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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