Austin Chemical Engineering

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Review Article

Engineering Yeast for Cellulosic Ethanol Production

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Received: August 11, 2015; **Accepted:** September 28, 2015; **Published:** October 06, 2015

Abstract

Biofuel, the alternative energy, aims to greatly reduce the carbon emissions on earth. Cellulosic ethanol is expected to replace the first generation biofuel made by agriculture crops, such as corn. Lignocelluloses degradation and fermentation efficiencies are the main limitations. Thus, the objective of consolidated bioprocessing is to engineer a "super" yeast with multiple cellulolytic enzymes, multi-sugars consumption, thermo-tolerance, toxin-tolerance and efficient ethanol production. The super yeast needs to be engineered to be suitable glycosylation, highly secretable, deficient protease, stress-tolerance and ethanol assimilation for cellulosic ethanol. Omics analysis, adaptive laboratory evolution and genome editing tools would accelerate yeast engineering not only for biofuel, but also applications in other biosynthetic areas.

Keywords: Biomass; Omics; Yeast; Fermentation; Cellulosic ethanol; Synthetic biology; Genome editing

Abbreviations

EU: European Union; CBP: Consolidated Bioprocessing; C5: Pentose; C6: Hexose; GH: Glycosyl Hydrolase; CRISPR: Clustered, Regularly Interspaced, Short Palindromic Repeats; Cas9: CRISPR-Associated Protein 9; SHF: Separate Hydrolysis and Fermentation; SSF: Simultaneous Saccharification and Fermentation; SSCF: Simultaneous Saccharification and Co-Fermentation

Introduction

Alternative fuels are important materials in battling ongoing green-house effects [1,2]. The EU passed a statement that asked car manufacturers to reduce the carbon emissions of their products. Since the 1970's, bio-ethanol has successfully been produced commercially using agriculture cultivates (especially in corn); benefits the green energy production in several food-supply countries, such as Brazil & USA. However, it enlarged the subsistence problem in other food-deprived countries [3]. Therefore, cellulosic ethanol produced by feed stocks became the new generation biofuel. Cellulosic ethanol from non-grain plant materials is used as one of the green energy to replace the fossil fuel which causes net carbon emissions and seriously affects climate change for decades.

The basic bioprocess for cellulosic ethanol is feed stocks degradation, sugar utilization and then fermentation (Figure 1). Feedstocks are composed by the main component of lignocelluloses, including celluloses, hemicelluloses, and lignin's. These polysaccharides could be degraded into sugars by kinds of cellulolytic enzymes. After lignocelluloses degradation, 1-2% C5 & C6 sugar mixtures (D-xylose, L-arabinose, glucose, lactose, etc) are hydrolyzed with the inhibitory compounds (acid/base, furfural, etc), formed during biomass pretreatment process. Yeast utilized these carbon sources can be further fermented and isolated in higher temperature for ethanol products.

An effective saccharification-fermentation biomass process for ethanol production is needed for the biorefinery. Among the kind of bioprocesses, Consolidated Bioprocessing (CBP) is often considered as the preferential process because it has the potential for high efficiency and low cost [4]. However, the rate-limiting step in the conversion of cellulose to fuels is feedstock degradation, especially in hydrolysis of cellulose, hemicelluloses & lignin to sugars. A great deal of effort has gone into the development of methods for conversion of cellulose to sugars through glycosyl hydrolase digestion [5-15]. Good degradation enzymes are always needed to increase the lignocelluloses hydrolysis in the biorefinery process [7,16-19]. Numerous cellulolytic enzyme cocktails, such as Cellic series of enzymes (Novozymes) and Accellerase series of enzymes (Genencor), were already applied for biomass hydrolysis in biorefinery [5,20-23].

Another improvement for CBP is to use efficient fermentation microbes, mainly yeast, which can carry out cellulase/hemicellulase production, hydrolysis, and fermentation to convert pentose sugars and glucose into ethanol [7,24-27]. To simplify the bioprocess for feed stocks conversion via CBP, for example, several good cell factories for both enzyme production and ethanol production were investigated, especially in yeast. Yeast has been a preferable host for fermentation since nineteen century when industrial factories used yeast to produce wine & beer. For example, *Saccharomyces cerevisiae* is the most common fermentation yeast and could tolerate higher alcohol percentage during fermentation [28]. However, thermo-sensitivity, over-glycosylation and secretion ability of yeast are still the limitations for CBP application and need to be addressed.

Yeast strain improvement for CBP

Several key approaches for both cellulase expression and ethanol production by consolidated bioprocessing were investigated [6,29-31]. Improving the efficiency of lignocellulosic breakdown requires engineering of yeast secretory pathway from system-wide metabolic analysis and DNA constructs for enhanced cellulase gene expression (Figure 2). Also, yeast exhibit high tolerance to severe stress due to inhibitory compounds [6]. Such improvements could be applied to industrial cellulosic ethanol as follows:

Deglycosylation and secretion improvement for better enzyme activity: Increasing the extracellular cellulases in *S. cerevisiae*

Citation: Tzi-Yuan Wang. Engineering Yeast for Cellulosic Ethanol Production. Austin Chem Eng. 2015; 2(2): 1018.

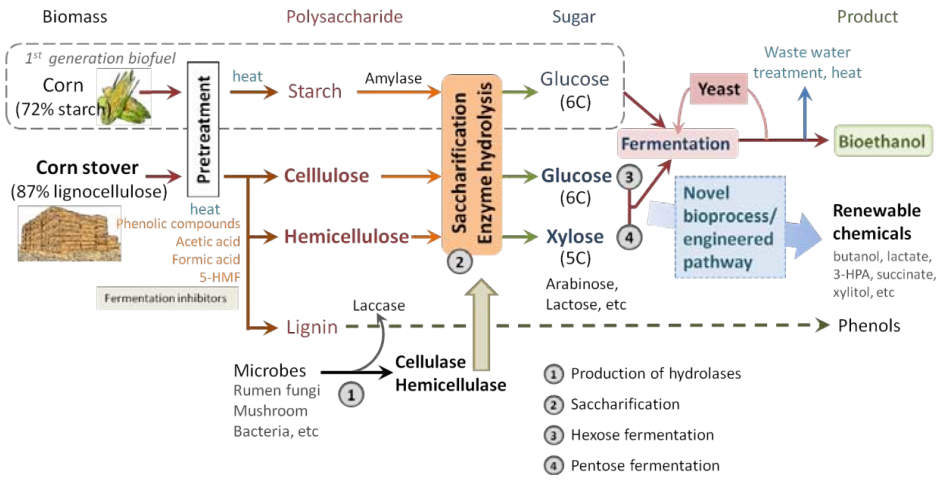

Figure 1: How to make cellulosic ethanol. There are typical hydrolysis and fermentation strategies: separate hydrolysis and fermentation (SHF with four-steps sequential process: (1) production of hydrolase +(2) saccharification +(3) hexose fermentation +(4)pentose fermentation), Simultaneous Saccharification and Fermentation (SSF with three step process: (1)+(2+3)+(4)), simultaneous saccharification and co-fermentation (SSCF with two step process: (1)+(2+3+4)), and Consolidated Bioprocessing (CBP with one-step process: (1+2+3+4)).

directly influences the efficiency of biomass breakdown. Both over- and under-glycosylation may alter the enzyme activity of cellulases in *S. cerevisiae* [32-34]. For example, knockout of the inherent glycosylation-related *MNN10* gene had a more than 6-folds increase in extracellular exocellulase activity [34]. In addition, blocking Golgi-to-endosome transport may force *S. cerevisiae* to export cellulases. Wang *et al.* (2013) showed yeast with deficient *VPS21* genes could increase extracellular cellulases activity by 6-fold and reduce the bioprocess time. Improving extracellular protein trafficking and gene expression can also accelerate the biomass degradation [6,34-37].

Cell surface engineering for better digestion: To degrade the feed stocks into sugars efficiently, feedstocks need to directly interact with cellulases/hemicellulases/ligninases. Therefore, artificial cellulosome is an alternative way to improve cellulose degradation [38-41]. For example, both clostridium and rumen fungi have CBM10 domain and cellulosome structure genes [13,42], which could be adapted for cell surface engineering in yeast [39,43].

Protease deficient strain for enzyme stability: Foreign proteins expressed in yeast are usually degraded by ubiquitin proteasome [44]. Thus, even though yeast could over-express cellulase proteins, the functional cellulase may not be exported out of the cell for cellulose breakdown. One needs to delete such proteases to prevent the heterologous cellulase degradation in engineered yeast [45-47]. For example, the proteins in *K. lactis* could be degraded because of its proteases [48]. Selected mutants or deficient proteases strain could be applied for heterologous proteins without degradation [49].

Multiple carbon utilization for CBP: More and better yeast sugar transporters could uptake sugar for efficient fermentation [30,50]. One way is to find out the yeast strains that could uptake various sugar, such as *Kluyveromyces marxianus* [51], *Pichia anomala* [52]. The other way is to engineer sugar pathway genes into yeast [29,53-55]. For example, C5 sugar pathway in *S. cerevisiae* could uptake xylose and glucose at once [29,56-58]; the same for C6 sugar pathway in *Pichia stipitis* [59]. Such recombinant yeasts could directly utilize these sugars for ethanol fermentation.

Increase ethanol production: Yeast ethanol assimilation is another mutant engineering method for CBP. Ethanol production is the final step to evaluate the efficiency of CBP bioprocess [54,60]. To increase ethanol production, for example, the Aspartic Protease gene (Asp) of *Neurospora crassa* expressed in industrial ethanol-producing yeast could increase both growth rate and viable yeast counts; also the recombinant strain of *S. cerevisiae* exhibits a higher ethanol yield [61]. Other approaches for a promising producer include mutagenesis and/or adaptive strains selected from feedstocks [62]. However, most wild yeasts also consume lots of ethanol, which reduce the production yield. Ethanol assimilation is one possible way to prevent yeast consume ethanol. Evolutionary adaptation or mutagenesis could select for such evolved yeast strains for high-yield ethanol production.

Tolerance adaptation for better yeast: Most yeast cannot have higher conversion ratio of ethanol due to thermo-intolerance [51], oxidative stress [63], and/or toxin-intolerance [51,52,64,65]. Therefore, adaptive evolution helps to select evolved strains that can grow at higher temperature and survive under quite amount of furfural or acid to reduce the energy cost during bioprocessing.

Immobilization and high cell density for efficient process: The other way to increase the efficiency is cell immobilization [66] and high cell density [67]. Biochemical engineers already have matured the techniques for efficient bioprocess. These methods enlarge the contact area with feedstocks and significantly reduce the fermentation time.

In short, an ideal yeast strain for CBP can secrete good glycosyl hydrolases for feedstock degradation, consume multiple sugars and tolerate high ethanol concentrations to optimize ethanol yields under harsh environment (high temperature and toxic conditions). Therefore, cellulosic ethanol bioprocess could be the economic scale-up for the industrial biorefinery in the near future.

Future direction

Yeast for cellulosic ethanol production is the main CBP

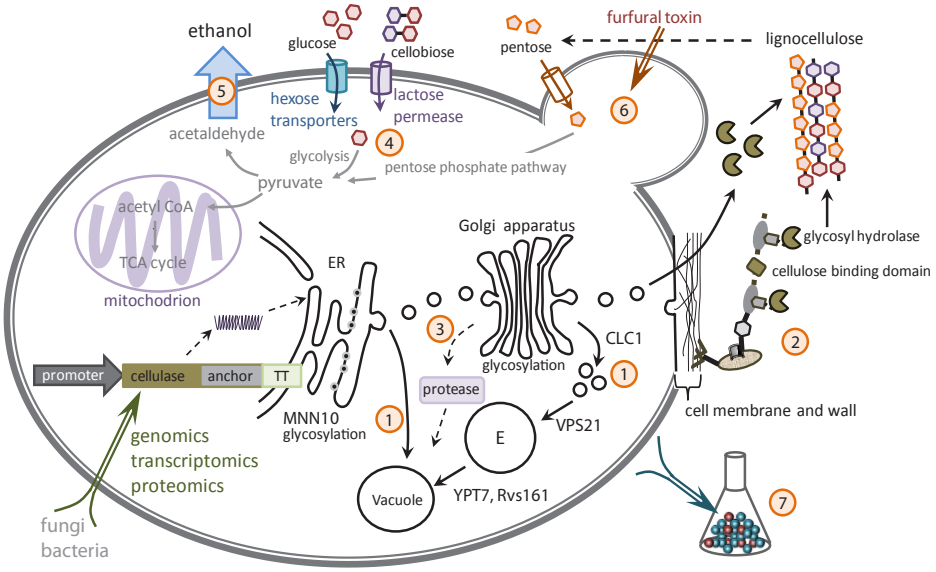

Figure 2: Strategies for CBP yeast engineering. (1) deglycosylation and secretion improvement; (2) cell surface engineering; (3) protease deficient strain; (4) multiple carbon utilization; (5) Increase ethanol production; (6) tolerance adaptation; (7) immobilization and high cell density.

for industrial application, not only because of its well-known fermentation efficiency, but also the simple genetic manipulation process. New bio-techniques recently accelerate yeast engineering to achieve cost-effective cellulosic ethanol for energy usage.

Omics approach reveals novel enzymes and fermentation engineering: Microbial strategies for degrading lignocellulose are diverse, but we know the enzymes involved in these processes are limited. Recent advances in genomics, metagenomics, transcriptomics, and secretomics offer a cost-effective approach to identify kinds of efficient cellulases and production from microbes [15,16,42,68-73]. Another approach is to identify efficient secretion and fermentation through metabolomes and transcriptome profiling of yeast, such as *S. cerevisiae, P. stipitis, K. marxianus, Zymomonas mobilis* [71,74,75]. By taking advantage of available 'Omics tools, this strategy is likely to succeed in generating CBP strains in the near future.

Genome editing accelerates yeast engineering: The CRISPR-Cas9 system makes genome editing easier in diploid, which has already succeeded in animals [76], plants [77] and microbes [78]. This new technology enables researchers to knock-out and/or mutate multiple genes at once in a yeast genome [79]. Designed yeast can utilize a set of genes or integrate new pathway genes faster than before. For example, a construction kit in *T. reesei* was established for high throughput generation of gene knock-outs [80]. Theoretically, the CRISPR-Cas9 system could engineer 5-10 pathway genes into yeast at once. Suitable CBP platform for cellulosic ethanol production will be realized soon through these genome editing tools.

Adaptive laboratory evolution for ideal CBP yeast: Environmental adaptation could easily change yeast phenotypes into thermo-tolerance and toxin-tolerance [81-83]. Such mutants selected by adaptive laboratory evolution could also reduce the energy wastes during fermentation. Thus, ideal CBP yeast could be constructed to fulfill all the needs for cellulosic ethanol production.

Conclusion

Ideal yeast is necessary for CBP, which new biotechnologies make it easier to create a suitable cell factory for cellulosic ethanol production. Yeast with secret able, high activity of glycosyl hydrolases and efficient, high-yield ethanol production will be essential not only to make cheaper cellulosic ethanol, but also to initiate new cell factory in agriculture and biomedical applications.

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