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Engineering of *Saccharomyces cerevisiae* as a consolidated bioprocessing host to produce cellulosic ethanol: recent advancements and current challenges

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Abstract
Lignocellulosic biomass, a rich and inexpensive source of fermentable and renewable carbon, is the most abundant material on earth. Microbial bioprocessing of lignocellulosic biomass to produce biofuels (bioethanol, biobutanol, biodiesel) is a sustainable blueprint to reduce our depleting energy reserves and carbon footprint. *Saccharomyces cerevisiae*, being an excellent industrial ethanologenic organism, is an ideal candidate to engineer as a consolidated bio-processing (CBP) host, a concept that integrates the different steps of cellulosic ethanol production, from hydrolysis of cellulose to glucose and fermentation of glucose to ethanol in one step. Owing to the developments in the field of genetic engineering and sequencing technologies, research in the past two decades have made pivotal achievements to realize CBP enabling yeast suited for industrial applications. However, overcoming major limitations such as incomplete substrate catabolism, low titres of heterologous protein expression, sub-optimal operational conditions and impediment due to toxic inhibitors/by-products accumulation is still challenging. This review focuses on the progress achieved in constructing *S. cerevisiae* to produce bioethanol in a CBP framework. The different techniques of developing cellulolytic yeast strains are initially explained followed by relevant strategies to tackle the key bottlenecks associated with the process. Additionally, engineering efforts towards designing hemicellulose-derived sugar utilizing yeast strains are discussed.

Keywords
S. cerevisiae, CBP, ethanol, cellulases, hemicellulose, xylose, fermentation, lignocellulosic biomass

1. Introduction

Two of the most pressing problems concerning the modern-day economy includes drastic changes in climatic conditions and fast depletion of non-renewable energy reserves. As global population continues to grow, the dependence on petroleum and fossil fuels has increased exponentially to meet the required industrialization and urbanization demands. Being non-renewable in nature, it has been estimated that fossil fuels will be exhausted by 2050. Although these fuels are majorly contributing to the world's energy supply, their non-sustainability is a big challenge to address. Further, their constant exploitation has raised major environmental concerns such as greenhouse gas emission, global warming, pollution etc. It is estimated that a further increase of even 2ºC in global average temperature can lead

to an extinction of millions of species accompanied with many forms of natural calamities (Mishra and Goswami, 2017). One of the most effective ways to reduce the environmental impact of current trends in energy exploitation is to implement the development and use of clean sustainable energy fuels (biofuels) derived from cheap and renewable feedstocks.

Lignocellulosic biomass (LCB) is the most abundant feedstock available on earth with annual production of ~200 billion tonnes. Being inexpensive and rich source of C6 (hexose) and C5 (pentose) fermentable sugars, it holds an immense potential for applications as feedstocks in the biorefineries and biofuel sector. Moreover, LCB does not interfere with food chain since it is non-edible. In general, LCB or biomass comprises of a wide variety of plant residues derived from agricultural, forest, fruit and vegetable processing wastes (Kuhad and Singh, 2008). Every year tons of waste material in the form of LCB is accumulated. These waste materials are either used as fodder for grazing animals or are burnt in open land causing massive environmental pollution and associated health risks due to the release of toxic gases. Additionally, burning plant waste emits fine particulate matter 2.5, a pollutant causing concerns in people with compromised lung functioning. The cost of air pollution due to the practice of waste burning in India is estimated to be $30 billion annually. Burning 1 tonne of rice crop wastes leads to a loss of nitrogen (5.5kg), phosphorous (2.3 kg), potassium (25 kg) and sulphur (1.2 kg) in the soil (Saini et al., 2015). Moreover, the heat from burning the biomass kills beneficial microbial populations of the soil, and rip off its carbon content significantly. To tackle this crisis, various policies at national levels are being formulated to resolve the problem of crop burning in India. One of the potential solutions to this problem is to develop technologies capable of converting these non-edible waste residues (lignocellulose) to a range of valuable products including biochemicals, biofuels etc to fully cover or supplement the ever-increasing energy demands.

LCB is primarily composed of three main polymers: cellulose, hemi-cellulose and lignin. These polymers interact with each other in a complex matrix of varying compositions depending on the type, age and source of biomass (Zoghlami and Paës, 2019). Hemicellulose and lignin are polymers surrounding the cellulosic fibres and act as physical barriers limiting the accessibility of enzymes (Bajpai, 2016). Hemicelluloses are heterogenous group of polysaccharides made up of different C6 and C5 sugars such as xylose, arabinose, galactose, glucose as primary sugar backbone substituted with different side-chains imparting an amorphous characteristic. It can be broadly classified into pentosans, such as xylan, a beta-(1,4)-linked repeating xylose (C5) monomers and glucans, such as mannans, xyloglucans, glucomannan and other higher order derivatives. Hemicellulose fractions are readily hydrolysed by dilute acid or steam explosion pre-treatment methods thereby exposing the cellulose layer. Lignin on the outermost layer, is a complex structure of cross-linked polymers containing phenolic compounds (coniferyl, coumaryl and sinapyl alcohol). It imparts rigidity and hydrophobicity to the structural backbone of the plant cell wall.

Cellulose is the main constituent of the plant cell wall providing structural support. It is a polymer of beta-D-glucopyranose units linked together by beta-(1,4)-glycosidic bonds with cellobiose as the fundamental repeating units. The cellulose chains are compactly packed together to make up microfibrils which further interact to form cellulose fibres. Depending on the degree of polymerization, cellulose chains in nature may contain 1000-15000 glucose units embedded in the lignocellulosic matrix contributing to its recalcitrance. Fermentable glucose can be released from cellulose chains upon chemical or enzymatic reactions breaking the intermolecular glycosidic linkages for downstream conversion to ethanol.

Ethanol, a simple organic compound, has multitude of uses as general solvent, medicine manufacturing, wine& beverage production and fuel molecule. The use of ethanol as biofuel is one of the most important market drivers currently in place. The global bio-ethanol market was evaluated to be around 100 billion litres in 2020, with an expected increase of 4% by

2021-2026[1]. Because of its clean, sustainable and biodegradable properties, countries are adopting various policies to either replace or supplement transportation fuels with bioethanol. Second generation (2G) bio-ethanol derived from LCB sources is a multi-step process consisting of (i) pre-treatment of the biomass to separate the cellulosic fractions (ii) production of cellulolytic enzymes (iii) hydrolysis of the pre-treated biomass rich in cellulose to release glucose and (iv) conversion of the resulting sugars using fermenting organisms to finally produce ethanol. Traditional bioprocessing approaches include separate hydrolysis and fermentation (SHF) and simultaneous saccharification and (co)-fermentation (SSF or SSCF). SHF involves independent steps of enzyme production, hydrolysis and fermentation in separate containers, providing an advantage of carrying out each of the steps under optimal conditions but largely suffers from the problem of feed-back inhibition of hydrolysing enzymes by accumulating sugars resulting in partial hydrolysis. The high concentration of sugars in the hydrolysate imposes osmotic stress on fermenting microorganisms and eventually affect production parameters. Due to the intensive energy requirement, expensive enzyme demands and high cost of operation, its industrial application is also limited. Alternatively, SS(C)F strategy combines hydrolysis and fermentation of sugars (hexose and pentoses). Since both the steps are carried out in a single vessel, as soon as the sugars are released, they are converted into ethanol avoiding the piling up of sugars at inhibitory levels. However, this process still suffers from challenges such as difficulty in optimizing the process parameters for hydrolysis and fermentation, presence of fermentation inhibitors and an overall low ethanol productivity (Ojeda et al., 2011). To circumvent these limitations, consolidated bio-processing (CBP) provides a sustainable strategy to integrate the three steps i.e., enzyme production, hydrolysis and fermentation in a single step process employing either a single or a consortium of microorganisms. CBP is potentially the most cost-effective concept as it reduces the need of exogenously added enzymes and facilitates the execution of all the individual steps simultaneously in one vessel. However, the strategy is majorly limited by the non-existence of any natural CBP enabling microorganism. While certain bacteria and fungus are excellent cellulase producers they show poor fermentation ability; on the other hand, fermentative organisms are non-cellulolytic. With an aim to design robust CBP hosts, researchers have engineered cellulolytic organisms to be ethanologenic (Ali et al., 2016; Anasontzis et al., 2011; Huang et al., 2014a; Tian et al., 2016) as well as ethanologenic organisms to be cellulolytic (Peña et al., 2018; Puseenam et al., 2015; Wang et al., 2018; S Yanase et al., 2010a; B. Zhang et al., 2015). Recent reports of engineering non-*Saccharomyces* hosts for CBP application are listed in **Table 1**.

Generally, important criteria for a robust CBP bioengineered host includes fast growth, broad range of substrate utilization, high ethanol productivity rate and tolerance towards performance inhibitors such as toxic by-products, high temperature, osmotic pressure. Among all the known microorganisms, *Saccharomyces cerevisiae* is a widely popular industrial host and many engineering efforts have been directed towards innovating tailored yeast cell factories to produce a variety of chemicals (Cripwell et al., 2020; Madhavan et al., 2021; Martínez et al., 2012; Pscheidt and Glieder, 2008). In this review, we have attempted to explain the recombinant techniques of developing cellulose utilizing yeast strains for the purpose of bioethanol production and the strategies applied to overcome critical bottlenecks with a special focus on synthetic biology driven engineering principles. Furthermore, we have covered recent studies on the expansion of the substrate utilization range in yeast and finally proposed the future directions for improving CBP prospects in this versatile host.

[1] https://www.mordorintelligence.com/industry-reports/bio-ethanol-market

2. Strategies to develop *S. cerevisiae* as a CBP host

Baker's yeast, *Saccharomyces cerevisiae*, is a widely used organism for baking, brewing and wine-making with the earliest evidence of its use by humans dating back 9000 years ago when yeast was first domesticated (Lahue et al., 2020). A single celled eukaryotic fungus, yeast has played an immense role in shaping the agricultural, topographical diversity and is slowly emerging as a prospective cell factory for generating commercially important products. Owing to its inherent (i) capacity of producing high ethanol titer, (ii) tolerance towards low pH, high sugar and ethanol concentrations (iii) resistance towards various inhibitors released from lignocellulosic hydrolysates and (iv) GRAS (generally regarded as safe) status, yeast makes an ideal candidate for CBP efforts. Additionally, the complete known genome sequence makes yeast tractable organisms to be easily manipulated using advanced synthetic biology and genetic engineering tools (Chen et al., 2018). Ethanol production from cellulose typically require synergistic actions of three important enzymes: i.e., endo-glucanase (EGL), which randomly cleaves internal β-glycosidic bonds in amorphous regions of cellulose to release cello oligosaccharide of different lengths; exo-cellulases (CBH1 and CBH2), which acts on reducing and non-reducing ends of EGL derived end products to release cellobiose; and β-glucosidase (BGL), which converts cellobiose into glucose (Barbosa et al., 2020). Many natural fungi, bacteria, protozoans capable of producing cellulases can be used to clone novel cellulase genes either in isolation or from environmental metagenomes for heterologous expression in this host (Jakeer et al., 2020; Pottkämper et al., 2009; Yang et al., 2016).

To develop yeast as a CBP organism, generally three different biotechnological strategies have been adopted: (i) secretion of multiple enzymes as free cellulases; (ii) surface display of the cellulases on the yeast cell wall; and (iii) assembling cellulosomes or minicellulosmes on the yeast cell surface (**Fig 1**). Notable efforts towards engineering of *S. cerevisiae* using these strategies are discussed below and summarized in **Table 2**.

2.1 Secretion of cellulases as free enzyme

Cellulases isolated from cellulolytic fungus or bacterial species are demonstrated to be recombinantly expressed in *S. cerevisiae* as free enzymes. Co-secretion of two or more cellulases have been effective in developing strains capable of growing and producing ethanol from various cellulosic feedstocks. In an early study, an endoglucanase (EGI) and a beta glucosidase (BGL1) from *Trichoderma reesei and Saccharomycopsis fibuligera*, respectively were expressed together in a *S. cerevisiae* strain enabling it to grow on phosphoric acid swollen cellulose (PASC) as the sole carbon source. Simultaneous expression of cellulose hydrolysing enzymes and fermentation of the released sugars into ethanol up to 1g/L was demonstrated in a single-step setup (Haan et al., 2007). Similarly, a non-cellulosomal endoglucanase (EngD) from a bacterial source, *Clostridium cellulovorans* and beta glucosidase (Bgl1) from *Saccharomycopsis fibuligera* were cloned under the control of yeast secretion signal MFα and overexpressed in a *S. cerevisiae* strain. EngD exhibited multienzyme properties possessing activities for endoglucanase, exoglucanase and xylanases along with a cellulose-binding domain (CBD), and is secreted as a free enzyme in its natural host. The engineered *S. cerevisiae* strain secreting high levels of EngD and Bgl1 could simultaneously saccharify and ferment 20g/L barley β-glucan to produce 9.15 g/L ethanol reaching above 80% of its theoretical maximum (Eugene Jeon et al., 2009). Another study from the same group engineered *S. cerevisiae* to co-secrete a different endoglucanase (EgE) from *Clostridium thermocellum* and a beta glucosidase (Bg) from *S. fibuligera* to produce

even higher titres of 9.67g/L of ethanol from barley βglucan along with growth and assimilation of other cellulosic materials such as carboxy methyl cellulose (CMC) and PASC (E Jeon et al., 2009). In a separate study, a CBP enabling yeast was engineered to secrete high levels of extracellular cellulases, specially cellobiohydrolase (CBH). In this study, three CBH enzymes i.e., CBH1 from *Aspergillus aculeatus* , CBH1 and CBH2 from *Trichoderma reesei* along with endoglucanase from *T. reesei* and beta glucosidase from *A. aculeatus* were sequentially integrated in yeast genome following delta integration method. The resultant strain secreting all the three CBH's along with EGL and BGL could produce the highest ethanol concentration of 28g/L from pre-treated corncob signifying the importance of CBH's diversity in hydrolysing complex biomass (Hong et al., 2014).
While the above studies used laboratory strains of *Saccharomyces* species, several research groups engineered industrial or natural yeast strains in a CBP configuration. For example, genes encoding cellulases (EGL, CBH and BGL) were successfully integrated into the chromosomes at ribosomal DNA and delta sites of a derivative of an industrial wine strain. The engineered cellulolytic yeast was able to utilize pre-treated corn stover and produce ethanol without the supplementation of any exogenous enzymes (Khramtsov et al., 2011). Another industrial yeast strain with high ethanol productivity and tolerance was engineered for genomic integration and constitutive secretion of *Trichoderma viridae* endoglucanase (EG3) and beta glucosidase (BGL1) for direct ethanol production from CMC (Gong et al., 2014). Similar efforts toward developing CBP yeast were directed towards exploring the genetically diverse yeast strains for optimum secretion of cellulases. In an elaborate study, thirty different natural isolates of yeast strains were screened for their higher cellulase secretion capacities and other secretion related stress tolerance. A superior strain from this study was later engineered to co-secrete endoglucanase and beta glucosidase in an optimum ratio, producing up to 4g/L ethanol from corn cob cellulose (Davison et al., 2019).

2.2 Surface display of cellulases

An alternative method for CBP engineering uses the surface display machinery in yeasts to express the recombinant proteins as anchored enzymes on the cell wall. Generally, the glycosylphosphatidylinositol (GPI) anchoring mechanism of yeasts are employed to construct fusion proteins encoding cellulases and the anchoring domain of the GPI protein. For example, α-agglutinin, which after maturation are immobilized on the yeast cell wall resembling a whole-cell biocatalyst. This technique offers several advantages over heterologous secretion of cellulases, for instance, the enzymes displayed on the cell surface are presented in close vicinity to each other which enhances their synergistic actions during saccharification and at the same time attenuates the requirement of repeated adsorption and desorption from the cellulose surface. This strategy also ensures that the displayed enzymes remain active as long as yeast continues to grow. Additionally, the engineered whole-cell biocatalysts can be easily separated and re-used in subsequent batches of fermentation, thereby, reducing the cost and time involved in culturing fresh yeast strains or supplying enzyme cocktails. In a pioneering study, *Saccharomyces cerevisiae* was engineered to co-display two cellulases (an endoglucanase from *Trichoderma reesei* and a beta glucosidase from *Aspergillus aculeatus*) using cell surface display technique. The resultant transformants could saccharify and ferment 45g/L barley β-glucan as a sole carbon source to produce 16.5g/L of ethanol within 48 hours of fermentation (Fujita et al., 2002). In a separate study, additional display of cellobiohydrolase (CBHII) of *T. reesei* in the above strain (co-displaying endoglucanase and beta glucosidase) induced synergistic and sequential degradation of an amorphous cellulose to efficiently produce high ethanol yield of up to 3g/L from 10g/L of phosphoric acid swollen cellulose (PASC) (Fujita et al., 2004). Like any other

recombinant expression system, surface display of heterologous enzymes can be optimized by controlling the copy number of the integrated genes, use of marker-less integration design or by specifically fine tuning the enzyme ratio for process maximization. For instance, a novel method called cocktail delta integration was developed to engineer yeasts displaying multi-enzymes components. In this technique, through repeated transformation, equimolar amounts of cellulase expression cassettes were integrated at the delta sites of yeast chromosomes simultaneously and transformants with the most optimum cellulose degrading activity were easily screened. Using this strategy, *S. cerevisiae* was designed to co-display an optimum ratio of three cellulases, endoglucanase and an exoglucanase from *T. reesei* and beta glucosidase from *A. aculeatus*, with potentially higher PASC degradation activity than conventional integration methods (Yamada et al., 2010a) .To further improve the cellulose-degradation activity, cocktail delta integration was applied to optimize cellulase expression in haploid yeast strains of opposite mating types. These strains were then mated to construct a diploid strain with augmented cellulase expression profiles. Compared to its haploid parent, the engineered diploid strain in this study exhibited six folds higher PASC degradation activity with a concomitant ethanol production of 7.6g/L and also displayed direct ethanol producing ability from pre-treated rice straw, without the supplementation of any exogenous enzymes (Yamada et al., 2011). Hence, cell surface engineering, owing to its effective increase in enzyme localization and recyclability, is a better suited method for a CBP host design than secretion of cellulases in free forms (Liu et al., 2016, 2015; S Yanase et al., 2010b).

Further efforts to improve yeast cell-surface application in CBP context have also been made, such as, optimization of promoter, anchoring elements (Inokuma et al., 2014), and displayed cellulase ratio, (Liu et al., 2017) co-expression of cellulases with swollenin protein (expansin-like proteins) (Nakatani et al., 2013) modification of fermentation system using high solid biomass loading (Matano et al., 2013, 2012) and expression of cellodextrin (cellobiose) transporters (Yamada et al., 2013). Yeasts engineered to co-express cellulase/cellodextrin transporter could produce 1.7-fold more ethanol from PASC compared to strains expressing the surface displayed cellulases only (Yamada et al., 2013). Recently, *S. cerevisiae* engineered to display combinations of cellulases were also employed to produce ethanol directly from ionic liquid pre-treated lignocellulosic biomass (Nakashima et al., 2011; Yamada et al., 2017). The ethanol production from 1-butyl-3methylimidazolium acetate [Bmim][OAc]-treated bagasse reached 0.8g/L in a single-pot fermentation setup in 96 hours, corresponding to 73.4% of its theoretical maximum. However, the efficiency of the engineered yeast was low towards hardwoods and only 18.3% and 21.2% of the theoretical ethanol yield was obtained after 72 hours of fermentation from [Bmim][OAc]-treated eucalyptus and cedar biomass respectively (Yamada et al., 2017).

2.3 Assembly of cellulases in cellulosomes

Many anaerobic bacteria and fungi have evolved to produce a complex multi-enzyme arrangement of cellulases, called cellulosomes, displayed on their cell surfaces to efficiently degrade cellulose in an energy limited environment (Bayer et al., 2004; Schwarz, 2001). The basic component of a cellulosome includes a structural scaffold consisting of at least one CBD and repeating units of cohesin modules acting as ‘launching pads’ for corresponding cellulases tagged with high-affinity dockerin domains. The CBD domain in the scaffold is responsible for cellulose latching and the spatial arrangement of the cohesin-dockerin pairs tethered with cellulases ensures an optimum synergy towards cellulose degradation. *S. cerevisiae* has been recently reconfigured to mimic these cellulosome machinery as a potential strategy for CBP applications. In an early study, functional assembly of three

cellulases as a 'minicellulosome' was demonstrated on the cell surface of *S. cerevisiae* engineered for cellulosic ethanol production. Briefly, a structural scaffold exhibiting three cohesin domains from *C. thermocellum*, *C. cellulolyticum* and *R. flavefaciens* were expressed in the yeast strains as cellulosomes. Upon incubation with *E. coli* lysate expressing cellulases (endoglucanase, exoglucanase and beta glucosidase) fused with respective dockerin domains in various combinations yielded a functional cellulosome on the yeast cell surface. The resulting yeast strain displaying the assorted cellulosome exhibited significantly enhanced glucose liberation and direct ethanol production from PASC, reaching a final ethanol concentration of 3.5g/L which was 2.6 folds higher than adding the same amounts of purified cellulases (Tsai et al., 2009). In a follow-up study, the same minicellulosomes were re-assembled using a synthetic yeast consortium. Four yeast strains were engineered to either display the tri-functional scaffolding or express individual cellulases tagged with the corresponding dockerin. Co-culturing of these yeast strains resulted in a species-specific assembly of minicellulosomes structure and the synergism of cellulose assimilation was easily fine-tuned by adjusting the ratio of different yeast populations in the consortium (Tsai et al., 2010). In a separate study of similar design, yeast cells were engineered for *in vivo* assembly and display of uni-, bi- and tri-functional cellulosomes with *T. reesei* EGII, CBHII and *A. aculeatus* BGL1. Yeast endowed with the tri-functional cellulosome showed enhanced enzyme-enzyme synergy and the ability to directly produce approximately 1.8g/L ethanol from PASC (Wen et al., 2010). Thus, reconstructing cellulosomes is a potential strategy to develop CBP-enabling organisms and several efforts to optimize the performance of such designs, like enzyme-enzyme synergy, enzyme proximity synergy, cellulose-enzyme synergy in yeast, has been explored in the recent past (Fan et al., 2012; Hyeon et al., 2010; Kim et al., 2013). A notable study addressing this aspect used an adaptive strategy to develop functional tetravalent designer cellulosomes on yeast cell surface. This novel design included (i) yeast surface bound anchoring scaffold containing two types of cohesin domains, (ii) two dockerin tagged adaptor scaffolds displaying additional cohesin-dockerin interaction sites to amplify the number of cellulase loadings and (iii) two dockerin tagged cellulase enzymes for cellulose breakdown. Yeast cells displaying this artificial cellulosome exhibited 4.2-fold enhancement in PASC hydrolysis compared to free enzymes and could also produce 2-folds more ethanol concentration compared to cells displaying divalent cellulosomes with similar enzyme loadings (Tsai et al., 2013).

3. Overcoming the impediments towards constructing *S. cerevisiae* as a CBP host

Although the vast number of studies conducted so far in developing CBP enabled *S. cerevisiae* is encouraging but translation of these endeavours in the industrial/commercial level has remained scarce. The design of an ideal CBP process requires the host organism to not only express copious amounts of cellulose solubilizing enzyme and accumulate high ethanol titre but also possess an effective mechanism to tolerate multiple toxic inhibitors, dynamic fermentation environment as well as by-products released during fermentation cycles (Dubey et al., 2016; Pandey et al., 2019). In spite of being highly ethanologenic, *S. cerevisiae* generally suffers from low heterologous cellulase productivity which is a prerequisite for disintegration of the cellulose structure and also displays a suboptimal inhibitor tolerance profile. Considering the economic motivation and sustainability of CBP technologies coupled with an immense advancements of synthetic biology/metabolic engineering tools, several research groups have contributed towards bringing down these limitations and ultimately enhancing bioethanol production in yeast. Some of the major strategies include optimization of the recombinant expression tools (promoter, transcription

factors, signal peptides), metabolic engineering of yeast protein secretion pathway and engineering a multi-inhibitor tolerance phenotype as discussed below.

3.1 Optimizing yeast expression toolbox

The most straightforward approach for developing a CBP yeast involves optimization of accessory elements (promoters, transcription factors, signal peptides etc) driving the expression of cellulases and a careful design strategy (episomal secretion vs chromosomal integration, copy numbers, cellulase ratio etc) (**Fig 2A**). Given that the cellulase promoters from native host are repressed by glucose, exploring different inducible, constitutive, and synthetic promoters to actively synthesize cellulase in yeast is the first logical step (Tang et al., 2020). Inducible promoters such as *GAL* and *CUP1* are commonly used for regulating high level gene expression in response to an inducer i.e., galactose and copper respectively. However, inducible promoters are not desirable for designing a CBP host as the use of additional inducers becomes too expensive for large scale culture. Constitutive promoters on the other hand such as *TEF1, GAPDH, PGK1, ENO1* maintain a relatively stable expression of the downstream genes with little or no effect of intra/extracellular stimuli. In a study conducted to characterize the expression profile, comparison of seven different constitutive promoters expressed during growth on glucose (*TEF1, ADH1, TPI 1, HXT7, GAPDH1, PGK1*, and *PYK1*) with strong inducible *GAL1* and *GAL10* promoters were performed. *TEF1* and *PGK1* promoters showed the most consistent activity and were equivalent to the strength of *GAL* inducible promoters (Partow et al., 2010). In a separate study, a library containing 15 promoters from *S. cerevisiae*, *Pichia pastoris*, and *Hansenula polymorpha* were screened for expression of an endoglucanase in *S. cerevisiae*. Gene fragments created by fusing endoglucanase with various promoters through promoter shuffling followed by CRISPR-δ-integration method were simultaneously applied to enhance the transcription levels of EG (Sasaki et al., 2019). A multifunctional cellulase called sestc was efficiently expressed in *S. cerevisiae* under the control of *Agaricus biporus GPD1* promoter. The recombinant strain was able to overexpress the cellulase enzyme and produce 7.53g/L ethanol from orange peel extract which was ~38 fold higher than the wild type *S. cerevisiae* (Yang et al., 2018). Alternatively, promoter engineering using random mutagenesis or knowledge-based design is a promising approach to precisely control enzymatic activities over a dynamic range. Beside promoters, other regulatory sequences such as transcription factors (TFs), the number of TF's binding site, terminators etc strongly influence gene expressions and are important engineering tools to broaden the control over recombinant enzyme expressions (Ito et al., 2015b). Recently a highly tuneable protein expression system with different combinations of the three regulatory elements- number of transcription activator binding sites, core promoter and terminator sequences were applied to express three cellulases (CBH1, CBH2 and EGL2) in yeast. Combinatorial screening of the above expression cassettes resulted in the identification of a superior transgenic strain secreting high levels of cellulases and subsequently accumulating high ethanol concentrations directly from crystalline cellulose (Ito et al., 2015a).

Another important tool regulating the expression of recombinant genes in yeast are the signal (leader) peptides, which in part, determines the trafficking of the secreted proteins. Various native, heterologous and synthetic signal sequences have been applied to optimize the protein secretion in yeast but generally these effects are protein specific. Commonly used leader peptides include signal sequence of yeast α-mating factor (*MFα*), invertase signal *SUC2*, inulinase *INU1* etc. For example, increased enzyme activity and cellulose utilization was observed in a yeast strain secreting an endoglucanase when the native secretion signal sequence of the cellulase was replaced by the mating factor α signal sequence, suggesting an

effective way to enhance cellulose derived ethanol (Zhu et al., 2010). Recently, the effectiveness of a novel signal peptide derived from *S. cerevisiae SED1* gene was demonstrated for enhanced secretion and cell-surface display of a *T. reesei* endoglucanase (EGII) and *A. aculeatus* beta glucosidase (BGL1). Recombinant strain secreting BGL1 exhibited 1.3- and 1.9-fold higher enzyme activity with the *SED1* signal peptide sequences compared to glucoamylase and MFα signal peptides, respectively. However, no significant activity difference was observed for EG II activity with *SED1* or *MFα* (Inokuma et al., 2016). Attempts to engineer synthetic signal peptides using rational design and adaptive evolution have also assisted in enhancing several recombinant proteins (Kjeldsen et al., 1997; J.A. Rakestraw et al., 2009). However, these synthetic peptides do not facilitate the secretion of every heterologous enzyme equally and thus require protein specific chaperone design. To address this limitation, a novel yeast genome-wide optimal translational fusion partner (TFP) screening system was developed that involves recruitment of an optimal secretion signal and a fusion partner to demonstrate the successful secretion of difficult-to-express proteins (Bae et al., 2015). In a following study, the broad repertoire of the optimum TFP's constructed previously were screened for the hypersecretion of cellulases from different sources in yeast. Secretion and enzyme activity of *Chrysosporium lucknowense* CBH2 by one of the TFP's were 2.4- and 1.4- fold more efficient than the native secretion peptide and MFα respectively. Similarly, other candidate TFP's were found to enhance the activities of *T. reesei* EGL with 6.2- and 1.3-fold and *S. fibuligera* BGL1 with 4.3- and 39.9- fold increase when compared with their native signal sequence and MFα counterparts respectively. Co-fermentation of the yeast strains secreting combinations of these cellulases was applied to approximately produce 14g/L ethanol from pre-treated rice straw in a CBP set-up (Lee et al., 2017).

Cellulase expression in yeast can be further controlled by optimizing the copy number and enzyme ratios for specific cellulosic substrates. Plasmid based overexpression offers high copy number of the heterologous enzymes but a constant need of selection pressure such as antibiotics or auxotrophic markers in the media and the genetic instability of the episomal plasmids itself limits its use in a large-scale culture. Therefore, as a substitute, chromosomal integration of the cellulase expression cassette ensures stability and marker-less expression of the heterologous enzymes. In an early study, twenty different integration sites in yeast were characterized to determine the expression levels of *LacZ* reporter gene, underlining the variation in the transcriptional levels between different chromosomal regions (Flagfeldt et al., 2009). However, the multicopy delta sites are generally targeted to increase the copy number and subsequently maintain high levels of cellulase expression in yeast (Mochizuki et al., 1994). Recently, an improved cocktail-delta-integration method was developed to simultaneously integrate several kinds of cellulases in a single yeast strain. Using this strategy, high cellulolytic yeasts with optimum cellulase copy number and enzyme expression ratio for PASC degradation were obtained (Yamada et al., 2010b). Several other studies emphasized the importance of synergistic actions of different cellulase enzymes as a superior determining factor than simple overexpression for efficient CBP of cellulosic substrates. Finally, gene codon optimization is also an effective strategy for improving the expression of several recombinant enzymes in yeast. Replacing the rarely used codons with the more preferential ones improves the rate and fidelity of the protein translation mechanism, thereby increasing the production of heterologous polypeptides for downstream processing. In a recent study, codon optimized version of genes encoding *Talaromyces emersonii* CBH1, *T. reesei* EGII and *A. aculeatus* BGL1 under the control of strong promoters and synthetic leaders were integrated at the delta sites of yeast genome using POT1 gene from *Schizosaccharomyces pombe* as the selection marker. Recombinant yeast strains with high cellulolytic activity on a range of cellulosic substrates were readily obtained which highlights the significance of combining multiple strategies including the role of promoters, signal

peptides, codon usage, cellulase synergy along with other metabolic engineering targets for effective production of target enzymes (Song et al., 2018).

3.2 Metabolic engineering of yeast secretory pathway

Despite the many positive attributes, the fundamentally low degree of protein production in *S. cerevisiae* poses a significant roadblock in developing this host for CBP application which pre-requisites the synthesis and secretion of multiple enzymes in large quantities. Protein secretion machinery is a complex multistep process whereby nascent polypeptides undergo co- or post- translational modification in different subcellular compartments before mature proteins are released in the extracellular milieu (**Fig 2B**). Briefly, all secretory proteins of *S. cerevisiae* are initially translocated in the endoplasmic reticulum (ER) for proper folding with the help of chaperones and foldase's along with passing through different quality checks such as signal peptide removal, glycosylation assisted stabilization, disulphide bond formation and proteolytic degradation of the misfolded proteins by eliciting the unfolded protein (UPR) response. Often, expression of foreign protein overburdens ER's folding capacity leading to an accumulation of aberrant intermediates which makes ER a crucial target of genetic modifications for improving the final product yield. For example, deletion of a single lipid-regulator gene *OPI1* inducing ER expansion in yeast, was shown to improve the secretion capacity of an important antibody by fourfold. In the same study, a folding factor overexpression library was screened and identified that an overexpression of the peptidyl-prolyl isomerase *CPR5* in the background of Δ*opi1* strain could further augment the product yield by 10 folds (de Ruijter et al., 2016). Engineering secretion leader peptides is another effective technique to optimize secretory pathway functioning (Brake, 1989). The leader sequence of *S. cerevisiae* mating factor alpha is routinely used for heterologous expression studies. It is composed of a 19-residue amino terminus pre-sequence that is cleaved by the signal protease following ER translocation, and a pro-segment that has numerous glycosylation sites, an ER export signal and Kex2 endoprotease recognition sequence. A number of studies have used directed evolution strategy for structural improvements of MFα and associated translocation of nascent proteins in the ER (Aza et al., 2021; J. Andy Rakestraw et al., 2009). Other ER engineering strategies include overexpression of signal recognition particles (SRP's), overexpression of chaperones, modulation of stress responsive genes and optimization of glycosylation sites and transport vesicles. In a recent study, a multi-level engineering approach was implemented to improve the extracellular activities of an endoglucanase, beta glucosidase and a small GFP tagged antibody by manipulating crucial steps of the early secretory pathway (Besada-Lombana and Silva, 2019). Briefly, ER processes such as translocation and protein folding, known to introduce bottlenecks, along with the ER to Golgi exit mechanism was focussed to improve the overall secretion. At first, peptide entry into the ER was improved by designing a hybrid secretory leader sequence (Ost1-pro-MFα1) and through a novel approach utilizing 3'UTR-mediated SRP pre-recruitment by substituting *CYC1* terminator with *Pmp1* or *Pmp2*. To further alleviate other potential limitations, engineering strategies were focussed on expanding ER membrane (by deleting *PAH1* gene), reducing retro-translocation machinery (by deleting *DER1* and consequently upregulating ERAD pathway) and enhancing exit recognition sites (by overexpressing *ERV29*). While the optimum combination of these individual strategies varied for the three tested proteins, a general improvement in protein secretion was observed in the strains carrying deletion of *PAH1, DER1* and *Erv29* overexpression demonstrating the importance of strain engineering (Besada-Lombana and Silva, 2019) . Similarly, a moderate overexpression of *SEC16*, which is involved in protein translocation/exit from ER to Golgi apparatus in yeast, was shown to improve the secretion of various hydrolytic enzymes (Bao et

al., 2017). Upon reaching Golgi compartments, these proteins undergo further modification by resident Golgi enzymes and the mature secretory proteins are subsequently sorted and dispatched to their destined location via clathrin-coated vesicles. Often, correctly folded heterologous proteins are frequently retained intracellularly, leading to their poor secretion which makes engineering of the trafficking pathway another major area of secretion improvement. Past studies have identified a number of targets such as deletion of *VPS10*, overexpression of *SSO1* or *SSO2*, overexpression of *SNC1* or *SNC2* as beneficial for overall protein secretion but these observations were majorly protein specific. To overcome this limitation, a recent study investigated the conserved vesicle trafficking process which requires soluble NSF receptor (SNARE) complex formation by *Sec1/Munc18* (SM) proteins whose role is less understood in literature. Out of the several SM proteins in yeast, an overexpression of *SEC1* and *SLY1* encoding SM proteins were identified to improve secretion for a broad range of proteins. Engineering Sec1p improved the trafficking between Golgi to cell membrane while Sly1p was responsible for vesicle fusion from ER to Golgi, thus demonstrating that engineering secretory pathway at the transit steps is indeed a novel strategy to enhance heterologous protein production in yeast (Hou et al., 2012).

Although the above studies provide several insights of engineering protein secretion pathways, a wider understanding of the cross-talks between secretory pathways with that of other cellular processes remains little understood. With the advancements in synthetic and systems biology, continuous efforts are directed towards unravelling the underlying mechanism of intracellular processes such as protein secretion pathway, to provide a more rational approach for engineering cell factories. For example, a recent study used RNA-seq to perform a transcriptional genome-scale analysis of several mutant yeast strains having fivefold varying protein secretion capacity for a recombinant protein (Huang et al., 2017). Although the number and types of mutation in these strains varied, the overall transcriptional regulatory response caused by these mutations were similar; additionally, the secretory pathway was shown to be affected by secondary cellular responses induced by these mutations. Using comparative genome scale transcriptional analysis, fundamental differences revealed that in the hypersecretory yeast strains the majority of genes related to glycolysis and TCA cycle were downregulated, whereas genes involved in lipid metabolism were knocked-out. Also, amino-acid biosynthesis genes were downregulated but amino acid transporters were upregulated. Additionally, the glutamate/glutamine biosynthetic pathway was upregulated, amino acid degradation pathway was deleted and the oxidative stress response was upregulated. Most interestingly hypoxia genes were significantly upregulated while the respiratory and mitochondrial functions were downregulated. Therefore, the mutant strains with increased protein synthesis exhibited reduced respiration, higher ethanol production, better stress adaptability and an overall reduction in final biomass yield. Most of the genes identified in this study demonstrated a common regulation pattern with the previous reported literature and thus specified a general guideline governing efficient protein secretion in yeast (Huang et al., 2017) . Similarly, using whole genome sequencing, mutagenized yeast libraries were screened for several genes involved in the trafficking pathway, histone deacetylase complex and carbohydrate metabolic processes as potential targets for improving protein secretion. Important modifications in the endosome-to-Golgi trafficking pathway i.e., deletion of *VPS*5 and *VPS*17 were proven to effectively reduce intracellular protein retention along with identification of several novel gene targets exhibiting synergistic effects on protein secretion mechanism (Huang et al., 2018, 2015).

3.3 Engineering yeast for multi-inhibitor tolerance

The complex architecture of LCB makes pre-treatment process necessary wherein cellulose, hemicellulose and lignin are segregated to enhance the digestion while limiting the degradation of monomeric sugars to toxic inhibitors (Baruah et al., 2018; Meng et al., 2013). However, commonly used pre-treatment methods involving high pressure, use of acid/alkali hydrolysis and/or steam explosion generates a variety of compounds (organic acids, furan and lignin derivatives) as by-products which are inhibitory to yeast growth and fermentation (Baruah et al., 2018; Kumar and Sharma, 2017). Although, *S. cerevisiae* has an efficient innate mechanism to tolerate pre-treatment derived inhibitors compared to other fermentative organisms, high concentration of these compounds interferes with host's membrane integrity, cellular machinery, protein synthesis, cell growth and ethanol production (Almeida et al., 2007). Therefore, yeast strains with an improved tolerance/detoxification process along with high ethanol yield are highly desirable for CBP applications. Typically, pre-treatment derived inhibitors can be classified into three main classes: (i) furans aldehydes (e.g., furfural and 5'-hydroxymethylfurfural (HMF)) (ii) weak acids (e.g., acetic acid, formic acid, levulinic acid) and (iii) phenolics (e.g., vanillin, coniferyl alcohol, *p*-coumaric acid, 4-hydroxy-3-methoxycinnamaldehyde etc) known to affect microbial fermentation ability. Furan derivatives (furfural and HMF), for instance, inhibits alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) along with several genes of glycolytic pathway and TCA cycle causing accumulation of reactive oxygen species (ROS) damaging organelle as well as chromatin/actin structure (Boyer et al., 1992). It also leads to an intracellular depletion of NAD(P)H and ATP levels and much of the cell's energy is spent in repairing this damage which either inhibits growth or result in longer lag phases affecting ethanol yield and productivity. The weak acids on the other hand causes intracellular anion accumulation and inhibits amino acid uptake from the extracellular medium. While low amounts of acids such as acetic or formic acid enhance ethanol production by stimulating anaerobic ATP production, diffusion of these compounds in high concentration causes rapid acidification of the cytosolic environment (low pH). The excess protons are pumped out from the cell by membrane ATPase via ATP hydrolysis until an equilibrium is maintained and consequently less energy is directed towards biomass and product formation (Casey et al., 2010; Guaragnella and Bettiga, 2021). However, the extent of inhibition depends upon the type of acids (formic acid > levulinic acid > acetic acid) present in the lignocellulosic hydrolysates. Finally, phenolic compounds consisting of several complex functional groups like aldehyde and ketone strongly affect the electro-chemical gradient of the cellular membranes causing impaired membrane functioning (**Fig 2C**) (Gu et al., 2019; Yan et al., 2019). *S. cerevisiae* possess a natural ability to metabolize some of these compounds in low concentration, for example, HMF and furfural can be reduced in a NAD(P)H dependent manner to their less toxic form (alcohols) under aerobic and anaerobic conditions; similarly, some of the phenolic compounds can be assimilated by native *PAD1* gene catalysing the decarboxylation of phenyl acrylic acids such as ferulic acid, cinnamic acid etc. Based on this existing mechanism, recent studies have focussed on further improving the tolerance profile using metabolic, genetic and evolutionary engineering strategies to develop robust strains resisting these inhibitors individually or in combination (Brandt et al., 2021; Westman et al., 2014; Xu et al., 2019). For example, the role of yeast transcription factors of multidrug resistance (MDR) pathway was investigated in providing resistance to lignocellulose derived inhibitors. Thirty deletion mutants and eight transformants overexpressing selected transcription factors of yeast MDR pathway were screened in the presence of spruce wood derived inhibitor cocktail (coniferyl aldehyde, 5-HMF and furfural). Candidates overexpressing *YAP1* and *STB5* were responsible for combating oxidative stress response, exhibited enhanced relative growth rates. While *YAP1* complemented *STB5* in conferring resistance towards HMF, it was solely responsible for resisting coniferyl aldehyde, a toxic

phenolic compound. Other transcription factors such as *DAL81, GZF3, LEU3, PUT3, WAR1* were also identified as important targets for inhibitor resistance (Wu et al., 2017). Transcription factor analysis also revealed the role of novel transcription factors- Sfp1p and Ace2p, in optimizing yeast strains for improved tolerance to mixed inhibitors. Overexpression of Sfp1p led to a fourfold improvement in specific ethanol productivity whereas Ace2p overexpression enhanced the rate by three times in the presence of acetic acid and furfural. Sfp1p was predicted to upregulate the transcriptional regulation of ribosomal genes in response to the oxidative stress caused by the inhibitors and Ace2p activated genes involved in cell separation and daughter-cell specific gene expression which could rescue growth inhibition induced by the toxic chemicals (Chen et al., 2016). Artificial transcription factor has also facilitated in exploring novel gene targets involved in stress modulation of yeast. An artificial zinc finger library (ZFP-TF) based screening of mutant strains identified three novel gene targets responsible for enhanced acetic acid tolerance. Among the three, deletion of *QDR3* significantly improved the glucose uptake rate and ethanol productivity in the presence of up to 5g/L of acetic acid in the fermentation media (Ma et al., 2015). In a separate study, differential expression analysis of *PRS3, RPB4* and *ZWF1* genes were studied in two industrial yeast strains in the presence and absence of lignocellulose derived inhibitors to establish their potential role and underlying molecular mechanism in inhibitor tolerance. *PRS3* overexpression improved the fermentation efficiency and ethanol productivity by 32% and 48% respectively. On the other hand, *ZWF1* and *RPB4* overexpression in the presence of acetic acid, HMF, furfural induced better adaptation to these inhibitors, but their overexpression did not improve fermentation performance in yeast (Cunha et al., 2015). ^{13}C metabolic flux analysis has been applied to evaluate the comparative metabolic reprogramming in two yeast strains with different degrees of tolerance towards acetic acid and furfural. By analysing the intracellular carbon flux, it was observed that under dual stress conditions both ATP and NAD(P)H levels drastically decrease indicating the potential benefits of upregulating energy and co-factor synthesis pathways for designing inhibitor tolerant phenotypes (Guo et al., 2016). Adaptive laboratory evolution (ALE) is another powerful method to naturally select for mutant strains with desired phenotypic characters and it has been widely applied to generate inhibitor tolerant yeast strains. In a novel adaptation strategy, yeast strains were evolved based on alternating cultivation cycles in the presence and absence of acetic acid for up to 55 generations. Whole genome sequencing of evolved strains identified subtle mutations in four genes (*ASG1, ADH3, SKS1 and GIS4*) as responsible for the acquired acetic acid tolerance (González-Ramos et al., 2016). More insights regarding acquired thermo-acidic tolerance in an *S. cerevisiae* strain were revealed in a recent study (Salas-Navarrete et al., 2022). Following an adaptive evolution on an increasing concentration of acetic acid (12 g/L) for one year, the evolved yeast strain was able to grow at 37°C in minimal media containing 2g/L acetic acid, pH 3 and accumulated 16 mmol per gram dry cell weight of ethanol. Using genome sequencing and reverse engineering in this strain, structural changes in genes involved in the glucose-mediated signalling cascade (*RAS2*), heat shock regulation (*HSF1*) and replication initiation (*SUM1*) were identified as key contributors of enhanced tolerance against thermal and acidic stress (Salas-Navarrete et al., 2022). In a separate study, adaptive evolution was applied to specifically evolve *GRE2* gene previously identified to confer highest tolerance by upregulating the conversion of furan aldehydes into alcohols. A yeast library consisting of PCR mutagenized *GRE2* variants was evolved in the presence of prolonged stress to allow the appearance of stronger mutants. Upon screening, a hyper tolerant GRE^{evol} carrying triple mutation in the gene (*P48S, I290V and D133D*) exhibited a remarkable improvement in its fermentation ability in the presence of a suite of inhibitors. The evolved *GRE2* gene together with elevated levels of extracellular potassium (K^+) and pH was sufficient to confer a “near-parity” production between inhibitor

laden and inhibitor free feedstock hydrolysates. This study demonstrated a functionally orthogonal and simple engineering strategy to develop multi feedstock tolerant yeast strains producing both cellulosic fuel and non-fuel products (Lam et al., 2021). Similarly, a modified ALE method called visualizing evolution in real-time (VERT) was developed to understand the molecular mechanism governing the tolerant behaviours in yeast. It offered a rational scheme to conveniently select mutants with differential levels of acquired resistance and growth rate enhancement on hydrolysates containing acetic acid, HMF and furfural. Transcriptomic analysis revealed significant upregulation of transcription factors involved in drug resistance and oxidative stress response pathways among mutants with enhanced tolerance to furan derivatives; suggesting the relation between oxidative stress and furan tolerance. Additionally, genes such as *ATP5*, *VMA3*, *VPH1* and *SPI1* were found to be commonly upregulated in mutants with higher tolerance to acetic acid (Almario et al., 2013). ALE has therefore proven very effective in rapidly producing microbial populations with novel phenotypes, suitable for dedicated applications. In an excellent review published elsewhere, recent studies on the deployment of ALE on diverse species of bacteria, fungi, and microalgae to boost their biotechnological significance have been described in detail (Mavrommati et al., 2022).

4. Engineering yeast to produce ethanol from hemicellulosic feedstock

Hemicellulosic polymers are the second most prevalent source of fermentable sugars in lignocellulosic biomass after cellulose. Pre-treatment and hydrolysis of hemicellulose release xylose as the main monomeric sugar, along with arabinose, galactose, rhamnose, etc (Amin et al., 2017; Kumar et al., 2009). Utilization of the broad repertoire of these sugars, especially xylose, is crucial for maximizing the potential of CBP technology. Although natural xylose-fermenting microbes exist, the poor ethanol yield and sensitivity towards fermentation inhibitors restrict their use in bioethanol production (Ochoa-Chacón et al., 2021). Contrarily, *S. cerevisiae* cannot utilize xylose naturally, shares the same transporter for xylose and glucose uptake, and suffers significantly from catabolite repression (Chandel et al., 2018; Meinander and Hahn-Hägerdal, 1997). Hence, genetic engineering of this host to produce ethanol from xylose as efficiently as from glucose has remained an utmost important area of research. Xylose assimilation occurs via two separate processes in native xylose-utilizing microorganisms, each involving a unique set of enzymes. First is the xylose reductase-xylose dehydrogenase (XR-XDH) pathway, which follows a two-step breakdown of xylose into xylulose. XR, in the presence of co-factor NADH or NADPH, initially reduces xylose to xylitol which is further oxidized to xylulose by XDH in the presence of co-factor NAD^+. Due to the different co-factor requirements and the amount of their intracellular availability, a redox imbalance typically leads to an accumulation of xylitol which reduces the efficiency of this pathway (Hou et al., 2009; Jeffries, 2006; van Vleet and Jeffries, 2009). Alternatively, the second pathway is the xylose-isomerase (XI) pathway, which directly converts xylose to xylulose without the requirement of any co-factors. Xylulose from both the above pathways then converts into xylulose-5-phosphate by xylulose kinase (XKS) and subsequently enters into the pentose phosphate pathway for further catabolism (Brat et al., 2009; Kuyper et al., 2005). We have recently discussed the various engineering approaches used to rebuild these pathways for the generation of xylose-fermenting *S. cerevisiae* strain and the different strategies applied to improve xylose utilization, such as eliminating cofactor specificities, development of pentose transporters, and native metabolic pathway engineering in detail elsewhere (Yusuf and Gaur, 2017). More developments on the subject can also be found in the recently published articles (Bae et al., 2021; Cunha et al., 2020b; Hou et al., 2017). Instead, in this review, we have focussed on studies that have engineered *S. cerevisiae* for

utilizing xylose and other non-cellulosic sugars to produce bioethanol in a mixed sugar state for CBP applications. For example, in an early study *S. cerevisiae* was engineered to simultaneously saccharify and ferment undetoxified rice straw hydrolysate consisting of hemicelluloses such as xylan, xylooligosaccharide, and cellooligosaccharide without the requirement of exogenous enzymes. The recombinant strain expressed XR, XDH from *Scheffersomyces stipitis* (formerly *Pichia stipitis*), and endogenous XKS to enable xylose assimilation. Next, in the same strain hemicellulose degrading enzymes consisting of *T. reesei* endoxylanase, *A. oryzae* β-xylosidase, and *Aspergillus aculeatus* β-glucosidase were surface displayed. The final engineered strain produced an ethanol titer of 8.2g/L after 72 hours of fermentation with a yield of 0.41g/g corresponding to 82% of theoretical yield (Sakamoto et al., 2012). In a separate study, *S. cerevisiae* was engineered to utilize xylan by recombinant expression of multiple xylan degrading and xylose assimilating enzymes. Endoxylanase encoding *xyn2* from *T. reesei* and xylosidase encoding *xlnD* from *A. niger* were co-expressed to enable the complete conversion of xylan into xylose. Further assimilation of xylose was facilitated by the expression of novel xylose isomerase (*xylA*) from *Bacteroides thetaiotaomicron* and xylulose kinase (*xyl3*) from *S. stipitis*, to bypass the cofactor requirement of the alternative XR-XDH pathway. The native aldose-reductase gene (*GRE3*) was also deleted to ensure minimum xylitol (by-product) accumulation. The resultant yeast strain showed enhanced growth and enzyme production under aerobic conditions as well as accumulated 9 g/L ethanol (90% of theoretical yield) under oxygen limitation when cultivated on xylose as the only carbohydrate source (Mert et al., 2016). Similarly, in a recent report, industrial workhorse strains of *S. cerevisiae* displaying thermal and inhibitor tolerance phenotypes were engineered to surface display hemicellulolytic enzymes and were equipped with optimized xylose utilization pathways for consolidated bioprocessing of corn-cob hemicellulose. The re-designed yeast cells were able to directly produce 11.1g/L ethanol from undetoxified hemicellulose hydrolysate derived from hydrothermally treated corn-cob feedstock without supplementation of commercial enzymes, thus acting as whole-cell CBP biocatalysts (Cunha et al., 2020a). In an effort to broaden the scope of expressing different lignocellulolytic enzymes, seven heterologous enzymes namely endoglucanase, β-glucosidase, cellobiohydrolase I and II, xylanase, β-xylosidase, and acetylxylan esterase were simultaneously secreted in single bioethanol producing industrial *S. cerevisiae* strain harbouring XI-based xylose-fermenting pathway. The engineered AC14 strain was able to utilize multiple lignocellulosic biomass and showed 94.5 FPU of secreted enzyme activity per gram dry cell weight of yeast without affecting the cell's glucose or xylose fermentation capacity (Claes et al., 2020). Such robustly performing cell factories are expected to serve as a cost-effective platform for the valorization of different biomass without the need for expensive commercial enzyme blends. Furthermore, *S. cerevisiae* has also been engineered to ferment arabinose, which represents the second most prevalent pentose sugar after xylose. Reconstruction of the arabinose utilizing pathways from heterologous fungal or bacterial species, expression of novel L-arabinose specific transporters, and metabolic engineering of native pentose phosphate pathway genes has facilitated *S. cerevisiae*'s ability to consume L-arabinose (Richard et al., 2003; Subtil and Boles, 2011; Wisselink et al., 2007; Ye et al., 2019). In a recent report, simultaneous co-utilization of xylose and arabinose in the presence of glucose was established in *S. cerevisiae* chassis by applying metabolic engineering and adaptive evolution. The resulting strain from this study consumed 24% more pentose sugar after 120 hours of fermentation on mixed sugar containing 20 g/L of xylose, arabinose, and glucose each (Wang et al., 2017). Similarly, a multi-sugar utilizing ability was conferred by additionally overexpressing three xylose and two arabinose metabolizing genes in a previously constructed xylose-fermenting yeast. The newly developed strain was able to consume and ferment a five-sugar mix containing glucose, galactose, mannose, xylose, and

arabinose in 72 hours with 72.5% increased ethanol yield (Bera et al., 2010). Many other studies have similarly reported the effectiveness of combining pentose and hexose sugar assimilation pathways in microbes for enhancing complete biomass utilization (Ha et al., 2011; Katahira et al., 2006; Wisselink et al., 2009; Zhang et al., 2015). Although the above studies provide encouraging results, it is generally recognized that expressing multiple heterologous enzymes places enormous metabolic stress on cells, resulting in reduced growth performances. Therefore, designing synthetic microbial consortiums that divide a multi-step metabolic pathway among more than one organism is being explored as a sustainable alternative (Caballero and Ramos, 2017; Tabañag et al., 2018). For instance, a consortium of three specialized *S. cerevisiae* strains was developed to ferment a glucose-xylose-arabinose mixture. The strains exclusively utilizing xylose or arabinose were constructed by eliminating hexose phosphorylation and subsequent rounds of evolutionary engineering (Verhoeven et al., 2018). The resulting consortium was able to rapidly consume glucose and arabinose in anaerobic batch cultures, however, xylose consumption was severely impaired in the presence of the three sugar mixtures. Consequently, extended rounds of anaerobic adaptive evolution on the three-strain consortium were carried out, which eventually improved the xylose consumption rate. The evolved consortium thereafter showed steady fermentation kinetics compared to single pentose utilizing strains over prolonged cultivation on mixed sugars (Verhoeven et al., 2018). Another recent study, demonstrated kinetic modelling to guide the design of co-culture conditions for two recombinant yeast strains consuming cellobiose and xylose respectively (Chen et al., 2018). The designed pipeline which was built on pure culture models included the effects of parameters including product inhibition, inoculum size, and starting substrate concentration. The predictions from this model correlated well with the results obtained from independent fermentation experiments. Using this model, simultaneous co-fermentation of 60g/L cellobiose and 20g/L xylose was achieved in *S. cerevisiae/ S. cerevisiae* co-culture. Many other interesting studies involving the application of diverse microbial consortiums for producing industrially important chemicals have been recently reviewed elsewhere (Gao et al., 2019).

5. Discussion and Future prospects:

The concept of CBP promises the most sustainable and cost-effective route for the biological conversion of lignocellulose to ethanol. Among all the microorganisms considered for lignocellulosic ethanol development by CBP route, *S. cerevisiae* has by-far attained an upper hand mainly due to its high ethanol productivity and amenability to genetic engineering. However, practical application of these achievements has been significantly limited by a number of factors governing the performance of yeasts in a CBP process. One of the most significant problem is the incomplete conversion of cellulose to mono-component sugars which can be partly attributed to the fact that natural crystalline cellulose is difficult to digest and often requires higher ratios of certain cellulases, specially cellobiohydrolases. Although the rate of heterologous enzyme production can be modulated by fine-tuning the expression elements or engineering *in vivo* metabolic pathways, the complex and dynamic nature of different cellulosic materials makes it impossible to design a universal CBP enabling yeast strain. Corollary to the above issue, inadequate production of cellulase titres in yeast also results in an overall reduction in hydrolysis efficiency, ultimately necessitating the addition of expensive commercial enzyme cocktails. To circumvent this problem, an in-depth understanding of the molecular cross-talks between different genes involved in the protein synthesis pathway is required to upregulate the production and secretion of functional cellulases. A second major limitation with *S. cerevisiae* is the sequential consumption of sugars from mixed blends of pre-treated biomass. Engineering the ability to co-utilize mixed

sugars (e.g., xylose and arabinose) in yeast is therefore imperative for the complete utilization of lignocellulosic hydrolysates. As described above sections, integration of xylose or arabinose utilizing pathways, expression of natural/synthetic non-glucose specific sugar transporters and construction of hybrid strains have been relevantly successful techniques in expanding the available substrates for yeast fermentation, but the production levels still need to improve. In the past several years, extensive research has been focussed on elucidating the glucose sensing and repression mechanism which controls most of the metabolic responses in yeast. Targeted reprogramming of key genes from these studies is urgently required to eliminate the diauxic growth behaviour of yeast by lifting off the glucose induced repression cascades. Additionally, the operational conditions required for ethanol production from lignocellulose by employing CBP-enabled *S. cerevisiae* host is also a major hindrance for its limited application in industrial setting. Since most of the commonly used cellulases exhibit an optimum activity at higher temperatures (50°C or higher), yeast can tolerate only up to a maximum of 40°C for growth and fermentation. This temperature difference significantly compromises the rate and efficiency of the cellulase performance in a CBP set-up. Thus, engineering efforts to design cold- adaptive cellulases is also crucial for the realization of CBP in practise.

In order to summarize, engineering *S. cerevisiae* as a CBP host to utilize renewable biomasses is the most prospective approach to reduce our dependency on non-renewable petroleum-based fossil fuels. Since its inception, research from all over the world have contributed to the development of this technology to produce ethanol at a competent TRY (titer-rate-yield) levels. Although *S. cerevisiae* owing to its multi-useful properties have been efficiently engineered to saccharify and ferment various biomasses along with increasing the endogenous inhibitor tolerance capacity, significant efforts to minimize the associated limitations must be pursued. In parallel, utilization of lignocellulosic biomass by other potential non-*Saccharomyces* yeasts such as oil-producing (oleaginous) yeasts can further help in replacing the petroleum-based diesel use (Valdés et al., 2020a, 2020b). Although in its nascent stage, a huge interest is currently invested in developing genetic engineering tools to rewire metabolic routes in these organisms towards lipid overproduction (Shi and Zhao, 2017). In this promising future, some of the most powerful solutions would come from the continuous advancements in synthetic biology and sequencing tools that will enable researchers to understand complex genetic nexus and develop more sophisticated bio-engineering principles to expedite our collective endeavours towards a sustainable future.

Author Contribution:

NAG and JS conceptualized the study. JS wrote the manuscript. VK, RP and NAG proof-read the manuscript.

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Conflict of Interest Statement

All the authors declare that there is no conflict of interest to disclose.

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Figure Legends

Fig 1: Engineering recombinant cellulolytic *S. cerevisiae* for production of ethanol by (a) secretion of free cellulases (b) surface display of cellulases and (c) display of cellulases in cellulosome complex.

Fig 2: Strategies to engineer *S. cerevisiae for* CBP applications. (A) Optimization of gene expression levels. Individual parts driving the gene expression (such as promoter, signal sequences, terminator sequences) followed by choice of transformation (episomal expression/chromosomal integration) and cellulase ratio optimization are shown as potential engineering target for downstream expression enhancement. (B) Engineering of protein secretion pathway in yeast. A general scheme of the pathway is shown with potential gene targets modulating enzyme expression. Gene targets whose overexpression improves the secretion is shown in green and gene deletion targets are shown in red. (C) Improving the tolerance capacity of yeast towards pre-treatment generated inhibitors. Consequential effects of the major inhibitory compounds (i.e., HMF, F-AL, weak acids and phenolics shown in blue) is represented in red. HMF:hydroxymethylfurfural; F-AL:furfural; WA:weak acids; RP:respiration pathway; FP:fermentation pathway; PDC:pyruvate decarboxylase; PDH:pyruvate dehydrogenase; ADH:alcohol dehydrogenase; CO2: carbon dioxide; Et-OH: ethanol; ROS:reactive oxygen species; ATP:adenosine triphosphate.

Table 1: Selected reports on engineering non-*Saccharomyces* sp. for CBP application

S. No	**Purpose**	***Microorganism***	**Genetic engineering strategy**	**Result**	**Ref.**
1	Engineering to establish or enhance the	*Thermoanaerobacterium saccharolyticum*	Elimination of acetate production pathway by deleting phosphotransacetylase (*pte*) and acetate kinase (*ack*) gene in the background of lactic acid deleted (*Δldh*) strain	Ethanol yield of 92% (33g/L)	(Shaw et al., 2008)

2	production of ethanol in cellulose utilizing microorganisms	*Clostridium thermocellum*	Elimination of H_2 production by deleting central hydrogenase maturase (*hydG*) and *ech* gene.	Ethanol yield of 64% of the theoretical yield from cellobiose	(Biswas et al., 2015)
3		*Clostridium thermocellum*	Engineering electron metabolism by overexpression of *rnf* (ion-translocating reduced ferredoxin:NAD^+ oxidoreductase) operon in strain with *hydG* gene deletion	Ethanol at 66% (5.1g/L) of the theoretical yield from Avicel	(Lo et al., 2017)
4		*Trichoderma reesei*	Three rounds of genome shuffling with *S. cerevisiae* by electroporation	Ethanol production of 3.1g/L on sugarcane baggase	(Huang et al., 2014b)
5		*Fusarium oxysporum*	Overexpression of phosphoglucomutase (*pgm*) and transaldolase (*tal*) gene	Ethanol at 95%(20g/L) of the theoretical yield from glucose	(Anasontzis et al., 2016, 2014)
6		*Myceliophthora thermophila*	Heterlogous expression of *S. cerevisiae ADH1* and overexpression of a glucose transporter *GLT-1* or cellodextrin transport system (*CDT- 1/2*). Promoter replacement of native *pyc* gene to downregulate the conversion of pyruvate to oxaloacetate.	Ethanol production of 11.3 g/L from cellobiose	(Li et al., 2020)
7		*Pyrococcus furiosus*	Heterlogous expression of monofunctional alcohol dehydrogenase (*AdhA)* from *Thermoanaerobacter sp* to construct the synthetic AOR/ AdhA pathway	Ethanol at 35% of theoretical yield from cellobiose	(Basen et al., 2014)
8	Engineering to confer cellulose utilization pathway in microorganism capable of producing ethanol/other biomass	*Zymomonas mobilis*	Heterologous expression of xylose isomerase (*xylA*), xylulokinase (*xylB*), transaldolase (*tal*), and transketolase (*tktA*) to engineer pentose metabolism	Ethanol at 86% of theoretical yield from xylose	(Zhang et al., 1995)
9		*Zymomonas mobilis*	Hetrologous expression of β-glucosidase gene from *EmRuminococcus* albus with *Z.mobilis* Tat signal peptide	Ethanol production of 0.49g/g of cellobiose	(Xia et al., 2019; Yanase et al., 2005)
10		*Pichia pastoris*	Surface display of a high-affinity minicellulosme by *in vitro* assembly of an endoglucanase	Ethanol production of 5.1g/L	(Dong et al., 2020)

	derived compounds		from *Clostridium thermocellum*, exoglucanase from *Yarrowia lipolytica*, β-glucosidase from *Thermoanaerobacterium thermosaccharolyticum* and a carbohydrate binding domain (CBM) from *Thermobifida fusca*	from carboxymethyl cellulose (CMC)	
11		*Kluyveromyces marxianus*	Surface display of *Trichoderma reesei* endoglucanase and *Aspergillus aculeatus* beta-glucosidase.	Ethanol at 92.2% (4.24 g/L) of the theoretical yield on beta-glucan.	(Shuhei Yanase et al., 2010)
12		*Saccharomyces pastorianus*	Secretion of *Trichoderma reesei* EGL, CBH and BGL gene	Ethanol production of 16.5g/L on PASC	(Fitzpatrick et al., 2014)
13		*Rhodotorula glutinis*	Secretion of *Trichoderma reesei* exocellulase *(CBH1 and CBH II), T. reesei* endoglucanase *EgIII* ; *Aspergillus niger* endoglucanase *(EgI* and *EglA)* and *Neocallimastix patriciarum* β-glucosidases (*BGS)*	Total cellulase activity up to 0.017 U/μg on commercial cellulose	(Pi et al., 2018)
14		*Lipomyces starkeyi*	Heterologous expression and signal peptide screening to secrete a *Trichoderma reesei* endoglucanase II (EG II) and a chimeric cellobiohydrolase I (TeTrCBH I) formed by fusion of catalytic domain of *Talaromyces emersonii* CBH I and CBM of *T. reesei* CBH I.	3.9 U/mg of purified EG II activity on CELLG3 substrate and up to 63.1% hydrolysis of corn stover cellulose by purified chimeric TeTrCBH 1	(Xu et al., 2017)

Table 2: Summary of the selected studies on engineering *S. cerevisiae* to produce cellulosic bioethanol.

Strategy	**Source & type of cellulase**	**Activity**	**Cellulose**	**Ethanol (g/L)**	**Fermentation time (hours)**	**Refs.**

Free secretion	*Trichoderma reesei* endoglucanase (EGI) & *Saccharomycopsis fibuligera* beta-glucosidase (BGL)	0.30 U mg^{-1} DCW for EGI & 0.48 U mg^{-1} DCW for BGL	PASC	1	192	(Riaan Den Haan et al., 2007)
	Clostridium cellulovorans endoglucanase (EGL) & *Saccharomycopsis fibuligera* beta-glucosidase (BGL1)	5.159 Ug^{-1} DCW for EGL & 1.862 UmL^{-1} for BGL	Barley beta-glucan	9.15	50	(Eugene Jeon et al., 2009)
	Clostridium thermocellum endoglucanase (EGL) & *Saccharomycopsis fibuligera* beta-glucosidase (BGL1)	1.03 U g^{-1} DCW for EGL & 4.97 U g^{-1} DCW for BGL	Barley beta-glucan	9.67	16	(E Jeon et al., 2009)
	Aspergillus aculeatus cellobiohydrolase (CBH1), *Trichoderma reesei* (CBH1, CBH2 and EGL) & *Aspergillus aculeatus* beta-glucosidase (BGL1)	np	Pre-treated corn-cob	28	168	(Hong et al., 2014)
	Trichoderma viride endoglucanase and beta-glucosidase (EG3 and BGL1)	2.34 U ml^{-1} for EG3 & 0.95 U ml^{-1} for BGL1	CMC	4.63	24	(Gong et al., 2014)
	Saccharomycopsis fibuligera beta-glucosidase (BGL) , *Trichoderma reesei* endoglucanase (EGII) & *Talaromyces emersonii* cellobiohydrolase (CBHI)	15 U g^{-1} DCW for BGL, 3.87 U mg^{-1} DCW for EGII & 7.32 U mg^{-1} DCW for CBHI	Pre-treated corncob	4.05	168	(Davison et al., 2019)

	Trichoderma reesei endoglucanase (EGII) & *Aspergillus aculeatus* beta-glucosidase (BGL)	3.64 U g^{-1} DCW for EGII & 5.94 U g^{-1} DCW for BGL1	Barley beta-glucan	16.5	50	(Fujita et al., 2002)
Cell surface display	*Trichoderma reesei* endoglucanase and cellobiohydrolase (EGII and CBH2) & *Aspergillus aculeatus* beta-glucosidase (BGL)	164.5 U g^{-1} DCW for BGL1	PASC	3	40	(Fujita et al., 2004)
	Trichoderma reesei endoglucanase (EGII), *Talaromyces emersonii* cellobiohydrolase (CBHI) & *Aspergillus aculeatus* beta-glucosidase (BGL)	24.2 U mL^{-1} for EGII & 4.7 U mL^{-1} for CBHI	PASC	2.90	96	(Liu et al., 2015)
	Trichoderma reesei endoglucanase and cellobiohydrolase (EG2, CBH2) & *Aspergillus aculeatus* beta-glucosidase (BGL)	234.1 mU g^{-1} wet cell of total PASC degrading activity	PASC	7.60	72	(Yamada et al., 2011, 2010b)
	Trichoderma reesei endoglucanase (EG2), *Talaromyces emersonii* cellobiohydrolase (CBHI), *Chrysosporium lucknowense* cellobiohydolase2 (CBH2) & *Aspergillus aculeatus* beta-glucosidase (BGL)	np	Pre-treated rice straw	1.50	96	(Liu et al., 2016)
	Trichoderma reesei endoglucanase and cellobiohydrolase (EG II and CBH II) & *Aspergillus aculeatus* β-glucosidase (BGL)	2.06 U g^{-1} DCW for EG2, 0.58 U g^{-1} DCW for CBH2 & 4.02 U g^{-1} DCW for BGL	PASC	2.10	60	(S Yanase et al., 2010b)

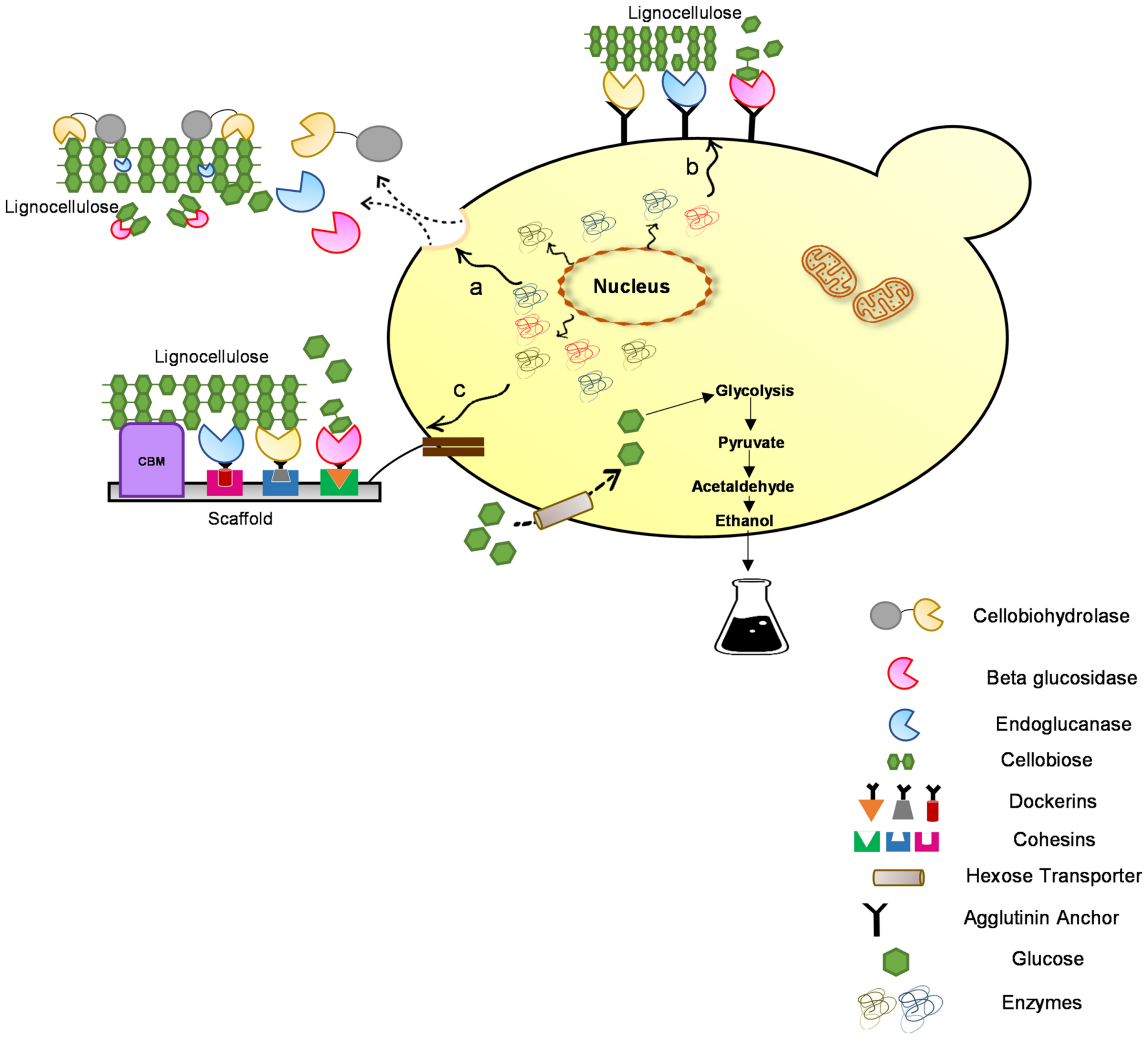
	Trichoderma reesei endoglucanase and cellobiohydrolase (EG and CBH) & *Aspergillus aculeatus* beta-glucosidase (BGL)	101.5 mU g^{-1} wet cells of total PASC activity	PASC	4.30	72	(Yamada et al., 2013)
	Trichoderma reesei endoglucanase and cellobiohydrolase (EG and CBH) & *Aspergillus aculeatus* beta-glucosidase (BGL)	np	Ionic liquid-swollen cellulose (ILSC)	2	160	(Nakashima et al., 2011)
	Trichoderma reesei endoglucanase and cellobiohydrolase (EG and CBH) & *Aspergillus aculeatus* beta-glucosidase (BGL)	np	Ionic liquid pre-treated bagasse	0.81	96	(Yamada et al., 2017)
Display of Cellulo-some	*Clostridium cellulolyticum* endoglucanase (EG) & *Clostridium thermocellum* beta-glucosidase (BGL)	np	PASC	3.5	48	(Tsai et al., 2009)
	Trichoderma reesei endoglucanase and cellobiohydrolase (EG and CBH) & *Aspergillus aculeatus* beta-glucosidase (BGL)	np	PASC	1.80	70	(Wen et al., 2010)
	Chimeric endoglucanase & *Saccharomycopsis fibuligera* beta-glucosidase (BGL)	np	CMC	3.45	16	(Hyeon et al., 2010)
	Clostridium cellulolyticum endoglucanase, cellobiohydrolase and beta-glucosidase (EGL, CBH and BGL)	np	AVICEL	1.42	96	(Fan et al., 2012)
	Clostridium thermocellum endoglucanase (EG), *Trichoderma reesei* cellobiohydrolase (CBHII) & *Aspergillus aculeatus* beta-glucosidase (BGLI)	790 mg/L of total reducing sugar	PASC	1.80	94	(Kim et al., 2013)

	Clostridium cellulolyticum endoglucanase (CelG) & *Clostridium thermocellum* beta-glucosidase (BglA)	nd	PASC	1.90	72	(Tsai et al., 2013)

np : enzyme activity in units not provided

HIGHLIGHTS

- Strategies to engineer yeast as a consolidated bioprocessing host to produce ethanol is outlined
- Recent biotechnological advancements to overcome the key limitation of this strategy is reviewed
- Future research directions for yeast-based bioethanol production are discussed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Lignocellulose
b
a
Nucleus
Lignocellulose
c
CBM
Scaffold
Glycolysis
Pyruvate
Acetaldehyde
Ethanol
Cellobiohydrolase
Beta glucosidase
Endoglucanase
Cellobiose
Dockerins
Cohesins
Hexose Transporter
Agglutinin Anchor
Glucose
Enzymes

Figure 1

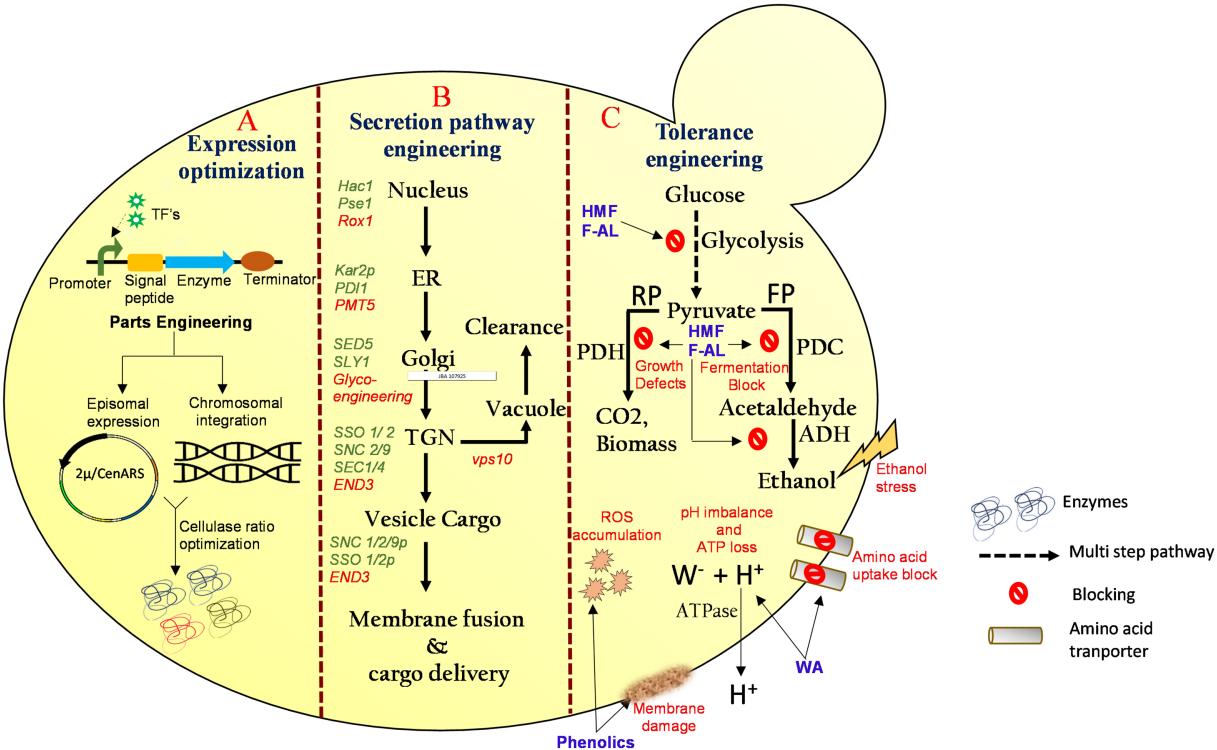
A
Expression optimization
TF's
Promoter
Signal peptide
Enzyme
Terminator
Parts Engineering
Episomal expression
Chromosomal integration
2μ/CenARS
Cellulase ratio optimization
B
Secretion pathway engineering
Hac1
Pse1
Rox1
Nucleus
Kar2p
PDI1
PMT5
ER
SED5
SLY1
Glyco-engineering
Golgi
Clearance
Vacuole
SSO 1/ 2
SNC 2/9
SEC1/4
END3
TGN
vps10
Vesicle Cargo
SNC 1/2/9p
SSO 1/2p
END3
Membrane fusion & cargo delivery
C
Tolerance engineering
Glucose
HMF
F-AL
Glycolysis
RP
Pyruvate
FP
PDH
HMF
F-AL
PDC
Growth Defects
Fermentation Block
CO2, Biomass
Acetaldehyde
ADH
Ethanol
Ethanol stress
ROS accumulation
pH imbalance and ATP loss
W- + H+
ATPase
H+
Amino acid uptake block
WA
Membrane damage
Phenolics
Enzymes
Multi step pathway
Blocking
Amino acid tranporter

Figure 2