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Advances in cellulosic conversion to fuels: engineering yeasts for cellulosic bioethanol and biodiesel production

Ja Kyong Ko[1] and Sun-Mi Lee[1,2,3]

Cellulosic fuels are expected to have great potential industrial applications in the near future, but they still face technical challenges to become cost-competitive fuels, thus presenting many opportunities for improvement. The economical production of viable biofuels requires metabolic engineering of microbial platforms to convert cellulosic biomass into biofuels with high titers and yields. Fortunately, integrating traditional and novel engineering strategies with advanced engineering toolboxes has allowed the development of more robust microbial platforms, thus expanding substrate ranges. This review highlights recent trends in the metabolic engineering of microbial platforms, such as the industrial yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica*, for the production of renewable fuels.

Addresses
[1] Clean Energy Research Center, Korea Institute of Science and Technology (KIST), Seoul 02792, Republic of Korea
[2] Clean Energy and Chemical Engineering, Korea University of Science and Technology, Daejeon 34113, Republic of Korea
[3] Green School (Graduate School of Energy and Environment), Korea University, Seoul 02841, Republic of Korea

Corresponding author: Lee, Sun-Mi (smlee@kist.re.kr)

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Introduction

Lignocellulosic biomass has gained significant attention as a promising renewable resource for biofuel production from an economic and environmental perspective. With recent technical advances and political and financial support, several companies have launched industrial-scale cellulosic bioethanol production plants with capacities of over 10 million gallons per year over the past few years [1]. Although this progress is encouraging, the economic feasibility of these pioneering facilities is not yet optimal, thus hampering the expansion of the cellulosic bioethanol industry and the production of other cellulosic biofuels.

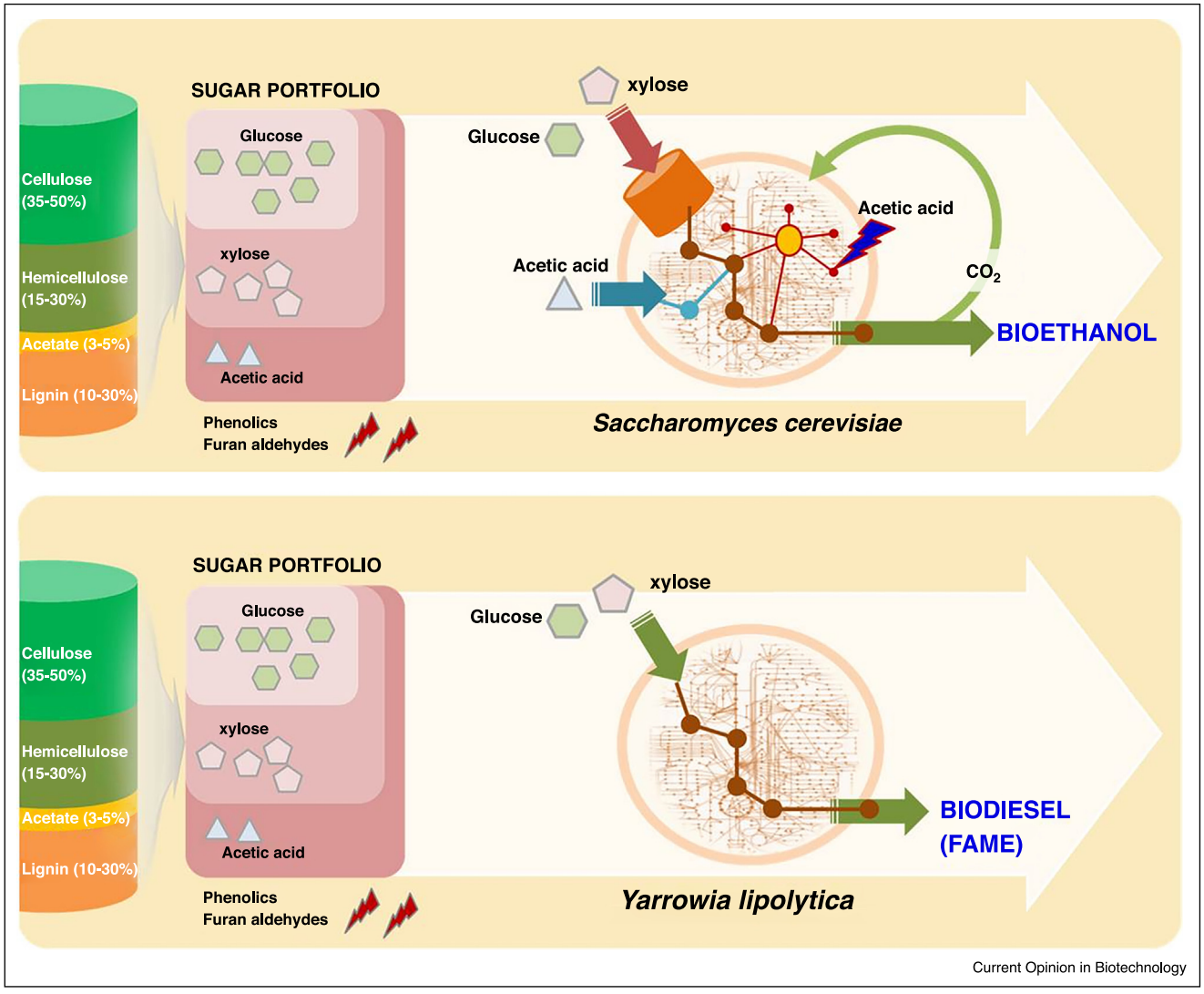
The economic viability of cellulosic biofuel production strongly depends on the performance of the microbial cell in utilizing a broad range of carbon substrates and handling toxic compounds derived from lignocellulosic biomass [2]. The main endeavors in the field of microbial engineering for cellulosic biofuel production have been the exploitation of robust microbial platforms with an expanded or improved carbon source portfolio (*e.g.* glucose, xylose, and acetic acid) and tolerance profiles (*e.g.* acetic acid, phenolics, and furfural); these profiles are directly related to biofuel productivity, titer, and yield [2]. Specifically, recent advances in the development of strains for cellulosic ethanol production have introduced a new era in cellulosic biofuel production by integrating traditional and novel engineering strategies encompassing not only metabolic pathways for the cellulosic biomass conversion into ethanol but also sugar transporters and gene regulatory networks. Moreover, the advanced metabolic engineering toolboxes have spurred the development of more powerful bioethanol producers, resulting in significantly improved production titers and yields. Recently, the cellulosic bioethanol production concept has been expanded to biodiesel production in which oleaginous yeasts are increasingly engineered as emerging biofuel production hosts and to other advanced biofuels (*e.g.* biobutanol) as well. Here, we present an overview of the recent advances and new trends in the engineering of microbial platforms for cellulosic biofuel production with a focus on the conventional biofuels, bioethanol and biodiesel, and their main producers, *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Figure 1).

Bioethanol: a path forward for the most conventional biofuel

New trend in the engineering of S. cerevisiae, the common bioethanol producer

The yeast *Saccharomyces cerevisiae* is a particularly attractive ethanol-producing microbial platform due to its well-characterized physiology, the availability of genetic tools and already proven industrial feasibility [3]. However, owing to its inability to assimilate xylose, the transition from 1st to 2nd generation bioethanol production has shown limited practical success, even with intensive metabolic engineering efforts. Over the past two years, the engineering strategies have expanded beyond the traditional concept of optimizing catabolic pathways, which focuses on certain key steps in the xylose catabolic pathway, to further improve cellulosic ethanol yield and/or productivity. The underscored

Figure 1



Current focuses on the engineering of yeasts for cellulosic biofuel production. Engineering approaches of *S. cerevisiae* to enhance bioethanol production from complex lignocellulosic biomass sources, including (a) transcription factor engineering for improved xylose catabolism and/or stress tolerance; (b) the development of xylose-specific transporters; and (c) non-sugar carbon source (acetate) catabolism. For cellulosic biodiesel production, *Y. lipolytica* is actively engineered to introduce the xylose catabolic pathway.

engineering strategies include (1) transporter and transcription factor engineering to boost the efficiency in carbon utilization and/or to improve stress tolerance and (2) reconsideration of acetic acid as a carbon source rather than an inhibitor. These transitions were largely contributed from evolutionary engineering in the era of affordable genome sequencing. Through this journey, unconventional engineering targets have also been revealed that have reinforced applicable engineering strategies. In the following section, we highlight the recent trends in the engineering strategies to develop more powerful cellulosic ethanol-producing strains of *S. cerevisiae* (Table 1).

Development of powerful mixed sugar co-fermenting yeast strain

Despite the improved xylose catabolic pathways, the limited xylose utilization led to engineering strategies being redirected to increase intracellular xylose transport. Directed evolution has led to the development of mutant xylose transporters with significantly improved xylose utilization rates and alleviated glucose repression. CiGXS1 $FIVFH_{497*}$, a mutant glucose/xylose co-transporter evolved from CiGXS1 of *Candida intermedia*, enabled *S. cerevisiae* to have a slightly faster xylose transfer rate than that of glucose while retaining the glucose transport rate at the wild-type level [4•]. Directed

Table 1

Recent metabolic engineering strategies used for bioethanol production by *S. cerevisiae*

Target	Strategy	Target gene	Ref.
Xylose transporter	Directed evolution for glucose/xylose co-transport	*CiGXS1*	[4•]
	Directed evolution for increased xylose transport	*AN25*	[5]
	Evolutionary engineering leading higher cell growth on xylose and partial glucose/xylose co-utilization	*HXT7*	[6]
	Targeted mutagenesis preventing ubiquitination of hexose transporter	*HXT36*	[7••]
	Engineering chimeric transporter preventing glucose induced degradation	*HXT2*	[8]
	Evolutionary engineering leading modified hexose transporter expression	*CYC8*	[9]
	Discovery of novel xylose transporters	*XltA, XltB*	[10]
Xylose catabolism	Evolutionary engineering improving xylose isomerase activity	*ASK10*	[11•]
	Evolutionary engineering altering intracellular metal homeostasis	*PMR1*	[12••]
	Evolutionary engineering upregulating pentose phosphate pathway	*PHO13*	[14]
	Evolutionary engineering; identifying novel targets[a]	*SNZ1, SNO1, SUL2*	[15]
	Coupling with CO_2 fixation/recycling pathway	*cbbM, sPRK, and GroEL-GroES*; *cbbL1-cbbS1, cfxP1*, and *HSP60-HSP10*	[16••,17]
Acetate catabolism	Cofactor engineering for redox balance in acetate catabolic pathway	*EhADH1*	[22]
	Cofactor engineering for increased cytosolic NADH formation	*gndA*	[23]
	Cofactor engineering for reduced glycerol production while maintaining osmotolerance	*gpsA*	[24•]
	Coupling with xylose metabolism	*AADH, SeACS*	[20,21]
Stress tolerance (inhibitors)	Global transcription machinery engineering	*HAA1*	[28]
	Global transcription machinery engineering	ZFP-TF library[b]	[29]
	Global transcription machinery engineering	*RPB7*	[30]
	Evolutionary engineering; identifying novel targets	*RTC3, ANB1, SFP1, ACE2, WHI2, ASG1, ADH3, SKS1, GIS4, GLO1, DOT5, CUP2, VMA7*	[26,31••,32–34]
	Overexpression of key enzymes in phenolics catabolic pathways	*ALD5, PAD1, ATF1, ATF2*	[35]

CiGXS1, glucose-xylose symporter from *Candida intermedia*; *AN25*, xylose transporter from *Neurospora crassa*; *HXT7*, hexose transporter; *HXT36*, chimeric transporter; *HXT11/2*, chimeric transporter; *CYC8*, general transcriptional co-repressor; *XltA* and *XltB*, novel xylose-specific transporters from *Aspergillus niger*; *ASK10*, stress response regulator; *PMR1*, Golgi Ca^{2+}/Mn^{2+} ATPase; *PHO13*, a member of haloacid dehydrogenase superfamily IIA; *SNZ1*, pyridoxal 5′-phosphate synthase; *SNO1*, glutaminase; *SUL2*, sulfate transporter; *cbbM*, Form-II Rubisco gene; *sPRK*, phosphoribulokinase from *Spinacia oleracea*; *GroEL-GroES*, shaperon from *Escherichia coli*; *cbbL1-cbbS1*, Form-I Rubisco gene; *cfxP1*, phosphoribulokinase from *Ralstonia eutropha; HSP60-HSP10*, chaperon; *AADH*, acetylating acetaldehyde dehydrogenase; *ACS*, acetyl-CoA synthetase; *ZWF1*, glucose-6-phosphate dehydrogenase; *GndA*, 6-phosphogluconate dehydrogenase from *Methylobacillus flagellates*; *gpsA*, glycerol-3P dehydrogenase from *Archaeoglobus fulgidus*; *seACS*, mutant ACS from *Salmonella enterica*; *HAA1*, transcription factor; *RPB7*, subunit of RNA polymerase II; *RTC3*, protein of unknown function involved in RNA metabolism; *ANB1*, mRNA translation elongation factor; *SFP1, ACE2*, transcription factor; *WHI2*, cytoplasmic globular scaffold protein; *ASG1*, transcriptional regulator; *ADH3*, mitochondrial alcohol dehydrogenase; *SKS1*, Putative serine/threonine protein kinase; *GIS4*, unknown function; *GLO1*, monomeric glyoxalase I; *DOT5*, nuclear thiol peroxidase; *CUP2*, copper-binding transcription factor; *VMA7*, subunit F of V1 peripheral membrane domain of V-ATPase; *ALD5*, aldehyde dehydrogenase; *PAD1*, phenylacrylic acid decarboxylase; *ATF*, alcohol acetyltransferase.

[a] Other target genes can be found in the paper reported by Zeng et al. [15].

[b] ZFP-TF, artificial zinc finger protein transcription factor.

evolution of a xylose-specific transporter AN25 from *Neurospora crassa* also significantly improved the xylose transport capacity (43-fold higher), which was limited in the wild type, while maintaining its high affinity for xylose [5]. Through adaptive evolution, a mutant xylose transporter of HXT7 (F79S) was obtained, supporting higher cell growth on xylose and partial co-utilization of glucose and xylose [6]. In addition to the alternation of xylose/glucose uptake rates, improving the half-life of xylose transporters has been beneficial for efficient co-fermentation. Triple mutations in the hexose transporter of Hxt36 (3K: K12, 35, 56R) have led to an increased xylose utilization efficiency by preventing ubiquitination [7••]. An engineered chimeric transporter of Hxt11/2 constrained the high glucose-induced degradation of HXT2, thus enabling efficient co-fermentation even in

the presence of inhibitors [8]. The modified hexose transporter expression landscape caused by the mutation in a general co-repressor of CYC8 (Y353C) has been found to improve xylose metabolism in *S. cerevisiae* [9]. Exploration of a set of xylose transporters from *Aspergillus niger* and *Trichoderma reesei* has offered many choices for transporter engineering by expanding the xylose transporter pool [10]. Xylose-specific transporters developed *via* different approaches are expected to play a vital role in advancing the co-fermentation of multiple sugars.

To develop better xylose catabolic pathways, a combination of evolutionary engineering and inverse metabolic engineering has been used to explore unknown remaining targets, such as *ASK10*, *PMR1*, and *PHO13*, that cannot be captured using rational approaches [11•,12••]. The mutation in a stress response regulator of Ask10 (M475R) was reported to improve xylose isomerase (XI) activity, which is one of the key rate-limiting factors in xylose-utilizing *S. cerevisiae* [13], by upregulating the transcription of molecular chaperones, which in turn contributed to the appropriate protein folding of XI [11•]. Altering intracellular metal homeostasis by PMR1 mutants was shown to stimulate metal-dependent XI activity and anaerobic growth on xylose [12••]. PHO13, which was recently revealed to be involved in the upregulation of pentose phosphate pathway, was also an unknown candidate frequently used for improving the xylose utilization system [14]. The recently revealed vitamin B and thiamine synthesis pathway genes and the transketolase gene (*TKL2*) [12••,15] may be the next engineering targets in the underexplored aspect of xylose metabolism engineering to develop xylose-utilizing *S. cerevisiae* at more advanced levels.

In addition to these strategies, coupling a CO_2 fixation or recycling pathway to the xylose catabolic pathway by introducing a PRK-Rubisco module is a novel strategy to achieve higher yields in cellulosic ethanol production, although the CO_2 recycling effects are minimal [16••,17]. Simultaneous utilization of cellobiose and xylose offers an effective strategy to circumvent glucose repression during the fermentation of cellulosic hydrolysates in which xylose utilization is synergistically improved [18,19].

Addition of a non-sugar to the yeast carbon source portfolio

As a major inhibitor in lignocellulosic hydrolysates, acetic acid has recently been added to the yeast carbon source portfolio by constructing a synthetic acetate catabolic pathway based on endogenous acetyl-CoA synthetase (ACS) and heterologous NADH-specific acetylating acetaldehyde dehydrogenase (AADH). The utilization of acetic acid as a non-sugar carbon source not only improves ethanol yield but also enables *in situ* detoxification of acetic acid in hydrolysates [20–23]. Thus, incorporating the more efficient acetic acid catabolic pathway in *S. cerevisiae* has been a potent strategy to improve cellulosic bioethanol production efficiency. The intrinsic limitation in NADH availability in the acetate-utilizing pathway was overcome by tuning the cofactor preference of AADH and overexpressing acetyl-CoA synthetase (ACS2) and glucose-6-phosphate dehydrogenase (ZWF1) [22]. Meanwhile, the incorporation of acetate as a redox sink in a redox imbalanced XR/XDH-based xylose-utilizing strain was shown to effectively enhance the metabolic flux of xylose to ethanol [20,21,23,24•]. Although the conversion of acetate into ethanol did not fully contribute to the higher ethanol yield, acetate co-consumption strains presented a promising strategy to overcome one key issue of the low ethanol yield in the XR/XDH-based xylose-utilizing strain.

Improving robustness of yeast strains with tolerance toward lignocellulosic inhibitors

Although acetate utilization accompanying *in situ* detoxification offers advantages, the yeast tolerance to acetic acid (and/or other inhibitors such as furan derivatives and phenolic compounds) should still be improved for the efficient fermentation of undetoxified lignocellulosic hydrolysates, particularly those containing a high level of inhibitors, at a low pH. Given that engineered xylose-utilizing strains are more susceptible to inhibitors [25–27], the development of stress-tolerant strains is more important to improve the overall yield in cellulosic biofuel production. Thus, transcription factors governing multiple genes have been engineered through directed evolution and modified complex phenotypes for tolerance to inhibitors. The mutant of a major transcriptional factor of HAA1 (S135F) conferred significantly improved acetic acid tolerance, demonstrating the potential of regulon-specific transcription factor engineering [28]. In a similar manner, a mutated artificial zinc finger-protein transcription factor (ZFP-TF) has generated acid-tolerant phenotypes [29]. By contrast, Qiu and Jiang (2017) modified the subunit RPB7 of RNA polymerase II to alter the global transcriptional profile, and they improved strain tolerances toward various types of inhibitors (such as acetic acid, furfural and phenolic compounds) [30]. Evolutionary engineering of inhibitor-tolerant strains has revealed genes such as *RTC3* and *ANB1* that can be used as the next engineering targets for the development of more robust mutant strains for cellulosic bioethanol production [26,31••,32–34]. Introducing catabolic pathways for the phenolic compounds derived from lignocellulosic biomass is another novel approach to develop an inhibitor-tolerant strain of *S. cerevisiae* [35].

Biodiesel: emerging microbes

Yarrowia lipolytica as an emerging biodiesel producer

An oleaginous yeast, *Yarrowia lipolytica*, is an emerging heterotrophic microbial platform for renewable biodiesel production primarily due to its lipid-producing capacity (up to 90% of the cell mass when grown on glucose [36])

and relative ease of metabolic engineering [2]. Over the past few years, the biomass and lipid yields of this microbial platform have been upgraded to produce 98.9 g/L of fatty acid methyl ester (FAME) from glucose with a yield of 0.269 g/g and a productivity of 1.3 g/L/h [37••]. Recently, *Y. lipolytica* has been engineered to secrete fatty acids into the culture media, allowing a further increase in the lipid accumulation capacity beyond its maximum (120% of its dry cell weight (DCW)) as well as reducing the cost of the energy-intensive steps of lipid extraction [38••]. These significant advancements in the capacity of *Y. lipolytica* to produce fatty acids have provoked growing interest in the metabolic engineering of this oleaginous yeast as a strong industrial host for cellulosic biodiesel production [36,37••,38••,39,40,41].

Pathway construction for non-glucose sugar utilization

As a more robust platform for industrial applications, there are challenges in extending the adaptability of *Y. lipolytica* to leverage a wide range of cellulosic substrates. In addition to the engineering efforts to expand the substrate range, as recently reviewed by Ledesma-Amaro and Nicaud [2], notable progress has been made in the development of xylose-utilizing *Y. lipolytica* over the past two years (Table 2).

The recent comprehensive investigation of the endogenous xylose catabolic pathway in *Y. lipolytica* partially explained its controversial innate capability of xylose utilization. In this study, the overexpression of endogenous XDH and XK enabled robust growth on xylose under high nitrogen conditions without the need for adaptation, revealing the bottleneck in its poorly expressed xylose catabolic pathway [42]. Through evolutionary engineering, the endogenous xylose catabolic pathway was temporarily activated, thus supporting cell growth on xylose as a sole carbon source, which was further increased by overexpressing XDH, the putative bottleneck [43]. In other approaches, the heterologous genes encoding XR and XDH from the well-characterized xylose-utilizing yeast *Scheffersomyces stipits* were overexpressed in *Y. lipolytica* to support a more efficient xylose catabolism [44••,45]. *S. cerevisiae* efficiently utilized xylose when it expressed XR and XDH; however, *Y. lipolytica* often required additional evolutionary engineering for sufficient growth [45] or experienced the divergence of metabolic flux into byproduct formation, such as xylitol and citric acid [44••]. This result suggests that more sophisticated xylose catabolism engineering is required to develop an efficient xylose-utilizing strain of *Y. lipolytica*. As an alternative approach to avoid the cofactor imbalance in the XR/XDH pathway, the successful construction of an XI pathway-based strain has not yet been reported, providing more opportunities for strain engineering to further improve the efficiency of cellulosic biodiesel production. With respect to the co-fermentation of glucose and xylose (or cellobiose), some groups have reported that a weaker glucose repression system was observed in *Y. lipolytica* [39,46], suggesting that the simultaneous mixed sugar utilization might be possible in *Y. lipolytica* without devoting extensive efforts to relieve the carbon catabolite system, as shown in other hosts (*e.g. S. cerevisiae*).

Y. lipolytica is considered to be a robust microbial platform regarding the utilization of undetoxified lignocellulosic hydrolysates for biodiesel production. In contrast to *S. cerevisiae*, this platform can naturally convert acetate to lipids [47,48], thus enabling *in situ* detoxification of the acetate-containing lignocellulosic hydrolysate during fermentation. In addition, the natural capability of *Y. lipolytica* to produce extracellular laccase can be harnessed to mitigate the inhibitory effects of lignin-derived phenolics on fermentation [49]. The development of cellulolytic *Y. lipolytica* has shown potential for the consolidated bioprocessing of cellulosic biodiesel production [50]. In a recent study on lipid production from lignocellulosic biomass by 32 oleaginous yeast strains, *Y. lipolytica* was reported to be among the top strains in terms of lipid productivity [51], suggesting that *Y. lipolytica* has an advantage as a promising cellulosic biodiesel production host. Given its relative ease of engineering with the increasing availability of engineering tools, such as the CRISPR-Cas9 system for genome editing [52], the prospects for *Y. lipolytica* in cellulosic biodiesel production appear promising.

Other oleaginous yeasts that can convert a wide range of substrates in lignocellulosic hydrolysates to lipids include *Rhodosporidium toruloides*, *Cryptococcus curvatus*, and *Lipomyces tetrasporus*, among others [51,53,54]. A recent study on the metabolic engineering of *R. toruloides* suggested this yeast as another alternative platform for producing cellulosic biodiesel [54] (Table 2).

Conclusion

To economically convert lignocellulosic feedstocks into biofuels, microbial platforms must possess cellular systems that can be modified to efficiently utilize a vast array of lignocellulose-derived carbon sources. Expanding the carbon source portfolio has attracted much interest and has been traditionally exploited by relying on rational and knowledge-driven technologies. Over the past two years, notable advances have been made, especially in the non-traditional fields, such as molecular transporters, gene regulation and stress responses, reflecting a path forward for the engineering of *S. cerevisiae* strains for cellulosic ethanol production. On the basis of the knowledge gained from *S. cerevisiae*, the development of efficient cellulosic biodiesel production strains of *Y. lipolytica* is likely to be accelerated. Moreover, continuously upgraded molecular engineering toolboxes, such as CRISPR-Cas9, and increasingly available multi-omics data serve as solid foundations leading to the emergence of industrially

Table 2

Recent results on strain development for cellulosic biodiesel production

Strain	Target pathway	Strategy	Genotype	Lipid content (% of DCW)	Lipid yield (g/g)	Lipid titer (g/L)	Lipid productivity (g/L/h)	Ref.
Y. lipolytica	Lipid accumulation	Reduce fatty acid degradation	*ΔPex10, Δmfe1, DGA1, leucine +, uracil +*	88 71	– 0.158[b]	16.1 25.3	0.2 0.21	[36]
		Flux push and pull: expression of d9-desaturase (SCD)[a]	*SCD, ACC1, DGA1*	67	0.234[b]	~55	0.707	[40]
		Rewire cytosolic acetyl-CoA pathway	*CAT2, ACC1, DGA1, leucine+*	72	0.229[b]	66.4	0.565	[41]
		Evolutionary engineering	*ΔPex10, Δmfe1, DGA1, leucine +, uracil+* (E26 strain)	87	0.243[b]	38.9	0.509	[39]
		Redox cofactor engineering	*ACC1, DGA1, GapC, MCE2* (ADgm strain)	66.8	0.269[b]	98.9	1.3	[37••]
	Lipid secretion	Mimic bacterial pathway for free fatty acid synthesis	*Δmfe1, Δfaa1, DGA2, TGL4, klTGL3*	120.4	0.20[b]	21	–	[38••]
	Xylose catabolism	Heterologous XR/XDH pathway/ starvation adaptation	*ssXYL1, ssXYL2*	–	–	15	0.19	[45]
		Heterologous XR/XDH pathway	*ssXYL1, ssXYL2, ylXYL3, Δpox1-6, Δtgl4, GDP1, DGA2*	–	0.08[c]	20.1	0.19	[44••]
R. tolulides	Lipid accumulation	Flux push and pull	*ACC1, DGA1*	61.1 43.4	0.23[b] 0.14[c]	16.4 9.5	0.08 0.03	[53]

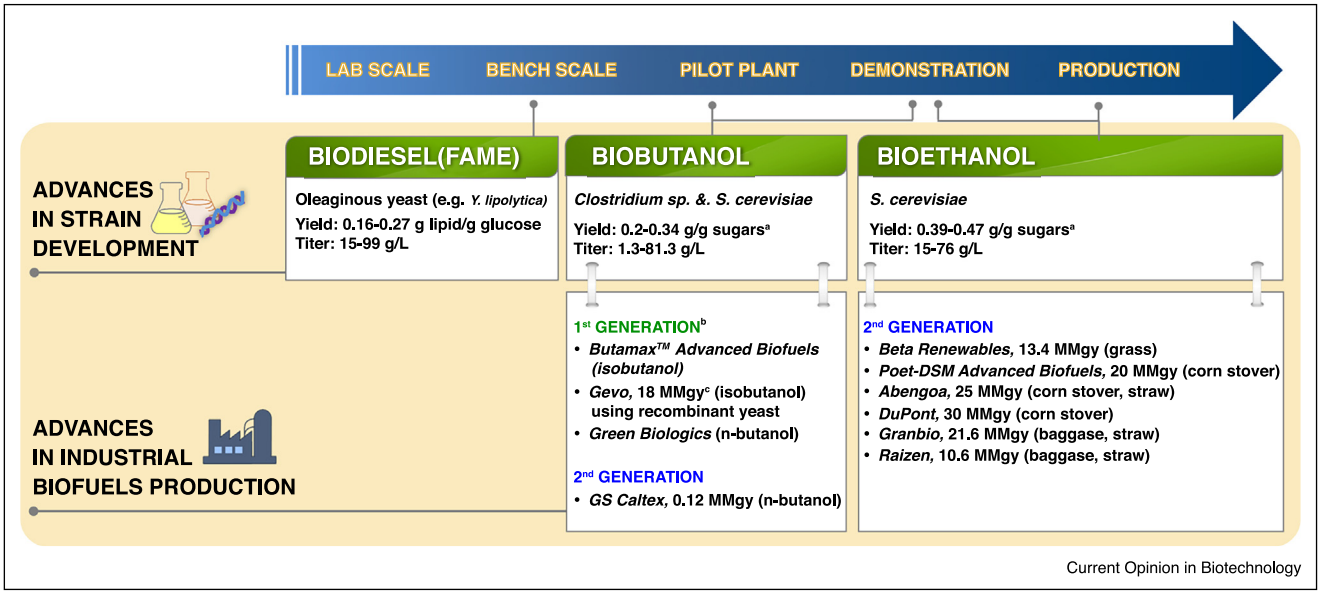
Pex10, peroxisome assembly protein 10; mfe1, multifunctional enzymes involved in beta-oxidation; SCD, delta-9 stearoyl-CoA desaturase; ACC1, acetyl-CoA carboxylase; DGA, diacylglyceride acyltransferase; CAT2, carnitine acyltransferase; GapC, Glyceraldehyde-3-phophate dehydrogenase from *Clostridium acetobutylicum*; MCE2, malic enzyme from *Mucor circinelloides*; faa1, acyl-CoA synthetase; TGL, triacylglycerol lipase; klTGL3, triacylglycerol lipase from *K. lactis*; ssXYL1, xylose reductase from *Scheffersomyces stipitis*; ssXYL2, xylitol dehydrogenase from *S. stipitis*; XYL3, xylulose kinase; pox1-6, acyl-CoA oxidases of peroxisomal β-oxidation; GDP1, G3P dehydrogenase.

[a] Conserved fatty acid regulator from specialized mammalian cell types.

[b] g lipid/g glucose.

[c] g lipid/g xylose.

Figure 2



Recent progress in the development of strains [27,57,58] and commercial-scale cellulosic biofuel production with a list of scale-up projects [1,56].
Yield (g product/g sugars[a]), product yield based on the multiple sugars derived from lignocellulosic hydrolysates.
Scale-up project sources[b], European Biofuels Technology Platform, http://www.biofuelstp.edu/cellulosic-ethanol.html.
MMgy[c], million gallons per year.

viable strains that efficiently convert lignocellulosic hydrolysates into biofuels. These advancements guarantee not only sustainability but also economic feasibility for the relevant companies (such as BP, DuPont, Gevo, GS Caltex, and Green Biologics), making the industrial production of cellulosic ethanol and butanol a realistic goal, with a vision toward lignocellulosic feedstocks [55–57] (Figure 2). Ultimately, the incorporation of these engineering strategies in the currently glucose-based advanced biofuel production systems may transform these facilities into advanced biofuel production plants in the future.

Conflicts of interest

None declared.

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