABZ*1			42.4 ± 10.9 µg/L	
M2	*ABZ*2			28.1 ± 10.2 µg/L	
M2	*DFR*1			33.3 ± 9.8 µg/L	
M2	*FOL*1			36.7 ± 7.2 µg/L	
M2	*FOL*3			27.1 ± 1.9 µg/L	
M2	*FOL*2/*ABZ*1			108.8 ± 20.7 µg/L	
M2	*FOL*2/*ABZ*2			110.3 ± 17.5 µg/L	
M2	*FOL*2/*DFR*1			77.5 ± 13.1 µg/L	
M2	*FOL*2/*FOL*1			85.7 ± 11.6 µg/L	
M2	*FOL*2/*FOL*3			113.6 ± 22.8 µg/L	
SQK2n	-	Bioreactor with Verduyn synthetic medium	Intracellular by HPLC	125 $\mu g/g_{CDW}$	(Hjortmo, Patring et al. 2008)
SQK2n	-	Bioreactor with YPD medium		45 $\mu g/g_{CDW}$	
SQK2n	-	Bioreactor with molasses as medium		38 $\mu g/g_{CDW}$	

For these reasons, in the present work genes involved in the folate and in the shikimate biosynthesis pathways (*i.e.*, *FOL*2, *ARO4*, *ARO*1, *ARO*2, *ABZ*1 and *ABZ*2),

were expressed separately or in additive combination and with different overexpression strategy in the same *S. cerevisiae* background. The obtained strains were all tested in the same condition of growth, using shake flasks and synthetic Verduyn medium. From the resulting engineered strains, we measured the total production of folate, with the help of a microbiological assay, and we also assessed intra- and extracellular production. This allowed us to describe the contribution of the different overexpression and on the different engineering strategies on the quantity of the folate produced and consequently to indicate the metabolic steps to unlock for further improving the production of bioactive folates.

In this first part of the study, we have created seven new strains with the intention of exceeding the folate production of the so called "F strain", which overexpresses only the *FOL*2 gene. None of the strains created succeeded in this, but this allowed us to understand that probably the bottleneck of this complicated metabolism could be the pool of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP), the two precursors of the shikimate pathway.

In literature there are several studies that aim to balance the intracellular quantity of PEP and E4P (Guo, Huang et al. 2020), (Zhu, Wang et al. 2021). We therefore considered the insertion of a heterologous pathway that allows the cleavage of fructose-6-phosphate (F6P) into E4P and acetylphosphate (AcP). In order to carry out these new modifications it was necessary to adopt a different engineering methodology, which allows to exploit integration sites selected in a previous work for maximizing gene expression (Jessop-Fabre, Jakočiūnas et al. 2016) while inserting bidirectional gene expression cassettes through the use of CRISPR/CAS9 technology (Maestroni et al., unpublished data). Therefore, starting from the parental strain, in which all the auxotrophies were complemented by the insertion of integrative plasmids (named WT), we again performed the overexpression of all the genes taken into consideration in the first part of the study (*FOL*2, *ARO4*, *ARO*1,

*ARO*2, *ABZ*1 and *ABZ*2) obtaining the EAFS strain. This strain has been further modified by inserting two heterologous genes encoding for phosphoketolase and transacetylase, obtaining the EAFSXP strain, which should be able to produce a larger quantity of E4P, solving the bottleneck described above. Growth and folate production of these two new strains were then evaluated, keeping the F strain as the reference. Changing the engineering strategy proved to be fundamental for the increase of folate production, more than the insertion of the heterologous pathway of phosphoketolase. The EAFS strain, in fact, shows higher productions and productivity in bioreactor than the EAFSXP strain, which are attested to be 620.0 $\pm$ 12.30 µg/L and 41.33 μg_{fol}/Lh respectively, among the highest observed in the literature using *S. cerevisiae* as *cell factory* for vitamin B_9 production.

MATERIALS AND METHODS

2.1 Yeast strains and growth conditions

The microorganisms used in this work were *Lactobacillus rhamnosus*, *Saccharomyces cerevisiae*, and *Escherichia coli*; details on the strains are reported in Table 2. All strains were stored at -80 °C in the presence of glycerol (20% v / v for yeast cells, 50% v / v for bacterial cells).

Table 2: Parental and engineered strains used in this study

Parental Strains

Strain	Genotype
Lactobacillus rhamnosus NRRL B-442	Wild Type
Saccharomyces cerevisiae CEN.PK 102-5B provided by Dr. P. Kötter, Institute of Microbiology, Johann Wolfgang Goethe-University, Frankfurt, Germany	*MAT*a *his3Δ1 ura 3-52 leu2-3/112 MAL2-8c SUC2*
Escherichia coli ATCC 68233 DH5α	*fhuA2Δ(argF-lacZ) U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

Engineered Strains

Strain	Overexpressed gene(s)	Plasmids used for the engineering
WT	-	pYX012, pYX022, pYX042
F	*FOL2*	pYX012, pYX022, pYX042 FOL
AFS	*ABZ*1$_{awri1631}$, *ABZ*2$_{QA23}$, *FOL*2, *ARO*4^{K229L}, *Aro*L, *ARO*2	pYX012 SHIK, pYX022 ABZ, pYX042 FOL
EAFS	*ABZ*1$_{awri1631}$, *ABZ*2$_{QA23}$, *FOL*2, *ARO*4^{K229L}, *Aro*L, *ARO*2	pG ABZ12 XI3, pG ALA2 XII5, pG F2A4 X4
EAFSXP	*ABZ*1$_{awri1631}$, *ABZ*2$_{QA23}$, *FOL*2, *ARO*4^{K229L}, *Aro*L, *ARO*2, *Bbxfpk, Ckpta*	pG ABZ12 XI3, pG ALA2 XII5, pG F2A4 X4, pG XP X3

The following media were used for the growth kinetics (2% agar was added to the solid media in plates):

- **YPD:** rich medium used for the (pre-) pre-cultivation of *S. cerevisiae*, consisting of (per liter): yeast extract 10 g, tryptone 20 g, glucose 20 g. When needed, the medium was supplemented with antibiotics G418 (500 mg/L) for the selection of the Cas9 plasmid, and/or nourseothricin (clonNAT) (100 mg/L) for the selection of the gRNA plasmid.
- **Verduyn** (Verduyn, Postma et al. 1992) used for *S. cerevisiae* production experiments: minimum medium, consisting of (per liter): $(NH_4)_2SO_4$ 5 g, KH_2PO_4 3 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, mineral stock 1X (EDTA 15 mg, $ZnSO_4 \cdot 7H_2O$ 4.5 mg, $CoCl \cdot 7H_2O$ 0.3 mg, $MnCl \cdot 4H_2O$ 1 mg, $CuSO4 \cdot 5H_2O$ 0.3 mg, $CaCl_2 \cdot 2H_2O$ 4.5 mg, $FeSO_4 \cdot 7H_2O$ 3mg, $Na_2MoO_4 \cdot 2H_2O$ 0.4 mg, H_3BO_3 1 mg, KI 0.1 mg) , vitamin stock 1X (D-biotin 0.05 mg, Ca-D-pantothenate 1 mg, nicotinic acid 1 mg, myo-inositol 25 mg, thiamine HCl 1 mg, pyridoxal HCl 1 mg, p-amino benzoic acid 0.2 mg). For bioreactor experiments all the concentrations listed above have been tripled (Verduyn 3X).
- **FACM 2X (Folic Acid Casei Medium):** (HiMEDIA Laboratories, Mumbai, India), used for the growth of *L. rhamnosus*. The medium was prepared by dissolving 9.4 g of FACM powder in 50 mL of water, then adding 100 mg of ascorbic acid; the solution was sterilized by filtration with a 0.22 µm filter (PrimoTM syringe filter Euroclone).
- **LB:** rich medium used for the growth of *E. coli*, composed of (per liter): yeast extract 5g, tryptone 10g, NaCl 10g. When needed, the medium was supplemented with 100 µg/mL ampicillin or 50 µg/mL kanamycin.

Yeast cultures were grown in Verduyn medium (Verduyn, Postma et al. 1992) with 2% (w/v) D-glucose as carbon source. All strains were grown in shake flasks at 30 °C and 160 rpm, with a flask volume:medium ratio of 5:1. Yeast cells were pre-grown until exponential phase in the same medium, and the growth curves were obtained by inoculating an initial optical density (OD) of 0.001, and monitoring growth spectrophotometrically (UV-1800; Shimadzu, Kyoto, Japan) at 660 nm. The total folate production was evaluated indirectly by a microbiological assay exploiting *Lactobacillus rhamnosus* as the test microorganism (Horne and Patterson 1988), as described in the dedicated section below.

2.2. Genes amplification and plasmids construction

All the primers used for gene amplifications and the obtained PCR products are listed in Table S2. The mutant gene *ARO*4^{K229L} was obtained by using *S. cerevisiae ARO*4 from the genomic DNA of the CEN.PK strain, extracted by standard methods (Sambrook, Fritsch et al. 1989) as template. Two different sets of primers (Fw flank*a*-ARO4 and Rev ARO4^{K229L}; Fw ARO4^{K229L} and Rev ARO4-flank*b*, Table S1) were designed and used to introduce the desired mutations (685A>T and 686A>T), and specific flanks complementary to the Triose Phosphate Isomerase gene promoter (TPI_p) and terminator (TPI_{ter}) regions present in the pYX012 vector (R&D Systems Wiesbaden, Germany) were also inserted. The two fragments obtained by PCRs were then assembled using the Gibson Assembly Cloning Kit (New England Biolabs, Ipswich, Massachusetts, USA) into the pYX012 vector, previously linearized with *Eco*RI, obtaining the pYX012*ARO*4 ^{K229L} vector.

*S. cerevisiae ARO*2 and *FOL*2 coding sequences were amplified by PCR using the genomic DNA from CEN.PK strain as a template. *E. coli AroL* coding sequence was amplified by PCR using as template the genomic DNA of the DH5α strain, extracted by standard methods (He 2011). The *ARO*2 PCR product, amplified with FW EcoRI

ARO2 and Rev BamHI ARO2 primers, was digested with *Eco*RI and *Bam*HI and ligated within the pYX042 vector (R&D Systems Wiesbaden, Germany) previously digested with the same enzymes, obtaining the pYX042*ARO*2 vector. The *FOL*2 PCR product, amplified with FW EcoRVFOL2 and Rev BamHIFOL2 primers, was digested with *Eco*RV and *Bam*HI and ligated with the pYX042 vector, previously digested with the same enzymes. The obtained vector pYX042*FOL*2, harboring *FOL*2 under the control of the TPI_p, was linearized with *Cla*I and directly used for yeast transformations. The *AroL* PCR product, amplified with FW EcoRI AroL and Rev BamHI AroL primers, was digested with *Eco*RI and *Bam*HI and ligated with the pYX022 vector (R&D Systems Wiesbaden, Germany), previously digested with the same enzymes, obtaining the pYX022*AroL* vector.

The "flank*a*-ARO4 ^{K229L}-TPI_{ter}-flank3", "flank3-TPI_p-AroL-TPI_{ter}-flank4" and "flank4-TPIp-ARO2-$TPIt_{er}$-flank5" fragments were obtained by PCR using as a template pYX012*ARO*4^{K229L}, pYX042*ARO*2 and pYX022*AroL*, respectively and using the primers described in Table 2. These fragments were then assembled into the pYX012 vector, previously linearized with *Eco*RI. The obtained vector pYX012*ARO*4 ^{K229L}*AroL ARO*2, harboring all the genes under the control of TPI_p, was linearized with *Bsm*I and directly used for yeast transformations.

The pYX022ABZ plasmid was obtained by cloning the $ABZ2_{qa23}$ and $ABZ1_{awri1631}$ genes obtained from the pMA plasmid ABZ (Thermo Fisher Scientific) within the pYX022 plasmid. The pMA ABZ plasmid was digested with BamHI-HF and NheI-HF to obtain a fragment of 3582 bp, purified from preparative gel and ligated in pYX022. The plasmid obtained was checked with an analytical digestion or an analytical PCR and was finally linearized with *Bsm*I and directly used for yeast transformations.

The strains EAFS and EAFSXP were created using the Easy MiSE tool kit (Maestroni et al., 2022) (Figure 3). In particular, the plasmids pG ABZ12 XI3, pG ALA2 XII5, pG F2A4 X4, pG XP X3, were created using the procedure described in Figure 3.

The phase 1 involves the construction of the so-called pEM plasmids, in which the different genes appropriately amplified with primers (primers from 9 to 20 Table S1) are inserted into the blue pGA background that have wings with Esp3I cleavage sites (as the reaction of golden gate will use this restriction enzyme). Furthermore, some genes require more amplicons in order to mutate the cleavage sites of restriction enzymes that are used in the kit (such as Esp3I, BsaI, NheI etc ...). Once the various pEM plasmids have been created, we proceed to phase 2 or the creation of the pG plasmids, in which the expression cassettes were literally built using the different pEMs with the genes to be overexpressed, and the various pGs created by Maestroni and co-authors who bring within them are promoters, terminators, linkers, regions of homology to chromosomes etc. By cutting with BsaI and the golden gate technique, the plasmids pG ABZ12 XI3, pG ALA2 XII5, pG F2A4 X4, pG XP X3 were thus obtained which will then be linearized with NheI and the product used for the transformation of *S. cerevisiae*. This is phase 3, and the transformation is carried out through the CRISPR/CAS9 technology described below, after which phase 4 begins, which involves the screening and selection of the engineered strains.

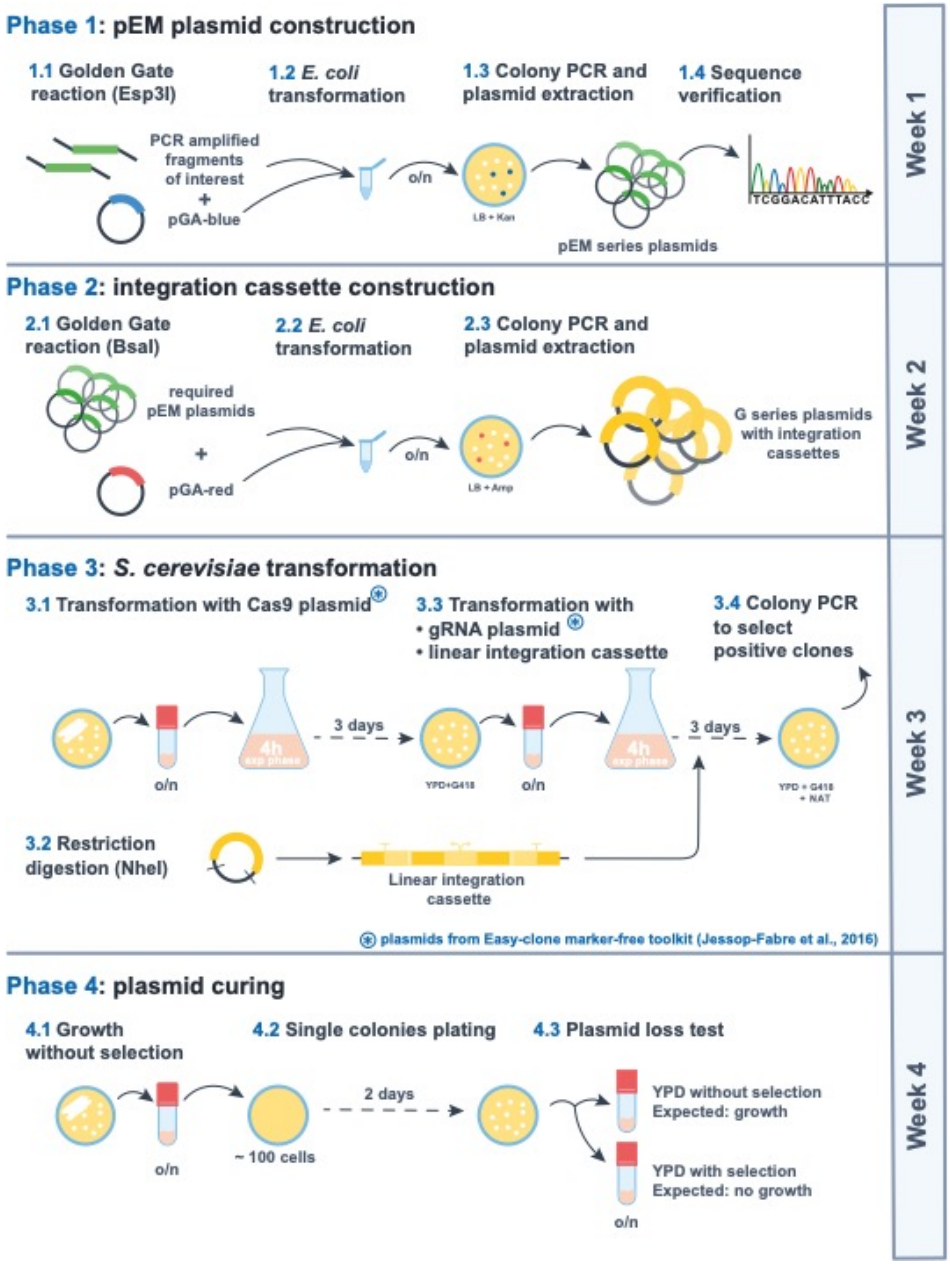

Figure 10: The engineering of S. cerevisiae cells with the EasyMISE toolkit. Figure from Maestroni et al., unpublished data.
Phase 1: the parts of interest (ORFs, promoters, etc.) are PCR-amplified using primers with appropriate flanking sequences and cloned into the pGA-blue acceptor vector via an Esp3I-based Golden Gate reaction. Phase 2: thanks to a BsaI-based Golden Gate reaction, the required parts from pEM plasmids are assembled into pGA-red backbone. Phase 3: S. cerevisiae parental strain is transformed with a Cas9 expression plasmid (3.1). The linear integration cassette is obtained treating G plasmid with NheI restriction enzyme (3.2). Cas9 expressing vector and gRNA helper plasmids are part of the Easy-clone marker free toolkit (Jessop-Fabre, Jakočiūnas et al. 2016). Phase 4: positive clones are plasmid cured in order to remove the gRNA helper plasmid and/or Cas9 vector by growing cells without selection (4.1), single colonies plating (4.2) and resistance loss test (4.3). The process can be repeated, according to the experimental design, to introduce additional integration cassettes.

2.3. Growth conditions in batch bioreactors

For batch fermentations, 2 L stirred tank bioreactors (BIOSTAT® A plus, Sartorius Stedim Biotech GmbH, Goettingen, Germany) equipped with Visiferm DO ECS 225 for pO2 measurement and Easyferm Plus K8 200 for pH measurement (both from Hamilton Bonaduz AG, Bonaduz, Switzerland) were used at a working volume of 1 L. The temperature was kept constant at 30 °C and pH was set to 5.5, maintained by automatic addition of 5M KOH and 5M HCl. The stirring rate was set to 300 rpm in cascade to maintain the oxygen concentration, which was set to 30% of saturation, to guarantee a completely aerobic condition to the cell culture. Filtered air (pore size 0.2 µm) was continuously sparged through the reactor at a flow rate of 1 vvm. Foam formation was controlled by the addition of an emulsifier agent (Triton 100X, Fisher reagents) and a silicon agent (Polydimetylsiloxane, Sigma-Aldrich). MCB was restreaked in YPD plates. A single colony was taken and grown in YPD liquid medium overnight. From this culture, individual aliquots of 1.3 mL were created and kept in 20% (v/v) glycerol at -80 °C (Working Cell Banks, WCB).

WCB were defrozen and inoculated directly in 50 mL glass tubes containing 5 mL of YPD and incubated for 8 h. Cells were then inoculated in 500 mL shake flasks in 100 mL of minimal medium for the intermediate cultures (starting OD 0.04) and grown overnight. For the inoculum, cells were harvested, washed twice with physiological solution (0.9% NaCl), and used to inoculate the bioreactor (starting OD 0.25). Samples were collected every three hours until 30 h and then at the end of the fermentations, stopped at 48 h.

2.4. Metabolites quantification

HPLC analyses were performed to quantify the amount of glucose, xylose, ethanol. Prior to analysis, all samples obtained in Par 5.3 were centrifuged (14000 rpm, 10', 4°C) and filtered with 0.2 µm PTFE filters (AISIMÔ CORPORATION CO., LTD). For the analysis of glucose, xylose, and ethanol, a Rezex ROA-Organic Acid H+ column (00H-0138-KO) 300 x 7.8 mm, 8 µm (Phenomenex, USA) coupled with a precolumn Micro-Guard Cation H+ refill cartridges 30 x 4.6 mm, 8 um (Biorad, USA) was injected with 20 µL of sample. The mobile phase was H_2SO_4 0.01 M pumped isocratically at a flow of 0.5 mL/min for 40 min. Column temperature was kept at 40 °C. Separated components were detected by a refractive index detector (RID) and peaks were identified by comparison with known reference standards dissolved in Ultrapure H2O (18 MΩ) obtained by using a Milli-Q purification system (Millipore, Bedford, USA). Calibration curves were prepared in a range between 20 and 0.625 g/L.

2.5. Folate microbiological assay

The extracellular or intracellular amounts of free folates (folate vitamers harboring at maximum three glutamyl units) produced by the yeast strain were determined indirectly by a microbiological assay, using *Lactobacillus rhamnosus* (NRRL culture collection, strain B-442) as the test microorganism. This bacterium is able to grow proportionally to the concentration of folic acid present in the medium: this allows to build a calibration curve that correlates the final OD reached by *L. rhamnosus* to the concentration of folates in the samples (Horne and Patterson 1988).

The microbiological assay was performed in 96-well microtiter plates, following a protocol adapted from Sybesma and colleagues (Sybesma, Starrenburg et al. 2003). The wells were filled by adding: 100 µL of twofold-concentrated Folic Acid Casei Medium (FACM, HiMedia, Mumbai, India), 100 µL of an unknown or reference sample in 0.1 M potassium phosphate buffer (pH 6.4) containing 1% (w/v) ascorbic

acid (Sigma-Aldrich, St. Louis, USA), and 20 µL of *the L. rhamnosus* inoculum. The plates were incubated at 37°C and the turbidity was measured after 18 hours using a multiscan spectrophotometer set at 595 nm (VICTOR^{tm} X3, PerkinElmer). Control wells were inoculated without *L. rhamnosus* to check the absorbance of the FACM medium, later subtracted from the absorbance of the samples.

The analysis of the total folate concentration (including forms with long chains of poly-glutamates) was performed as for free folate but after enzymatic deconjugation of the folate samples with rat serum (Sigma-Aldrich, St. Louis, USA) as source for γ-glutamyl hydrolase activity. The purified rat serum was added to the folate samples at the final concentration of 20% (v/v) (Sybesma, Starrenburg et al. 2003). After 3 hours of incubation at 37°C, the enzyme was inactivated by heating for 5 min at 100°C. Samples were cooled down and after centrifugation at 14000 rpm for 20 min at 4 °C, the supernatant was collected and used for the microbiological assay, as described above.

2.6. Yeast transformation

In this study, 2 different transformation protocols were used based on the use of pYX plasmids or the easy MiSE tool kit. For the auxotrophy complementation (pYX) the transformation takes place starting from yeast cells in the exponential phase (OD660 between 0.8 and 1). The culture is transferred into a 50 mL tube and centrifuged (4000 rpm, 8 '); the supernatant is discarded and the pellet is washed with 20 mL of dH_2O. The pellet is resuspended in 1 mL of 100 mM LiAcetate and transferred to an eppendorf. After centrifugation (14000 rpm, 20 s), the supernatant is removed and the pellet resuspended in a volume of 100 mM LiAcetate equal to 3 times the volume of the pellet. The transformation takes place using 50 µL aliquots of this solution. After centrifugation (14000 rpm, 20 s), the pellet is resuspended in 360 µL of the transformation mix (240 µL PEG 50% v / v, 36

µL 1M LiAcetate, 25 µL salmon sperm DNA single strand (211 mg / mL, denatured at 99 °C for 5 min), 20 µL of dH2O, and 0.1-10 µL of the plasmid of interest diluted (pYX linearized) with dH_2O in a final volume of 50 µL). The solution is vortexed for 1 minute until complete resuspension of the pellet, and incubated at 30 °C for 30 min. After the thermal shock (42 °C, 30 min), the solution is centrifuged (6000 rpm, 20 s) and the pellet is resuspended in 1 mL of dH2O; this wash is repeated twice. The solution is centrifuged (8000 rpm, 3 min), the cells are resuspended in 200 µL of supernatant, and plated on an appropriate selective medium. The plates are incubated at 30 °C.

Yeast transformants were obtained exploiting the EasyClone-MarkerFree tool kit constructs created by Maestroni and co-authors (Maestroni et al., 2022).
The gRNA helper vectors (natMX as dominant marker) and the Cas9 plasmid pCfB2312 (kanMX as dominant marker) come from the EasyClone-MarkerFree vector set, a gift from Irina Borodina (Addgene kit #1000000098). All the transformations were performed following the EasyClone- MarkerFree manual. The starting yeast carrying the Cas9 plasmid (pCfB2312) was transformed adding in the mix of transformation only with 500 ng of Cas9 expression vector and selected into YPD+G418 media. Plasmids with integration constructs were linearized with NheI, the integration fragments were gel-purified and transformed along with a gRNA helper vector, into yeasts already carrying the Cas9 plasmid (pCfB2312) adding in the mix of transformation 500 ng of gRNA helper vector and 500 ng of linearized integration vector. Correct integration of the vectors into the genome were verified by colony PCR.
Once positive clones were obtained and verified, the gRNA helper vector was removed by optimizing the curing protocol as follows: a single colony was inoculated in 5 mL YPD + G418 at 30 °C, 160 rpm overnight. Then about 100 cells

were plated on a YPD + G418 plate and incubated at 30 °C for 2 days. To verify the gRNA helper and Cas9 expression vectors loss, single colonies were grown overnight in 2 different media: YPD with G418, YPD with clonNAT: cells without gRNA helper and Cas9 expression vectors will not be able to grow on media with clonNAT and G418.

2.7. Colony PCR

To perform colony PCRs, at least 5 different *E. coli* colonies were picked for each transformation plate and dissolved i) in 20 µL of growth media with the proper antibiotic as a colony back-up and ii) into the PCR tube with the appropriate PCR mix. To boost cell disruption, the initial denaturation step must last at least 5 minutes. The positive *E. coli* clones are then inoculated starting from the 20 µL liquid cultures prepared at the beginning.

To perform colony PCRs on *S. cerevisiae* colonies, genomic DNA was extracted following the LiOAc-SDS optimized procedure of Lõoke et al. (Lõoke, Kristjuhan et al. 2011). Once obtained the genomic DNA, 1 µL of the supernatant was used as PCR template. The positive clones were then inoculated in the correct growth media.

Wonder Taq DNA polymerase (Euroclone) was used on a ProFlex PCR System (Life technologies) to perform colony PCR reactions.

2.8. Real Time - qPCR

Quantitative PCR was performed in order to assay *FOL*2, *ARO*4, *AroL*, *ARO*2, *ABZ*1 and *ABZ*2 overexpression. Aliquots of yeast cells were collected from cultures in exponential phase, and total RNA prepared using an *ZR Fungal/Bacterial RNA MiniPrepTM Kit* (ZYMO RESEARCH, Irvine, USA). Reverse transcription was performed on 1 μg of total RNA as a template, using *iScriptTM cDNA Synthesis Kit* (BIO-RAD, Hercules, USA). RT-qPCR was carried out to amplify *FOL*2, *ARO*4, *Aro*L and *ARO*2 cDNAs using *LunaScript RT SuperMix Kit* (New England Biolabs, Ipswich, USA). Actin cDNA was also amplified for normalization. Relative expression was calculated using the $\Delta\Delta C_t$ method.

2.9. Statistical analysis

All statistical analyses, where P-values are indicated, were performed using a two-tails, unpaired, heteroscedastic Student's t-test.

RESULTS AND DISCUSSION

3.1 Engineering of *S. cerevisiae* and evaluation of folates production

The information in the literature regarding the production of folate with *S. cerevisiae* are many, but there are many differences between the various studies. Some introduced genetic modifications to increase the folate title in wine production (Liu, Walkey et al. 2016). Other studies show how folate production changes based on the growth medium used (Hjortmo, Patring et al. 2008) and other studies bioprospecting the diverse natural strains of *S. cerevisiae* and other microorganisms to evaluate differences in vitamin B_9 production (Hjortmo, Patring et al. 2005). In this study we tried to standardize as much as possible the chassis and operating conditions: first of all, the experiments were carried out on Verduyn synthetic minimal medium because, as reported by Hjortmo and co-authors, it allows to increase the folate titers produced (Hjortmo, Patring et al. 2008). The second fundamental element is the overexpression of the *FOL*2, which guarantees the greatest increase in folate production as reported by Liu and co-authors (Liu, Walkey et al. 2016).

The folate biosynthesis pathway is divided in two main branches (Figure 2) of reactions leading to the synthesis of folate skeleton, not considering the glutamate moiety: FOL2p convert GTP into 7,8-dihydropterin-6-methyldiphosphate and therefore it is involved in the first biosynthetic step of the folate pteridine moiety (Figure 1).

The other branch starts from E4P and PEP, the precursors of the shikimate pathway, which has the chorismate as its final intermediate which can be converted into pABA, the second building block of vitamin B9 (Figure 1).

Chorismate derives from the shikimate pathway, which branches to the production of other metabolites, such as aromatic amino acids. As a consequence, it is well known that the enzymes of this biosynthetic route are highly regulated, both at transcriptional and translational/post-translational level (Luttik, Vuralhan et al. 2008). The first step of the pathway is catalysed by 2-dehydro-3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (EC 2.5.1.54): the two isoenzymes in *S. cerevisiae* are encoded by the *ARO*4 and *ARO*3 genes. Aro4p and Aro3p enzymes are feedback-inhibited by tyrosine and phenylalanine, respectively (Luttik, Vuralhan et al. 2008). To enhance the level of activity, the mutated version *ARO*4^{K229L}, a feedback-resistant variant of DAHP synthase (Hartmann, Schneider et al. 2003), was introduced in the CEN.PK strain. In *S. cerevisiae*, DAHP is then converted into 5-O-(1-Carboxyvinyl)-3-phosphoshikimate by the pentafunctional enzyme codified by *ARO*1, while in other organisms such as *E. coli* the five steps are catalysed by monofunctional enzymes (Rodriguez, Kildegaard *et al.* 2015). In literature it is reported that the third step (the conversion of shikimate into shikimate-3-phosphate) has the highest flux-control among the five steps (Rodriguez, Kildegaard et al. 2015). Therefore, *AroL* from *E. coli*, encoding the shikimate kinase, was overexpressed in the CEN.PK strain. In addition, we overexpressed the *ARO*2 gene, involved in the last step of the shikimate pathway, in which the 5-P-(L-carboxyvinyl)-3-phosphoshikimate is converted into chorismate.

Therefore, chorismate enters in the folate biosynthesis pathway and it is converted in pABA through the activity of two enzymes encoded respectively by *ABZ*1 and *ABZ*2. The overexpression of these two genes should decrease the chorismate fraction used for the synthesis of aromatic amino acids and increase the chorismate fraction converted to pABA and, consequently, the amount of folate. In this study we overexpressed two variants of homologous genes, *ABZ*1$_{awri1613}$ and *ABZ*2$_{qa23}$, isolated from the strains of winery *S. cerevisiae* AWRI1631 and QA23, selected as

they are able to guarantee a greater flow than the variant copies, as reported in Averesch's work (Averesch, Winter et al. 2016). Thereby we obtained the so-called AFS strain, in which the genes *ScARO4*^{K229L}, *EcAroL*, *ScARO2*, Sc*FOL2*, Sc*ABZ1*$_{awri1613}$ and Sc*ABZ2*$_{qa23}$ were overexpressed simultaneously under the strong and constitutive TPI promoter.

Strains WT, F and AFS were grown in Verduyn minimal medium with 20 g/L of glucose, in 100 mL flasks. We firstly evaluated the peak of folate production, analyzing only the extracellular production. As can be seen in Figure 4, the productions reach their maximum with the exit from the exponential phase and the entry into the stationary phase, confirming previous works (Hjortmo, Patring et al. 2008).

These preliminary results show that as regards the analysis of extracellular folates, the F strain is confirmed as our positive control, in fact with a production of 17.5 ± 0.79 ng/mL is able to produce twice more than AFS (7.7 ± 0.89 ng/mL) and five times more than the WT strain (3.3 ± 0.42 ng/mL).

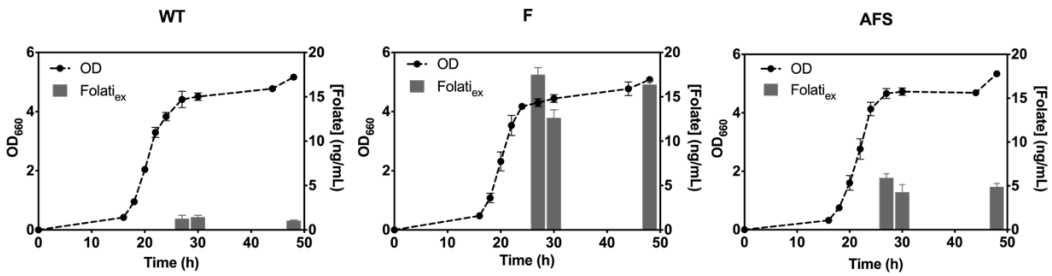

Figure 11: Growth kinetic of WT, F and AFS strains and extracellular folate production in different time points. Dashed line with a full black circle represents the absorbance at 660 nm. Grey bars show extracellular folate production. Values reported are the mean ± standard deviation of three independent experiments.

We then evaluated the total folate productions of the three strains WT, F and AFS taking the samples at the beginning of the stationary growth phase. Figure 5 shows the results obtained, in particular the height of the bars shows the total folate content which is given by the sum of monoglutamate folate (solid bar) and polyglutamate folate (empty bars) measured both intra and extracellularly. Both

the F and AFS strain increased their folate production compared to the WT strain. It is important to observe that the strain F is able to produce more monoglutamate folate than AFS, but when compared, the total folate concentrations obtained are comparable, 34.0 ± 3.66 ng/mL and 38.7 ± 3.04 ng/mL respectively. This means that the overexpression carried out in the AFS strain led to a metabolic change in the production of the vitamers, increasing the polyglutamate folate pool that reach about 31 ng/mL compared with the less result of the strain F, 21 ng/mL.

However, the purpose of these engineering was to increase the total folate production and therefore also exceed those obtained with the F strain. The results obtained by RTqPCR confirm that the genes involved are overexpressed even if the fold changes obtained do not possess very high values (Table S2 and S3). In fact, all genes have been placed under the control of the TPI promoter and this could have undesirable effects on expression. Furthermore, the different cassettes integrated in the genome are close to the genes of leucine, uracil and histidine: these genetic loci might be less accessible to transcription once the exponential growth phase is over as they are no longer essential for the vitality of the cells. Another explanation for the obtained results could lie in the fact that the real bottlenecks of this pathway could be the precursors of the shikimate pathway, E4P and PEP.

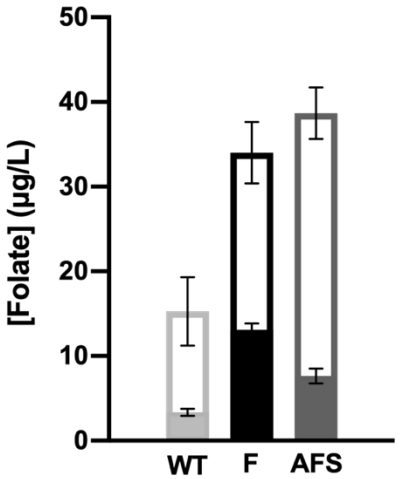

Figure 12: Total folate productions by WT, F and AFS strains. Solid bars show monoglutamate folate titles while the empty bars carry out polyglutamate folate concentrations. Values reported are the mean ± standard deviation of three independent experiments.

3.2 New engineering approach and phosphoketolase overexpression

It is possible that the AFS strain has an impairment linked to the type of overexpression technology or to the limited quantity of the precursors of the shikimate pathway, E4P and PEP. We therefore decided to work on both aspects in parallel:

1. Inserting two heterologous genes to balance the metabolic flux between E4P and PEP.
2. Adopting a new strategy for gene integration and overexpression.

1) Numerous studies performed in yeast (Zhu, Wang et al. 2021), (Curran, Leavitt et al. 2013) (Suástegui, Guo et al. 2016), (Liu, Yu et al. 2019) confirm that the flow towards the shikimate pathway is limited by the availability of precursors. In particular, less than 8% of the PEP enters the shikimate pathway (Guo, Huang et al.

2020), and the amount of E4P is at least an order of magnitude lower than the amount of PEP (Zhu, Wang et al. 2021). In addition, it is also necessary to consider the imbalance between the quantities of PEP and E4P. Furthermore, DAHP synthase (encoded by *ARO*3 and *ARO*4) - the first enzyme of the shikimate pathway and limiting step (Guo, Huang et al. 2020) - shows an affinity for PEP (in terms of K_m) 4-7 times greater than E4P. Finally, E4P was described as the limiting substrate also in other microorganisms, in particular *E. coli*, *Bacillus subtilis*, and *Corynebacterium glutamicum* (Liu, Yu et al. 2019).

In literature there are several strategies to increase the availability of precursors, with a particular focus on chorismate, which can be summarized in two main approaches. On the one hand, it is possible to increase the carbon flux through PPP by acting on some of the key genes such as *TKL1, ZWF1, TAL1*, and/or *RKI1* (Liu, Yu et al. 2019); on the other hand, it is possible to overexpress a heterologous phosphoketolase (*PHK*), which catalyses the conversion of fructose-5-phosphate (F6P) (or xylose-5-phosphate (X5P)) to erythrose-4-phosphate (E4P) (or glycerol-3-phosphate (G3P)) and acetate (Guo, Huang et al. 2020).

Therefore, to overcome the intrinsic scarcity of E4P, we implemented a heterologous phosphoketolase (PHK) pathway (Figure 6). This consisted of a phosphoketolase - from *Bifidobacterium breve* (*Bbxfpk*) - that irreversibly cleave F6P into E4P and Acetil-phosphate (AcP), and a phosphotransacetylase - from *Clostridium kluyveri* (*Ckpta*) - to convert AcP into acetyl-CoA (Liu, Yu et al. 2019).

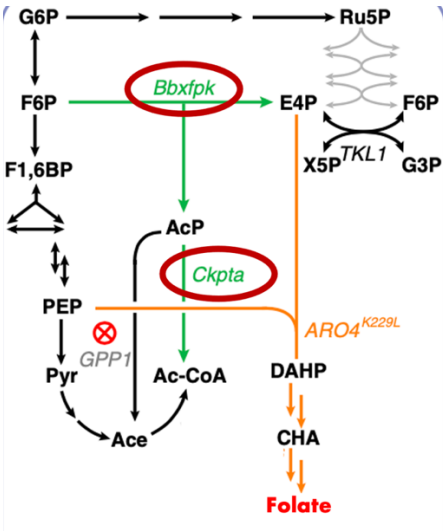

Figure 13: Heterologous phosphoketolase (PHK) pathway. G6P → Glucose-6P; F6P → Fructose-6P; F1,6BP → Fructose1,6 bisphosphate; PEP → Phosphoenolpyruvate; Pyr → Pyruvate; Ace → Acetate; Ac-CoA → Acetyl-CoenzymeA; AcP → Acetylphosphate; Ckpta → phosphotransacetylase from Clostridium kluyveri; Bbxfpk → phosphoketolase from Bifidobacterium breve; Ru5P → Ribulose-5P; E4P → Erythrose-4P; X5P → Xylulose-5P; G3P → Glyceraldehyde-3P. Image adapted from Liu, Yu et al. 2019.

2) The use of Easy MiSE tool kit a new system developed in our lab by Maestroni and co-authors was taken into consideration, this allows to integrate genes into the genome with bidirectional cassettes, controlled by different strong and constitutive promoters, in previously studied and optimized locus and with a CRISPR/CAS9 marker free technology (Jensen, Strucko et al. 2014). This allows to increase the effectiveness of the over-expressions and it also has the advantage of being very fast and precise. Hence, we reconstructed the EAFS strain, in which *ABZ*1$_{awri1613}$, *ABZ*2$_{qa23}$, *FOL*2, *ARO*4^{K299L}, *Aro*L, and *ARO*2 genes were integrated into the genome through the use of the CRISPR/CAS9 system. Moreover, starting from the strain EAFS the two genes of the PHK pathway were integrated obtaining the strain EAFSXP.

The respective folate productions of the strains F, EAFS and EAFSXP were monitored firstly during a growth kinetic on Verduyn medium, in shake flasks at 30 °C and 160rpm. The results are reported in Figure 7: folate production of these

two new strains exceeds by about two times that one of the *FOL*2 overexpressing strain (46.6 ± 5.04 µg/L), reaching a production of 81.2 ± 6.95 µg/L and 76,7 ± 4.37 µg/L respectively, after 28h from the inoculum, time point in which all the strains enter in stationary phase. These results highlight the importance of the genome locus selection for the integration of overexpressing cassette genes and show the effectiveness of the Easy MiSE tool kit. The addition of the two exogenous genes *Bbxfpk* and *Ckpta* reduces the growth of the strain and increases the production of acetate (data not shown). This confirms what has been seen in previous studies. AcP - produced by phosphoketolase after F6P cleavage - can be converted into Ac-CoA through the inserted exogenous transacetylase: however, *S. cerevisiae* has two endogenous variants of glycerol-3-phosphatase, encoded by the *GPP*1 and *GPP*2 genes, which are able to convert AcP into acetate. This would primarily justify the increased accumulation of acetate in the EAFSXP strain. The elevated acetate concentration increases the ATP-requirement for ion homeostasis and reduces the biomass yield (Bergman, Hellgren et al. 2019). Thus, it is difficult to evaluate the effect of this heterologous pathway without the deletion of the *GPP*1 gene (Liu, Yu et al. 2019), which will be considered for future steps of this study.

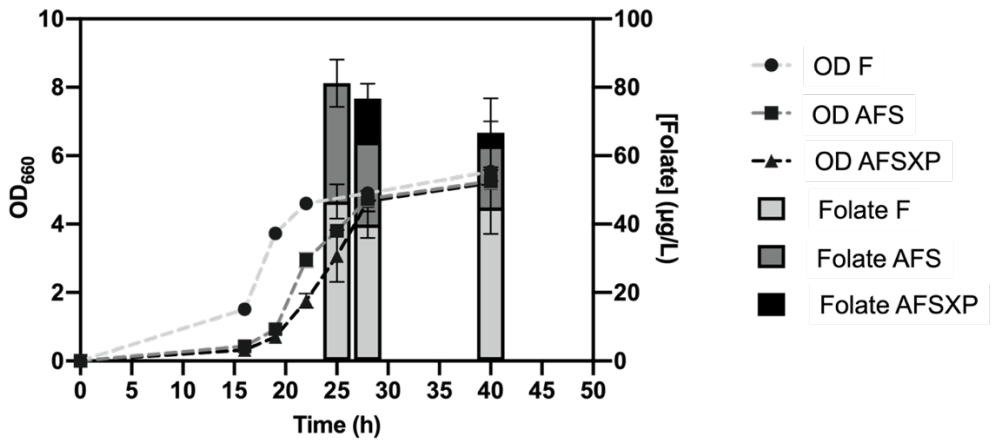

Figure 14: Fermentation profile and folate production of the strains F, EAFS and EAFSXP. On the left axis of the ordinates is reported the OD (F in light grey dashed line, EAFS in grey dashed line and EAFSXP in black dashed line); the right axis of the ordinates shows the production of folate (F in light grey bars, EAFS in grey bars and EAFSXP in black bars). Values reported are the mean ± standard deviation of three independent experiments.

3.3 Bioreactor fermentation and folate productions

In order to better characterize fermentation profile and folate production of EAFS strains we moved the production at bioreactor scale using defined minimal Verduyn 3X medium. In this medium all the quantities of nutrients were tripled and therefore the final glucose concentration was 60 g/L: this also allowed us to extend the fermentation time in bioreactor to better characterize the growth and the by-products profile. The EAFS strain was inoculated from the pre-culture to obtain an initial OD of 0.5. As shown in Figure 8, after 15 hours from inoculation, the strain is able to metabolize all the sugar provided, reaching 16.6 OD (blue full circle) with a growth rate of 0.41 h^{-1}. The EAFS strain also produces high concentrations of by-products, in particular ethanol, acetate and glycerol (21.4, 2.1 and 3.8 g/L, respectively). As can be seen from both the absorbance curve (blue dashed lines) and that of the CO_2 produced (black lines), EAFS cells, once glucose is finished, enter in the diauxic phase in which they begin to consume the by-products produced during the exponential phase - ethanol, acetate and glycerol - reaching 43.3 OD.

Thanks to the CO_2 profile, we analysed the folate production in the four different phases of growth; at the end of the exponential phase, at the beginning and at the end of the second growth diauxic phase and in the late stationary phase. The production peak is obtained at the end of the exponential phase, similarly to what observed in flasks and to what reported in the literature (Hjortmo, Patring et al. 2008) and is attested to be 620.0 ± 12.30 µg/L. EAFS folate productivity, calculated as micrograms of folate produced on liter per hour, is 41.33 μg_{fol}/Lh.

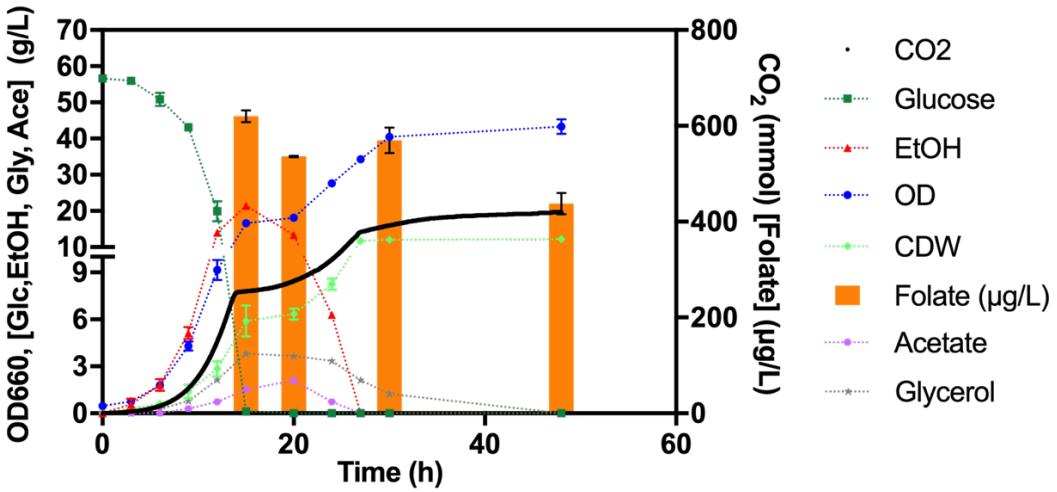

Figure 15: Fermentation profile and folate productions of S. cerevisiae strains EAFS in bioreactor on minimal Verduyn3X medium, with 60 g/L of Glucose as carbon and energy source. Values are the mean ± standard deviation of independent experiments

Despite the by-products, we obtained the highest production and productivity of vitamin B_9 among those reported in the literature for *S. cerevisiae* (Figure 9). Nonetheless, as reported in the table, the yield (10,94) - expressed as micrograms of folate produced per grams of sugar consumed - is lower than those obtained in 2L bioreactors with Verduyn2X and 20 g/L of glucose by Hjortmo and co-authors, which was 18 µg/g. The optimization of a fed-batch process could increase the biomass and product yield.

Table3: Folate productions of S. cerevisiae in different medium.

Microorganism	Medium	[Sugars](g/L)	Volume	Folate Production (µg/L)	Productivity (µg/Lh)	Yield (µg/g)	Ref
S. cerevisiae	YPD	Glc 20	2L bioreactor	250	12.5	12.5	Hjortmo, Patring et al. 2008
	Verduyn 2X	Glc 20		360	18.0	18.0	
	SBM	Suc 68		150	7.5	2.21	
***S. cer.* EAFS**	Verduyn 3X	Glc 60		620.0 ± 12.30	41.3	10.94	This study

CONCLUSIONS

In the first part of this work, we overexpressed six different genes involved in the biosynthesis of folate, exploiting previously described mutated yeast genes and a bacterial gene: *ABZ*1$_{awri1613}$, *ABZ*2$_{qa23}$, *FOL*2, *ARO*4^{K299L}, *Aro*L, and *ARO*2. In the AFS strain we observed higher levels of poly-glutamate folate in respect to that produced by F train, but the total productions were comparable. These strains might have limitations due the fact that overexpressed genes were all under the control of the same constitutive promoter and harbored in multicopy plasmids.

To improve the productions, we have studied in parallel the use of a new engineering strategy exploiting a kit developed in the laboratory by Maestroni and co-authors in which all the genes mentioned above were integrated and overexpressed in different genetic locus, previously characterized for being highly transcribed, through the use of CRISPR/CAS9 marker free technology (Maestroni et al., 2022). Moreover, we considered the insertion of two additional heterologous genes aimed at balancing the flow between E4P and PEP, the two precursors of the shikimate pathway.

Interestingly, the new strain EAFS, despite harboring the same overexpression of AFS strain produces higher amount of folate, even exceeding those of strain F, confirming the importance of integration sites for reaching stable and productive strains. As EAFSXP strain was producing amounts of folates comparable to those of EAFS, only this strain was further characterized in bioreactor. The strain reached a production of 620.0 $\pm$ 12.30 µg/L, which is the highest production of vitamin B_9 reported in the literature for *S. cerevisiae*. Further improvement could be achieved by further engineering the strain (for example by decreasing the competitive pathways towards folates production, such as the ones leading to the biosynthesis of aromatic amino acids, or by increasing the pool of GTP), and/or by optimizing the fermentation protocol, both in terms of media composition and fermentation strategy. It is important that for the first time an engineering strategy allowed to ameliorate the folate production in *S. cerevisiae* cells over the productions obtained with *FOL2* overexpression, which was reported in all the other studies present in literature. This opens to the possibility to study further bottlenecks, for evaluating the true potential of this yeast in the production of Vitamin B_9.

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SUPPLEMENTARY TABLES

Table S1: List of primer used in the study

N°	Name	Sequence (5'-3')	DNA template for PCR	PCR product
1	Fw flank*a*-ARO4 Rev ARO4^{K229L}	CTACAAAAAACACATACAGGTCAGAAATGAGTGAATCTC AGCAACACCATGCAAAGTAACACC	*S. cerevisiae* gDNA	*ARO4*^{K229L} first 725 bp
2	Fw ARO4^{K229L} Rev ARO4-flank*b*	GGTGTTACTTTGCATGGTGTTGCT CCCTAGGATCCATGGTGTGTACGTTACATATATCA	*S. cerevisiae* gDNA	*ARO4*^{K229L} last 485 bp
3	Fw EcoRI ARO2 Rev BamHI ARO2	ATTAGAATTCGCATGTCAACGTTTGGGAA AATGGATCCTCTTTAATGAACCACGGATC	*S. cerevisiae* gDNA	*ARO2 (ORF)*
4	FW EcoRV FOL2 Rev BamHI FOL2	TACAGGATATCATGCATAACATCCAATTAGT AATGGATCCTCAAATACTTCTTCTTCCTA	*S. cerevisiae* gDNA	*FOL2* (*ORF*)
5	FW EcoRI AroL Rev BamHI AroL	ATTCGAATTCCGATGACACAACCTCTTT AATGGATCCAAAATCAACAATTGATCGTC	*E. coli* gDNA	*AroL* (*ORF*)
6	Fw flank*a*-ARO4 Rev ARO4 TPI$_{ter}$-flank1	CTACAAAAAACACATACAGGTCAGAAATGAGTGAATCTC GAGATAGAAATTTGACATTCCTAGACAAAGACAAAAAAAAAAAG GG	pYX012*ARO4* ^{K229L}	flank*a*-*ARO4*^{K229L}-TPIter-flank3
7	Fw flank1-TPI$_p$AroL Rev AroL TPI$_{ter}$-flank2	GAATGTCAAATTTCTATCTCTTTCGTCTTCAAGAATTGGGGATCTA GTATTTAGTTAGTACTTAAGGAACAAAAGCTGGAGCTAGACAAAG AC	pYX022*AroL*	flank3-TPI$_p$-*AroL*-TPI$_{ter}$-flank4
8	Fw flank2-TPI$_p$ARO2 Rev ARO2 TPI$_{ter}$-flank3	CTTAAGTACTAACTAAATACCGTCTTCAAGAATTGGGGATCTACGT ATG GGGCCCTAGGATCCATGGTGGGAACAAAAGCTGGAGCTAGACAA AG	pYX042*ARO2*	flank4-TPIp-*ARO2*-TPIt$_{er}$-flank5
9	Fw_FOL_L_DE Rv_FOL_L_DE	TGCCAACGTCTCATGGTCTCCGCTCATGCATAACATCCAATTAGTG C TCGATCCGTCTCAGGTCTCCAACTCTCAAATACTTCTTCTTCCTAAA AG	pYX042*FOL2*	FOL2.L
10	Fw_ARO4_R_GH ARO4_Mut1_Rv	TGCCAACGTCTCATGGTCTCCATTCATGAGTGAATCTCCAATGTTC TCGATCCGTCTCATCAGCCAAGTATTGAGGAG	pYX012 SHIK	ARO4^{K229L}.R1
11	ARO4_Mut1_Fw Rv_ARO4_R_GH	TCGATCCGTCTCACTGATTTGGTTTCCTTCG TCGATCCGTCTCAGGTCTCCCGGTCTATTTCTTGTTAACTTCTCTTC	pYX012 SHIK	ARO4^{K229L}.R2
12	Fw_AROL_L_DE Rv_AROL_L_DE	TGCCAACGTCTCATGGTCTCCGCTCATGACACAACCTCTTTTTCTG TCGATCCGTCTCAGGTCTCCAACTTCAACAATTGATCGTCTGTG	pYX012 SHIK	AROL.L
13	Fw_ARO2_R_GH ARO2_Mut1_Rv	TGCCAACGTCTCATGGTCTCCATTCATGTCAACGTTTGGGAAACTG CCTTTTCGTCTCTAGGGGTC	pYX012 SHIK	ARO2.R1
14	ARO2_Mut1_Fw ARO2_Mut2_Rv	TGCCAACGTCTCACCCTAGAGATGAAAAGGATAG TCGATCCGTCTCAGTAGGCAAGTTTCTCACGAC	pYX012 SHIK	ARO2.R2
15	ARO2_Mut2_Fw Rv_ARO2_R_GH	TCGATCCGTCTCACTACCGGTCTTGGTGAGC TCGATCCGTCTCAGGTCTCCCGGTTTAATGAACCACGGATCTGG	pYX012 SHIK	ARO2.R3

16	Fw_ABZ1_L_DE	TGCCAACGTCTCATGGTCTCCGCTCATGCTGTCCGATACAATTG	pYX022	ABZ1.L1
	ABZ1_Mut1_Rv	TCGATCCGTCTCATCAATAGTCCTATCCAATCG	ABZ	
17	ABZ1_Mut1_Fw	TCGATCCGTCTCATTGATAGAGATCCCATATAC	pYX022	ABZ1.L2
	ABZ1_Mut2_Rv	TCGATCCGTCTCAAACTGCTTAACGTAAGTTG	ABZ	
18	ABZ1_Mut2_Fw	TCGATCCGTCTCAAGTTCGAGGTTTCTGAAGAC	pYX022	ABZ1.L3
	Rv_ABZ1_L_DE	TCGATCCGTCTCAGGTCTCCAACTCTACATGAAAATTTGCAAGTTG	ABZ	
19	Fw_ABZ2_R_GH	TGCCAACGTCTCATGGTCTCCATTCATGAACGAATTTTTTTTATTAG ATG	pYX022 ABZ	ABZ2.R1
	ABZ2_Mut1_Rv	TGCCAACGTCTCAATTCAGAGAAGTGCTGAAG		
20	ABZ2_Mut1_Fw	TCAGCACGTCTCTGAATG	pYX022	ABZ2.R2
	Rv_ABZ2_R_GH	TCGATCCGTCTCAGGTCTCCCGGTTCAATATTTTGTCTTCACTGTTC	ABZ	

Table S2: Real Time qPCR results of the strain F in which only FOL2 gene is overexpressed.

	WT	F		T.TEST	
	FOL2	*FOL2*		0,032	*
	1	1,40			
	1	2,10			
	1	1,40			
	1	1,60			
	WT	F			
Media fold change	1,00	1,63			
DEV.ST	0,00	0,33			

Table S3: Real Time qPCR results of the strain AFS in which the genes ABZ1$_{awri1613}$, ABZ2$_{qa23}$, FOL2, ARO4^{K299L}, AroL, and ARO2 are overexpressed.

	WT	AFS								
		ABZ1	*ABZ2*	*FOL2*	*ARO4*	*ARO2*	*AroL*	Ttest		
	1	1,99	2,24	1,28	3,98	1,73	264280,66	ABZ1	0,163	
	1	0,90	1,66	1,14	4,24	1,56		ABZ2	0,054	*
	1	2,16	5,25	2,81	5,43	3,04		FOL2	0,101	
	1	1,30	3,06	2,33	6,97	3,65		ARO4	0,002	*
	1	0,91	1,74	1,14	3,99	1,65		ARO2	0,036	*
	WT	AFS								
		ABZ1	*ABZ2*	*FOL2*	*ARO4*	*ARO2*	*AroL*			
media	1	1,45	2,79	1,74	4,92	2,33	264280,66			
dev.st	0	0,59	1,48	0,78	1,29	0,96	#DIV/0!			

OUTLOOKS

At the end of my bachelor's degree, I was very undecided whether to continue with my studies or not. Two experiences mainly convinced me to go on: the first was the internship at the IndBiotech lab, in which I approached for the first time a real and concrete research project. The second was a job interview at Solvay, a chemical multinational company. During the interview I was asked this question: "This is a chemical company, why should we need a biotechnologist?". My answer was: "biotechnology can change the world, every process present here in Solvay can be converted into a biotechnological process and this is my goal". Obviously, I was not hired: but in that spontaneous and sincere response I understood that despite nothing can be changed in a short run, still with a PhD I could have added to my words more relevant weight for the near future. Biotechnology can offer a better alternative in respect to a classical chemical process, while in other cases green chemistry can be the right choice for implementing a traditional chemical workflow. In the future these two possibilities will more and more be seen as complementary and not as mutually exclusive, and I believe that this will allow us to match the best option in terms of environmental and economical sustainability.
Thus, I arrived here, this is my doctoral thesis and I was very lucky because it fully reflects my thinking and my idea of sustainability.

The Green Deal is certainly an important step for the European Union to safeguard the environment and consequently my future and that of future generations. This thesis work focuses on the development of possible new technologies that can be adopted in the context of the circular economy, for the creation of sustainable and net-zero impact processes.

In particular, I focused the study on the concept of biorefineries, as, in my opinion, they are a key enabling technology for valorizing and upcycling industrial waste destined for disposal, such as lignocellulosic biomass. This technology is promising to substitute the traditional way of producing products spanning from low to high added value such as biofuels, chemicals, building blocks, enzymes, cosmetic ingredients, dyes, nutraceuticals, drugs. In short, the biorefineries, if properly developed and studied, can be inserted in every industrial sector to replace the chemical processes currently present and not very sustainable from an environmental point of view.

Specifically, in chapter 1, I explored how the ENEA methodology can be used on the territory to put different companies in contact with each other. These companies could share every type of resource, from the inputs to the outputs they possess, but also technological knowledge: these were subsequently analyzed in order to create operating manuals, delivered to individual companies, in which the possible cases for the creation of industrial symbiosis are described. This method can be of great help also for universities to facilitate the creation of bridges between academia and industry and establish collaborations aimed at solving specific and real problems. In this PhD work it was very useful to understand, for example, which residual biomasses of the agro-food sector can be destined for valorisation through biorefinery. I then deepened the study with the production of Vitamin B_9, an essential component for our diet which today is produced almost exclusively by chemical synthesis. This production can be seen as a case study, but it also represents a potential real target for the development of innovative biobased microbial processes. I worked with two different yeasts capable of producing it naturally, *Scheffersomyces stipitis* and *Saccharomyces cerevisiae*.

S. stipitis was selected for its notable ability to use different sugars, both five and six carbon atoms ones, a fundamental skill for enhancing the value of residual

biomass, in which the sugars composition is different. We therefore studied and optimized the growth on minimal synthetic medium, emulating the sugar compositions of pre-treated lignocellulosic residues. We have obtained excellent results, as this microorganism is the best *wild type* producer of folate reported in literature. In fact, on a mixed synthetic medium of glucose and xylose we obtained good results in terms of production (3.36 mg/L), yield (112.00 µg/g) and productivity (124.00 µg/Lh), if compared to known literature. Further metabolic and/or process engineering would further increase these numbers and make the process significand and robust enough to be tested in a pilot plant. Thanks to the knowledge acquired in this study, we were able to valorize three different residual biomasses, unfermented grape marcs (UGM), chosen thanks to the study conducted with ENEA, molasses and sugar beet pulp (SBM and SBP), already present and used in the laboratory. The best production was obtained on SBM, 188.20 µg/L, while the best yield (9.54 µg/g) was observed using SBP as growth medium. As expected, however, production, yield and productivity obtained from waste biomass are much lower than those obtained in synthetic soil, but as demonstrated with other processes of production, the more the physiological features of the cell factory are understood and studied, the more the process can be ameliorated.

S. cerevisiae was selected for its easy genetic manipulation, using it as a model to observe which genes should be considered to increase natural folate production. Also in this case by optimizing engineering strategies we obtained the highest production (620.00 µg/L) when compared with those present in the literature, but with lower yield (10.94 µg/g). In the future, other genome modifications will certainly be designed and tested, and the fermentation process will be optimized to increase production, yield and productivity of Vitamin B_9 in this host. All the acquired information can be implemented in *S. stipitis* to further increase its current

natural production. This would allow us to carry out an analysis of economic and environmental sustainability, on a laboratory scale, a work that can be of great help to understand whether or not to scale the process to pilot plants.

This doctoral thesis must be a starting point - not an arrival - for the development of a technology in line with the needs that the future imposes on us.