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Minireview

Interdependence between lignocellulosic biomasses, enzymatic hydrolysis and yeast cell factories in biorefineries

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Summary

Biorefineries have a pivotal role in the bioeconomy scenario for the transition from fossil-based processes towards more sustainable ones relying on renewable resources. Lignocellulose is a prominent feedstock since its abundance and relatively low cost. Microorganisms are often protagonists of biorefineries, as they contribute both to the enzymatic degradation of lignocellulose complex polymers and to the fermentative conversion of the hydrolyzed biomasses into fine and bulk chemicals. Enzymes have therefore become crucial for the development of sustainable biorefineries, being able to provide nutrients to cells from lignocellulose. Enzymatic hydrolysis can be performed by a portfolio of natural enzymes that degrade lignocellulose, often combined into cocktails. As enzymes can be deployed in different operative settings, such as separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF), their characteristics need to be combined with microbial ones to maximize the process. We therefore reviewed how the optimization of lignocellulose enzymatic hydrolysis can ameliorate bioethanol production when *Saccharomyces cerevisiae* is used as cell factory. Expanding beyond biofuels, enzymatic cocktail optimization can also be pivotal to unlock the potential of non-*Saccharomyces* yeasts, which, thanks to broader substrate utilization, inhibitor resistance and peculiar metabolism, can widen the array of feedstocks and products of biorefineries.

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Introduction

Biorefineries can be described as 'the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat)' (IEA Bioenergy Task42, 2014). Biorefineries indeed aim to provide a broad portfolio of products alongside classical bio-based molecules such as biofuels or biogas (European Commission, 2018; Rosales-Calderon and Arantes, 2019; Stegmann *et al.*, 2020). Consistently, they are considered as one of the key technologies in the circular bioeconomy scenario, presenting different opportunities and challenges across countries, as they need to be organically integrated in the territories' landscape and infrastructure.

In order to widen possible outcomes of biorefineries and, in some cases, minimize environmental impacts, it is possible to exploit microorganisms, the so-called microbial cell factories, whose role is to convert the provided biomass(es) into the desired product(s) (Dahiya *et al.*, 2018). As a consequence, it is crucial that nutrients released from biomasses can match microbial requirements. In the case of lignocellulosic biomasses (LCBs), constituted by cellulose, hemicellulose and lignin in different ratios, a pre-treatment step to open-up the recalcitrant macromolecular structure is followed by enzymatic hydrolysis. This step is preferred to chemical treatment (Galbe and Wallberg, 2019) (e.g. acid) as enzymes operate under conditions that are more compatible with microbial growth. Different hydrolyses can

generate different mixtures of sugars and other nutrients, both in terms of composition and relative quantities. Notably, enzymes can be applied in two quite distinct processes, namely, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), in order to match microbial cell factories' characteristics (Kawaguchi *et al*., 2016). Here, we recapitulate recent literature on this subject, and our aim is to underline the tight correlation between biomass, enzymes and yeast cell factories: optimization of the enzymatic cocktail and its operative conditions can unlock the potential of a lignocellulosic biomass and/or of a yeast cell factory (Fig. 1). This synergy is crucial for assessing or improving the viability of the overall process and therefore its economic feasibility (Pellis *et al*., 2018). This review is a qualitative description on the importance of a link between LCBs composition, choice of enzyme cocktail and selection of yeast species and strains that need to be considered in an integrated fashion to enable the development of an efficient process.

LCBs as preferred feedstock in biorefineries

LCB as a recalcitrant and uneven feedstock

Annually, about 1.3 billion tons of LCBs are generated all around the world but only a small fraction is exploited to produce biochemicals (Baruah *et al*., 2018). For these reasons, by 2030, the bio-based consortium aims to replace the 30% of the overall chemical production in the EU with biomolecules derived from biomass (Hassan *et al*., 2019).

The main examples of LCBs are plant straw, coconut husk, corn stover, sugarcane bagasse and woody materials in general. The structure of lignocellulose comprises three main biological polymers, lignin (20–30%), hemicellulose (20–40%) and cellulose (40–50%), held together by different types non-covalent bonds and covalent cross-linkages (Hosseini Koupaie *et al*., 2019). Cellulose, the most abundant LCB polymer, is composed of β-D-glucose units linked by β-(1,4) glycosidic bonds, with cellobiose as the fundamental repeating dimeric unit. Around 500–1400 glucose molecules compose the cellulose chains forming the microfibrils that are embedded in the lignocellulosic matrix, which, in turn, makes it resistant to enzymatic hydrolysis (Zoghlami and Paës, 2019). Hemicelluloses are composed of heterogeneous groups of biopolymers containing various monosaccharide subunits to form xylans, xyloglucan, mannans and glucomannans (McKendry, 2002). They are amorphous with very little physical strength but act as a physical barrier for enzyme accessibility. Lignin, responsible for the hydrophobicity and structural rigidity, binds hemicelluloses, which, in turn, adhere to cellulose microfibrils in the cell wall (Zoghlami and Paës, 2019). It is a complex amorphous heteropolymer of phenylpropanoid building units (Agbor *et al*., 2011). Sugar release from LCBs is influenced by factors such as total lignin content, lignin composition and structure (Santos *et al*., 2012). In addition to blocking access to (hemi)cellulases, the hydrophobic structural features of lignin can also irreversibly adsorb enzymes during hydrolysis (Zeng *et al*., 2014). Cellulose and hemicelluloses are linked together through hydrogen bonds, while lignin is covalently linked to hemicelluloses to form lignin-carbohydrate complex (LCC). There are five different types of LCC bonds (phenyl glycosides, benzyl ethers, γ-esters, ferulate/coumarate esters and acetal linkages) that involve the 4-OH and 4-O positions of the lignin moieties (Tarasov *et al*., 2018). The interaction between lignin and cellulose microfibrils and/or hemicelluloses, driven by LCC linkages, reduces the area of cellulose accessible for enzymes, significantly affecting the enzymatic hydrolysis of LCBs (Du *et al*., 2014).

Before the enzymatic hydrolysis, a pre-treatment step is required to destabilize the recalcitrant structure of

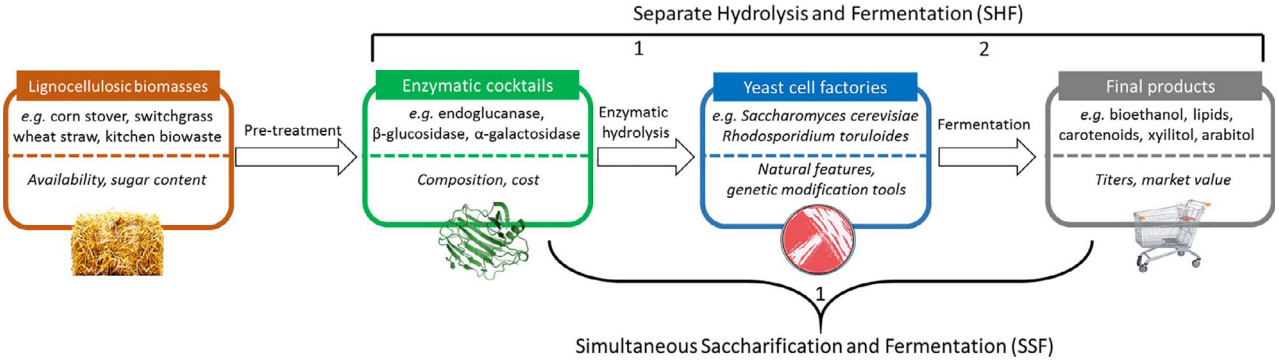

Fig. 1. Overview of the processes/factors involved in the conversion of lignocellulosic biomass into final products in second-generation biorefineries. For each step of the overall process (coloured boxes), the main parameters to be considered when establishing a (yeast-based) biorefinery are indicated under the dotted line. Pre-treatment is needed to weaken the intertwined structure of LCBs prior to enzymatic hydrolysis. In SHF, hydrolysis and fermentation are performed as sequential steps, whereas in SSF, they are combined into a single one.

LCBs, thereby enabling access of the enzymes to their substrates. Depending on the type of LCB used and the end product of interest, there are several physical, chemical, thermal and biological pre-treatment methods that can also be used individually or in combination (Hosseini Koupaie *et al*., 2019). Some of the preferred pre-treatment methods used in second generation biorefineries are listed in Fig. 2, together with examples of enzymes used in processes for specific products, which might change for matching different combinations of biomasses and microbial cell factories.

In any biorefinery, the choice of pre-treatment method is determined in part by the type of LCB and the infrastructure available, but it must also consider subsequent steps as these are differentially affected by particular pre-treatments (Baruah *et al*., 2018). Acid or alkaline-based methods may lead to the release of carboxylic acids, phenolic compounds and furans, whereas the main by-products of the pre-treatment by hydrothermal processing are acetic acid and furan aldehydes (i.e. furfural and hydroxymethylfurfural) (Jönsson and Martín, 2016; Kim, 2018). Enzyme cocktails can be inhibited by solubilized aromatic compounds, such as phenols, as well as solid components like lignin and residual hemicellulose (Jönsson and Martín, 2016). Pretreatment methods such as steam explosion lead to minimal release of furanic compounds but releases acetyl groups from lignocellulose that normally are linked to the hemicellulose moiety, exacerbating the impairment of enzymes and microbial activity (Sun *et al*., 2016). There is a considerable ongoing effort to integrate knowledge of the potential negative effects of pre-treatment into process development to ensure that efficient enzyme hydrolysis and subsequent microbial fermentation are possible. This can include the development of enzyme cocktails and microbial strains that are less sensitive to inhibition by by-products from particular types of pre-treatment.

Hydrolysis of LCBs by enzymatic cocktails: unity is strength

According to the International Union of Biochemistry and Molecular Biology, most of the (hemi)cellulases and other polysaccharide degrading enzymes are grouped in the family of O-glycoside hydrolases (GH) and further sub-classified into different families based on primary structure of catalytic domains (Houfani *et al*., 2020). Given the variation between LCBs from different sources, and the heterogeneity that arises following pre-treatment, exploiting the natural biodiversity of GHs is pivotal to address the hydrolysis of the different components of these matrixes. Cellulases and hemicellulases, often combined in cocktails, are employed to hydrolyze cellulose to cellobiose and glucose and hemicellulose to diverse pentose and hexose sugars (Houfani *et al*., 2020). Multiple factors such as temperature, pH, rate of mixing, substrate concentration, enzyme loading and addition of surfactants influence dimeric and monomeric sugar yields from LCBs (Sarkar *et al*., 2012). The complete degradation of cellulose can occur by combined and simultaneous action of three distinct classes of cellulolytic enzymes, namely, endoglucanases, cellobiohydrolases and β-glucosidases. Endoglucanases are involved in the cleavage of internal β-glucosidic bonds, thereby providing accessible cellulose chain ends to cellobiohydrolases. These enzymes release cellobiose, which is further hydrolyzed to glucose by β-glucosidase (Yennamalli *et al*., 2017). In contrast, as the composition of hemicellulose varies depending on the type of LCB, with multiple different monomeric units and bond types, hemicellulolytic enzymes are accordingly more diverse.

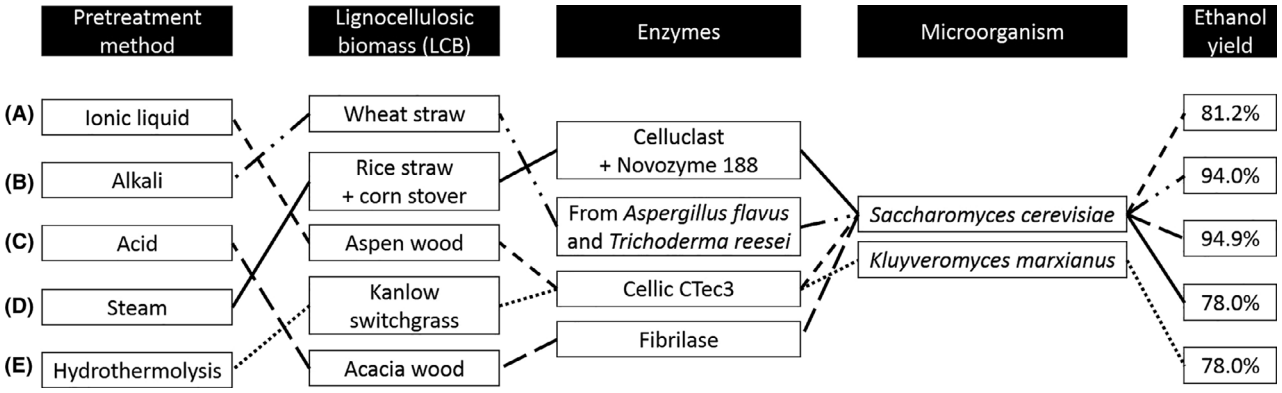

Fig. 2. Examples of pre-treatment methods used in combination with enzymatic hydrolysis and microbial fermentation to obtain ethanol. Equal line styles allow to reconstruct experimental data reported in literature, and without this guideline, it would be not possible to forecast the correct combinations. Ethanol yields displayed are reported as percentage, considering 100% the theoretical yield. References: (A) (Goshadrou *et al*., 2013), (B) (Singh and Bishnoi, 2012), (C) (Lee and Yu, 2020), (D) (Nielsen *et al*., 2020) and (E) (Suryawati *et al*., 2008).

For example, endo-β-1,4-xylanase is involved in breaking down internal bonds of xylan, a major polymer found in hemicellulose, leading to the release of xylo-oligosaccharides, the non-reducing ends of which can then be hydrolyzed by β-xylosidase. Accessory enzymes such as α-L-arabinofuranosidase, α-glucuronidase, α-galactosidase, acetylxylan esterase and ferulic acid esterase (Maitan-Alfenas *et al*., 2015) are often important to increase sugar yields from hemicellulose during the saccharification process (Robl *et al*., 2013). The correct combination and ratio of hydrolytic and accessory enzymes is very important to effectively liberate the monomeric sugars and to reduce the inhibitory effect of lignin (Van Dyk and Pletschke, 2012). For example, corn stover pretreated by ammonia fibre expansion, hydrolyzed by using six core enzymes (cellobiohydrolase 1, cellobiohydrolase 2, endo-β-1,4-glucanase, β-glucosidase, endo-β-1,4-xylanase 3 and β-xylosidase), led to a glucose yield of 38.5%; when these enzymes were combined with five accessory enzymes from *Trichoderma reesei* (endoglucanase II and endo-β-1,4-xylanase 2, produced endogenously, and endoglucanase IV, α-glucuronidase and arabinosidase 2 from recombinant *Komagataella phaffii*), the yield increased to 52.1% (Banerjee *et al*., 2010).

Furthermore, the use of additives such as non-catalytic proteins and surfactants may reduce lignin adsorption of enzymes and improve the interaction between cellulases and cellulose fibres, thereby enhancing the overall hydrolysis of LCBs (Xu *et al*., 2019).

Numerous bacterial and fungal species produce (hemi)cellulases, with the filamentous fungal genera *Trichoderma* and *Aspergillus* of particular interest (Maitan-Alfenas *et al*., 2015). *Trichoderma* ssp. are widely exploited owing to their natural ability to produce two cellobiohydrolases, five endoglucanases and three endoxylanases, but they have lower β-glucosidase activity (Bischof *et al*., 2016). Efficient β-glucosidase producers are *Aspergillus* ssp., being therefore able to complement the missing activity (Sarkar *et al*., 2012). Nevertheless, there is a need to explore other fungal strains such as anaerobic gut fungi that are known to possess a wide range of biomass degrading enzymes (Usmani *et al*., 2021). For example, enzymatic formulation from Neocallimastigomycota (anaerobic gut fungi) displayed a 300% increase in xylan degradation activity, compared to the commercial *Aspergillus* enzyme formulations, compared to the commercial *Aspergillus* enzyme formulations (Solomon *et al*., 2016). The lack of a single natural microbial species capable of secreting all the required cellulolytic enzymes in high titers and balanced ratios for efficient enzymatic hydrolysis of lignocellulosic biomass has necessitated the use of blends of enzymes from several microorganisms (Maitan-Alfenas *et al*., 2015). This has led to the development of formulated enzymatic cocktails, with Novozymes and Du-Pont Genencor among the leading commercial producers (Adsul *et al*., 2020).

Enzymatic cocktails have a pivotal role in improving the efficiency of biomass hydrolysis by reducing the amount of enzymes and time required to convert all the carbohydrates into fermentable sugars and having the possibility to function at high substrate loadings (Adsul *et al*., 2020). Furthermore, auxiliary enzymes like lytic polysaccharide monooxygenase (LPMO), copper-enzymes that catalyze oxidative cleavage of glycosidic bonds, are often part of commercial enzymatic cocktails. Indeed, the addition of LPMOs to cocktails has led to a significant reduction in the cost of the enzymatic process (Johansen, 2016) and is an effective way of increasing the digestibility of structural carbohydrates (Duque *et al*., 2021). Rodriguez and co-workers reported an increase in cellulose release from various LCBs (sugarcane bagasse, corn stover and wheat straw) when LPMOs were used alongside enzymatic cocktail Cellic CTec2, permitting the use of hydrothermal pretreatment rather than organosolv and alkaline ones (Rodríguez-Zúñiga *et al*., 2015).

The formulation of cocktails can be designed to exploit synergistic effect of enzymes such as cellulase, xylanase and pectinase. Considering the hydrolysis of sugarcane bagasse, it was reported that replacing 20% of cellulase by xylanase led to an increase in glucose yield by 6.6%, 8.8% and 9.5% in sugarcane bagasse pretreated by steam explosion, NaOH and H_2O_2 respectively (Li *et al*., 2014). These observations suggested that glucose release is positively affected by the degree of synergism between cellulase and xylanase, and it is also dependent on hydrolysis time (Li *et al*., 2014). In a study based on steam-treated sweet sorghum bagasse, when the combination of enzymatic cocktails (cellulases – Cellic CTec2 and endoxylanases – Cellic HTec2) was optimized by response surface methodology (RSM), sugars yield increased by 20% (Pengilly *et al*., 2015). Regarding corn stover pretreated by steam explosion, it was noticed that the use of commercial cellulase (Spezyme CP) in combination with cellulase from *Aspergillus fumigatus* led to a 26% increase in the conversion of glucan to glucose compared to the use of the sole cocktail (Wang *et al*., 2012). Furthermore, corn stover pretreated by ammonia fibre expansion (AFEX) resulted in glucose and xylose yields >80% and 70%, respectively, after enzymatic hydrolysis by a cocktail containing cellulases, xylanses and accessory enzymes, and the addition of accessory hemicellulases further increased xylose yields by 20% (Gao *et al*., 2011). The use of enzymatic cocktail containing exoglucanase (Cel7A), endoglucanase (Cel5A) and two endoxylanases (XYN10A,

XYN11A) in synergism along with swollenin, a non-hydrolytic disruptive protein, led to a significant increase in xylose yields from steam pretreated corn-stover by >300%, by enhancing enzymatic access to the hemicellulose fraction, increasing in turn cellulose accessibility as well (Gourlay *et al*., 2013).

Considering the importance of combining pretreatments and cocktail compositions, and the still largely unexplored potential of enzymes biodiversity, it is evident that the next decade will witness large improvements in LCBs exploitation.

Combination of microbial enzymes and cell factories in LCBs-based biorefineries

Together or separate?

Up to now, we introduced diverse LCBs, pretreatment principles and enzymes as elements to be combined for obtaining the desired media to be fermented by microbial cell factories in a bioprocess. However, the timing of hydrolysis and fermentation has not yet been discussed. In the last decades, two main types of processes have been developed: SSF and SHF. These processes, where enzymes and cells are working in a single (SSF) or in separate (SHF) vessels, have pros and cons in terms of efficiency, duration, presence/release of inhibitory molecules and downstream processing of the final product (Choudhary *et al*., 2016; Kawaguchi *et al*., 2016; Haldar and Purkait, 2020). There are several examples in the literature of second-generation bioethanol production by *S. cerevisiae* based on SHF or SSF processes where distinct enzymes are used resulting in different ethanol yields (Fig. 3).

An obvious advantage of SHF is the possibility to choose the optimal conditions for both enzymatic hydrolysis and fermentation steps as they are temporally and spatially separated. Temperature, for example, is a key parameter as efficient enzymatic cocktails often have optimum activity at around 50 °C, whereas *S. cerevisiae* fermentation is optimal at 30 °C (Choudhary *et al*., 2016). In addition, in a SHF process, it is possible to eliminate by centrifugation the water insoluble solids (WIS) that cause poor homogenization of the liquid medium and can impair yeast growth (Ask *et al*., 2012). Although it is not a consideration regarding bioethanol production, separation of WIS is also advantageous if not indispensable when the final product is intracellular (Öhgren *et al*., 2007). Nevertheless, SHF also displays drawbacks related to both the biological process and the economics. On the biological side, inhibition of enzyme activity caused by the mono- and disaccharides products can reduce yields, and conversely, very successful hydrolysis can deliver medium with sugar concentrations that are problematic for batch fermentation because of osmotic stress. Economically, in SHF, there is higher capital expenditure (CAPEX) because of the need for different vessels, and the prolonged process time with the additional risk of undesired fermentations by contaminants that may reduce the economical sustainability of the process (Kawaguchi *et al*., 2016; Haldar and Purkait, 2020).

In SSF, yeast cell factories can metabolize sugars concurrently with their release from the biomass, thus

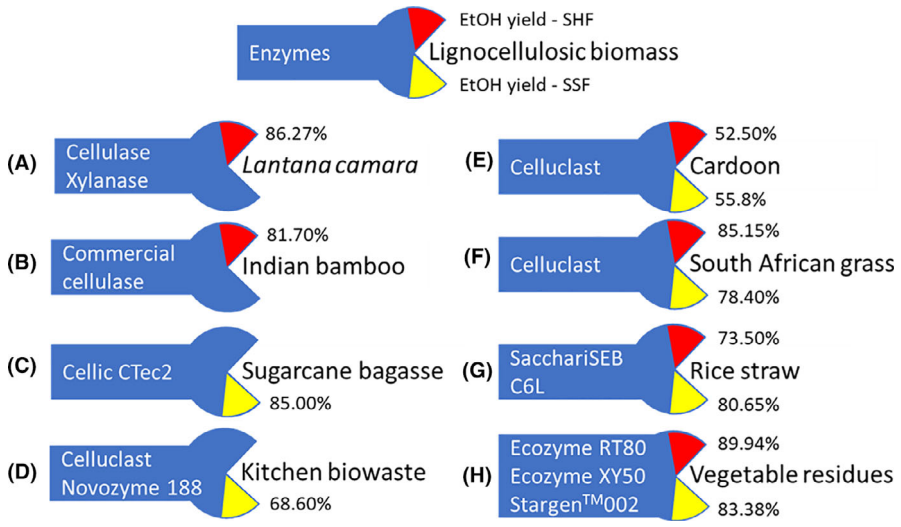

Fig. 3. Selected examples of second-generation bioethanol production with *S. cerevisiae* as cell factory by SHF or SSF processes. The scheme reports the enzyme used, the biomass utilized as substrate, the different fermentation strategies and the corresponding ethanol yields (reported as percentage, considering 100% the theoretical yield). References: (A) (Kuila and Banerjee, 2014), (B) (Sindhu *et al*., 2014), (C) (Unrean *et al*., 2016), (D) (Ntaikou *et al*., 2018), (E) (Fernandes *et al*., 2018), (F) (Burman *et al*., 2019), (G) (Mishra *et al*., 2016) and (H) (Mithra *et al*., 2019).

mitigating both the osmotic stress and the inhibitory effect of (simple) sugars on the enzymes (Choudhary *et al*., 2016; Kawaguchi *et al*., 2016; Haldar and Purkait, 2020). Another advantage is that the low titer of glucose may facilitate consumption of other saccharides by the yeast, which is often impaired by the catabolic repression caused by glucose. One important factor to consider is the effect of high solids loading (>20% w/v), which can affect cell viability and the action of enzymes: the compromise between the amount of sugars and inhibitors must be considered (Wu *et al*., 2018; Da Silva *et al*., 2020). Nevertheless, the major drawback is that it is necessary to carry out the process at temperatures that are far below the optimum for the hydrolytic enzymes. One option to maximize the efficacy of enzyme hydrolysis in an SSF process is to replace *S. cerevisiae* with a more thermophilic yeast that can ferment at a higher temperature (Choudhary *et al*., 2016).

In deciding whether to implement an SHF or an SSF process, it is necessary to consider multiple variables. Several studies have attempted to perform this type of comparison to determine if SHF or SSF better suited for particular processes (Dahnum *et al*., 2015; Rodrigues *et al*., 2016; Wu *et al*., 2018; Ben Atitallah *et al*., 2019; Mithra *et al*., 2019; Bertacchi *et al*., 2020). For example, a comparison of processes for the production of carotenoids by the yeast *Rhodosporidium toruloides* from *Camelina sativa* meal hydrolysate found that SSF was able to guarantee the highest titer of the final products (Bertacchi *et al*., 2020). Although generally negative, in this case, it was speculated that the presence of growth inhibitory WIS may actually trigger the production of scavenger molecules like carotenoids. This illustrates why bespoke analysis of each production process is necessary. Modelling and statistical tools can assist in this decision-making process. For example, in bioethanol production, response surface methodology (RSM) was used to infer the optimum conditions for SSF and SHF (Althuri and Banerjee, 2019), whereas empirical equations modelling served to determine glucose and ethanol titers in both processes (Burman *et al*., 2019).

By implementing these studies, we are constantly learning and therefore designing further optimization, among which it is notable to mention the hybrid solution of starting a process as a suboptimal SHF, followed by SSF with a reduced or optimized enzyme loading (US9187390B2).

Non-Saccharomyces *yeasts in LCB-based second generation biorefineries*

Despite the widespread application of *S. cerevisiae* in second-generation bioethanol production, one of the main roadblocks is its preference to glucose, thereby lacking the ability to consume different C5–C6 sugars present in the lignocellulosic biomass. Cultures based on *S. cerevisiae* strains engineered to preferentially and singularly metabolize either glucose, xylose or arabinose have been developed (Verhoeven *et al*., 2018), but this work is still closer to the proof of concept phase than to production. Different is the case of a mixed cultivation of *S. cerevisiae* and *S. stipitis* (naturally able to consume pentose sugars), developed for an SHF/SSF processes for the production of ethanol from kitchen biowaste (Ntaikou *et al*., 2018): here, the consortium might be more effective at industrial scale, with a caveat related to the limited ethanol tolerance of *S. stipitis*.

In the presented scenario and moving beyond bioethanol, yeast biodiversity can offer advantages that are not yet fully exploited and, in some cases, also poorly explored. Prominent examples are the co-consumption of hexose and pentose sugars (which includes both sugar transporters and catalytic enzymes), the native production of enzymes for the hydrolysis of LCB-derived polymers/oligomers, the resistance towards inhibitory compounds arising from the pre-treatment and hydrolysis of the biomass, and finally, the natural ability to transform substrates into the desired products.

The ability to withstand various growth inhibitors often derives not only from the product but also from the pre-treatment and hydrolysis steps of LCBs (Sitepu *et al*., 2014; Pandey *et al*., 2019). Oleaginous yeasts often display good resistance towards classic inhibitors present in lignocellulosic hydrolysates (Sitepu *et al*., 2014; Poontawee *et al*., 2017; Osorio-González *et al*., 2019). For example, *S. cerevisiae* growth is inhibited by 0.8 g l^{-1} of furfural, whereas several oleaginous yeasts (e.g. *R. toruloides* and *Y. lipolytica*) can withstand 1 g l^{-1} of this toxic compound (Sitepu *et al*., 2014). In this context, different yeasts of the *Yarrowia* clade were tested for their ability to withstand several inhibitory compounds derived from acid-pretreated switchgrass hydrolyzed with a mixture of Cellic Ctec2 and HTec2 cocktails (Quarterman *et al*., 2017). Acetic acid, which is detached from lignocellulose by pre-treatment (Jönsson and Martín, 2016), is an example of such an inhibitor as it can impair metabolism and microbial growth. Whereas *S. cerevisiae* needs to be engineered or evolved for acetic acid tolerance (Martani *et al*., 2015; Ko *et al*., 2020), species like *Zygosaccharomyces bailii* are able to withstand acetic acid, with a minimum inhibitory concentration of 375–550 mM (whereas *S. cerevisiae* of 80–150 mM) and to consume it for its own growth, even in the presence of glucose (Kuanyshev *et al*., 2017; Palma *et al*., 2018). C*andida tropicalis* also showed the ability to withstand furfural (Wang *et al*., 2016) up to 1.5 g l^{-1}, being therefore more resistant than *S. cerevisiae* (Pandey *et al*.,

2019) and to produce xylitol from several residual biomasses (Eryasar and Karasu-Yalcin, 2016; Mattam *et al*., 2016). Furthermore, in respect to the aforementioned limitations of SSF processes, there are studies that compare the performance of species like *Blastobotrys adeninivorans, Pichia kudriavzevii* and *K. marxianus* with that of different *S. cerevisiae* strains (Choudhary *et al*., 2016).

Figure 4 lists some other recent examples of different non-*Saccharomyces* yeasts deployed to produce valuable products with specific biomass and enzymatic cocktails, employing the natural ability of those species. Indeed, their broader substrate range can be coupled not only to the production of ethanol (which still remains one of the most investigated products when demonstrating industrially relevant products) but also of biodiesel or more generally single cell oil, as in the case of oleaginous yeasts (Poontawee *et al*., 2017; Carsanba *et al*., 2018; Sreeharsha and Mohan, 2020). It is interesting to mention that synthetic consortia can be considered to perform the enzymatic hydrolysis, allowing implementation of a hybrid SHF/SSF without the addition of external enzymes. This is the case of sugarcane bagasse triggering the secretion of endoglucanase, β-glucosidase and xylanase in a co-culture of *S. cerevisiae* and *C. tropicalis* (Qadir *et al*., 2018).

One important comment relates to pentose and hexose sugars co-consumption: in most cases, this is difficult to achieve, and strain engineering or synthetic consortia needs to be accurately assisted by bioprocess engineering, adjusting cultivation parameters, feeding, dimension and ratio of the inoculum to maximize productivity. The concerns related to the fermentation time are not only linked to the overall costs of the process but also to the fact that the accumulation of the final product is in most of the case detrimental to the cells, if not toxic.

Non-*Saccharomyces* yeasts are therefore an important biological reservoir of biodiversity that can be applied to the development of second-generation biorefineries, in order to widen our horizons beyond the common exploitation of *S. cerevisiae* for bioethanol production, and to maximize the portfolio of enzymatic cocktails available on the market.

Conclusions

Biorefineries will play a pivotal role in the development of a sustainable global bioeconomy. Efficient biorefineries will integrate biomass, bespoke enzyme cocktails and specific cell factories. Although the traditional focus has been on cell factory design, the critical need to exploit second-generation biomasses to achieve sustainability is now a major driver of research. Indeed, this leads to somewhat of a paradigm shift since, in these scenarios, the substrate achieves equal importance to the product: a process that does not use residual

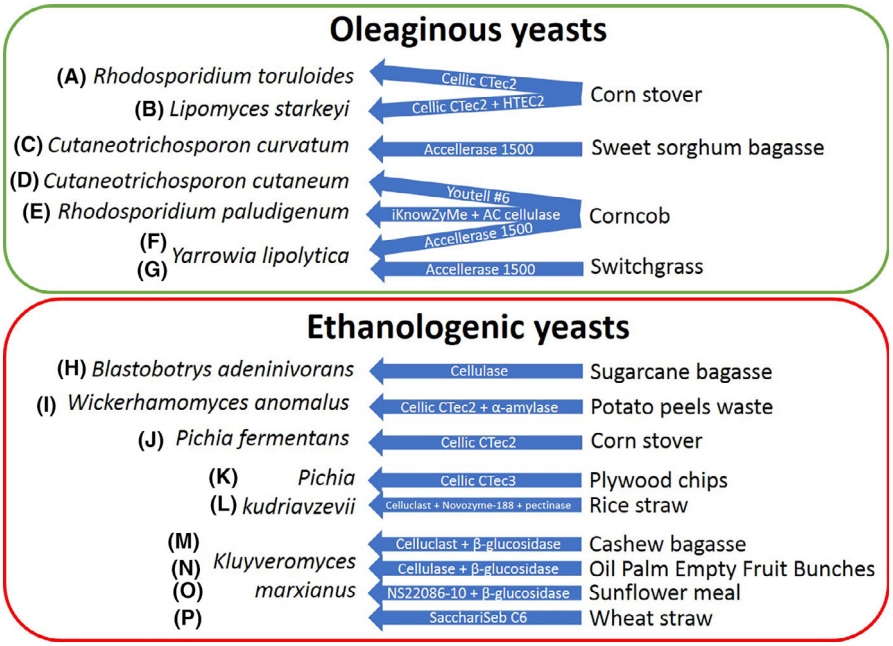

Fig. 4. Biodiversity of non-*Saccharomyces* yeasts in second-generation bioprocesses. Two different and representative yeast metabolisms (lipidogenesis and alcoholic fermentation) are reported together with LCBs and enzymatic cocktails used in some selected examples. References: (A) (Fei *et al*., 2016), (B) (Pomraning *et al*., 2019), (C) (Liang *et al*., 2012), (D) (Gao *et al*., 2014), (E) (Chaiyaso *et al*., 2019), (F) (Kahr *et al*., 2015), (G) (Quarterman *et al*., 2017), (H) (Antil *et al*., 2015), (I) (Ben Atitallah *et al*., 2019), (J) (Mierzejewska *et al*., 2019), (K) (Yuan *et al*., 2017), (L) (Oberoi *et al*., 2012), (M) (Rodrigues *et al*., 2016), (N) (Sukhang *et al*., 2020), (O) (Camargo *et al*., 2014) and (P) (Saini *et al*., 2015).

biomasses will struggle to deliver sustainability. This adds a substantial variable that was little considered in traditional fermentations and first-generation bioprocesses. It also creates new opportunities since there is increased scope to exploit microbial diversity, and in the case of yeasts, non-*Saccharomyces* yeasts because of their properties related to substrate specificity, inhibitor tolerance and growth parameters. The metabolism of these yeasts can then be exploited to produce new products, often with better efficiency that *S. cerevisiae*. The third pillar in second-generation biorefineries is the enzyme that links the biomass to the cell factory. This is a crucial area of ongoing investigation that considers the enzymes and how they are applied in either SHF or SSF processes. The use of enzyme cocktails to treat LCBs is now in routine, but considerable work is still required to decide on the best enzyme formulation, and the range of options is still too limited. It is important to recognize that the best enzyme cocktail is the one that can deliver the optimum sugar mix to a specific cell factory microbe when starting from a particular LCB source. This is also possible because of the improved knowledge on yeast biodiversity and the constant development on enzymes potential. It is implicit in this that considering any of the components in isolation cannot achieve the best outcome. At the moment, each bioprocess needs to be designed from first principles, but it is hoped that, as experience grows, it will become possible to develop framework principles that facilitate rational selection of the components of a biorefinery. In this case, it will be possible to reduce the cost and time needed to develop new second-generation biorefineries, including those that operate on a modest scale.

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Conflict of interest

The authors declare that there is no conflict of interest.

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