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Article

Utilizing a wastewater-based medium for engineered *Saccharomyces cerevisiae* for the biological production of fatty alcohols and carboxylic acids to replace petrochemicals

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SUMMARY

Personal care items, pharmaceutical formulations, food additives, detergents, plasticizers, and industrial solvents manufactured from artificially synthesized acids or petrochemicals can be produced with more sustainable methods. The metabolic engineering of microbial hosts, specifically the heterotrophic yeast *Saccharomyces cerevisiae*, using a cost-effective wastewater-based growth medium could potentially provide a solution. Upregulation of the fatty acid biosynthesis (FAS) pathway present in this species is key to increasing all fatty acid-derived products. Three plasmids containing genes responsible for the production of the enzymes acetyl-CoA carboxylase (ACC1), fatty acid synthase (FAS), and fatty acid reductase (Far1), were transformed into *S. cerevisiae* cells using the PEG-LiOAc method. Transformation was conducted separately and together to optimize efficiency. We hypothesized that transformed cells would display more varied fatty acid and fatty alcohol profiles, and an increased ability to grow in a modified wastewater-based medium while degrading dissolved organics. A spectrophotometric assay predicated on the oxidation of NADPH to $NADP^+$ was performed to determine the activity of the overexpressed enzymes. High performance liquid chromatography (HPLC) analysis underscored the presence of C-16 and C-18 fatty alcohols and fatty acids present in the yeast. Finally, a gas chromatography-mass spectrometry (GC-MS) analysis portrayed a reduction in organic compounds in wastewater media that was metabolized by *S. cerevisiae* while evidence of ethanol production via fermentation was seen. The study adds to research on renewable energy alternatives, but more importantly, demonstrates an effective method in which *S. cerevisiae* can biologically produce valuable specialty and commodity chemicals to mitigate petroleum use while removing organic content from wastewater.

INTRODUCTION

In recent years, biofuels have received growing interest as an alternative fuel source due to concern about the environmental impact of fossil fuels. While extensive research has been done on utilizing microalgae for biofuel production, methods available constrain its prospects as a source for future energy. The commercial production of biofuel is not yet economically viable due to slow rates of production from microalgae and the expensive apparatus needed for algal growth, namely gas exchange equipment. Some yeast species, though in need of carbon sources, can produce lipids at higher efficiencies compared to microalgal species. Therefore, transitioning to yeast, which can be grown anaerobically, provides a promising alternative for fuel production for the future (1).

Currently, there are two practical bio-based options that can replace fossil fuels at least to some degree: bioethanol for gasoline and biodiesel for diesel (2). *Saccharomyces cerevisiae,* or baker's yeast, remains the preferred cell factory for production of bioethanol. While *S. cerevisiae* is responsible for nearly 100% of all bioethanol produced, its promise as a source of fatty alcohols, key in the production of specialty and commodity items, has not been exploited (3). In general, the growth of oleaginous (fatty acid-containing) yeast species is slower compared to that of bioethanol-producing yeast like *S. cerevisiae*, and biolipid production by oleaginous yeasts requires a long period to produce any optimal yield (1).

One of the major advantages of *S. cerevisiae* is the vast knowledge available about its metabolism, which has opened opportunities for metabolic engineering approaches (3). Biolipids, including triacylglycerol produced by oleaginous yeast, are one of the most important feedstocks for biodiesel production. The first step towards increasing the fatty alcohol production potential, and thereby commodity chemical potential of *S. cerevisiae*, is the upregulation of the fatty acid biosynthesis (3). In fatty acid biosynthesis, fatty acids are generated from three hydrocarbon chains esterified with a glycerol backbone (4). Malonyl-CoA is used in this process to elongate acyl-CoA chains and is synthesized by the enzyme acetyl-CoA carboxylase (ACC1) (5). Overexpression of the gene encoding for this rate-limiting enzyme could possibly increase production of malonyl-CoA and fatty acid-derived products. Second in the biosynthesis pathway, C-16 fatty acid (palmitic acid) assembly is initiated with the use of fatty acid synthase (FAS) (6). Finally, the introduction of a synthetic fatty acid reductase (Far1) allows for the reduction of fatty acids to fatty alcohols (7,8). Fatty alcohols provide an ideal class of chemicals that can replace petrochemicals in specialty and commodity chemical production due to their amphipathic nature, and act as non-ionic surfactants.

While using yeast for lipid and ethanol production has

	Time (minutes)						
	0	5	10	15	20	25	30
Control	0.0543	0.34578	0.84203	0.234235	0.10777	0.045443	0.02435
ACC	0	0.17153	0.38653	0.13897	0.04467	0.031241	0.022435
FAS	0	0.15342	0.345924	0.124422	0.05362	0.04257	0.02907
FAR	0	0.124215	0.27688	0.116478	0.05589	0.04864	0.025651
ACC + FAS	0	0.09535	0.19245	0.105852	0.04648	0.039842	0.024385
ACC + FAR	0	0.066346	0.165624	0.07324	0.04467	0.031241	0.018493
ACC + FAS + FAR	0	0.024364	0.121234	0.05866	0.03362	0.02257	0.01947

Table 1: Average absorbance of transformed *S. cerevisiae* cell lysates (ten replicates of separate 30 minute intervals). Values are given in absorbance units.

distinctive advantages in terms of lipid quality, cultivation ease, and overall productivity, the heterotrophic nature of yeast like *S. cerevisiae* reduces its competitiveness as a biodiesel source (10). It is this reason that there have been attempts to utilize wastewater as a free source of water, nutrients, and even carbon sources (11). The practical use of wastewater requires a prior treatment that eliminates contaminants and most significant malevolent bacteria, as well as balances nutrient composition (11).

Here, *S. cerevisiae* was metabolically engineered for enhanced fatty alcohol and free carboxylic acid production while utilizing wastewater as a cost-effective nutrient culture source. We show that if the *ACC1* (encoding a deregulated acetyl-CoA carboxylase), *FAS*, and *Far1* (*Mus musculus*) genes are overexpressed in *S. cerevisiae in vitro* via plasmid-based expression, then fatty acid, fatty alcohol, and ethanol content accumulated. By the same token, it was also predicted that if mixed liquor was utilized as a carbon source, after sterilization and nutrient balance, then yeast cell viability persisted in wastewater media, and organics in the media were degraded (12).

RESULTS

Determination of activity of ACC1, FAS, and Far1 enzymatic products

We used a lithium acetate heat shock method to transform the three plasmids containing the *ACC1, FAS, Far1* genes and confirmed transformation with selection for the URA-3 or LEU-2 nitrogenous base markers. However, to ensure enzymatic activity of acetyl-CoA carboxylase, fatty acid synthase, and fatty alcohol reductase, we monitored the oxidation of NADPH to $NADP^+$ indicative of three steps involving the enzymes in the *S. cerevisiae* fatty acid metabolism. Around 10 minutes after the start of the reaction, the oxidation of NADPH to $NADP^+$ for all *S. cerevisiae* strain lysates was lowest in the wildtype control yeast based on elevated absorbance values (**Figure/Table 1**). Transformed *S. cerevisiae* cell lysates had a consistently lower absorbance than the control, with the lowest observed in the *ACC1 + FAS + Far1* strain (one-tailed t-test, $p<0.05$). This indicated that not only was NADPH being converted to $NADP^+$ in the FAS pathway, but it also suggested greater enzymatic activity in the transformed cells (**Figure/ Table 1**).

Quantification of fatty acid metabolites

We used high-performance liquid chromatography (HPLC) to demonstrate the presence of C-16 and C-18 fatty alcohols and fatty acids, which we induced with the overexpression of *ACC1*, *FAS*, and *Far1*. Before running cell pellet extracts containing lipids from engineered strains, we analyzed standards of hydroxy-palmitic acid, palmitic acid, 1-hexadecanol, and stearyl alcohol, and recorded elution times to compare against the samples. We ran a control *S. cerevisiae* strain which outputted minimal detection of palmitic acid (**Figure 2**). However, in the strain transformed with just the *Far1* gene, C-16 fatty alcohol, 1-hexadecanol, and palmitic acid were identified (**Figure 3**). Furthermore, the strain that was transformed with the *ACC1, FAS*, and *Far1* genes demonstrated concentrations of 1-hexadecanol, hydroxy palmitic acid and in fact, even C-18 fatty alcohol, stearyl alcohol was identified (**Figure 4**). This HPLC analysis provided evidence of intracellular fatty-derived components.

Gas chromatography-mass spectrometry (GC-MS)

We used gas chromatography using a silica-based capillary column connected to a spectrometric interface to quantify metabolites present in metabolized mixed liquor. The data suggested, in comparison between the two tables, that

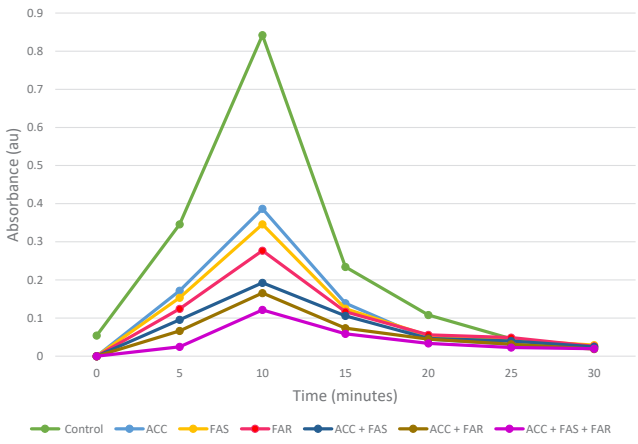

Figure 1. Enzymatic activity of S. cerevisiae. The reduction of NADPH to $NADP^+$ was seen around 10 minutes into the reaction for all *S. cerevisiae strain* lysates. Optimal absorbance was indicated at the peak of the reaction (where NADPH was being reduced). Values are given in absorbance units.

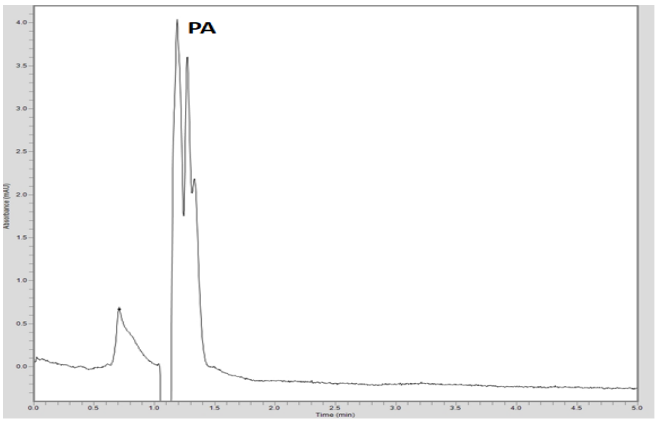

Figure 2. Fatty-derived products of non-transformed cells. A control *S. cerevisiae* strain was run through the silica-based column, which outputted minimal detection of palmitic acid and hydroxy-palmitic acid. PA refers to palmitic acid.

an overall reduction in phenolic, volatile organic compounds (VOCs), and aromatics was seen, while concentrations of succinic acid, malic acid, and oxalic acid (all carboxylic acids) and increased concentrations of acetone and ethanol were identified after "treatment" with yeast (**Table 2 and 3**). This indicates an ability of *S. cerevisiae* to effectively remove organics from wastewater while utilizing the organics to grow. We found acetone, ethanol, methanol, and acetaldehyde in much larger quantities than other metabolites, while phenolic compounds (namely pentachlorophenol and 4-tert-Butylphenol) and aromatics (benzene, bromomethane, and chloroethane) were found in much smaller quantities (**Table 3**).

DISCUSSION

The goal of this study was to successfully transform the *ACC1*, *FAS*, and *Far1* plasmids into *S. cerevisiae* to create a viable microbial strain that could treat wastewater while also producing specialty and commodity chemicals in the form of fatty alcohols. In this project, we examined the role of the fatty acid biosynthesis pathway in the production of lipids and bioethanol, and the potential use of wastewater organic content as a suitable nutrient source for yeast-related industries. To address this, we successfully transform *S. cerevisiae* with plasmids the enzymes acetyl-CoA carboxylase, fatty acid synthase, and fatty acid reductase, as demonstrated by the spectrophotometric enzymatic assay. Transformed *S. cerevisiae* demonstrated increased oxidation of NADPH to $NADP^+$ compared with non-transformed strains. A final lipid extraction and HPLC analysis allowed for the identification of stearyl alcohol, 1-hexadecanol, hydroxy-palmitic acid, and palmitic acid, supporting the notion that the transformation of the plasmids would allow for the overexpression of the three enzymes (first hypothesis). An analysis utilizing GC-MS of a viable *S. cerevisiae* culture in wastewater-based media indicated a reduction VOCs, aromatic hydrocarbons, and phenolics, while carboxylic acids and ethanol concentrations were determined. We demonstrate that the hypotheses, which stated that plasmid overexpression would lead to fatty alcohol production and yeast viability would persist in wastewater-based media while organics were degraded, were effectively supported based on successful transformation and expression of the three recombinant plasmids and reduction of volatile organics in mixed liquor used as growth media.

Thus, we demonstrate the creation of metabolically engineered *S. cerevisiae* strains that successfully grew in a nutrient-balanced, synthetic wastewater-based media. While the study reinforces the ability of *S. cerevisiae* to replace algae in renewable energy developments, the implications are far-reaching. The project effectively underscores the value of microbial hosts, in that they can produce important bio-based chemicals through engineering of cell metabolism. The fatty alcohols, fatty acids, and carboxylic acids identified in the project can be used to produce a variety of important items, including cosmetics, shampoos, toothpastes, concrete additives, pest control formulations, pharmaceutical formulations, food additives, textiles, biopolymers and bioplastics, detergents, industrial solvents, and plasticizers. Furthermore, conventional production of these very same fatty-acid derived chemicals using petroleum or artificial synthesis is environmentally harmful. As such, the prices of these chemicals fluctuate with the price of petroleum and will become more expensive and scarce as the non-renewable petroleum supply dwindles.

Furthermore, the utilization of wastewater as a cost-effective media is key to implementing chemical production from yeast on a commercial scale. Our wastewater method is less expensive in comparison to standard yeast peptone dextrose-based full medium, as expensive nutrients and trace element supplements are not needed. We further reveal that while *S. cerevisiae* utilizes the media to grow, the study reveals that a plethora of organics were degraded in the wastewater. Excreted acids and ethanol can be harvested from the wastewater with industrial processes like liquid-liquid microextraction, and bioethanol factories that already currently use *S. cerevisiae* can be retrofitted to produce these

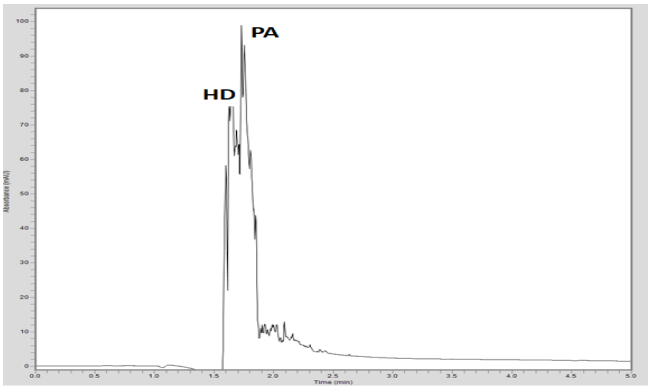

Figure 3. Fatty-derived products of FAR-transformed cells. In the *S. cerevisiae* strain transformed with just the *Far1* gene, concentrations of C-16 fatty alcohol, 1-hexadecanol, and palmitic acid were identified. PA refers to palmitic acid and HD refers to hexadecanol (C-16 fatty alcohol).

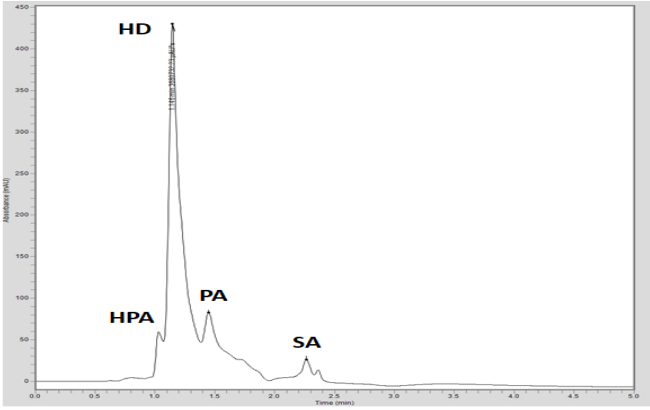

Figure 4. Fatty-derived products of ACC1, FAS, and FAR-transformed cells. The strain that was transformed with the *ACC1*, *FAS*, and *Far1* genes demonstrated a presence of 1-hexadecanol, and in fact, even C-18 fatty alcohol, stearyl alcohol was identified. PA refers to palmitic acid, HPA refers to hydroxy-palmitic acid, HD refers to hexadecanol (C-16 fatty alcohol), and SA refers to stearyl alcohol.

chemicals in metabolically engineered yeast. Expensive wastewater treatment processes to remove organic content could possibly be reduced or eliminated. To address any pathogenic organisms that could be harvested with the yeast, after lipid extraction, dry cell mass can be heated to high temperatures and pressures (350°C and 21,000 kPa).

In the future, analyzing the composition of wastewater through field analysis could improve the means to balance nutrient composition. In this project, overexpressing different genes involved in the production of triacylglycerols, linear saturated, unsaturated, aromatic dicarboxylic acids, and fatty acid methyl esters would allow for the creation of *S. cerevisiae* strains with the potential to replace a great number of artificial and environmentally harmful chemical production methods. Utilizing oleaginous yeast species in addition to *S. cerevisiae* could identify other microbial candidates for specialty and commodity chemical production. To develop commercially viable chemicals, processes such as dispersive liquid–liquid microextraction, cross-current liquid-liquid microextraction, or designing hydrophobic ionic liquids could allow for the isolation of organic acids (carboxylic acids, excreted fatty acids, and ethanol) from fermentation broth while also purifying biochemicals from dry yeast mass.

There were several limitations that impeded the investigation. While carboxylic acids were identified in wastewater GC-MS analysis, a metabolic engineering approach was not developed to increase the concentrations of these biochemicals. It will also be important to test wastewater from numerous sources to expand applicability beyond domestic wastewater treatment. While fatty alcohols and fatty acids were identified, the cost of completely purifying them into a commercially viable form can be calculated to assess economic feasibility. Lastly, GC-MS analysis involved preset parameters, including column thickness and size, mass spectrometer detector, flow rate, and carrier gas used, due its limited accessibility. Method development could have led to cleaner metabolite identification.

Compound	RT (min)	LOQ (ng/L)	Concentration (ng/L)
Acetone	2.02	7500	8400
Ethanol	3.16	7500	14300
Methanol	2.71	7500	7900
Acetaldehyde	1.66	8400	10200
Pentachlorophenol	10.29	1.2	4.5
4-Tert-Butylphenol	5.74	1.5	5.4
Bisphenol A	14.13	0.3	3.8
Triphenyl-phosphate	18.92	0.2	2.3
Chloromethane	9.61	0.6	4.1
Benzene	5.54	0.4	3.9
Bromomethane	5.99	0.8	4.6
Chloroethane	6.78	1.4	5.7
Chlorobenzene	7.61	0.7	3.6
Succinic Acid	ND	ND	ND
Malic Acid	ND	ND	ND
Oxalic Acid	ND	ND	ND

Table 2. Wastewater analysis before treatment. Select organic compounds identified in the initial wastewater obtained. In analysis of wastewater used before inoculation of *S. cerevisiae*, a host of volatile organics, aromatics, and phenols were identified. The table includes retention times (RT) and the limits of quantification (LOQ) for the organic compounds portrayed. ND indicates that the select organic compound was not detected.

METHODS

Saccharomyces cerevisiae (Carolina 173620) was first inoculated into 25 mL of defined medium (YPD) containing yeast extract (0.5 g/L), 20 g/L glucose, 5 g/L $(NH4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ adjusted to pH 6 (7). After sterilization, 2 mL/L trace element solution and mL/L of vitamin solution were added. The culture was grown for

Compound	RT (min)	LOQ (ng/L)	Concentration (ng/L)
Acetone	1.98	7500	11900
Ethanol	3.2	7500	27500
Methanol	2.77	7500	9500
Acetaldehyde	1.62	8400	20200
Pentachlorophenol	10.21	1.2	4.6
4-Tert-Butylphenol	5.76	1.5	2.7
Bisphenol A	ND	ND	ND
Tri-phenyl-phosphate	ND	ND	ND
Chloromethane	9.57	0.6	3.3
Benzene	5.58	0.4	1.5
Bromomethane	5.94	0.8	4.8
Chloroethane	6.78	1.4	4.6
Chlorobenzene	7.53	0.7	2.3
Succinic Acid	10.81	1.5	2.8
Malic Acid	13.48	1.1	2.3
Oxalic Acid	11.69	0.8	1.9

Table 3. Wastewater analysis after treatment. Select organic compounds identified after S. cerevisiae cells were inoculated in the wastewater-based medium over 4 days at 30°C. Phenolics, volatile organic compounds (VOCs), and aromatics can be seen, while concentrations of succinic acid, malic acid, and oxalic acid (all trace carboxylic acids from metabolic pathways) were also found. The table includes retention times (RT) and the limits of quantification (LOQ) for the organic compounds portrayed. ND indicates that the select organic compound was not detected.

4 days under lights at 25°C with shaking at 150 r.p.m, and spectrophotometry (OD600) was utilized to monitor cell density.

The *ACC1*, *FAS* and *Far1* genes were obtained as recombinant plasmids which all contained a URA-3 or LEU-2 selection marker, and an IPTG inducible marker. *DH5 alpha* cells transformed with plasmids containing *FAS, Far1, or ACC1* were procured from GenScript (*FAS* and *Far1*) and Addgene (*ACC1*). Once the bacteria were propagated using ampicillin as the selection marker for cells containing the respective plasmids, the plasmid DNA was then extracted. An overnight culture was pelleted after growing the LB medium to mid-log phase overnight at 37°C. Using a Monarch® Plasmid DNA Miniprep Kit, the DNA was isolated into 50 µl of elution buffer and quantified at 260 and 280 nm.

A lithium acetate (LiOAc) transformation procedure was used to insert the plasmid DNA into the *S. cerevisiae* cells. Denatured salmon sperm DNA, lithium sorbitol (LiSorb), heat shock (at 30°C and 42°C), and PEG (polyethene glycol) were used to enhance transformation efficiency. Cells were then grown on nitrogen base agar with supplements to take advantage of the URA-3 and LEU-2 selection markers.

We conducted a spectrophotometric assay to assess the activity of the *ACC1*, *FAS*, and *Far1* gene products in cell lysates (the acetyl CoA carboxylase, fatty acid synthase, and fatty acid reductase enzymes) (6). To do this, the reduction of NADPH to $NADP^+$ was analyzed by measuring absorbance at 340 nm. A reaction mix consisting of malonyl CoA, acetyl-CoA, NADPH and PBS was created. This was added to cell extracts containing protein that was extracted with lysis buffer. Over the span of 15 minutes, the absorbance of the solution was taken in 5-minute intervals. Concentrations of enzyme in each cell lysate was calculated using Beer's Law ($A = \varepsilon l c$, with extinction coefficient 6220 $mol^{-1}cm^{-1}$). Therefore, a higher absorbance indicated a smaller activity of the enzyme, as NADPH was reduced less frequently.

To verify the presence of C-16 and C-18 fatty alcohols and fatty acids, a high-performance liquid chromatography (HPLC) was conducted. Lipids were extracted from cell pellets using hexane and petroleum ether. HPLC analyses were performed using a silica-based column with a flow rate of 1 mL/min, a gradient composition, and compound standards of palmitic acid, hydroxy-palmitic acid, 1-hexadecanol, and stearyl alcohol were used.

Finally, mixed liquor (stage 1 wastewater containing solids and dissolved organic material) was collected from a local wastewater facility. Once collected, a modified wastewater-based media was created by adding nutrients, including vitamin B12, D(+) –biotin, nicotinic acid, calcium pantothenate, and thiamine-HCl $2H_2O$. Trace elements were composed of HCl (1 mL/L), $FeCl_2$-$4H_2O$ (15 mg/L), $ZnCl_2$ (7 mg/L), $MnCl_2$-$4H_2O$ (1 mg/L), $CuCl_2$ -$2H_2O$ (0.02 mg/L), NiCl2-$6H_2O$ (2.4 mg/L), and Na_2MoO_4 -$2H_2O$ (0.36 mg/L). Once the wastewater media was prepared, *S. cerevisiae* cells were grown in the media at 30°C over a period of 4 days and monitored for cell viability using spectrophotometry (OD600). Then, the wastewater was analyzed for total phenols, total phosphorus, total nitrogen, chemical oxygen demand, pH, purgable aromatics and purgable organics, C4 carboxylic acids, ethanol, and total hydrocarbons. A gas chromatograph connected to a mass spectrometer interface (GC-MS) was used to analyze for organic and aromatic compounds in association with a trap-and-purge method. We created a standard curve for each identified organic compound using external standards at varying concentrations. Each standard curve was constructed based on outputted peak area of the external standard, and concentrations of the organic compounds identified were subsequently determined. Phenols were identified after extraction from wastewater using methylene chloride and using a 2-propanol solvent.

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