Metabolic Engineering of Yeast for Bioethanol Production

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Increased human population and the rapid decline of fossil fuels resulted in a global tendency to look for alternative fuel sources. Ethanol has been the primary fossil fuel alternative due to its low carbon emission rates, high octane content and comparatively facile microbial production processes. As a well-known platform microorganism and native ethanol producer, baker's yeast *Saccharomyces cerevisiae* has been the primary subject of interest for both academic and industrial perspectives in terms of enhanced ethanol production processes. Metabolic engineering strategies have been primarily adopted for direct manipulation of genes of interest responsible in mainstreams of ethanol metabolism. To overcome limitations of rational metabolic engineering, an alternative bottom-up strategy called inverse metabolic engineering has been widely used. In this context, evolutionary engineering, also known as adaptive laboratory evolution (ALE), which is based on random mutagenesis and systematic selection, is a powerful strategy to improve bioethanol production of *S. cerevisiae*. Metabolic and evolutionary engineering strategies are intertwined and many metabolically engineered strains for bioethanol production can be further improved by powerful evolutionary engineering strategies as well as the recent advancements in directed genome evolution, including CRISPR-Cas9 technology.

bioethanol | biofuel | directed genome evolution | ethanol tolerance | ethanol production | metabolic engineering | evolutionary engineering | *Saccharomyces cerevisiae* | adaptive laboratory evolution

1. Metabolic Engineering of Yeast for Bioethanol Production

Since the first introduction of metabolic engineering in 1991 by Bailey as a new scientific discipline [1], there have been significant developments in genetic engineering tools and techniques which enabled researchers to engineer or transfer microbial metabolic pathways with higher efficiency. Specific and targeted changes can be made to a DNA sequence of interest, by a technique known as site-directed mutagenesis that can be utilized for point substitution, deletion, and insertion mutations. Site-directed mutagenesis can be easily performed by using polymerase chain reaction (PCR). As an extensively used technique, the PCR-based gene targeting method includes introduction of exogenous DNA into the host cell by various transformation methods and manipulation of the host genome by the native double-stranded break (DSB) repair system [2]. Another common approach for site-directed mutagenesis is cassette mutagenesis in which a synthetic double-stranded DNA 'cassette' containing desired mutations is introduced into a plasmid vector between two restriction sites [3]. However, the major limitation

for this approach is the availability of suitable restriction sites that flank the site to be mutated. Most recently, the powerful CRISPR-Cas9 genome editing technology has been extensively used in metabolic engineering research. The CRISPR-Cas9 technology uses artificially engineered nucleases to create specific double-stranded breaks at a desired locus, and single or multiple gene editing is achieved by the cell native repair system [2].

1.1. Lowering ATP Yield

The first-generation bioethanol production involves utilization of conventional feedstocks like glucose or sucrose [4]. Glucose is catabolized through the Embden–Meyerhof–Parnas (EMP) pathway in *S. cerevisiae* where one mole of glucose is metabolized into two moles of pyruvate and two moles of ATP. The ethanol yield of this pathway is between 90–93%, while the maximal biomass yield is around 7% [5]. ATP is used for growth at the expense of glucose which is not converted to ethanol. Thus, cell growth or biomass formation can be described as a by-product of the first-generation ethanol production. Lowering ATP yield during alcoholic fermentation reduces substrate conversion to biomass and thereby increases ethanol yield [6]. This goal can be achieved using rational metabolic engineering strategies by introducing futile cycles, decreasing the ATP stoichiometry of yeast glycolysis, and modifying the structure and energy coupling of disaccharide metabolism and transport [7] For example, to overcome the tight regulation of phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FBPase) futile cycle, a bacterial (*Escherichia coli*) FBPase insensitive to fructose-2,6-bisphosphate inhibition was expressed in *S. cerevisiae* which resulted in an increase in ethanol yield by 8.8%, along with an increase in yeast biomass, while decreasing ATP levels by 31–39%, compared to the wild-type strain [8].

1.2. Sustainable Reduction of Glycerol Formation

In addition to biomass, glycerol is another primary by-product of first-generation bioethanol production, where approximately 5% of the sugar feedstock is converted to glycerol during industrial bioethanol fermentation [9]. Under anaerobic conditions, glycerol synthesis compensates for the depletion of NAD+ by re-oxidizing excess NADH from growth reactions [9]. Glycerol 3-phosphate dehydrogenase is a key enzyme for glycerol production which is encoded by *GPD1* and *GPD2* genes [10]. Fine-tuning of *GPD1* and *GPD2* expression without disrupting the regeneration of NAD^+ can increase the ethanol yield. More recently, 90% decrease in glycerol production and 15% increase in ethanol yield on sugar were achieved in *S. cerevisiae*, compared to the reference strain, by deletion of *GPD2* and heterologous expression of Calvin-cycle enzymes PRK (phosphoribulokinase) and RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), to enable the use of CO_2 as an alternative electron acceptor for the reoxidation of NADH [11].

1.3. Prevention of Bacterial Contamination

Bacterial contamination is also an important factor that may reduce the yield and productivity of bioethanol fermentations. Most commercial ethanol fermentation facilities regularly experience chronic and unpredictable acute bacterial infection due to continuous yeast propagation and non-sterile fermentation conditions which halts the fermentation process [12],[13]. In a recent study, yeast cell surface display technology was used to inhibit *Limosilactobacillus fermentum* strains in *S. cerevisiae* corn mash fermentation. For this purpose, *S. cerevisiae*

EBY100 strain was used to anchor a recombinant peptidoglycan hydrolase, the lactobacilli phage endolysin LysKB317, with the a-agglutinin proteins Aga1p–Aga2p. The resulting recombinant *S. cerevisiae* strain expressing LysKB317 showed 83.8% decrease in bacterial cell counts, improved ethanol production and reduced levels of lactic and acetic acid [14].

1.4. Introduction and Optimization of Xylose Assimilation Pathway

The second-generation bioethanol production involves utilization of lignocellulosic biomass that is rich in pentose sugars, such as D-xylose and L-arabinose. However, as *S. cerevisiae* cannot naturally utilize pentose sugars, the introduction of specific pentose metabolic pathways to *S. cerevisiae* has been the major goal of rational metabolic engineering studies for second-generation ethanol production. For xylose assimilation, two different pathways have been introduced to *S. cerevisi*ae by metabolic engineering strategies: the oxidoreductase pathway and the isomerase pathway [15][16]. There are various successful examples of introducing the two-step oxidoreductase pathway in *S. cerevisiae* by rational metabolic engineering. Cadete et al. [17] reported 0.40 g g^{-1} cell dry weight (CDW) ethanol yield and 0.33 g g^{-1}CDW h^{-1} productivity in *S. cerevisiae* by expressing *XYL1.2* (xylose reductase) from *Spathaspora passalidarum* that can also use NADPH as a cofactor, but prefers NADH; and *S. stipitis XYL2* (xylitol dehydrogenase) that can use NADH as a cofactor but prefers NADPH. The *S. cerevisiae* TMB 3044 strain used in that study had an overexpressed xylose utilization pathway and virtual absence of XR activity (Δ*gre3*) as a background [17]. The introduction of the isomerase pathway in *S. cerevisiae* by rational metabolic engineering involves heterologous expression of genes encoding xylose isomerase (XI) from various microorganisms. XI gene (*xylA*) from the bacterium *Burkholderia cenocepacia* was successfully expressed in *S. cerevisiae*. The developed strain had a 5-fold increase in xylose consumption and over 1.5-fold increase in ethanol production in a medium containing a glucose-xylose blend which resembled sugar cane bagasse hydrolysates [18].

1.5. Increasing Stress Tolerance

Another challenge of the second-generation bioethanol production is the inhibitory effects of toxic compounds that are released upon pretreatment of lignocellulosic feedstocks. These compounds include furfural, 5-hydroxymethyl-furfural (HMF), weak acids such as acetic acid and phenolics [19]. Thus, diverse metabolic engineering strategies have been employed to improve the tolerance of *S. cerevisiae* against these compounds and increase its ethanol yield and productivity. For example, Almeida et al. [20] developed an HMF-tolerant *S. cerevisiae* strain by overexpressing alcohol dehydrogenase genes (*ADH1* and *ADH6*) to reduce HMF to less toxic compounds.

2. Evolutionary Engineering of Yeast for Bioethanol Production

As an inverse metabolic engineering strategy, evolutionary engineering or ALE is based on random mutagenesis and selection in repeated batch or chemostat cultivations in the presence of a selective pressure that favors a desired microbial phenotype. To increase the genetic diversity of the initial microbial population of selection, physical or chemical mutagenesis can be applied, such as UV and ethyl methanesulfonate (EMS) mutagenesis [21].

However, there are also successful examples of evolutionary engineering, in which the selection experiments were performed without prior physical and chemical mutagenesis, particularly if the selective pressure itself may have highly mutagenic characteristics, as in the case of evolutionary engineering of caffeine-resistant *S. cerevisiae*, where the high concentrations of caffeine used as the selective pressure were highly mutagenic, such that highly caffeine-resistant evolved strains were obtained without prior mutagenesis of the parental strain by UV or EMS mutagenesis [22]. Following evolutionary selection experiments, the evolved strains with the desired phenotypes are then isolated and characterized, to understand the genetic basis of their phenotypes. High-throughput screening methods and omics technologies are required for these purposes [21]. Rational metabolic engineering applications may cause perturbations on specific metabolic pathways and produce rate-limiting steps on metabolism which may result in a decrease in viability and growth rate of the engineered strains. For this purpose, rational metabolic engineering and evolutionary engineering strategies are commonly combined to further increase the robustness of metabolically engineered strains [23].

2.1. Increasing Growth Rate and Viability

Short generation time is a key parameter for evolutionary engineering studies, for a time-efficient selection of evolved strains. In addition, strain improvement without a growth advantage over the background strains generally experiences challenges in the evolutionary selection procedure. Thus, increase in growth rate and viability through evolutionary engineering is a vital process both for generating robust strains and for environmental fitness [23]. In a previous study, evolutionary engineering was applied on *S. cerevisiae* for improved growth rate on galactose, a common sugar in nonfood crops, as the sole carbon source. Upon 62 days of selection in galactose-containing medium, three evolved strains with 24% increased specific growth rate on galactose and higher ethanol yield were isolated. It was suggested that the mutation found in the *RAS2* gene was responsible for the increased specific growth rate on galactose [24].

2.2. Decreasing By-Product Formation

Biomass and glycerol are two major by-products that can decrease ethanol yield during bioethanol production. In a recent study on decreasing biomass by evolutionary engineering, heterologous hexose-proton symporters were first expressed in *S. cerevisiae*. The resulting metabolically engineered strain was then further adapted to anaerobic growth by an evolutionary engineering strategy, based on gradually decreasing oxygen levels from 100% air to 100% N2 in a sequential batch reactor. The final evolved strains had a 17.2% increased ethanol yield, along with a 44–47.6% decrease in biomass formation [25].

2.3. Improving Utilization and Transport of Sugars

A major aim of rational metabolic engineering studies for second-generation ethanol production is to introduce specific metabolic pathways in *S. cerevisiae* for the utilization of pentose sugars. Evolutionary engineering strategies are usually combined with metabolic engineering approaches to further improve such metabolically engineered strains that can utilize pentose sugars. For example, dos Santos et al. [26] first metabolically engineered a robust industrial *S. cerevisiae* strain by including genes related to pentose metabolism. They then applied

evolutionary engineering to that strain for optimal xylose utilization, and the resulting evolved strains had an improved yield of 0.46 g ethanol/g xylose. Whole genome sequencing of the evolved strains revealed that *ISU1* gene encoding a scaffold protein for the assembly of iron-sulfur clusters and *SSK2* gene that is a member of MAPKKK signaling pathway are crucial for the regulation of xylose fermentation [26].

2.4. Increasing Tolerance to Ethanol and Lignocellulosic Inhibitors

During industrial bioprocesses, yeast cells are faced with diverse environmental stress conditions. Thus, stress-resistance or robustness is a highly desirable trait for industrial yeasts [27]. However, as stress resistance is a multigenic and complex trait, evolutionary engineering has been a more suitable and efficient strategy than rational metabolic engineering to obtain yeast cells with high resistance against diverse stress factors. In the research group, for example, genetically stable *S. cerevisiae* cells resistant to multiple-stresses [27], oxidative stress [28], silver stress [29], starvation stress [30] and 2-phenylethanol stress [31] were successfully obtained using evolutionary engineering, and characterized by omics technologies. Although *S. cerevisiae* is widely used for bioethanol production, high concentrations of ethanol affect cell and mitochondrial membrane, cause elevated reactive oxygen species (ROS) levels and decrease cell viability and ethanol yields [32]. A haploid laboratory strain of *S. cerevisiae*, CEN.PK 113-7D, was significantly improved by evolutionary engineering, using serial batch cultivation with gradually increasing ethanol levels. The resulting evolved strains could resist up to 12% (v/v) ethanol, a concentration at which the reference strain could not survive. They also had significantly higher ethanol productivity and titer than the reference strain during aerated fed-batch cultivation, and increased glycolytic and ribosomal protein abundance and lower respiratory activity, compared to the reference strain, based on proteomic and transcriptomic results. The study also showed that evolutionary engineering under ethanol stress triggered diploidization of the parental strain during early steps of the selection procedure, at about 7% (v/v) ethanol stress level [33].

In second-generation bioethanol production, during the pretreatment steps of lignocellulosic feedstock such as acid hydrolysis, a significant amount of by-products are formed that have inhibitory effects on yeast cells. These toxic inhibitors include furfural, 5-hydroxymethyl-furfural (HMF), phenolic compounds and weak acids such as acetic acid [34]. Liu and Ma [35] investigated the transcriptomic responses of a furfural and HMF-tolerant, evolved strain, upon exposure to these inhibitors. The comparative transcriptomic analysis results revealed some key pathways such as the cell wall response, endogenous and exogenous cellular detoxification pathways and specific transcription factors like Yap1, Met4, Msn2/4 and Pdr1/3 as the main differentiated components of the inhibitor-tolerant strain, which may have a role in the complex genetics of HMF and furfural tolerance [35]. Phenolics are another major group of inhibitors found in lignocellulosic hydrolysates. One of the most toxic phenolic inhibitors found in lignocellulosic hydrolysates is coniferyl aldehyde that can reduce the performance of *S. cerevisiae* cells up to 80%, at a concentration of 1.4 mM [36]. Hacısalihoğlu et al. [37] successfully developed a highly coniferyl aldehyde-resistant *S. cerevisiae* strain by evolutionary engineering. The evolved strain could rapidly convert coniferyl aldehyde, and was also resistant to other phenolic inhibitors, including ferulic acid, vanillin and 4-hydroxybenzaldehyde. Comparative transcriptomic and genomic analysis of the evolved strain revealed major changes in protein homeostasis, cell wall integrity pathways, response to oxidative stress and oxidoreductase

activity, and mutations in some genes encoding key transcription factors, such as *PDR1*, *GLN3* and *CRZ1*, which may be involved in coniferyl aldehyde resistance [37]. Weak acids are also important inhibitors found in lignocellulosic hydrolysates. As acetic acid is one of the most common weak acids, it is desirable to develop *S. cerevisiae* strains that are tolerant to acetic acid. In a recent study, evolutionary engineering strategies have been successfully employed to obtain thermo-acid tolerant and acid-tolerant *S. cerevisiae* strains. The evolved strains could grow in minimal media containing 12 g/L acetic acid at pH 4 and 30 °C, and produced high levels of ethanol, up to 29.25 ± 6 mmol/gDCW/h. Whole-genome sequencing and transcriptomic analyses revealed mutations and expression changes in key genes involved in the RAS-cAMP-PKA signaling pathway (e.g., *RAS2*) and the heat shock transcription factor (*HSF1*). Reverse engineering results indicated that *RAS2* mutation conferred acid tolerance and *HSF1* mutation conferred thermotolerance [38].

3. Challenges of Evolutionary Engineering for Bioethanol Production

Although evolutionary engineering is generally more advantageous than rational metabolic engineering as it does not require extensive knowledge about the phenotype of interest, there are still some challenges. A major challenge of evolutionary engineering is the “trade-off” situation of the engineered strains. In evolutionary biology, the trade-off is a common concept and it is accepted as a cost of adaptation, which is an important issue in evolutionary engineering studies, particularly for industrial purposes [21],[39]. Caspeta & Nielsen [40] reported that genetic adaptations of yeast to high temperatures resulted in decreased growth rate at ancestral temperatures and reduced cellular functions, while ethanol production was improved. Another challenge of evolutionary engineering is the long time requirement for cultivation and stress application cycles during systematic selection. It is a tedious and labor-intensive work, as the cell growth and response to the applied stress must be continuously monitored for a long time. Partially or fully automated systems can overcome these difficulties. For example, Radek et al. [41] performed evolutionary engineering in an automated microtiter plate format and Wang et al. [42] used a microbial microdroplet culture platform for their evolutionary engineering experiments.

4. Future Directions for Evolution-Based Metabolic Engineering of Yeast for Bioethanol Production

Owing to recent developments in directed genome evolution strategies, strain development and modification of microbial genomes has sped up. As an inverse metabolic engineering strategy, the most challenging step of evolutionary engineering is the identification of the genetic basis that confers the selected phenotype [21]. Due to the highly interconnected genotypic—phenotypic information flow, comparative analysis and understanding of cellular processes and the molecular basis of complex phenotypes are possible through multi-omics approaches [43]. Evolutionary engineering studies can be combined with targeted directed evolution approaches to construct more precise mutant libraries in a shorter period of time and analyse genomic modifications faster through high-throughput-sequencing methods at population level [44]. A typical example for targeted directed evolution is the generation of transcription factor mutation libraries through global Transcription Machinery Engineering (gTME) and

directional screening of target phenotype [45]. gTME and site-saturation mutagenesis on the gene encoding the transcription factor *SPT15* were recently used to increase the ethanol yield of *S. cerevisiae*. The improved strains had up to 28.5% increase in ethanol yield, and 127 amino acids were identified to have an important role in the binding efficiency of Spt15 [46]. As a revolutionary gene modification technique, Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPRs) and CRISPR-associated (Cas) proteins is a fast, precise and efficient targeted genome editing tool with minor disadvantages like possible off-targets. CRISPR-mediated genome editing techniques have also been applied to introduce specific changes that are expected to improve bioethanol production of *S. cerevisiae*. A recent application of CRISPR-Cas9 technology to improve bioethanol production and reduce byproduct formation involved deletion of the *S. cerevisiae GPD2*, *FPS1*, *ADH2*, and *DLD3* genes by CRISPR-Cas9 approach. The genes were knocked-out sequentially by using targeted gRNAs for these genes, nuclease Cas9-NTC and donor DNA. The resulting strain with deletions in all four genes had 18.58% increased ethanol content and decreased contents of the byproducts glycerol, acetic acid and lactic acid by 22.32%, 8.87% and 16.82%, respectively. Transcriptomic analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis results revealed that the upregulated and downregulated genes of the engineered strain were mainly enriched in carbohydrate energy metabolism, and acid metabolic pathways, respectively [47]. For producing serial and combinatorial genomic diversity, Multiplex Automated Genome Engineering (MAGE) is a rapid directed evolution technique. It can introduce genomic mutations in many locations simultaneously, by using automated devices [48]. This system uses cDNA libraries that cover the whole genome of the microorganism and encode overexpression and knockdown mutations. These modular parts were introduced into the *S. cerevisiae* genome by using the CRISPR-Cas system and robotic automation. The successive iteration of the system and selection against acetate tolerance, glycerol utilization or isobutanol production accelerated the evolutionary selection procedure [49]. The genome-scale CRISPR interference (CRISPRi) system, which uses a deactivated Cas enzyme that can only bind to a target sequence and decrease its expression, can also be used to generate genome-scale knockdown libraries. Using this technique, whole genome can be targeted with an inducible library and optimized specifically to yeast spacer design rules. Owing to the use of inducible library design, dosage-sensitive and dosage-insensitive genes can be targeted similarly, unlike the previous studies. As well as library construction, the screening of individual strains can be done through amplicon sequencing, using the gRNAs like barcodes [50].

The current limitations and future prospects of evolutionary approaches imply that the increased use of automated culture systems for evolutionary selection experiments, targeted directed evolution approaches such as gTME, combinatorial genomic diversity (MAGE) and CRISPR-based genome editing tools will all speed up evolutionary engineering research and lead to significant improvements in industrial bioethanol production by *S. cerevisiae*.

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