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Engineering yeast for utilization of alternative feedstocks

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Realizing the economic benefits of alternative substrates for commodity chemical bioproduction typically requires significant metabolic engineering of common model organisms, such as *Saccharomyces cerevisiae*. A growing toolkit is enabling engineering of non-conventional yeast that have robust native metabolism for xylose, acetate, aromatics, and waste lipids. *Scheffersomyces stipitis* was engineered to produce itaconic acid from xylose. *Yarrowia lipolytica* produced lipids from dilute acetate at over 100 g/L. *Cutaneotrichosporon oleaginosus* was engineered to produce omega-3 fatty acids and recently was shown to accumulate nearly 70% lipids when grown on aromatics as a carbon source. Further improvement to toolkits for genetic engineering of non-conventional yeast will enable future development of alternative substrate conversion to biochemicals.

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Introduction

Most bioprocesses use refined glucose or glucose-rich saccharides; however, the use of alternative substrates for biochemical production can have distinct advantages. Here, we define an alternative substrate as a less refined substrate, less commonly used substrate, process waste, or substrate not normally metabolized in nature. As feedstocks are a significant cost in commodity chemical bioproduction, cheaper alternative feedstocks can improve process economics [1]. Furthermore, alternative substrates can have higher theoretical yield for particular products [2], have improved sustainability and marketability [3], or may be the most abundant substrate in a resource-poor setting [4] (Tables 1 and 2). Therefore, depending on the available resources and desired products, alternative substrates warrant strong consideration.

This review focuses on recent advances in engineering non-conventional yeast for alternative substrate metabolism. Compared to bacteria, yeast have a longer history in biochemical production, are not prone to phage infection, and generally have higher tolerance to inhibitory compounds [5]. Furthermore, the eukaryotic cell physiology enables greater chemical diversity through specialized compartments (organelles) and post-translational modifications [6–8]. The focus on non-conventional organisms is motivated by our general philosophy of finding and engineering the best microbe for the job. This requires consideration of more than just *S. cerevisiae*, as there are several yeasts that have evolved complex phenotypes more suited for economic bioproduction using alternative substrates [7,9,10]. One must examine multiple competing factors, including tolerance to substrates and products, metabolism of alternative substrates, and metabolism leading to product formation. The choice of *S. cerevisiae* is often motivated by the availability of genetic engineering tools; however, we now have a rapidly increased ability to produce similar toolkits for non-conventional and non-model yeast, which greatly expands on the possible starting points for strain engineering [11–15]. We propose this will ultimately speed strain development, and enable titers and productivities far more difficult to access with conventional hosts.

The major alternative carbon substrates of interest include: carbon dioxide, methane, acetate, glycerol, xylose, aromatics, and fatty wastes. As carbon dioxide, methane, and glycerol have been extensively reviewed elsewhere [16,17], we omit them from this manuscript. We also briefly discuss recent progress using alternative nitrogen and phosphorous substrates.

Engineering xylose metabolism

Xylose is abundantly available from the hydrolysis of hemicellulose; however, organisms capable of efficiently consuming this pentose are poorly developed. Xylose metabolism commonly uses the oxidoreductase pathway where D-xylose is converted to xylitol by xylose reductase (XR), and then to xylulose by xylitol dehydrogenase (XDH). Xylulose is then converted to xylulose-5-phosphate by a xylulokinase (XK) and enters the pentose phosphate pathway (dashed blue box in Figure 1). An

Table 1

List of alternative substrates, their common sources, and benefits of utilization

Substrate	Source	Benefits	Ref.
Xylose	Lignocellulosic biomass	Readily available; cost-effective; better theoretical yield than glucose in some cases.	[18]
Aromatics	Lignin and waste effluent	Widely-available; hinders contamination through toxicity; remediation.	[38]
Acetate	Syngas, gasification of organic material	Underutilized byproduct; presents efficient conversion to acetyl-coA	[35]
Fats	Animal waste, plant oil processing waste	Large waste stream of animal and plant oil processing; better theoretical yield than glucose in some cases.	[42]
Urea	Urine	Plentiful; cost-effective; remediation	[48]
Cyanamide	Non-natural	Inhibits contamination	[49]
Phosphite	Non-natural	Inhibits contamination	[49]

Table 2

Theoretical yields for various products derived from alternative feedstocks as compared to glucose

Substrate	Source	Theoretical Yield (g/g substrate)		Product	Ref.
		Alternative	Glucose		
Xylose	Lignocellulosic biomass	0.34	0.32	Lipids	[21]
		0.51	0.51	Ethanol	[9]
Aromatics	Lignin and waste effluent	N.D.	–	–	–
Acetate	Syngas, gasification of organic material	0.17	0.32	Lipids	[35]
Fats	Animal waste, plant oil processing waste	N.D.	–	–	–

N.D. represents no data reported.

alternative pathway common in prokaryotes uses a xylose isomerase (XI) to convert D-xylose directly to xylulose.

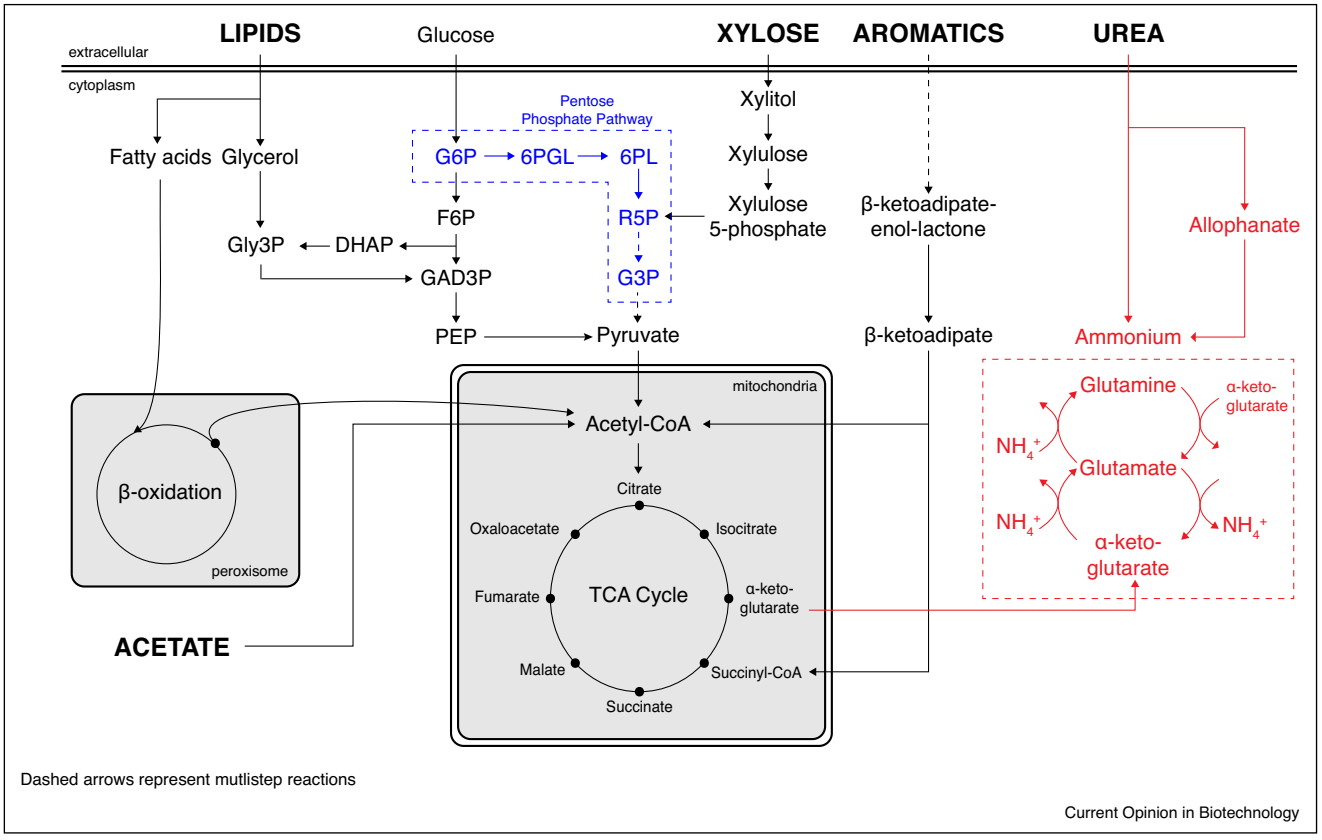
S. cerevisiae is generally considered a non-xylose metabolizing yeast. Heterologous expression of the oxidoreductase pathway from *Scheffersomyces stipitis* in *S. cerevisiae* has proven difficult because of redox imbalances and pathway bottlenecks [18,19]. Additionally, poor activity of XI requires adaptation [18,19]. A recent study successfully engineered aerobic growth of *S. cerevisiae* on xylose utilizing XI from *Piromyces*, and discovered that a single mutation in the XI enabled anaerobic growth on xylose without necessitating adaptation [20]. Alternatively, native xylose metabolizing yeast already have efficient growth on xylose. Genome shuffling in *S. stipitis* resulted in strain TJ2-4, which produced 21.9 g/L of ethanol from 50 g/L xylose [9]. A separate study resulted in 1.52 g/L itaconic acid production through heterologous expression of cis-aconitase carboxylase and overexpression of native aconintase. The recent development of a rapid computational method to identify centromere sequences in non-conventional yeast resulted in stable plasmids for heterologous gene expression in *S. stipitis* [14••]. Further expansion of genetic engineering tools is expected to continue accelerating the use of *S. stipitis* for biochemical production.

Cutaneotrichosporon oleaginosus, previously known as *Trichosporon oleaginosus* and *Cryptococcus curvatus*, is a viable candidate for industrial xylose bioconversion to lipids and oleochemicals [21]. This oleaginous yeast accumulated 40% of its biomass as lipids while utilizing xylose as the sole carbon source, with identical substrate uptake rates and lipid accumulation compared to glucose [22]. Using a limited genetic toolkit consisting of a single promoter, terminator, and agrobacterium transformation, *C. oleaginosus* was engineered for omega-3 eicosotrienoic acid production [23••]. Improved genetic engineering tools will be necessary for making this host useful for industrial scale production from xylose.

The model oleaginous yeast, *Yarrowia lipolytica*, has recently been engineered for xylose metabolism, either by overexpressing a cryptic endogenous oxidoreductase pathway or by heterologous expression of *S. stipitis* genes [24–26,27•]. In our study, overexpression of endogenous XDH and XK genes enabled robust xylose growth without the need for adaptation [27•]. We recently showed that additional overexpression of an endogenous XR led to growth rates approaching those for glucose, and transport of xylose was not rate limiting (unpublished). Significant tools have been developed to enable genetic tractability of this oleaginous yeast, including a CRISPR-Cas9 system [13••], promoter libraries, and regulated hybrid promoters [12•,28–30].

Nearly two decades of work to engineer *S. cerevisiae* to use xylose for ethanol production have been challenging and required substantial rewiring of metabolism. Valorization of xylose may benefit from further work with natural xylose metabolizing yeast. Overall, we suggest the choice of organism is no longer as limited as it once was, and that

Figure 1



Overview of alterative substrate utilization pathways. This figure outlines the metabolic pathways required to use glucose or various alternative substrates as feedstocks. Dashed arrows represent multi-step reactions, the blue dashed box represents the pentose phosphate pathway, and the red dashed box represents nitrogen metabolism.

less metabolic engineering is required for xylose conversion to a bioproduct.

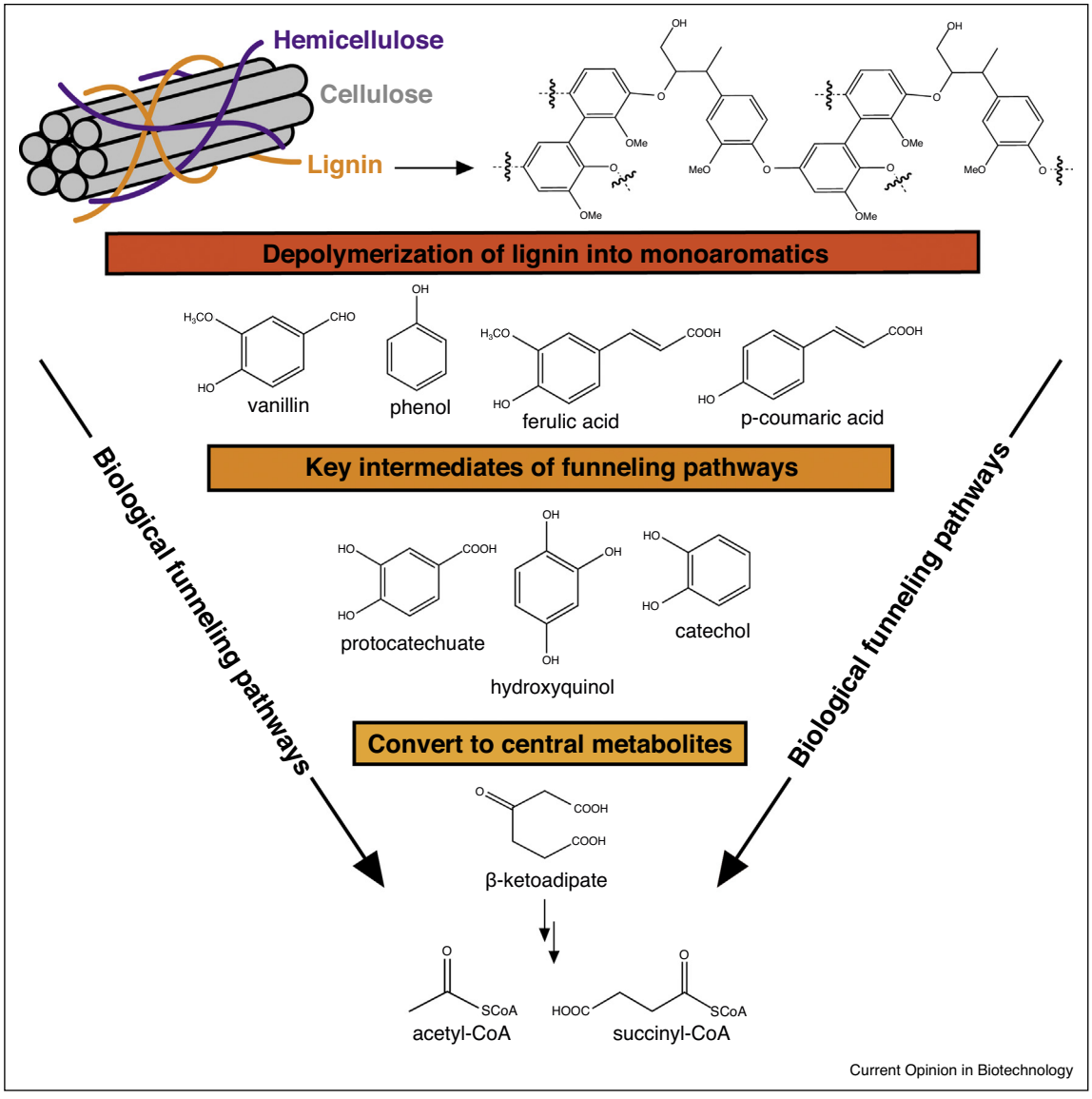
Engineering acetate and acetic acid metabolism

Acetate and acetic acid are abundantly available from low-cost sources such as anaerobic digestion, gasification of organic material, lignocellulosic hydrolysates, and syngas [31–33]. It is directly converted into acetyl-CoA by acetyl-CoA synthetase and serves as a precursor for biosynthesis of amino acids, ketoacids, polyphenols, and fatty acids. Metabolism of acetate is energetically expensive due to proton pumping required for maintaining homeostasis when acetic acid is deprotonated at physiological pH. Deletion of *GPD2*, which encodes the major glycerol-3-phosphate dehydrogenase isoenzyme in anaerobic, low-osmolarity cultures, strongly activated acetate reduction to ethanol in *S. cerevisiae* [34]. Overexpression of a heterologous acetylating-acetaldehyde dehydrogenase allowed assimilation of acetate, decreased glycerol formation, and increased ethanol production without altering growth rate.

Efficient lipid accumulation requires high flux through acetyl-CoA. Acetate is therefore an attractive substrate for oleaginous yeasts, such as *C. oleaginosus* and *Y. lipolytica*. *Cutaneotrichosporon oleaginosus* was grown in batch cultures with 30 g/L acetate resulting in 73.4% lipid accumulation and a titer of 4.2 g/L. When cells were grown with corn stover hydrolysate containing 19.2 g/L glucose, 9.2 g/L xylose, and 15.9 g/L acetate at a pH of 7.0, cells accumulated a lipid titer of 9 g/L while metabolizing glucose and acetate simultaneously, and metabolizing all three substrates completely by 60 hours [35]. Further improvements are expected with increased access to *C. oleaginosus* genetic engineering tools.

An engineered 'obese' strain of *Y. lipolytica* grown on 30% (v/v) acetic acid produced a lipid titer of 51 g/L and lipid accumulation of 61%. In the same study, a dilute 3% (v/v) acetic acid media was used with a cell recycling scheme and led to a lipid titer of 46 g/L and accumulation of 59% [31]. This was later made into a semi-continuous process and combined with model-guided feed control leading to a lipid titer of 115 g/L, accumulation of 59%, and productivity of 0.8 g/L-hour [36••].

Figure 2



Representation of biological funneling pathways for aromatics. Biological systems overcome the heterogeneity of lignin and other aromatic sources by utilizing funneling pathways. These pathway funnels enable metabolism of many aromatic compounds into a few key intermediate metabolites, which are then converted into central metabolites.

Engineering aromatics metabolism

Valorization of lignocellulosic biomass has largely focused on biomass-derived sugars and disregarded lignin-derived aromatics. Currently, 50–70 million tons of lignin are produced by pulp and paper mills each year. Only 2% is recovered for non-fuel purposes, while the rest is burned for its heating value [37]. Another major source of aromatic compounds include phenolics in industrial wastewater effluents [38]. The major barrier to using aromatics as an alternative feedstock is the lack of microbes able to tolerate and metabolize these compounds.

Significant efforts have been made to engineer *S. cerevisiae* for production of aromatic compounds; however, very few have explored aromatic metabolism. *S. cerevisiae* tolerates up to 1.4, 1.8, and 9.7 mM coniferyl aldehyde, ferulic acid, and p-coumaric acid. Tolerance of aromatics results from conversion of these inhibitory compounds into less toxic metabolites. Interestingly, ethanol yields were not impacted by growth with both glucose and any of the phenolic compounds [39]. Further characterization revealed *S. cerevisiae* has endogenous genes involved in coniferyl aldehyde metabolism. Aldehyde dehydrogenase, phenylacrylic acid decarboxylase, and alcohol

acetyltransferases were overexpressed to create strain *APT_1*. This strain had the highest growth rate in 1.8 mM ferulic acid and performed similarly to the control strain in 9.7 mM p-coumaric acid [40].

Lignin composition varies with source biomass, and depolymerized lignin can contain a great number of aromatic monomers at differing concentrations. Funneling pathways overcome substrate variability and enable conversion to central metabolites (Figure 2). Recently, non-conventional organisms *Rhodotorula toruloides* and *C. oleaginosus* were shown to be able to metabolize a wide variety of aromatic compounds, suggesting they may be proficient at coping with the heterozygosity of depolymerized lignin. We recently showed that *C. oleaginosus* could metabolize aromatic compounds, including 1 g/L resorcinol and p-hydroxybenzoic acid, at a rate similar to 1 g/L glucose [41••]. We also observed weaker growth in 2 g/L phenol, p-coumaric acid, syringic acid, and ferulic acid (unpublished data). Fed-batch experiments using resorcinol as the sole substrate led to lipid accumulation nearly 70% lipids by weight [41••]. The combination of native glucose, xylose, and aromatic metabolism coupled with tolerating lignocellulose hydrolysis byproducts makes *C. oleaginosus* a promising microbial host for one-pot utilization of biomass hydrolysates. Such a characteristic has been unidentified in any other microbial platform, and reinforces the need to engineer downstream production pathways into non-conventional organisms, rather than both upstream and downstream metabolic pathways.

Rhodotorula toruloides is an oleaginous yeast that is gaining popularity due to its combination of lipid accumulation and beta-carotene production. A recent report demonstrated this yeast could fully metabolize 2 g/L p-coumaric acid, p-hydroxybenzoic acid, ferulic acid, and benzoic acid, similar to *C. oleaginosus*, which is significantly higher than reported values for *S. cerevisiae*. Vanillic acid was found to be toxic at these concentrations, although the authors do not report testing lower concentrations [42]. While xylose and aromatic metabolism rates are slower in *R. toruloides* compared to *C. oleaginosus*, one must carefully weigh the benefits of beta-carotene production when choosing between these two hosts.

Engineering waste lipids metabolism

An abundant, low-cost, and underappreciated alternative substrate is waste lipids, including rendered animal fats and plant oil processing effluents. To use waste lipids as a substrate, the microbial host must be able to both metabolize fats and tolerate potentially inhibitory compounds, such as salts, alkanes, and toxic fatty acids. Oleaginous yeast of the genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Rhizopus*, *Cutaneotrichosporon*, *Lipomyces*, and *Yarrowia* often possess the lipase activity and tolerate such impurities in these industrial wastes. For example, *Y. lipolytica* has been used to convert waste animal fat into more industrially useful compounds. When fed an industrial waste fat primarily composed of stearic acid (C18:0), *Y. lipolytica* was able to produce shorter and unsaturated fatty acid species, including palmitic (C16:0), oleic (C18:1), and linoleic acids (C18:2) at up to 20 wt%, 20 wt%, and 7 wt%, respectively. During this conversion, 14 g/L of citric acid was also produced. Under these conditions, lipid titers can exceed 50% DCW [43,44]. Additional peptone resulted in 18 g/L citric acid production [45]. Olive mill wastewater is a substantial source of oils, sugars, and polyphenolics, and has resulted in citric acid titers of 19 g/L and cellular lipid content of 48 wt% [46•]. It should be noted that the salt and polyphenolic content of olive mill waste tends to inhibit growth of many other microorganisms.

Dairy waste, containing up to 40% lipids and 3% protein by weight, has recently been shown to support growth of *Aspergillus oryzae* and *Neurospora intermedia*. On this media, the organisms produced up to 6 wt% ethanol and a high-protein biomass (48 wt%) useful for feed applications [47]. In the examples thus far, the products are native to the yeast and require no additional genetic engineering to synthesize. Random mutation of *Y. lipolytica* has resulted in a strain capable of producing the sugar alcohol erythritol from waste cooking oil [48]. Titers as high as 22.1 g/L at a yield of 0.74 g/g cooking oil were achieved, representing a practical demonstration of efficient production from a waste substrate.

Engineering other alternative substrate metabolism

In addition to the alternative carbon sources mentioned above, alternative sources for other nutrients also exist (dashed red box in Figure 1). For large-scale bioprocesses, pure substrates represent a substantial cost. On an equivalent nitrogen basis, urea is more cost-effective compared to ammonium sulfate. Furthermore, urea is abundantly available in urine, typically at concentrations of 10–20 g/L. We recently demonstrated equivalent or more efficient growth of *Y. lipolytica* using urea, synthetic urine, and untreated human urine compared to ammonium sulfate (unpublished). Another recent study showed that *Lactobacillus* also grows on fresh urine [49]. Taken together, it is clear that urea and urine show promise as alternative substrates.

Recently, *S. cerevisiae* and *Y. lipolytica* were engineered to grow using cyanamide as a nitrogen source [50••]. Cyanamide is not normally metabolized by industrial microbes. This was accomplished by heterologous expression of a cyanamide hydratase from *Aspergillus niger* and evolution of the resultant strain. In a similar fashion, phosphite dehydrogenase expression and laboratory evolution allowed for the growth of the same yeasts on media

containing potassium phosphite as the major source of phosphorous.

In addition to cost, use of atypical nutrient sources brings with it another advantage: competition. Phosphite and cyanamide media is less permissive of growth and, as such, more resistant to contamination. The authors demonstrated this by challenging the engineered yeast with the contaminating species, *Kluyveromyces marxianus* CBS 6556. In standard media, *K. marxianus* was highly competitive, however, on phosphite/cyanamide, it was functionally dead. Adaptive strategies such as this are well-suited to non-model organisms as they require minimal a priori knowledge and have a directly screenable phenotype.

Perspectives

Increasing interest and use of non-conventional organisms such as *Y. lipolytica* have been motivated by their capacity for particular types of products, such as fatty acids. We already have seen these systems leveraged for the production of fatty acid derivatives, such as omega-3 fatty acids, ricinoleic acid, and alkanes. It will not likely be long before researchers start to leverage non-conventional yeast to make products, such as polyketides, that are derived from high flux precursors, such as malonyl-CoA. The next logical step is to consider organism choice based on both the intrinsic capabilities to make products as well as the capability to use a particular alternative substrate.

The use of alternative substrates is alluring. In particular, the potential to lower feedstock costs, in some cases increase theoretical yield, and enhanced sustainability provides a strong economic incentive. Nevertheless, there are still technical barriers to overcome for utilizing alternative feedstocks. For example, when using aromatics, the general toxicity of these substrates must be alleviated. Strategies to understand the basis of toxicity and cellular engineering to overcome toxicity will likely be needed. Furthermore, there is likely to be some investment of time and money into developing a well-annotated genome sequence and robust toolkit of vectors, selectable markers, and genome editing that enable metabolic engineering efforts. However, rapidly advancing genomics and synthetic biology capabilities have significantly reduced this burden.

Conflict of interest

The authors declare no competing financial interests.

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