Microbial contamination is an obstacle to widespread production of advanced biofuels and chemicals. Current practices such as process sterilization or antibiotic dosage carry excess costs or encourage the development of antibiotic resistance. We engineered *Escherichia coli* to assimilate melamine, a xenobiotic compound containing nitrogen. After adaptive laboratory evolution to improve pathway efficiency, the engineered strain rapidly outcompeted a control strain when melamine was supplied as the nitrogen source. We additionally engineered the yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica* to assimilate nitrogen from cyanamide and phosphorus from potassium phosphate, and they outcompeted contaminating strains in several low-cost feedstocks.

Supplying essential growth nutrients through xenobiotic or ecologically rare chemicals provides microbial competitive advantage with minimal external risks, given that engineered biocatalysts only have improved fitness within the customized fermentation environment.

Microbial bioconversion of plant biomass is a promising route to sustainable liquid fuels and chemicals. For compatibility with society’s petroleum-based infrastructure, researchers have used metabolic engineering to produce “drop-in” molecules that meet existing petrochemical standards (1, 2). However, despite many technical advances enabling the production of biofuels and biochemicals, their large-scale production at competitive economic cost is lagging. One of the major obstacles for new bioprocesses is microbial contamination, which negatively affects yield, productivity, and operability. When scaling up new bioprocesses, contamination can be a major barrier to successful operation (6).

Conventional biofuel and biochemical fermentations use naturally occurring organisms that are highly competitive in their specific operating environment. For example, *Saccharomyces cerevisiae*, which is used to produce over 100 billion liters of ethanol per year (7, 8), is well suited to exploit the environment provided by modern fruit (9), where it rapidly converts high concentrations of simple sugars to ethanol. Ethanol inhibits many competing microbes and allows the more tolerant *S. cerevisiae* to dominate the fermentation. By co-opting this naturally evolved competitive advantage, bioethanol producers avoid the need for feedstock refining and sterilization, enabling cost-competitive biofuel production. However, even with this natural competitive advantage, ethanol producers often resort to antibiotic treatment to halt the growth of contaminating bacteria (10, 11), particularly *Lactobacilli*, that tolerate high ethanol concentrations (12).

A consequence of manipulating their central metabolism to produce advanced biofuels and biochemicals is that engineered microorganisms often have reduced growth rates or other competitive impairments. We sought to address this challenge by endowing biocatalysts with an engineered competitive advantage. We did this by supplying the macronutrients nitrogen and phosphorus (which are required by all microorganisms for growth) through low-cost xenobiotic or ecologically rare compounds and metabolically engineering their complementary assimilation pathways (Fig. 1). This combination creates an environment where the engineered biocatalyst becomes the dominant microorganism without solely relying on naturally evolved ecological fitness. We have term this strategy “robust operation by utilization of substrate technology” (ROBUST).

To test the ROBUST strategy, we engineered *Escherichia coli* ATCC 10798 with a synthetic six-step pathway for the conversion of melamine, a xenobiotic compound containing 67% nitrogen by weight, to ammonia and carbon dioxide. We demonstrated the utilization of melamine’s six nitrogen atoms ([figs. S1 and S2] through expression of *Acidovorax avenae*, *Pseudomonas* sp., *Rhodococcus sp.*, *E. coli*, and *S. cerevisiae* enzymes that had previously been characterized for the bioremediation of triazines (13–16). When transformed with a low-copy plasmid carrying the melamine utilization pathway, *E. coli* grew with melamine at a maximum rate of 0.12 ± 0.02 hour⁻¹.

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**BIOENGINEERING**

**Metabolic engineering of microbial competitive advantage for industrial fermentation processes**

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**Microbial contamination is an obstacle to widespread production of advanced biofuels and chemicals. Current practices such as process sterilization or antibiotic dosage carry excess costs or encourage the development of antibiotic resistance. We engineered *Escherichia coli* to assimilate melamine, a xenobiotic compound containing nitrogen. After adaptive laboratory evolution to improve pathway efficiency, the engineered strain rapidly outcompeted a control strain when melamine was supplied as the nitrogen source. We additionally engineered the yeasts *Saccharomyces cerevisiae* and *Yarrowia lipolytica* to assimilate nitrogen from cyanamide and phosphorus from potassium phosphate, and they outcompeted contaminating strains in several low-cost feedstocks. Supplying essential growth nutrients through xenobiotic or ecologically rare chemicals provides microbial competitive advantage with minimal external risks, given that engineered biocatalysts only have improved fitness within the customized fermentation environment.**

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**Fig. 1. The ROBUST strategy.** Macronutrients essential for microbial growth are supplied in the form of xenobiotic or ecologically rare chemicals. Metabolic pathways enabling macronutrient assimilation are engineered in the desired biocatalyst (blue cells), establishing them as the dominant microorganism over nonutilizing contaminants (brown and red cells) inside the industrial bioreactor environment. NAD+, oxidized nicotinamide adenine dinucleotide; NADH; reduced nicotinamide adenine dinucleotide.
We next performed adaptive laboratory evolution spanning about 100 generations in defined medium containing 0.5 mM melamine; we subsequently isolated a colony, designated strain NS163, with a threefold increase in growth rate to 0.40 ± 0.04 hour⁻¹ (n = 3).

Plasmid isolation from NS163 and reintroduction into wild-type E. coli resulted in an immediate growth rate on melamine of 0.4 hour⁻¹ (Fig. S3). Sequencing of the parental and reisolated plasmids identified a single point mutation in the evolved plasmid, resulting in an arginine-to-serine change at amino acid 352 of the E. coli gene guaD, which encodes ammeline deaminase, the second of six steps required for complete melamine degradation. That E. coli guaD is a target of evolutionary selection is perhaps not surprising, given that guaD is a “housekeeping” enzyme with primary activity for guanine deamination and is thought to catalyze ammeline deamination as a side activity (17).

To establish competitive advantage, the melamine utilization pathway ideally operates at a rate that makes ammonium available for rapid growth, but not so fast that free ammonium is excreted. With strain NS163, no free ammonium was detected during growth on melamine (fig. S4), although the degradation intermediate cyanuric acid did accumulate to about 13% of the total melamine fed, suggesting that further pathway optimization is possible. Growth on melamine also acted to maintain plasmid stability (fig. S5).

In coculture experiments in defined medium with melamine as the nitrogen source, E. coli strain NS163 rapidly outcompeted a control E. coli strain carrying the pACYC177 reference plasmid (Fig. 2A). The utilization pathway additionally conferred growth on melamine to the industrially relevant E. coli B, MG1655, and Crooks strains (fig. S6).

Next, we engineered S. cerevisiae to utilize cyanamide, a nitrile-containing compound, and we engineered both S. cerevisiae and the oleaginous yeast Yarrowia lipolytica to utilize phosphite, a phosphorus-containing chemical-industry intermediate. For the former, we expressed a cyanamide hydratase (18) gene homolog from Aspergillus niger to convert cyanamide to biologically accessible urea. After adaptive evolution, this strain grew with cyanamide as the sole nitrogen source. The process was repeated for the conversion of phosphite to biologically accessible phosphate by expression of bacterial phosphite dehydrogenase (19) and adaptive evolution (supplementary text and fig. S7). This resulted in S. cerevisiae strain NS856, which was able to simultaneously use cyanamide as a nitrogen source and potassium phosphate as a phosphorus source. We additionally expressed phosphite dehydrogenase in Y. lipolytica wild-type (strain NS324) and lipid-overproducing backgrounds (strain NS92), which enabled phosphite medium–based growth and lipid production at rates comparable to those in phosphate medium (fig. S8), without the need for adaptive evolution.

In competition with a control S. cerevisiae strain, NS586 demonstrated superior fitness in cyanamide-containing media (Fig. 2B), despite baseline growth of the control strain. Likewise, the Y. lipolytica phosphite-assimilating strain NS324 outcompeted a control strain in phosphite-containing media (Fig. 2C).

For the supply of nitrogen and phosphorus through synthetic compounds to be economically viable, additional costs must be minimal and more than offset by the use of lower-cost processing methods and feedstocks. The compounds
in this study are derived from bulk industrial chemical precursors and represent a cost that is comparable to that of industrial antimicrobial additives (table S1) and superior to that of sterile fermentation (table S2). We sought to apply ROBUST to minimally refined, low-cost feedstocks. We evaluated sugarcane juice and wheat straw lignocellulosic hydrolysate, two broadly available industrial feedstocks, for ROBUST fermentation with *S. cerevisiae* strain NS586. Sugarcane juice is composed primarily of simple sugars, but also contains some free amino acid-bound nitrogen and 0.1 to 0.6 g of phosphorus per kilogram of dry matter, or 1.7 to 12.5% of the phosphorus necessary for complete aerobic conversion of the sugars to yeast biomass (table S3). We tested batch fermentation of 2% w/v sugarcane juice supplemented with media containing 4 mM potassium phosphate or potassium phosphite. To these media, strain NS586 and *Kluyveromyces marxianus* CBS 6556, a fast-growing spoilage yeast (20), were co-inoculated at a 10:1 ratio. Despite the presence of a low level of naturally occurring phosphate, in the phosphate-supplemented medium, NS586 outcompeted *K. marxianus* for sugar utilization (Fig. 3A). With variability in raw feedstock supplies, ROBUST may be more or less effective at different naturally occurring phosphate concentrations. For a given set of operating conditions (e.g., biocatalyst loading, growth rate, fermentation time), a maximum natural phosphate concentration may be established for reliable operation; blending raw sugarcane juice with refined sugar could be used to keep phosphate below this level.

Wheat straw lignocellulosic hydrolysate is under evaluation for the production of bioethanol and biodegradables, and it requires nitrogen supplementation for complete growth of *S. cerevisiae* (fig. S9). We co-inoculated *S. cerevisiae* NS586 and *K. marxianus* CBS 6556 at a 10:1 initial ratio with 5 mM urea or cyanamide in 2% w/v glucan.
Fig. 4. ROBUST-enabled grain-to-lipid fermentation. (A) Dry-mill corn fractionation enables low-cost separation of food- and animal feed–quality germ and fiber from fractionated corn mash. (B) Simultaneous saccharification and fermentation in fractionated mash co-inoculated with lipid-overproducing \( Y. \) lipolytica NS392 (engineered for phosphite utilization) and contaminating \( S. \) cerevisiae strain Ethanol Red at a 10:1 initial ratio, with potassium phosphate supplying phosphorus. (C) Fermentation under identical conditions, except with potassium phosphate supplying phosphorus. In (B) and (C), CFU counts are reported as means ± SD (n = 4). (D) Lipid accumulation, reported as fatty acid methyl ester (FAME), at the end of fermentation.

REFERENCES AND NOTES

16. Materials and methods are available as supplementary materials on Science Online.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/353/6299/583/suppl/DC1

Materials and Methods

Supplementary Text

Figs. S1 to S11

Tables S1 to S6

References (26–59)

Database S1

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Xenobiotics to the rescue
Contaminating microorganisms can be highly detrimental to the large-scale fermentation of complex low-cost feedstocks, such as sugarcane or dry-milled corn for biofuels or other industrial purposes. The challenge is that foreign organisms have to be inhibited without using antibiotics because of concerns about spreading antibiotic resistance. Shaw et al. engineered bacteria and yeast to use rare compounds as sources of nutrients (see the Perspective by Lennen). Engineering the common biocatalyst Escherichia coli, for example, to consume melamine as a nitrogen source allowed it to outcompete contaminating organisms. Similarly, engineering yeast to use cyanamide for nitrogen or phosphite for phosphorus also improved competitive fitness.

Science, this issue p. 583; see also p. 542

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