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Neither 1G nor 2G fuel ethanol: setting the ground for a sugarcane-based biorefinery using an iSUCCELL yeast platform

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ABSTRACT

First-generation (1G) fuel ethanol production in sugarcane-based biorefineries is an established economic enterprise in Brazil. Second-generation (2G) fuel ethanol from lignocellulosic materials, though extensively investigated, is currently facing severe difficulties to become economically viable. Some of the challenges inherent to these processes could be resolved by efficiently separating, and partially hydrolysing the cellulosic fraction of the lignocellulosic materials into the disaccharide cellobiose. Here we propose an alternative biorefinery, where the sucrose-rich stream from the 1G process is mixed with a cellobiose-rich stream in the fermentation step. The advantages of mixing are threefold: 1) decreased concentrations of metabolic inhibitors that are typically produced during pretreatment and hydrolysis of lignocellulosic materials; 2) decreased cooling times after enzymatic hydrolysis prior to fermentation; 3) decreased availability of free glucose for contaminating microorganisms and undesired glucose repression effects. The iSUCCELL platform will be built upon the robust *Saccharomyces cerevisiae* strains currently present in 1G biorefineries, which offer

competitive advantage in non-aseptic environments, and into which intracellular hydrolyses of sucrose and cellobiose will be engineered. It is expected that high yields of ethanol can be achieved in a process with cell recycling, lower contamination levels and decreased antibiotic use, when compared to current 2G technologies.

Keywords: ***Saccharomyces cerevisiae*****, sucrose, cellobiose, biorefinery, fuel ethanol**

INTRODUCTION

Sugarcane is considered the most efficient crop for fuel ethanol production and a major player in energy diversification and sustainable development. Production of fuel ethanol from a mixture of sugarcane juice and molasses has been termed first-generation (1G) ethanol, fuel ethanol or bioethanol. In this non-aseptic and anaerobic process, the yeast *Saccharomyces cerevisiae* converts sugars into ethanol with typical yields around 90% of the theoretical maximum, which is equal to 0.511 g ethanol per g of hexose equivalent. The fermentation medium, known as 'must' in the industrial jargon, is prepared by mixing sugarcane juice - the liquid stream obtained from directly milling sugarcane - and diluted molasses, a dark brown viscous liquid generated as a by-product of edible sugar production. The fibrous residue left after sugarcane juice extraction, known as bagasse, is normally burnt in furnaces, which, depending on the efficiency of the boiler, not only provides the energy necessary to run the biorefinery (in the form of high pressure steam and electricity), but also generates revenue by exporting excess electricity to the national grid. Thus, in a typical Brazilian sugarcane-based biorefinery, three major products are generated: sugar, ethanol and electricity. Normally, the bioethanol plants have excess production capacity installed, to allow some flexibility in the sugar to ethanol production ratio, which can be finetuned depending on the prices of these commodities in the international market. The current sugarcane-based biorefinery has been comprehensively discussed, from different perspectives, in several articles (Abreu-Cavalheiro and Monteiro 2013; Della-Bianca *et al*. 2013; Furlan *et al*. 2013; Gombert and van Maris 2015; Lopes *et al*. 2016; Vaz 2017; Ceccato-Antonini 2018; Paulino de Souza *et al*. 2018).

The sugarcane plant is composed of stem and straw (green tops and dry leaves). The stem – used for milling to obtain the sugarcane juice – represents 80 to 85% of the total plant biomass (Carvalho-Netto *et al*. 2014), and consists of 70% water, 16% sugars, and 14% fibre (or bagasse), whereas the remaining fractions of sugarcane (straw) are composed of lignocellulose. Since the cost of this raw material represents a major part of the final production costs of fuel ethanol, it would be very important to utilise the entire sugarcane plant in a more efficient way. One of the most popular strategies targets the use of the lignocellulosic fraction of sugarcane for the production of ethanol in a so-called second-generation (2G) process (Soccol *et al*. 2010; Canilha *et al*. 2012; dos Santos *et al*. 2016).

In the 2G process, part of the cellulose/hemicellulose-rich sugarcane bagasse is diverted to produce additional volumes of ethanol, which occurs without any expansion of the cultivation area. For this to happen, the recalcitrant lignocellulosic matrix must be broken down and hydrolysed, before yeast can ferment the sugars. Naturally, this will come at the expense of electricity generation (Dias *et al*. 2011; Furlan *et al*. 2013; Tapia Carpio and Simone de Souza 2019). On the other hand, since nearly 95% of sugarcane is presently harvested mechanically in the Central-South region of Brazil (Bordonal *et al*. 2018) encompassing 91% of the total planted area, additional lignocellulosic biomass such as sugarcane leaves, has been made available in the recent years. Furthermore, in many industrial units, the boilers currently employed to convert heat to electricity could be replaced with more efficient variants, thereby less bagasse would have to be burnt to generate the same amount of electricity (Dias *et al*. 2016). To give a more quantitative impression, ethanol production could be increased up to 50%, if all the sugarcane bagasse and straw were fermented to ethanol (Somerville *et al*. 2010; Pereira *et al*. 2015).

There are two 2G sugarcane-based biorefineries currently in operation in Brazil, but they are still struggling financially. GranBio, located in Alagoas state (Northeast region) uses energycane as a raw material. Energycane accumulates less soluble sugars and has more fibre per hectare than conventional sugarcane. Raízen, located in São Paulo state (Southeast region) uses lignocellulosic residues from sugarcane as their raw material and the 2G process site is integrated with the 1G fuel ethanol producing unit. Some recent works have discussed the main aspects of 2G sugarcane-based biorefineries (dos

Santos *et al*. 2016; Sindhu *et al*. 2016; Valdivia *et al*. 2016; Jansen *et al*. 2017; Polizeli *et al*. 2017).

Some of the challenges involved in the 2G process are: 1) pretreatment and hydrolysis of lignocellulosic biomass releases compounds, such as acetic acid, furfural, and hydroxymethylfurfural, among others, which inhibit yeast in a concentration-dependent manner (Taherzadeh, Niklasson and Lidén 1997; Taherzadeh *et al*. 2000; Jönsson, Alriksson and Nilvebrant 2013; Jönsson and Martín 2016); 2) enzymatic hydrolysis preceding the fermentation step is typically carried out at a higher temperature (45 to 50 °C) (Canilha *et al*. 2012; Zabed *et al*. 2017) than the fermentation step (30 to 35 °C) (Abdel-Banat *et al*. 2010; dos Santos *et al*. 2016), necessitating the cooling of the hydrolysate before yeast can be inoculated, resulting in decreased productivities and/or increased equipment costs; 3) high cost of the enzymes required for the hydrolysis of pretreated biomass (Klein-Marcuschamer *et al*. 2012; Liu, Zhang and Bao 2016); 4) decreased productivities (or increased fermentation times), due to the preferential use of glucose by yeast, to the detriment of the remaining carbon sources present in the medium (e.g. xylose), which is still the case even for engineered strains (Kim *et al*. 2012; Jansen *et al*. 2017); 5) contamination events during fermentation, and the inherent need to rely on antibiotics (or other antimicrobials) to minimise the bacterial load, which leads to higher costs and also environmental and public health issues. Infection is a persistent issue in current 1G biorefineries (Shaw *et al*. 2016; Ceccato-Antonini 2018), and the presence of the universal carbon and energy source glucose in the medium certainly aggravates this problem.

In this mini-review article, we propose an alternative strategy to the stand-alone 2G fuel ethanol process, successfully addressing some of the challenges listed above. The raw material would still be sugarcane, but the principle is to combine the process streams from 1G and 2G biorefineries to yield a mixed stream. The 1G stream remains the same as that in current sugarcane-based biorefineries: a mixture containing sugarcane juice and molasses with sucrose as the predominant sugar constituent. However, for the 2G stream, our strategy is to have a few upstream steps that can separate the lignin and the hemicellulosic fraction in sugarcane bagasse to yield a cellulose rich fraction for hydrolysis. This could be achieved e.g. by pretreating the biomass with organosolvents that can delignify and solubilise the hemicellulose fraction present in the biomass to yield cellulose rich solids that have better enzymatic digestibility (Sun *et al*. 2016;

Zhang, Pei and Wang 2016; Matsakas *et al*. 2019). The further use or conversion of the lignin and hemicellulose fractions is outside the scope of this mini-review, but there are different proposals in the literature (Canilha *et al*. 2013; Ragauskas *et al*. 2014; Vardon *et al*. 2015; Beckham *et al*. 2016; Arora, Sharma and Kumar 2018; Liao *et al*. 2020). The cellulose rich fraction, in turn, can be partially hydrolysed to cellobiose, instead of a complete hydrolysis to glucose as is normally performed in a 2G process, since the iSUCCELL yeast chassis we propose here is capable of hydrolysing cellobiose into glucose (intracellularly). The partial hydrolysis of cellulose into cellobiose eliminates the cost of supplementing the enzyme cocktail with β-glucosidase (BGL, production cost ~ 310 USD/kg of enzyme), the key enzyme component that breaks down cellobiose to glucose (Ferreira, Azzoni and Freitas 2018). By mixing the 1G and 2G streams, the time required for cooling the 2G stream (typically around 50 °C) before fermentation (~ 30 °C) can commence, will be reduced. Furthermore, the concentration of metabolic inhibitors will decrease according to the mixing proportion. Finally, by hydrolysing cellulose incompletely, the release of glucose is minimised, creating a less favourable environment for the spread of contaminants, and avoiding the undesired effects of glucose repression over the consumption of other carbon sources by yeast.

We name our strategy iSUCCELL, for intracellular sucrose and cellobiose utilisation and it uses a mixture of 1G stream and partially hydrolysed 2G stream. This is achieved by metabolic engineering of industrial yeast strains that have better tolerance to metabolic inhibitors than wild or laboratory strains and have a favourable track record in non-aseptic processes with cell recycling including acid treatment. This is accomplished by introducing active transport systems for sucrose and cellobiose, followed by their hydrolysis in the cytosol via intracellular invertase and heterologously expressed BGL. The intracellular hydrolysis avoids/decreases the release of glucose in the extracellular environment and results in increased ethanol yield on sugars due to different energy conservation schemes (Basso *et al*. 2011). It should be noted, though, that a techno-economic assessment of this strategy is out of the scope of this article.

THE CONCEPT OF A SUGARCANE-BASED BIOREFINERY USING iSUCCELL YEAST CHASSIS

Process-related aspects

The emergence of lignocellulosic ethanol contributed enormously in our current vision of a biorefinery. Nowadays, research initiatives on sugarcane-based 2G ethanol are oriented towards a synergy with the 1G process, aiming at promoting the transition of Brazilian bioethanol plants into true biorefineries, with the ability to process all fractions of sugarcane and the potential to produce other, higher-value compounds. The integration of 2G processes into 1G sites already in place has shown advantages over stand-alone 2G technologies, improving the overall efficiency and energy balance of the plant (Dias *et al*. 2012; MacRelli, Mogensen and Zacchi 2012; Erdei *et al*. 2013; Lennartsson, Erlandsson and Taherzadeh 2014; Losordo *et al*. 2016). Process integration benefits from unit operations that are common to both 1G and 2G processes. Moreover, the availability of sugarcane bagasse on-site reduces cost and operational issues related to logistics, transportation, and storage of the lignocellulosic material (Soccol *et al*. 2010; Dias *et al*. 2012; Furlan *et al*. 2013; Losordo *et al*. 2016). Although there is a necessity to leave part of the straw in the field to augment soil carbon and to reduce soil erosion and water loss (Leal *et al*. 2013), a small portion of this straw could be transported to the plant and used in the boilers for cogeneration while the rest is diverted towards ethanol production (Dias *et al*. 2011; Furlan *et al*. 2013).

Different configurations have been evaluated for an integrated sugarcane-based 1G+2G fuel ethanol process, which include different combinations of the fermentation medium. A 1G+2G sugar stream can be generated by mixing sugarcane juice with either the hydrolysed C6 liquor (SJ+C6) (Dias *et al*. 2012; Furlan *et al*. 2013; Mariano *et al*. 2013), the hydrolysed C6 and C5 liquors (SJ+C6+C5) (Dias *et al*. 2013), or molasses and the C5 liquor (SJ+M+C5) (Losordo *et al*. 2016). Regardless of the configuration adopted, the 2G fraction requires prior pretreatment and hydrolysis to release the sugars before being mixed with the 1G sugar stream. The commercial enzyme preparations currently available for these applications contain a mixture of hydrolytic enzymes collectively known as cellulases and hemicellulases, although a set of other ancillary enzymes have also been identified as important accessory proteins (Sun *et al*. 2015). The cellulase complex, mostly produced from mutant strains of the fungus *Trichoderma reesei*, includes three types of enzymes working in a synergistic manner:

endoglucanases (EGs), cellobiohydrolases (exoglucanases, CBHs), and BGLs. As a result of the action of EGs and CBHs on cellulose, the disaccharide cellobiose is released as the main product, before being further hydrolysed into glucose by BGLs. Cellobiose is a strong inhibitor of both EGs and CBHs, and BGL alleviates this inhibition by cleaving cellobiose into two glucose molecules (Singhania *et al*. 2013). However, the BGL activity of *T*. *reesei* is also limited by product-inhibition from glucose (Chen, Hayn and Esterbauer 1992). Traditional commercial cellulase preparations, such as Spezyme® CP (Genencor) and Celluclast® 1.5L (Novozymes), contain low amounts of BGLs, causing the accumulation of cellobiose and subsequent product-inhibition of cellulases (Berlin *et al*. 2007; Pryor and Nahar 2010; Hu, Arantes and Saddler 2011; Qing and Wyman 2011; Agrawal *et al*. 2015, 2018; Rodrigues *et al*. 2015). Hence, to overcome this limitation and improve the rate and extent of saccharification, these commercial formulations are commonly blended with additional BGL, generally sourced from *Aspergilli*, such as the commercial BGL preparation Novozyme 188 (Berlin *et al*. 2007; Hu, Arantes and Saddler 2011; Zhai, Hu and Saddler 2016), which despite being less sensitive to feedback inhibition (Riou *et al*. 1998; Decker, Visser and Schreier 2001; Rajasree *et al*. 2013), represents additional costs to the process (Liu, Zhang and Bao 2016; Ferreira, Azzoni and Freitas 2018). Strategies to alleviate inhibition of cellulases by cellobiose or glucose via site-directed mutagenesis are being extensively investigated (Atreya, Strobel and Clark 2016; Guo, Amano and Nozaki 2016). The newest generations of cocktails, e.g Cellic CTec® series (Novozymes) and Accellerase® 1500 (Genencor), have improved significantly, containing many accessory enzymes leading to improved sugar conversions. Nevertheless, the cost of enzymes for manufacturing low value-added products such as ethanol is still significant and needs to be minimised.

In 2G processes, complete degradation of cellulose into glucose is a requirement since *S*. *cerevisiae* is not capable of utilising partially hydrolysed cellulose or cellodextrins (Lynd *et al*. 2002). However, in the last few years a paradigm involving partial hydrolysis of cellulose has begun to emerge (Galazka *et al*. 2010; Chen 2015; Parisutham *et al*. 2017), involving yeast platforms that can assimilate cellodextrins directly. Yet, intensive efforts in strain engineering and optimisation are mandatory to unravel ways to integrate the heterologous pathway into the endogenous metabolism,

since process parameters such as high yield and productivity as well as strain robustness are vital for the success of this endeavour (Chen 2015).

The concept of an integrated 1G+2G fuel ethanol process and the approach of partial cellulose hydrolysis can be combined into a new type of sugarcane-based biorefinery, which can deliver fuel ethanol from the 1G+cellobiose-rich sugar streams, as well as offer possibilities for product diversification from the C5 and lignin-rich fractions of sugarcane bagasse (Rabelo *et al*. 2011; Mariano *et al*. 2013; Ferreira Silva *et al*. 2014; Vardon *et al*. 2015; Beckham *et al*. 2016; Arora, Sharma and Kumar 2018; Unrean and Ketsub 2018) (Figure 1), opening the doors for new markets, spreading the risks, and increasing revenues (UNCTAD 2016).

The 1G stream, prepared by mixing sugarcane juice and molasses at various proportions, contains different concentrations of minerals, organic nutrients, and toxic compounds. While the juice is minerals-deficient (11-16% of total sugars on a wet-weight basis, with ~90% of sucrose and ~10% of glucose and/or fructose), molasses provides a minerals-rich syrup with up to 65% (w/w) of total sugars, with approximately 80% sucrose and ~20% glucose and fructose, in equal proportions (Wheals *et al*. 1999; Lino, Basso and Sommer 2018). On the other hand, it is expected that a typical 2G stream generated using commercial BGL-poor cellulase cocktails, e.g. Celluclast® 1.5L, on a mixture of pretreated sugarcane bagasse and straw, will release glucose with a ~30% yield (w/w) (Ávila, Forte and Goldbeck 2018). Thus, considering the numbers above, in the process proposed here, the 1G+cellobiose-rich stream would consist mainly of sucrose and cellobiose (C12 sugars), together with small amounts of monosaccharides (glucose and fructose, C6 sugars).

Since both sugar streams are processed at different temperatures (~30 °C for the 1G stream and ~50 °C for the cellobiose-rich stream), their mixture would decrease not only the cooling time necessary for subsequent fermentation to start, but also the energy and water usage. Furthermore, a mixed 1G+cellobiose-rich broth would decrease the stress imposed on yeast by inhibitors that are formed during lignocellulosic pretreatment, compared to a conventional 2G process, due to dilution of the cellobiose-rich (hydrolysate) stream with the 1G stream, vastly improving yeast performance (de Andrade *et al*. 2013; Erdei *et al*. 2013).

Yeast-related aspects

The new sugarcane-based biorefinery proposed here requires new yeast platforms capable of both utilising the different sugars and tolerating the inhibitors present in the combined 1G+cellobiose-rich stream. Although this could be accomplished by different strategies, we propose the iSUCCELL platform, where sucrose and cellobiose are metabolised via active transport and intracellular hydrolysis using an engineered yeast (Figure 2). This approach involves the release of monomers inside the cell, and relies on the use of current industrial strain backgrounds commonly found in sugarcane-based biorefineries for metabolic engineering, due to their inherent robustness under industrial conditions (Basso *et al*. 2008; Della-Bianca and Gombert 2013; Della-Bianca *et al*. 2014). The advantages of this strategy in the context of yeast metabolism and process feasibility are summarised in Table 1.

ENGINEERING THE iSUCCELL YEAST CHASSIS FOR A NEW SUGARCANE-BASED BIOREFINERY

Disaccharide utilisation by *S. cerevisiae*

Disaccharides such as sucrose (α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside or β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside), maltose (α-D-glucopyranosyl-(1→4)-α-D-glucopyranose), cellobiose (β-D-glucopyranosyl-(1→4)-β-D-glucopyranose) and lactose (β-D-galactopyranosyl-(1→4)-β-D-glucopyranose) are commonly encountered glucosides in yeast biotechnology. There is evidence that at least 151 yeast species are capable of fermenting sucrose, whereas 827 can grow on this sugar and 859 can grow on cellobiose, from a total of 1270 species tested for these phenotypes (Kurtzman, Fell and Boekhout 2011). In *S*. *cerevisiae*, the utilisation of sucrose is quite peculiar, since this sugar can be hydrolysed both extra- and intracellularly. In contrast, the utilisation of maltose (another α-glucoside) in *S*. *cerevisiae* is exclusively intracellular. Interestingly, β-glucosides such as cellobiose and lactose are not natural substrates for *S*. *cerevisiae* owing to the absence of assimilatory pathways.

The iSUC component

Due to the importance of sucrose as a substrate for industrial biotechnology, the metabolism of this sugar in yeast has interested the scientific community for decades. More recently, aspects involving sucrose and *S*. *cerevisiae* have been reviewed by

Marques *et al*. (2016). Sucrose catabolism in *S*. *cerevisiae* is initiated by either its hydrolysis outside the cells via invertase (encoded by the *SUC2* gene) - followed by assimilation of glucose and fructose via facilitated diffusion - or active transport of sucrose across the plasma membrane via sucrose-H^+ symporters, in which case it is followed by hydrolysis within the intracellular environment using the cytosolic form of invertase (Santos *et al*. 1982; Stambuk *et al*. 1999; Batista, Miletti and Stambuk 2004). Besides invertase, at least two additional sucrose-hydrolysing enzymes classified as α-glucosidases have been identified in *S*. *cerevisiae*, namely maltases (Malx2) (Khan, Zimmermann and Eaton 1973) and isomaltases (Imax) (Marques *et al*. 2017). Additionally, different permeases have been identified as sucrose-H^+ symporters in *S*. *cerevisiae*: a general α-glucoside-H^+ symporter encoded by the *AGT1* gene (which is different from the *MAL11* gene, in contrast to what is reported in the yeastgenome.org database (Trichez *et al*. 2019)), and the maltose-H^+ symporters encoded by *MALx1* genes (where *x* represents the locus number) (Stambuk *et al*. 1999; Stambuk, Batista and de Araujo 2000).

These two modes of sucrose metabolism (extra- or intracellular) in *S*. *cerevisiae* have different energetics, a feature which has been explored by researchers to improve product yields on sucrose. When sucrose is fermented via the extracellular pathway, 4 mol ATP are formed per mol of sucrose, while only 3 mol ATP (25% lower) are produced when one mol of sucrose is metabolised intracellularly (Figure 2). This difference in ATP yield is due to the energy requirement of the proton-coupled symporter, which leads to the indirect expenditure of 1 ATP per proton taken up along with sucrose to maintain pH homeostasis in the cytoplasm. Theoretically, a decrease in the ATP yield will divert a higher fraction of the carbon-source towards ethanol formation, thus increasing the ethanol yield on sucrose compared to its fermentation via the extracellular pathway. This fundamental concept was indeed demonstrated by Basso and collaborators (Basso *et al*. 2011) in a strategy that involved metabolic and evolutionary engineering approaches, resulting in a strain that displayed an 11% higher ethanol yield on sucrose, when compared to the parental strain, which mainly metabolised sucrose via the extracellular pathway. After molecular analysis of the improved phenotype, the authors revealed that up-regulation and duplication of the *AGT1* gene had occurred.

It can also be envisaged that intracellular sucrose hydrolysis (iSUC) might have additional advantages in the context of industrial sugarcane-based processes. Since fructose utilisation in *S*. *cerevisiae* is less favoured than glucose utilisation, unconsumed fructose is often found at the end of fermentation processes, which represents economic losses (Berthels *et al*. 2004). Furthermore, the presence of extracellular fructose may favour the proliferation of heterofermentative bacteria in the context of Brazilian sugarcane-based biorefineries. This type of contaminants showed preference for this sugar, over glucose, in cultures containing equal amounts of both monosaccharides (Basso *et al*. 2014). Thus, the intracellular sucrose assimilation route prevents the direct formation of fructose in the extracellular environment, potentially minimising this problem. Some fructose might still diffuse out into the medium if it is not rapidly metabolised. Thus, metabolic engineering of downstream steps in metabolism, such as overexpression of hexokinase, might be necessary to circumvent this potential issue.

The iCELL component

Cellobiose utilisation by *S*. *cerevisiae* can only be achieved by genetically modifying this organism. Analogously to sucrose metabolism, cellobiose utilisation can be accomplished by either extracellular hydrolysis and internalisation of the monosaccharides released or via import of the disaccharide and intracellular hydrolysis/phosphorolysis. The hydrolytic reaction (regardless of the location) is performed by BGLs. To achieve cellobiose utilisation, initial studies focused on expressing secretable or surface-displayed BGLs from diverse yeast and fungal origins (Machida *et al*. 1988; McBride *et al*. 2005; van Rooyen *et al*. 2005; Guo *et al*. 2011). However, these strategies generate extracellular glucose, which can have at least the following consequences: 1) higher risk of bacterial contamination (mainly in non-aseptic processes); 2) repression of the catabolism of other sugars present in the medium, and 3) end product inhibition of BGLs by glucose.

In 2010, Galazka *et al*. reported for the first time a *S*. *cerevisiae* strain expressing an intracellular cellobiose-metabolising pathway consisting of a cellodextrin transporter (CDT-1 or CDT-2) and a BGL (GH1-1) from the cellulolytic fungus *Neurospora crassa*. The engineered strains were able to grow on cellobiose and on longer cellodextrins, as well as to produce ethanol (Galazka *et al*. 2010). Despite the slow performance of the engineered strains, this breakthrough study paved the way for numerous publications aiming at developing efficient biocatalysts for cellobiose

fermentation. One of the approaches aimed at exploring novel cellodextrin transporters and intracellular BGLs in order to confer *S*. *cerevisiae* cells the ability to ferment cellobiose more efficiently. In this regard, diverse cellobiose permeases from yeast or from other fungi different from *N*. *crassa* have been successfully expressed in *S*. *cerevisiae* (Sadie *et al*. 2011; Ha *et al*. 2013b; Li *et al*. 2013; Zhang *et al*. 2013; Bae *et al*. 2014; dos Reis *et al*. 2016; Casa-Villegas, Polaina and Marín-Navarro 2018; Nogueira *et al*. 2018), as well as alternative intracellular BGLs from yeast, bacteria and fungi (Bae *et al*. 2014; Fan *et al*. 2016; Casa-Villegas, Polaina and Marín-Navarro 2018) (Table 2).

Besides cellobiose hydrolysis, BGLs may also catalyse a transglycosylation reaction (Bohlin *et al*. 2013), as transient accumulation of extracellular cellodextrins was observed during cellobiose-xylose co-fermentation (Ha *et al*. 2011a). Transglycosylation might be triggered when cells accumulate high levels of intracellular cellobiose (Kim *et al*. 2014a). Although the accumulated cellodextrins can be reutilised later on, the productivity of the overall process may be compromised due to the slower uptake of cellodextrins compared to cellobiose (Ha *et al*. 2011a). Hence, intracellular BGLs with reduced transglycosylation activities are crucial for better utilisation of cellobiose.

Much success has been achieved with the heterologous expression of the *N*. *crassa* pathway in *S*. *cerevisiae* (CDT-1 + GH1-1), followed by laboratory evolution (Wei *et al*. 2015; Hu *et al*. 2016; Oh *et al*. 2016) or combinatorial engineering approaches (Du *et al*. 2012; Eriksen *et al*. 2013; Yuan and Zhao 2013) (Table 2). Interestingly, the improved phenotypes reported by Du *et al*. (2012), Yuan and Zhao (2013), and Hu *et al*. (2016), regardless of the methodology employed, involved strains with higher mRNA levels of CDT-1 and GH1-1, compared to the parental strains, indicating a dose-dependent behaviour. Moreover, besides absolute values, an optimised GH1-1/CDT-1 gene expression ratio in the improved strains were also critical. This adjusted ratio probably led to a decreased intracellular accumulation of cellobiose, consequently decreasing the transglycosylation activity of GH1-1 and the cellodextrin accumulation in the medium (Hu *et al*. 2016; Oh *et al*. 2016). In accordance with this, two studies from the same lab reported that their evolved strains harboured higher copy numbers of

CDT-1 and GH1-1 in their genomes, when compared to the unevolved strains (Wei *et al*. 2015; Oh *et al*. 2016). In addition, the evolved strain of Oh and co-workers (Oh *et al*. 2016) showed a GH1-1/CDT-1 copy number ratio similar to that of Yuan *et al*. (2013) (2:1 vs 2.5:1); it should be noted that Yuan and co-workers measured the mRNA levels. In contrast, the optimised strain obtained by Eriksen *et al*. (2013) had gene expression levels comparable to the wild-type strain, but involved overall higher CDT-1 and GH1-1 protein activities. Although no mutations were found in the coding regions of the CDT-1 and GH1-1genes in the previous reports (with the exception of Eriksen *et al*. 2013), single amino acid substitutions in cellobiose transporters were identified after evolutionary engineering in strains carrying other cellobiose-degrading pathways, such as CDT-1 + SdCBP (cellobiose phosphorylase from *Saccharophagus degradans*) (Ha *et al*. 2013a), HXT2.4 (putative hexose transporter from *Scheffersomyces stipitis*) + GH1-1 (Ha *et al*. 2013b), and CDT-2 + SdCBP (Kim *et al*. 2018) (Table 2). These mutations were found to be responsible for enhanced transport activities.

Transcriptional and metabolite profiling have revealed that yeast cells fermenting cellobiose are subjected to severe physiological changes, compared to cells fermenting glucose, as reflected in the activation of mitochondrial function and a decrease in amino acid biosynthesis, and in a carbon starvation-like state of the plasma membrane ATPase (Pma1) (Lin *et al*. 2014; Chomvong *et al*. 2017). Furthermore, when cultivated in cellobiose medium, yeast cells accumulate high levels of trehalose and of intermediate metabolites in the γ-aminobutyrate (GABA) shunt pathway, improving the strain's tolerance to oxidative stress (Kim *et al*. 2014b; Yun *et al*. 2018).

Co-fermentation of cellobiose and xylose/galactose has been investigated to eliminate the challenges inherent to the presence of glucose in sugar mixtures (Kim *et al*. 2012). Glucose represses the transcriptional machinery responsible for the consumption of alternative sugars (Gancedo 1998; Kayikci and Nielsen 2015). However, when the disaccharides are hydrolysed intracellularly, glucose repression is minimised, enabling the co-consumption of cellobiose and xylose (Li *et al*. 2010; Ha *et al*. 2011a), leading to an increase in ethanol productivity. Moreover, when a mixture of cellobiose and xylose was supplemented with a small amount of glucose (< 10% of total sugars), the performance of the engineered strain was not affected (Li *et al*. 2010; Ha *et al*. 2011a), indicating that an intracellular cellobiose-hydrolysing (iCELL) strain would probably perform well under industrial conditions, as glucose will always be present in small

amounts in the partially hydrolysed 2G stream. Alleviation of glucose repression was also observed in mixtures of cellobiose and galactose, yielding higher ethanol productivity in comparison to the sequential utilisation of sugars in a mixture of glucose and galactose (Ha *et al*. 2011b).

Although fuel ethanol has been the main product of interest in most of the studies discussed above, the formation of other biotechnological compounds has also been reported using the iCELL approach, e.g. 2,3-butanediol (Nan *et al*. 2014), lactic acid (Turner *et al*. 2016), and biosurfactants (Jayakody *et al*. 2018).

The iSUCCELL chassis

As detailed above, intracellular disaccharide utilisation in *S*. *cerevisiae* has been evaluated for a single disaccharide or for a disaccharide combined with the co-consumption of one or more monosaccharides. Our approach involves the intracellular utilisation of two disaccharides, namely sucrose and cellobiose, in a single yeast chassis (Figure 2). When these two disaccharides are metabolised via symport and intracellular hydrolysis under anaerobic conditions, there is a 25% decrease in free-energy conservation, namely 4 to 3 mol ATP per mol of sugar, when compared to an extracellular route in which the released monosaccharides are transported via facilitated diffusion across the cell membrane (Basso *et al*. 2011). This platform could be used to ferment the sugars present in an industrial *must* obtained by mixing a sugarcane-based medium (juice and/or molasses), as currently used in Brazilian 1G biorefineries, and cellobiose-rich hydrolysates from the cellulosic fraction of sugarcane bagasse (or even from other lignocellulosic raw materials).

S. *cerevisiae* strains currently adopted by the Brazilian fuel ethanol industry have demonstrated high tolerance towards the stressors/inhibitors typically present both in a 1G and in a 2G context (Della-Bianca *et al*. 2013, 2014; Pereira *et al*. 2014; Cola *et al*. 2020). Two of the most widely employed strains in industry, namely *S*. *cerevisiae* PE-2 and CAT-1 (both are diploids), have already been engineered for xylose fermentation (Romaní *et al*. 2015), highlighting their potential for genetic manipulation, as well as for their use in a 2G process. Recently developed synthetic biology tools, such as the RNA-guided endonuclease mediated CRISPR/Cas method, should be leveraged to facilitate remodelling of native sucrose metabolism and the introduction of the heterologous cellobiose pathway. It should be noted that the CRISPR/Cas system has

already been applied with great success both in laboratory and in industrial strains, enabling simultaneous introduction of multiple genetic modifications into the yeast genome without the need for multiple selectable markers (Stovicek, Holkenbrink and Borodina 2017; Lian, HamediRad and Zhao 2018).

Although the disaccharides sucrose and cellobiose could be cleaved intracellularly either via hydrolysis or via phosphorolysis, the iSUCCELL strategy proposed here involves hydrolysis, since this route benefits from decreased free-energy conservation, which in turn results in higher ethanol yields on sugar (Basso *et al*. 2011) (Figure 2). In order to achieve intracellular sucrose hydrolysis, the invertase-encoding *SUC2* gene could be either modified to constitutively and exclusively express the intracellular form of invertase (Basso *et al*. 2011), or be completely deleted, as this Δsuc2 strain would still hydrolyse sucrose via intracellular α-glucosidases (Dário 2012; Franken *et al*. 2013; Bahia *et al*. 2018). Since duplication of the *AGT1* gene proved crucial for improved sucrose fermentation in the evolved i*SUC2* strain developed by Basso *et al*. (2011), overexpression of native *AGT1* under a stronger, constitutive promoter or introduction of extra *AGT1* copies might also be necessary.

To engineer *S*. *cerevisiae* for intracellular cellobiose hydrolysis, a heterologous cellobiose transporter and an intracellular BGL need to be expressed in the platform strain. It is noteworthy that most of the attempts for cellobiose fermentation in *S*. *cerevisiae* involve the use of episomal plasmids, hampering the applicability of these strains in large-scale industrial conditions. However, since the cellobiose pathway comprises only two genes, chromosomal integrations should not be a complex task with the efficient and well-developed CRISPR-based methodologies, offering precise control over gene stability and copy number (Da Silva and Srikrishnan 2012). Additional metabolic adjustments might be needed for the iSUCCELL yeast to achieve the productivities required for industrial applications. In this sense, adaptive laboratory evolution, systems biology, reverse engineering, and other combinatorial approaches could be useful. For instance, integration of CDT-1 and GH1-1 genes in multiple copies at a *ca*. 1:2 ratio could contribute to a faster fermentation of cellobiose (Oh *et al*. 2016). Integration of the mutated HXT2.4 (A291E) cellobiose transporter from *S*. *stipitis* could be implemented to harness the improved kinetic properties of this permease (Ha *et al*. 2013b). Additionally, modulation of two native transcription factors (overexpression of *SUT1* and deletion of *HAP4*) can be leveraged to speed cellobiose fermentation (Lin *et*

al. 2014). Finally, if necessary, to avoid leakage of monosaccharides to the medium after intracellular hydrolysis, overexpression/finetuning of downstream steps, e.g. those catalysed by hexo- and/or glucokinases, might also be required.

OUTLOOK/CONCLUSION

2G technologies are vital for producing additional amounts of fuel ethanol from existing feedstock, and for mitigating the deleterious effects of climate change. Despite extensive research and development, 2G fuel ethanol is yet to become a commercial success. The approach proposed here is unique in that it combines 1G and 2G process technologies with strain engineering for intracellular utilisation of sucrose and cellobiose, in robust *S*. *cerevisiae* strains currently used in the Brazilian fuel ethanol industries. The 'iSUCCELL' strategy utilises a mixture of 1G stream and a partially hydrolysed, cellobiose-rich 2G stream as a substrate and confers competitive advantages to both the microbe and the process, compared to currently existing strategies. The use of this strategy decreases the availability of free glucose that can be used by contaminating microbes, decreases the cooling time of the feed stream prior to fermentation (avoiding either decreased productivities or higher capital costs), decreases the process time through the co-consumption of sugars because of the absence of glucose repression, and finally, decreases the concentration of metabolic inhibitors that hinders the performance of yeasts. Modern CRISPR-based engineering technologies should be employed for initial strain engineering, after which other approaches, such as laboratory evolution combined with reverse engineering, should be exploited for metabolic fine-tuning. CRISPR-based metabolic engineering of industrial diploid strains has been successfully demonstrated for cellobiose (Ryan *et al*. 2014) and glycerol (Klein *et al*. 2016) utilisation, as well as for the production of 3-hydroxypropionic acid (Jessop-Fabre *et al*. 2016), S-adenosyl-L-methionine (Liu *et al*. 2019), and lactic acid from either glucose (Stovicek, Borodina and Forster 2015) or xylose (Lian *et al*. 2018). We hope the scientific community and eventually the fuel ethanol companies will embrace these proof of concepts to pursue scale-up, and possibly implement the iSUCCELL strategy in existing biorefineries.

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CONFLICT OF INTEREST

The authors declare they have no conflicts of interest involved in this work.

LIST OF ABBREVIATIONS

1G	First-generation
2G	Second-generation
Agt1	α-glucoside-H^+ symporter
BGL	β-glucosidase
C12	Disaccharide sugars
C5	Pentose sugars
C6	Hexose sugars
CBH	Cellobiohydrolase
Cdt	Cellodextrin transporter
CDT-1/CDT-2	Cellodextrin transporter from *Neurospora crassa*
EG	Endoglucanase
GH1-1	β-glucosidase from *Neurospora crassa*
Hxt	Hexose transporter from *Saccharomyces cerevisiae*
HXT2.4	Putative hexose transporter from *Scheffersomyces stipitis*
iCELL	Intracellular cellobiose hydrolysis
Imax	Isomaltase
iSUC	Intracellular sucrose hydrolysis
iSUCCELL	Intracellular sucrose and cellobiose hydrolyses
M	Molasses
Malx1	Maltose-H^+ symporter
Malx2	Maltase
Pma1	Plasma membrane H^+-ATPase
SdCBP	Cellobiose phosphorylase from *Saccharophagus degradans*
SJ	Sugarcane juice
Suc2	Invertase enzyme

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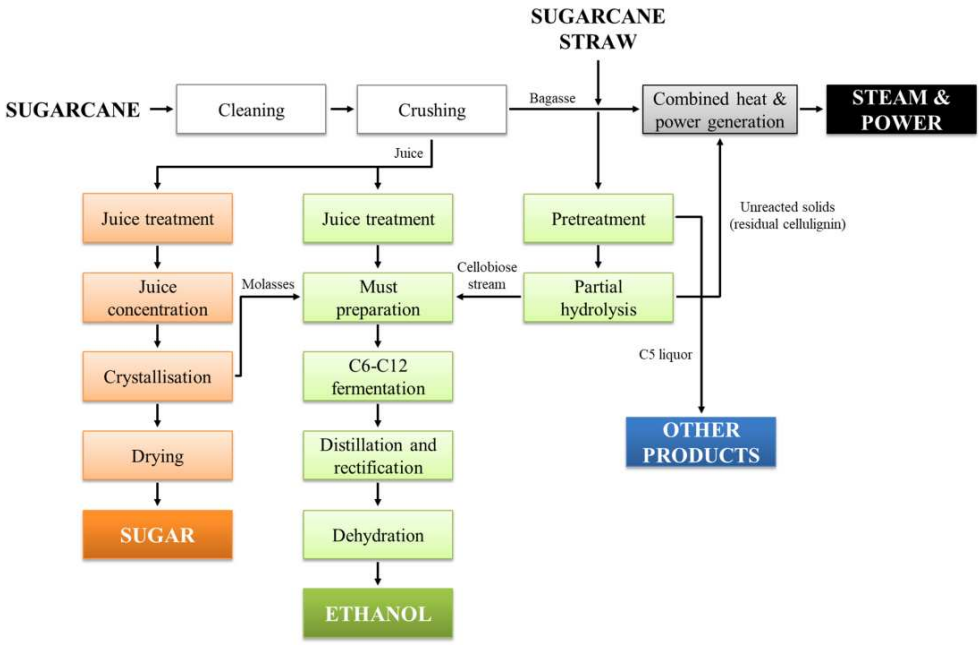

Figure 1. Schematic representation of a sugarcane-based biorefinery, in which the iSUCCELL yeast platform proposed here is applied (in the C6-C12 fermentation step). For this to occur, after pretreatment of lignocellulose, the cellulosic fraction is only partially hydrolysed to cellobiose, which is mixed with the 1G stream (Juice treatment). The end products of the biorefinery are highlighted (dark coloured rectangles). Adapted from Mariano *et al*. 2013.

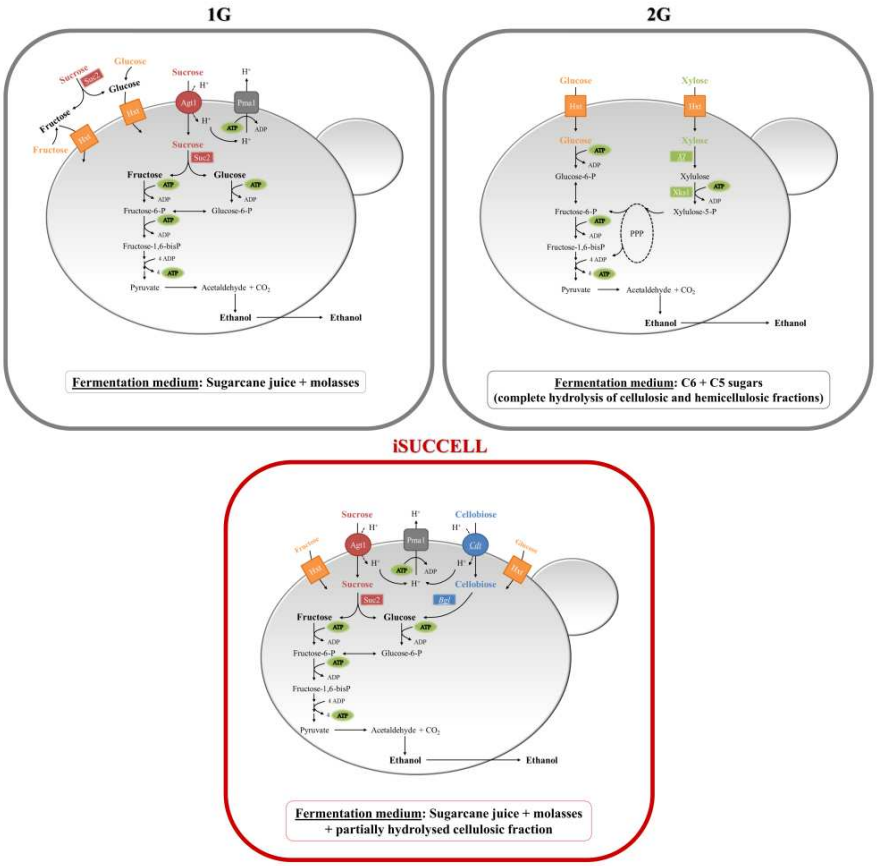

Figure 2. Comparison between yeast strains from 1G and 2G processes with the iSUCCELL yeast proposed as a platform strain for an integrated sugarcane-based fuel ethanol process involving partial cellulose hydrolysis to cellobiose. The 1G fermentation medium contains C12 (sucrose) and some C6 (glucose and fructose) sugars. The 2G medium presented here consists of a typical stream containing C6 (glucose) and C5 (xylose) sugars as a result of a complete hydrolysis of the cellulosic and hemicellulosic fractions. The engineered 2G strain expresses a heterologous xylose isomerase (XI) to convert xylose into xylulose, which is subsequently phosphorylated into xylulose-5-phosphate by native xylulokinase (Xks1). The fermentation medium for the iSUCCELL yeast contains C12 (sucrose and cellobiose) and small amounts of C6 (glucose and fructose) sugars, as a result of a combined 1G+cellobiose-rich medium. Sucrose and cellobiose uptake are mediated by sucrose and cellobiose-proton symporters (Agt1 and Cdt, respectively) followed by hydrolysis of the disaccharides via intracellular hydrolases (Suc2 and Bgl, respectively) and proton extrusion by the plasma membrane ATPase Pma1. Uptake of glucose, fructose and xylose is mediated by native hexose transporters (Hxt). The iSUCCELL strain conserves 3 ATP for each disaccharide (sucrose or cellobiose) consumed, which results from glycolysis via the Embden-Meyerhof glycolytic pathway (+4 ATP/disaccharide) and proton extrusion via Pma1 (-1 ATP/disaccharide). Heterologous proteins are indicated by names in italics and underlined. PPP: non-oxidative pentose-phosphate pathway.

Table 1. Process and yeast-related advantages of an integrated sugarcane-based biorefinery using iSUCCELL yeast chassis.

Process or yeast-related challenges for fuel ethanol production	Advantage of the integrated sugarcane-based biorefinery using iSUCCELL yeast chassis
Different process temperature for ligno-cellulose hydrolysis (~50 °C) and fermentation (~30 °C)	A mixed 1G+cellobiose-rich stream reduces the cooling time between saccharification and fermentation, increasing productivity and/or saving equipment costs*
Operational costs owing to the use of enzymes	Supplementation with additional BGL in the cocktail is not required
High concentration of inhibitors in the 2G stream	A mixed 1G+cellobiose-rich stream dilutes the inhibitors' concentrations, minimising their harmful effects on yeast*
High incidence of bacterial contamination during fermentation	The intracellular hydrolysis of sucrose and cellobiose minimises the accumulation of extracellular glucose, reducing both the level and diversity of contaminants and subsequently the use of antibiotics and other antimicrobials
Ethanol yield on sugars	Active transport of sucrose and cellobiose present in the mixed stream, followed by intracellular hydrolysis to ethanol and CO_2, yields 3 net ATP/disaccharide, whereas their metabolism by extracellular hydrolysis leads to 4 ATP/disaccharide**. This decreased ATP yield leads to a higher fraction of the substrate being converted into ethanol (Basso *et al*. 2011)

Fructose accumulation leads to incomplete 1G fermentation because of low affinity of hexose transporters for fructose	When sucrose is hydrolysed intracellularly, the presence of unutilised extracellular fructose is minimised (Berthels *et al*. 2004)
Strain robustness	Use of robust industrial strains (commonly found in sugarcane-based 1G ethanol plants) as chassis for the iSUCCELL strategy eliminates the need to engineer alternative yeast strains (Della-Bianca and Gombert 2013; Pereira *et al*. 2014; Cola *et al*. 2020). Many of the strains currently employed in the Brazilian fuel ethanol industry are diploid (Della-Bianca *et al*. 2013) and should be amenable to engineering using e.g. CRISPR, capitalising on their innate robustness.

[*] These advantages do not rely exclusively on the iSUCCELL approach and could in principle be met by any other strategy involving an integrated 1G+2G process.

[**] Glucose and fructose fermentation into ethanol and CO_2 yields 2 ATP per mole of hexose. Thus, 4 ATP are formed when sucrose or cellobiose are metabolised via extracellular hydrolysis. When these disaccharides are metabolised via intracellular hydrolysis, 1 ATP per disaccharide is required to expel the proton that is taken up by the sucrose or cellobiose-H^+ symporters, reducing the total ATP yield to 3 ATP for each disaccharide.

Table 2. Cellobiose fermentation performances of different engineered *S. cerevisiae* strains*

Growth conditions	Improvements post-metabolic engineering?	Growth rate [1/h]Growth rate [1/h]	Cellobiose consumption rate [g/(L·h)]Cellobiose consumption rate [g/(L·h)]	Ethanol production rate [g/(L·h)]Ethanol production rate [g/(L·h)]	Ethanol yield on cellobiose [g/g]Ethanol yield on cellobiose [g/g]	Reference
30 °C, anaerobic, ODinitial = 2.0 30 °C, anaerobic, ODinitial = 2.0	No	NS	≈ 0.167a≈ 0.167a	≈ 0.075a≈ 0.075a	0.441 ± 0.001 get/gglu0.441 ± 0.001 get/gglu	Galazka et al., 2010Galazka et al., 2010
30 °C, 150 rpm, aerobic, ODinitial = 0.45 30 °C, 150 rpm, aerobic, ODinitial = 0.45	No	0.11 ± 0.005	0.139b0.139b	NS	NS	Guo et al., 2011Guo et al., 2011
30 °C, oxygen-limited, ODinitial = 1 30 °C, oxygen-limited, ODinitial = 1	No	NS	1.667b1.667b	0.7	0.42	Ha et al., 2011aHa et al., 2011a
NS	No	NS	NS	0.37 ± 0.01	0.34 ± 0.01	Ha et al., 2011bHa et al., 2011b
30 °C, 100 rpm, oxygen-limited, ODinitial = 130 °C, 100 rpm, oxygen-limited, ODinitial = 1	Combinatorial transcriptional engineering	NS	2.18	0.74	0.39	Du et al., 2012Du et al., 2012
30 °C, 100 rpm, oxygen-limited, ODinitial = 0.230 °C, 100 rpm, oxygen-limited, ODinitial = 0.2	Directed Evolution	0.102 ± 0.002	2.65 ± 0.02	1.00 +/- 0.03	0.436 ± 0.004	Eriksen et al., 2013Eriksen et al., 2013
30 °C, mild agitation	No	NS	1.542b1.542b	0.533b0.533b	NS	Yamada et al., 2013Yamada et al., 2013

30 °C, 100 rpm, oxygen-limited, ODinitial = 130 °C, 100 rpm, oxygen-limited, ODinitial = 1	Directed Evolution + promoter engineering	NS	2.50 ± 0.21	0.89 ± 0.01	0.37 ± 0.01	Yuan et al., 2013Yuan et al., 2013
30 °C, oxygen-limited, ODinitial = 130 °C, oxygen-limited, ODinitial = 1	Evolutionary engineering	NS	1.72 ± 0.11	0.74 ± 0.05	0.44	Ha et al., 2013aHa et al., 2013a
30 °C, oxygen-limited, ODinitial = 130 °C, oxygen-limited, ODinitial = 1	Site-directed mutagenesis	NS	3.18	1.16	NS	Ha et al., 2013bHa et al., 2013b
30 °C, oxygen-limited, ODinitial = 130 °C, oxygen-limited, ODinitial = 1	No	NS	2.30	0.87	NS	Ha et al., 2013cHa et al., 2013c
30 °C, 80 rpm	No	NS	1.02 ± 0.06	0.30 ± 0.01	0.38 ± 0.01	Bae et al., 2014Bae et al., 2014
30 °C, 220 rpm, strict anaerobic, ODinitial = 2030 °C, 220 rpm, strict anaerobic, ODinitial = 20	Evolutionary engineering (in previous report)	NS	3.6 ± 0.05	1.5 ± 0.03	NS	Chomvong et al., 2014Chomvong et al., 2014
30 °C, 100 rpm, oxygen-limited, ODinitial = 130 °C, 100 rpm, oxygen-limited, ODinitial = 1	No	NS	≈ 2.21b≈ 2.21b	0.53 ± 0.01	0.39 ± 0.01	Kim et al., 2014Kim et al., 2014
30 °C, anaerobic, ODinitial = 1030 °C, anaerobic, ODinitial = 10	Directed evolution	NS	2.7 ± 0.02	1.1 ± 0.01	0.45 ± 0.01	Lian et al., 2014Lian et al., 2014

39 °C, 200 rpm, oxygen-limited, biorreator, ODinitial = 1	Evolutionary engineering	NS	3.04	1.50	0.49	Hu et al., 2016
30 °C, 100 rpm, oxygen limited, ODinitial = 1	Evolutionary engineering	NS	2.73 ± 0.01	1.09 ± 0.00	0.40 ± 0.00	Oh et al., 2016
30 °C, 100 rpm, oxygen limited, ODinitial = 1	Evolutionary engineering	NS	NS	0.70 ± 0.01	0.42 ± 0.01	Kim et al., 2018
30 °C, 100 rpm, ODinitial = 1	No	NS	NS	0.36 ± 0.01	0.33 ± 0.01	Kim et al., 2019