The future of bioethanol

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Chapter

THE FUTURE OF BIOETHANOL

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ABSTRACT

Yeasts have been domesticated by mankind before horses. After the mastering of alcoholic fermentation for centuries, yeasts have become the protagonist of one of the most important biotechnological industries worldwide: the

production of bioethanol. This chapter will initially present some important challenges to be overcome in this industry, both in first and second generation biofuel production. Then, it will briefly revisit some advances obtained in recent years. Finally, it will present and discuss some opportunities, in the scope of metabolic engineering and synthetic biology, that will likely be present in the future of bioethanol.

Keywords: Bioethanol, Yeast, Metabolic Engineering, Synthetic Biology

CHALLENGES AND PERSPECTIVES IN FIRST GENERATION ETHANOL

The global production of bioethanol is expected to increase in the coming years due to increasing investment and governmental policies to incentivise biofuels. Bioethanol, or fuel alcohol, can significantly decarbonise our future energy needs, since they can alleviate the negative impacts of greenhouse gas emissions from fossil fuels, and thus constrain global climate change. For this reason, new distilleries will be built in the coming years, with production expected to surpass the mark of 110 billion liters of ethanol by 2023 (Figure 1).

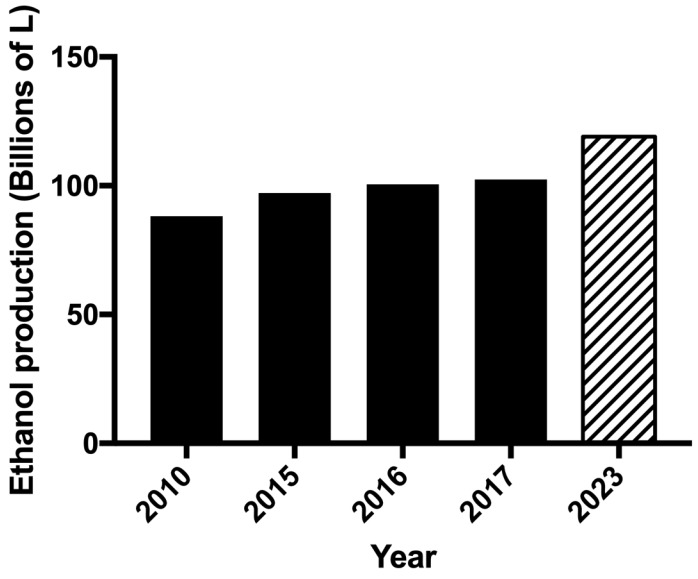

Figure 1: Realised (black bars) and estimated (dashed bar) global ethanol production (Renewable Fuels Association 2019).

The two major producers of first-generation ethanol include Brazil and the US, who are responsible for 26% and 58% of global ethanol production from sugarcane and maize, respectively. Although these are considered mature industries, there is still much room for improvement. Examples include the selection of more stress-tolerant yeast strains isolated from the industrial process itself (Basso et al. 2008; Della-Bianca et al. 2013), or strain improvement strategies of metabolic and evolutionary engineering (Gombert and van Maris 2015; Walker 2011).

Stress Factors in Fermentation Processes

In view of the high levels of ethanol at the end of each fermentation, alcohol is one of the main stress factors that deleteriously affect yeast cells during industrial fermentation. In the case of Brazilian ethanol production, the cell recycling process further exacerbates the stressful effects of ethanol, given the cumulative stress that cells face in subsequent fermentation batches. Despite these limitations, fermentations to a high ethanol concentration are extremely desirable in the context of industrial practices, since they allow reductions in water consumption (yeast dilution and must preparation) and energy expenditure during the distillation stage, thus favouring overall sustainability of industrial processes. In distilleries, the final ethanol content is limited by the inherent tolerance of the yeast strains present during fermentation, together with high temperatures and acidity that exacerbate ethanol stress (Basso, Basso, and Rocha 2010).

The high international price of sugar has led the sugar industry to prioritize the production of sucrose, resulting in the generation of more exhausted molasses. Such impure molasses exerts a more pronounced inhibitory effect on yeast fermentation due to more intensive sugar thermo-degradation. Thus, new yeast strains should be selected to handle variable molasses substrates, even when operating with normal ethanol titres.

Due to the nature of the industrial process of ethanol production, aseptic conditions are very difficult to achieve, and so fermentations operate in the presence of bacterial contaminants. In addition to redirecting sugars that could be used by yeast in ethanol production, these bacteria also produce metabolites (such as lactic and acetic acid) which are harmful to the fermentative performance of yeasts. As a result of bacterial contamination, negative effects include a reduction in ethanol yield, increased yeast flocculation, increased foaming and reduced yeast cell viability (Basso et al. 2014; Ceccato-Antonini 2018). Flocculation induced by the presence of bacteria impairs the efficiency of centrifugation and reduces the contact surface between the yeast cells and the fermentation medium. In turn, excessive

foam formation, caused by the presence of bacteria, increases the costs of the process due to the use of antifoams. Finally, the antibiotics used to control contamination not only increase the costs of the process, but also make dry yeast co-products unfit for commercialization as a food supplement.

High-Gravity Fermentation

There is currently widespread interest in the use of very high gravity fermentations for industrial alcohol production, mainly due to the associated reduction in production costs. It is also expected that this technology will improve environmental sustainability by reducing water and energy consumption. Thus, it is still possible to increase the efficiency of first-generation fuel ethanol processes by adopting this technology. Fermentations that result in high concentrations of ethanol would not only benefit energy balance, but would also result in a significant reduction in the volume of vinasse and the costs of its transportation (for fertilisation of sugar plantation) (Braga et al. 2017). In addition, higher levels of ethanol could suppress bacterial growth during fermentation and, therefore, decrease the requirement for antibiotics to control such contaminations. Nevertheless, high gravity fermentation using cane molasses as substrate will require special strains with simultaneous improvements in osmotic and ethanol tolerance.

CHALLENGES AND PERSPECTIVES IN LIGNOCELLULOSIC ETHANOL

As mentioned in the previous chapters of this textbook (please refer to Chapter 1), Brazil stands out as one of the countries that has implemented a large-scale program of renewable fuels, with many environmental, economic and social advantages. However, in order for ethanol produced in the country to remain the most competitive, it is absolutely necessary to continue investing to improve the production process as well as expanding the sources of raw material for the production of this important source of renewable energy. Within this context, we now highlight exciting opportunities in the exploitation of lignocellulosic materials, such as sugarcane bagasse. This feedstock is a low-cost raw material available and already generated as a co-product of sugar mills and distilleries. Because many technological and political challenges are expected for first generation ethanol production, the future of bioethanol lies in exploiting non-food (lignocellulosic) residues for its production (Walker 2011).

Conversion of second generation lignocellulosic biomass such as sugarcane bagasse poses several technological challenges, but also opportunities. The pre-treatment process, which aims to make the biomass amenable to efficient attack by hydrolytic enzymes, can release pentoses and oligomers that can be fermented by selected yeast strains. Many interesting advances has been made in *S. cerevisiae* for efficient lignocellulosic-derived sugar utilization, including xylose, arabinose, cello- and xylo-oligosaccharide fermentation (van Maris et al. 2006; Walker 2011; Galazka et al. 2010; Li et al. 2015). However, many of these advances are still in development, and not yet tested worldwide at fully industrial scale. The lignocellulose substrate also contains toxic compounds generated during pre-treatment, such as aldehydes, organic acids and phenols (Hahn-Hägerdal et al. 2006). Since lignocellulosic hydrolysates present low sugar and lack of yeast organic and mineral nutrients, addition of cane molasses compensates for these deficiencies, and allow fermentations with higher ethanol titres. Thus, due to the presence of these inhibitors (from molasses and hydrolysate), stressful effects even more intense than that already presented by molasses itself will be imposed on the fermenting yeast. It is well known that even the most appropriate strains available for industrial fermentation do not tolerate musts formulated solely with molasses. Therefore, new strains will be required, with a superior tolerance phenotype as compared to production strains, to conduct the fermentations of a new substrate, making fermentation efficient for second generation ethanol.

A trend that is appearing on the horizon of second generation ethanol processes is the option to implement all strategies presented above in non-Saccharomyces yeasts with industrially interesting properties, such as those displaying high-temperature- and low-pH-tolerant phenotypes (Jansen et al. 2017). Moreover, co-feeding of additional, low-value carbon sources might be explored as a strategy to further increase ethanol production.

According to Jansen et al. (2017), yeast-based consolidated bioprocessing remains a ‘holy grail’ in lignocellulosic ethanol production, but considerable progress is already being made in engineering cellulolytic bacteria for efficient ethanol production. It would be extremely valuable from an industrial standpoint if a simple mechanical pre-treatment with biomass deconstruction and fermentation by a single organism could be coupled to match the robustness of yeasts under industrial-relevant conditions.

Jansen et al. (2017) have compiled specific conversion rates, such as specific xylose consumption rate and specific ethanol production rates, from studies performed using synthetic and real-life hydrolysate media (Table 1). While ‘academic’ yeast platforms do exhibit high ethanol yields in hydrolysates, conversion rates under such conditions are significantly lower than in synthetic

media. According to the authors, improving kinetics and robustness in industrial hydrolysates is of utmost importance in the development of industrial yeast strains.

Table 1: Fermentation parameters in cultures of *S. cerevisiae* strains engineered for pentose fermentation, grown in lignocellulosic hydrolysates and in synthetic medium (Based on data compiled by Jansen et al. (2017)).

Yeast strain (*S. cerevisiae*)	**Pathway**	**Feedstock**	**Fermentation conditions/nitrogen source**	**$Y_{Eth/S}$ (g g^{-1})**	***q*glucose (mmol g^{-1} h^{-1})**	***q*ethanol (mmol g^{-1} h^{-1})**	***q*xylose (mmol g^{-1} h^{-1})**
TMB3400	XR/XDH	Spruce	Anaerobic/Yeast extract	0.41	0.021	0.005	0.005
GLBRCY87	XR/XDH	Corn stover	Semi-anaerobic/Urea	0.28	1.4	0.27	0.04
MEC1122	XR/XDH	Corn cob	Semi-anaerobic/Urea and Yeast extract	0.3	n.d.	0.12	0.25
RWB218	XI	Wheat straw	Anaerobic/Ammonium sulphate	0.47	1.58	1.0	0.32
GS1.11-26	XI	Spruce	Semi-anaerobic/Amino acids	0.43	2.46	0.3	0.11
LF1	XI	Corn stover	Semi-anaerobic/Urea	0.41	0.57	0.34	0.23
S. cerevisiae (various strains, including most of the above ones)	XI and XR/XDH	Synthetic media with glucose and xylose as the sole carbon source	Anaerobic/ Ammonium sulphate	0.39 ± 0.05		0.26 ± 0.22	0.64 ± 0.51

A STEP FURTHER IN BIOETHANOL PRODUCTION: THE POWER OF METABOLIC ENGINEERING FOR IMPROVING ETHANOL PRODUCTION

The power of metabolic engineering, coupled or not to other ancillary molecular genetic tools such as laboratory adaptive evolution, will be critical to improve the production of bioethanol. Some illustrative examples aimed at improving ethanol production are depicted in Figure 2, with further examples described in greater detail below.

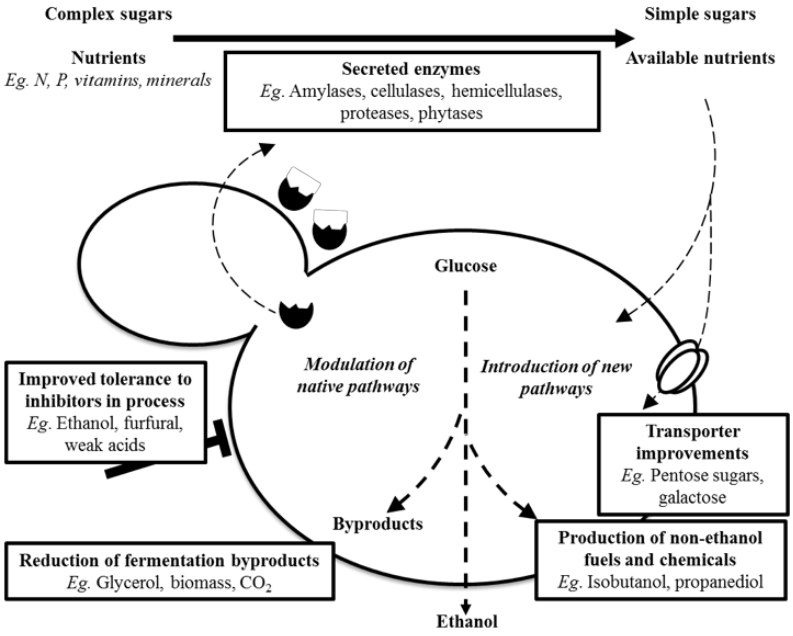

Figure 2: Yeast strain improvement opportunities for fuel alcohol fermentations (Reproduced from Argyros and Stonehouse, 2017 with permission).

Decrease in Free-Energy Conservation for Yield Improvement

In chemo-heterotrophic organisms, which comprise the majority of industrial microorganisms, the Gibbs free-energy available during the dissimilation of the energy source (which is also often source of carbon) is consumed in biosynthesis reactions and in cell maintenance. This free-energy is mostly temporarily stored in

the chemical bonds of adenosine triphosphate (ATP). In *S. cerevisiae*, the synthesis of ATP is intrinsically linked to the formation and excretion of ethanol during fermentative metabolism. Thus, any metabolic engineering strategy that forces cells to direct a major part of the carbon and energy source to ATP synthesis will result, at least in theory, in a higher ethanol yield, concomitantly with decreases in yields of other compounds (glycerol, for example) and yeast biomass.

Normally yeasts use the enzyme invertase to hydrolyse sucrose (the most abundant carbon source of sugarcane must) in the extracellular environment. The glucose and fructose generated in this way are transported into the cells by facilitated diffusion (without free-energy expenditure). When replacing this native mechanism by sucrose uptake through co-transport with protons and subsequent intracellular hydrolysis of this disaccharide, there is a 25% decrease in the conservation of free-energy (moles of ATP per mole of sugar consumed), since the cells are forced to expend energy to remove the protons from the intracellular environment to maintain homeostasis. Thus, by reducing the amount of ATP generated in the assimilation of sucrose, a greater deviation of sucrose is expected for the formation of ethanol as compated to the native mechanism (Figure 3). Combining this metabolic strategy with laboratory evolution, Basso et al. (2011) have shown it was possible to increase the conversion factor of sucrose in ethanol by 11% compared to the original phenotype.

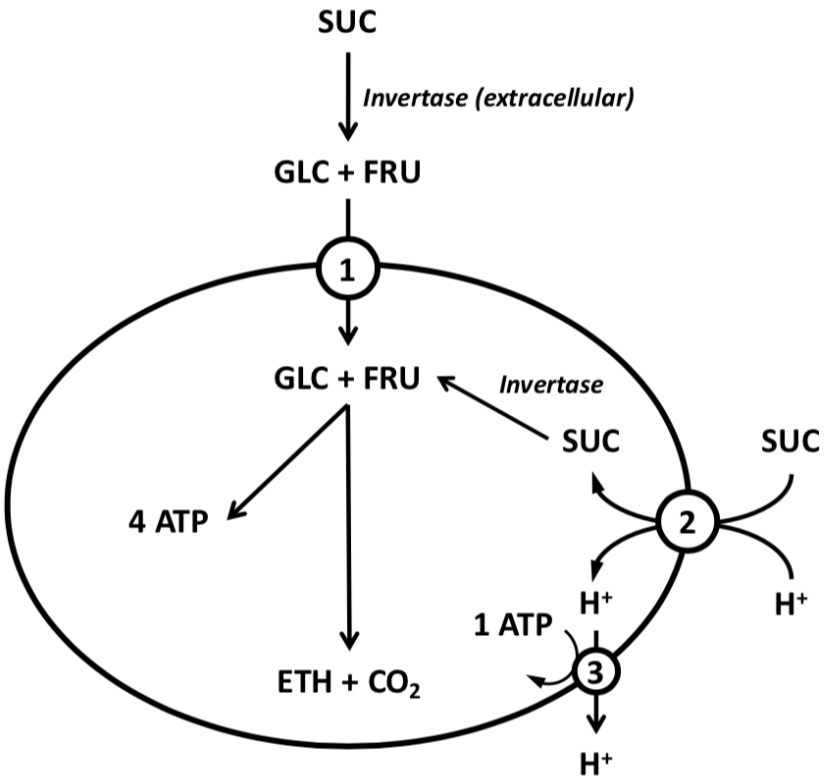

Figure 3: Sucrose metabolism in *S. cerevisiae*, highlighting the extracellular and the intracellular pathways. Legend: SUC: sucrose; GLC: glucose; FRU: fructose; ETH: ethanol; 1: Hxtp hexose transporters; 2: Agt1p or Malx1p transporters; 3: H^+-ATPase transporter.

Expanding Substrate Range

The production of ethanol from lignocellulosic sources, also known as second generation ethanol, requires the fermentation of the hemicellulosic fraction of the biomass, which is rich in pentoses such as xylose and arabinose. However, the growth of genetically modified *S. cerevisiae* strains for the metabolism of xylose is generally compromised by problems in the redox balance of the NAD^+ and $NADP^+$ cofactors of the xylose reductase and xylitol dehydrogenase enzymes, or by the low xylose isomerase activity, depending on the strategy of metabolic engineering in question.

In this context, Sonderegger and Sauer (2003) demonstrated the application of evolutionary engineering to select for a strain of *S. cerevisiae* capable of growing in xylose under anaerobic conditions. In this case, a recombinant line (expressing the xylose reductase and xylitol dehydrogenase enzymes), capable of efficiently growing xylose under aerobic conditions, was slowly adapted in a chemostat to grow under microaerobic conditions, and then subjected to the situation of anaerobiosis. This procedure, which totalled 260 generations of selection, is a strong indication that multiple mutations were necessary to obtain this new phenotype.

Kuyper et al. (2004) and Kuyper et al. (2005) demonstrated the possibility of using metabolic engineering strategies associated with evolutionary engineering to obtain a strain of *S. cerevisiae* capable of efficiently converting xylose into ethanol. In this instance, a line expressing the enzyme xylose isomerase was submitted to serial transfers in medium containing xylose as the only carbon source. Subsequently, in a repeated batch regime, another stage of evolutionary engineering was started, under increasing conditions of oxygen limitation, until the point of full anaerobiosis. At the end of this selection, a mutant capable of growing in xylose under strict anaerobic conditions was obtained.

These examples demonstrate the applicability of adaptive evolution strategies in generating new yeast strains with valuable characteristics for second generation bioethanol production.

Reduction of By-Product Formation

Under anaerobic conditions, the growth of the yeast used in the production of biofuels is accompanied by the formation of glycerol, which results in a decrease in substrate conversion to ethanol and lower fermentation yield. However, formation of glycerol is essential to reoxidize excess NADH formed during cell growth and also to protect cells from osmotic stress, which are common in industrial processes.

In a promising example, CO_2 was used as the final electron acceptor generated in biomass biosynthesis reactions, as an alternative to glycerol (Guadalupe-Medina et al. 2013). In this instance, atmospheric CO_2 was reduced to ethanol through the insertion of two plant genes, responsible for the synthesis of two enzymes of the Calvin Cycle, phosphoribuloquinase and Rubisco. This strategy resulted in a 90% reduction in glycerol formation and a 10% increase in ethanol yield.

Another very interesting example, with proven effectiveness for industrial application, is the case of the SucraMax™ yeast strain (Argyros & Stonehouse, 2017), developed by Lallemand/Mascoma. The strain is claimed to reduce glycerol

by-product formation between 20-30% in sugarcane-based fermentations. Furthermore, ethanol yield is also claimed to increase by 2-3% compared to regular strain fermentations.

For corn-based fermentation, the same company has developed another yeast strain named TransFerm®. This strain is able to secret a glucoamylase (GA) and thus reduces the amount of purchased enzyme addition (Argyros & Stonehouse, 2017). To illustrate the potential benefits of this strategy, a large corn ethanol plant typically spends more than US$ 1 million on purchased glucoamylase in a single year. This strategy has been followed, and further developed by other companies, such as Novozymes, Lesaffre and Global Yeast.

During industrial strain development for ethanol production, several important parameters should be considered when developing new strains. For example, in the case of sugarcane- and corn-based fermentations, it is of paramount importance to compare strains on a representative industrial feedstock. Tests could then be conducted in vessels that do not allow oxygen to enter the system once the fermentation has started. Importantly, the scalability of yeast biomass production needed to supply industry demand has to taken into account during strain selection.

THE ETHANOL PLANT AS A BIOREFINERY

Ethanol production can offer new economic opportunities in terms of lessening dependence on energy imports, especially for developing countries. Nevertheless, the origin of the raw materials used for its production should be sustainable and must not threaten biodiversity or food security (Walker 2011). In this respect, cereal crops are not the most suitable alternatives, since they may compete with global food requirements. Sugarcane and other grasses, on the other hand, do not bring such issues, but are of limited capacity to meet an envisaged dramatic increase in bioethanol production for the years to come. Consequently, the future for bioethanol lies in exploiting lignocellulosic residues, comprised of raw materials generated from agriculture, industry and forestry activities (Walker 2011).

Microbial cell factories may be used to produce biofuels and so replace hydrocarbons with renewable biomass (Zhou, Kerkhoven, and Nielsen 2018). The development of these cell factories will benefit from metabolic engineering strategies that have been developed in the recent past or are still under development. This is mainly because there is still a great lack of knowledge about how their metabolism is regulated. Microbial cells have evolved to grow and survive within their natural habitats. When these cells are modified in order to employ them in an industrial biotechnological process, several complex cellular factors come into

play, which often act against the establishment of the phenotype sought by the strategy of metabolic engineering.

Many of the current applications and studies are still based on a few microbial species, especially the bacterium Escherichia coli and the yeast *S. cerevisiae*. The prospect is that other cell factories will become the target of metabolic engineering with increasing frequency, especially exploring characteristics important for industrial applications, such as tolerance to extremes of pH or temperature.

As knowledge about the cellular systems used in metabolic engineering advances and is deposited and organized in public databases on the internet, the process of modifying a given cell for a biotechnological application should become increasingly prevalent. The use of computational algorithms, machine learning and big data interpretation for the integration of all the information available will be increasingly important. Thus, Systems Biology and Synthetic Biology certainly have and will play a key role in these activities, including the possibility of synthesizing entire molecules of DNA in vitro and, perhaps, we will eventually reach the first fully synthetic cell (see Section "THE POWER OF SYNTHETIC BIOLOGY").

FIGHTING WITH BACTERIAL CONTAMINANTS DURING ETHANOL PRODUCTION

Fuel ethanol fermentation is one of the largest industrial biotechnological processes in the world. However, in view of the nature of the process and the large volumes processed, aseptic conditions are never achieved. Therefore, bacterial contamination is a concurrent problem in industrial fermentations. This is regarded as a major drawback that deviates sugars away from ethanol formation and lead to detrimental effects upon yeast fermentative performance, such as reduced ethanol yield, yeast cell flocculation, and low yeast viability (de Oliva-Neto and Yokoya 1994; Narendranath et al. 1997; Bayrock and Ingledew 2004; Basso et al. 2014; Rich et al. 2018).

Bacterial contaminants found during ethanol fermentation comprise mainly lactic acid bacteria (LAB) (Narendranath et al. 1997; Lucena et al. 2010), probably because of their higher tolerance towards acidic pH and ethanol titres when compared to other microorganisms (Kandler 1983; Skinner and Leathers 2004). Studies that investigated the identity of these contaminants during yeast fermentation in Brazilian ethanol plants found that *Lactobacillus* spp. was the most abundant genus (Lucena et al. 2010). In the case of cereal-based fermentations, such

as corn ethanol, the major contaminants are also *Lactobacillus* strains (Narendranath et al. 1997; Walker and Walker 2018).

Contaminating lactic acid bacteria are traditionally classified in two major metabolic sub-groups according to the pathway used to metabolize hexose sugars: homo- and heterofermentative (Kandler 1983). In general, bacteria isolates from industrial fermented sugarcane substrates have shown to include both types (Costa et al. 2008).

Homofermentative bacteria catabolize hexoses via the so-called Embden-Meyerhof-Parnas (EMP) pathway, in which 1 mol of hexose results in the formation of 2 mol of lactic acid and 2 mol of ATP. In comparison, another pathway is active in heterofermentative bacteria: the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway (Kandler, 1983). In this case, hexoses are converted to equimolar amounts of lactic acid, ethanol or acetate, and carbon dioxide, yielding 1 mol of ATP per mol of hexose fermented (Axelsson et al. 1993; Cogan and Jordan 1994). With the conversion of acetyl phosphate to acetate instead of ethanol, an additional ATP can be produced. Then, regeneration of surplus NAD^+ must be achieved by an alternative electron acceptor. Under aerobic conditions, oxygen may serve as the electron acceptor (Cogan and Jordan 1994), but under anaerobic or even oxygen-limited conditions, fructose is reduced to mannitol, serving as an electron sink (von Weymarn, Hujanen, and Leisola 2002). There is also a third classification group that, differently from the homofermentative strains that cannot metabolize pentose sugars, can ferment these sugars using an inducible phosphoketolase pathway, producing lactate and acetate (Kang, Korber, and Tanaka 2013).

Modulating the Bacterial Microbiome in the Ethanol Industry

To tackle the issue of bacterial contamination during ethanol fermentation and to avoid the emergence of bacterial resistant strains due to antibiotic usage, novel antimicrobial strategies are appearing in the horizon of ethanol distilleries. One such interesting example is the modulation of the bacterial microbiome in the ethanol plant.

Rich et al. (2018) identified a group of 26 LAB strains, previously isolated from corn ethanol plants, which restored the ethanol yield that was hampered by the presence of *L. fermentum* to the level of that without contamination. The beneficial bacterial strains were *L. plantarum*, *L. casei* and *L. pontis*, and the benefit was strain-specific. It is believed that specific bacterial cell-cell antagonism is governed by the production of small peptides, such as bacteriocins. However, this strategy remains to be tested in industrial scale sugarcane-based fermentations.

HOW TO FIND THE YEASTS OF TOMORROW

As mentioned in Section "CHALLENGES AND PERSPECTIVES IN FIRST GENERATION ETHANOL", it is expected that the fuel ethanol production industry will continue to grow, especially sugarcane-based bioprocesses. One of the reasons for this efficiency is the biochemical evidence that near 93% of fermented sugar free-energy still remains in the produced ethanol molecule. Yeast cells therefore charge only 7% of the available energy for in the process of converting sugar into a liquid fuel. These figures testify to just how efficient this bioprocess is for fuel production.

The 1G ethanol production process, at least in Brazil, is experiencing changes in the feedstock itself. The recently introduced sugarcane mechanical harvesting method has resulted in increasing levels of polysaccharides (starch), aconitic acid and phenolic compounds in molasses. This is because tips and leaves of sugar cane are also collected during mechanical harvesting. Aside from the inhibitory effects of phenolic compounds, and perhaps aconitic acid, these compounds require a more intensive chemical treatment of cane juice to remove colour during crystal sucrose production, leaving more residual compounds in the yeast substrate (molasses).

Musts prepared entirely with molasses exhibit some toxic effects to yeast, especially during the process of reusing yeast. Only a few strains with desirable tolerance are available, although these are unsuitable for many distilleries as few distilleries operate under high- gravity fermentation conditions.

Where can we find these new robust yeast strains? To answer this question, laboratory improvement of strains by hybridisation followed by selection and adaptive evolution have previously been performed, but such strains are still under laboratory evaluation (Basso, 2015). On the other hand, some distilleries have been operating for several years with high ethanol titres and musts entirely formulated with molasses. Karyotyping and other molecular tools has revealed yeast population of indigenous strains replacing the starter yeast strain. These recent observations follow the same trend documented more than two decades ago, when more robust strains in relation to the starter strains were selected from the industrial process (Basso et al. 2008).

It is evident that the great biodiversity of indigenous yeasts found in fermentation vats are continuously propagating during cell recycling. Two fermentation cycles per day during a season of 200-250 days allow for ca. 60-70 generations, where adapted strains can evolve and be selected. Therefore, searching for prevalent and persistent strains in industrial environments seems to be an efficient approach to select for desirable strains with multi-tolerant traits.

But again, the Brazilian industrial process can provide a remarkable contribution. Indeed, an ongoing experiment in a Brazilian distillery (São Luiz Mill, São Paulo State), operating for many years with 11-12% (v/v) ethanol and using vigorous agitation and molasses substrate, was able to finalize fermentation within a record time of 4 hours, operating under cell recycling in a continuous process. Under such unusual physiological conditions, dominant strains were isolated and showed to be more tolerant and with higher fermentation rates than the (replaced) starter strain (CAT-1). This short fermentation time was possible due to the use of fast fermenting strains and intensive agitation, and it could significantly contribute to an increase in productivity and reduction in equipment costs (such as increasing production capacity) (Cunha, Zimak and Basso, unpublished results).

In another occasion, isolated strains presenting very high sugar consumption rates (in a fed-batch process) were able to sustain very low sugar levels during must feeding. In both cases an additional benefit is expected: attenuation of bacterial growth due to improved competition by the yeast strain to the same substrate.

In these cases (as observed two decades ago), the improved strains evolved in the fermentation vats under conditions established by the distillery. The laboratory task was then to identify suitable strains, access their physiological and technological traits, and evaluate their fermentation capability in the following seasons. As physiological conditions are rather unique to each distillery, selected strains are likely to adapt for each individual distillery.

THE POWER OF SYNTHETIC BIOLOGY

Introduction

Synthetic biology is an emerging field of research with excellent potential to improve bioethanol production from renewable sources. A significant and ongoing reduction in the cost of synthesising DNA, as well as corresponding advances in supporting technologies, have driven developments in this field over the past two decades (Cameron, Bashor, and Collins 2014). These new capabilities have enabled the design and construction of multiple synthetic genes, pathways and even entire genomes (Gibson et al. 2010).

The overall goal of synthetic biology is to improve predictive capacity and reconcile complexity in biological systems; and, ultimately, introduce new-to-nature gain-of-function. Although no consensus exists in terms of definition (Calvert 2010; Rodrigues and Rodrigues 2017), the field of synthetic biology may be broadly described as the engineering of biology, incorporating concepts such as modularity, standardisation, automation, hierarchies of abstraction, robustness and

orthogonality (Giese et al. 2013; Rodrigues and Rodrigues 2017). These approaches, coupled with the development of new enabling technologies, distinguish synthetic biology from genetic engineering, and have ultimately advanced our ability to translate an idea into a product with real-world potential (Figure 4).

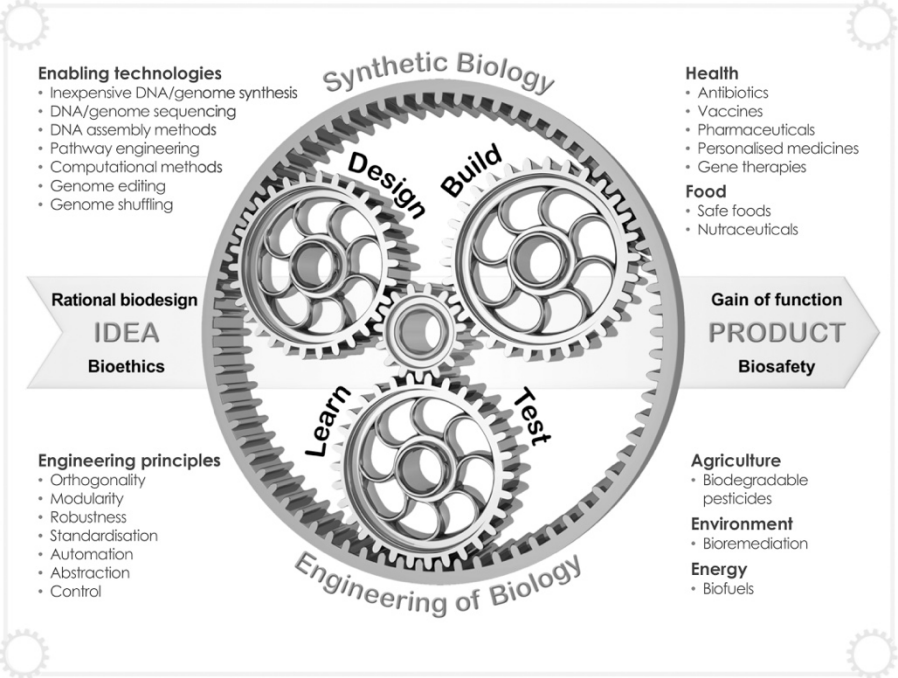

Figure 4: Synthetic biology applies rational engineering principles at the molecular level. New technologies and approaches have enabled researchers to look beyond the traditional 'tinkering'-based approaches of genetic engineering, and significantly reduce the amount of time required to translate an idea into a product or application. These approaches are commonly based on the Design-Build-Test-(Learn) cycle. Reproduced with permission from Walker and Pretorius (2018).

Engineering *S. cerevisiae* for Bioethanol Production

In the context of bioethanol production, synthetic biology research focuses on the yeast, *S. cerevisiae*. Aside from existing industrial benefits such as stress tolerance and a natural propensity to produce ethanol; *S. cerevisiae* has a well-understood physiology, powerful homologous recombination machinery and

accompanying collection of genetic tools. For further information, (Liu, Zhang, and Nielsen 2019) review the synthetic biology of yeast in general and (Tsai et al. 2015) describe its applications for biofuel production.

New tools include precise genome editing techniques, such as the CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-associated protein-9 nuclease) system, which may be used to precisely alter, remove or insert genes. CRISPR-Cas9 may also be used to introduce whole metabolic pathways (Stovicek, Borodina, and Forster 2015; Stovicek, Holkenbrink, and Borodina 2017), and simultaneously alter, or introduce, multiple genes in a multiplexed manner (Horwitz et al. 2015). CRISPR-Cas9 editing is simple to perform, inexpensive, and has the advantage of allowing the simultaneous modification of multiple copies of the same chromosome in aneuploid or polyploid industrial yeast strains.

Recent examples of CRISPR-Cas9 include the modification of a polyploid industrial strain to introduce a xylose pathway for the improved production of ethanol from cellulose hydrosylates (Lee et al. 2017). In this instance, an NADH oxidase enzyme from Lactococcus lactis was introduced to overcome a redox imbalance resulting in xylitol accumulation. CRISPR-Cas9 has also been performed in an automated manner to systematically introduce modifications to the *S. cerevisiae* genome, leading to improvements in acetic acid tolerance (automated multiplex genome-scale engineering in yeast; (Si et al. 2017)). Furthermore, CRISPR-Cas9 may be applied to introduce alterations to genes conferring industrially-important traits. This can be performed by applying pooled-segregant whole-genome sequence analysis to identify the causative alterations associated with improved ethanol, temperature and acetic acid tolerance (Deparis et al. 2017).

Yeast 2.0

Advances in de novo DNA synthesis have led to projects with increasing scope and ambition. The Sc2.0 (*S. cerevisiae* version 2.0) project currently lies at the forefront of eukaryotic synthetic biology, and is undertaking rapid progress towards the complete construction of the world's-first synthetic eukaryote genome (Pretorius and Boeke 2018). Significant design alterations include the introduction of specialised LoxPSym sites throughout the genome, enabling an inducible system called SCRaMbLE (Synthetic Chromosome Recombination and Modification by LoxP-mediated Evolution) to re-arrange the genome into unique variations. SCRaMbLE may be applied to generate a cell population with theoretically a near-infinite number of genome permutations (Shen et al. 2016), providing researchers with a tool to improve our understanding of the genetic complexity associated with

improved yeast strain phenotypes. For example, (Blount et al. 2018) employed SCRaMbLE to improve growth on xylose, revealing potential non-intuitive solutions to intracellular redox balance. Furthermore, SCRaMbLE may be used to improve cellular fitness in response to industrial stressors such as heat, ethanol and acetic acid (Luo et al. 2018).

Lignocellulose Processing

It remains a significant challenge to produce high yields of ethanol from lignocelluose following its biological conversion into fermentable substrates. Despite this challenge, improvements to lignocellulose bioprocessing is a major focus of research in synthetic biology. One advantage includes the rapid design and DNA synthesis of modular, codon-optimised biological parts (genes) which may be recovered from any organism of choice. A large library of these biological parts may then be rapidly tested to identify the optimal individual gene, or combination of genes, for improved biomass degradation and solvent tolerance (French 2009). Furthermore, it may also be advantageous to combine both synthetic biology and metabolic engineering approaches for lignocellulose processing. (Wei et al. 2015) demonstrated the introduction of both a hexose and pentose fermentation pathway and an acetic acid reduction pathway into *S. cerevisiae* for substrate co-utilisation (Wei et al. 2015). This approach led to higher biomass utilisation and substantially higher yield compared to control strains.

An interesting trend in recent years has been to tether pathway enzymes to the yeast cell surface. Engineering of the cell surface for improved consolidated bioprocessing was reviewed by Chen (2017) and Hasunuma and Kondo (2012). In one example, the attachment of four cellulose degrading enzymes onto the yeast cell surface was shown to improve production of bioethanol directly from rice straw (Liu et al. 2016). Yields were later improved by altering the ratio of cellulases combined with a novel screening strategy (Liu et al. 2017). Furthermore, (S. L. Tsai, Goyal, and Chen 2010) applied a synthetic cellular consortia of four different yeast strain variants expressing a minicellulosome on the cell surface to convert cellulose to ethanol. This synthetic biology approach achieved an ethanol yield corresponding to 93% of the theoretical maximum. Finally, the tethering of a phytase to the yeast cell surface was also shown to reduce the environmentally-damaging phytate phosphorus and improve the rate of yeast cell growth (Chen et al. 2016).

The Future of Synthetic Biology: Thinking Outside the Bioethanol Box

The field of synthetic biology is still very much in relative infancy and so it is difficult to predict the state-of-the-art in the years ahead. It's unclear if synthetic biology will provide a singular 'magic bullet' to improve bioethanol production, and so a combination of metabolic engineering, directed evolution and synthetic biology approaches may be required. However, synthetic biology will likely be key to unlocking unexpected research avenues and will provide new technological solutions in the years to come.

The synthetic biology toolbox and approaches used are constantly evolving. For example, the compartmentalisation of metabolic pathways in synthetic organelles may be one strategy to maximise local substrate concentration, sequester toxic products and create unique biochemical environments (Lau et al. 2018). Furthermore, the refactoring of entire metabolic systems with improved variants may lead to more energy-efficient yeast strains (Kuijpers et al. 2016). Finally, the Yeast 2.0 project lends weight to the intriguing notion that it may be possible to 'print' synthetic chromosomes with defined characteristics in future.

Despite the proven benefits of the *S. cerevisiae* workhorse, there is also potential in the field of synthetic biology to engineer "non-standard" organisms. These organisms often display beneficial characteristics, such as exceptional thermotolerance and rapid growth of the yeast *Kluyveromyces marxianus* (even up to a reported 50oC on xylose-rich hydrolysate (Kumar et al. 2015)) to the broad substrate range and high secretion capacity of *Kluyveromyces lactis* and *Pichia pastoris* (Syn. *Komagataella phaffii*) (Gellissen et al. 2005). Despite inefficient homologous recombination and the oxygen requirement of Crabtree-negative yeast, new tools are being developed to engineer such strains (Wagner and Alper 2016). The use of synthetic biology and metabolic engineering approaches for nonconventional yeasts in biofuel production has been reviewed previously (Madhavan et al. 2017).

Future work may also be applied to feedstock material, such as developing plants with reduced lignin content (Rodrigues and Rodrigues 2017). Furthermore, it may be possible to increase the yield of plant biomass for first-generation and second-generation biofuels. By applying synthetic biology approaches, (South et al. 2019) recently reported the introduction of a metabolic pathway into a transgenic tobacco plant line, ultimately boosting productivity by 40%. It is easy to envision future transgenic crops with a significantly improved capacity for photosynthesis and resulting ethanol yield.

Synthetic biology must still address and overcome numerous existing and future hurdles. These include the ongoing challenge of producing commercially-viable ethanol yields from lignocellulose (Walker and Walker 2018), the stressors and burdens of scaling-up designer organisms to industrial fermentation and additional challenges such as the economics of algal bioethanol production (Jagadevan et al. 2018). Despite these considerations, the field of synthetic biology holds great promise and may well prove to be a disruptive technology in the years ahead.

CONCLUDING REMARKS

Yeasts are considered the most important, and at the same time, the least understood player in ethanol production processes (Walker and Walker 2018). Particularly in sugar cane bioethanol plants, it is of paramount importance to control production of secondary fermentation metabolites (e.g., reduce glycerol biosynthesis) in order to increase sugar conversion yield and to improve glycolytic flux (Gombert and van Maris 2015). For second generation ethanol production, metabolic engineering is imperative to build strains of *S. cerevisiae* able to utilize xylose and arabinose as fast as hexose sugars (van Maris et al. 2006; Jansen et al. 2017).

It is now possible to obtain over 20% v/v ethanol in industrial fermentations with proper nutrients. However, if yeasts are stressed, stuck and sluggish fermentations can be encountered. It is thus essential for optimizing alcoholic fermentations to understand aspects of yeast cell physiology, particularly when exploiting lignocellulosic substrates for second-generation bioethanol production (Walker and Walker 2018).

Very importantly, in non-targeted strain improvement strategies (such as evolutionary engineering, strain hybridization, genome shuffling), it is crucial to maintain selective pressure on all relevant phenotypes of strain performance, to avoid trade-offs between sugar fermentation kinetics and/or stress robustness (Jansen et al. 2017; Demeke et al. 2013).

In spite of all challenges associated with ethanol production and the various limitations imposed by factors that affect yeast performance, many opportunities exist to improve this process (Table 2). For example, the enormous diversity of strains that evolve in the fermentation vessel of the characteristic recycle processes that operates in Brazilian plants constitutes a valuable source of new yeast varieties that have been, and will be, prospected in future to facilitate the continual improvement of industrial processes (Basso et al. 2008; Della-Bianca et al. 2013). In addition, many studies on the physiology, microbiology, and genetics of

microorganisms in Brazilian laboratories, will also provide information on the trophic relationships between yeasts and bacteria in the fermentation environment. Such research will undoubtedly enhance the underlying science that will lead to improved industrial ethanol production.

Table 2: Selected strategies for improving yeast fermentations. Adapted from (Walker and Walker 2018).

Improvement aspect	**Improvement strategies**
Optimising yeast nutrition	Control of nutrient bioavailability in industrial feedstocks for yeast fermentations (sugar, nitrogen, vitamin and mineral sources). Adopt high-gravity fermentation processes and reduce inhibitory chemicals from fermentation substrates. For 2G bioethanol, exploit GM yeast strains to ferment pentose sugars.
Mitigating yeast stress	Employ cell physiological approaches, including culture preconditioning, to minimize yeast stress caused by temperature, pH, osmotic pressure. Simultaneous saccharification and fermentations (SSF) can be employed for starch-to-ethanol processes to alleviate glucose-induced osmotic stress.
Enhancing yeast fermentative metabolism	Production of secondary fermentation metabolites such as glycerol can be reduced using metabolic engineering. GM strains of *S. cerevisiae* can be constructed to improve glycolytic flux and ferment xylose and arabinose. Increase of product yield by manipulating free-energy conservation.
Yeast strain improvement	Selection of naturally robust, and non-flocculent, yeast strains for bioethanol fermentations. Exploit genetic manipulation, and potentially synthetic

	biology, for new yeast strains especially for second-generation bioethanol fermentations.
Controlling microbial contamination	Bioethanol plants to be operated hygienically, including when necessary the use of antimicrobials. Use of beneficial microbes.
Bioprocess optimisation	Fermentable feedstock preparation, fermentation and distillation processes, together with and downstream technologies to be properly monitored and controlled to avoid ethanol losses.

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Important Note Regarding Chapter References

Please see the **Chicago Manual Style** reference guide included with your instructions.

Within text, the references should be either author identified **or** reference number identified and placed in square brackets on the line, e.g. [37].

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