Lignocellulosic Fermentation of *Saccharomyces cerevisiae* to Produce Medium-chain Fatty Alcohols

Katherine Elizabeth Bland

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Ryan Senger, Chair

Chenming Zhang

Silke Hauf

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ABSTRACT

The effects of climate change have made the need to develop sustainable production practices for biofuels and other chemicals imminent. The development of the 'green' economy has also led to many industries voluntarily improving the sustainability of the products they produce. The microbial production of fatty acid-derived chemicals allows for the opportunity to reduce petroleum-based chemicals in the marketplace. However, for microbial produced chemicals to be industrially competitive, significant work is needed to improve the production capacity of industrial strains. There are a number of bottlenecks and challenges related to the production of various fatty acid derivatives that need to be addressed.

One of these key challenges relates to the source of the fermentation feedstock. While sources such as corn or sugar cane are currently common, these feedstocks compete with food supply and require nutrient-rich soils. The use of lignocellulosic feedstocks is preferred to combat this issue, however these feedstocks present their own unique challenges. Pretreatment is required to release fermentable sugars, and this process also results in various fermentation inhibitors released into the solution. A better understanding of how engineered strains utilize these fermentable sugars as well as improving resistance to the inhibitors will help to improve the chemical production capacity of these chemical products. This work will focus on describing key bottlenecks related to fatty acid-derived products, while also evaluating proposed solutions to these bottlenecks.

Keywords: ^{13}C-MFA, Green Chemistry, Sustainable Production, Fermentation, *Saccharomyces cerevisiae*, lignocellulosic biomass

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GENERAL AUDIENCE ABSTRACT

Currently, many common household products and plastics are developed using petroleum-based components. From plastic bottles to common cosmetics, these contain ingredients that are derived from petroleum. In order to combat our reliance on petroleum for these every day products, it is essential to develop alternate sources for these materials. A potential source involve using plant material and by-products to produce these same compounds that we are able to produce from petroleum.

While there has been significant research to produce useful products such as bioethanol from corn, this is not an ideal crop. Corn requires more water and space than other crops such as grasses. In addition, these grasses can grow in soil that food crops are unable to grow in, so we don't utilize valuable land to develop common household products. However, these grasses are much more difficult to treat and process in order to form these basic chemical ingredients.

In order to use grass-based crops, it is possible to engineer organisms such as yeast to process the raw material into valuable chemical precursor. This work aims to genetically engineer yeast in order to produce some of these chemical precursors from a grass-like feedstock. In addition, this work also analyzes how physical characteristics of yeast affect the final product formation. Finally, a model was developed to show how yeast ferments corn-like and grass-like feedstocks differently.

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Background

Sustainable Bio-based Production

With the signing and ratification of the Paris Agreement in 2016, the nations of the world agreed that climate change needs to be addressed internationally. In addition to carbon emission mitigation, this process will require the development of more sustainable energy and chemical production. As of 2017, 150 nations have implemented renewable energy targets for electricity generation [1]. Biofuels have potential to be part of the solution to attain these renewable energy goals. For example, many small island nations rely on diesel to power large, centralized electrical grids [2]. The local availability of biofuels could play a role in the development of smaller, decentralized grid systems and could be used to provide grid stability. In addition, bio-based production of chemicals and even electricity from waste products has the potential to significantly improve the energy efficiency of waste treatment [3].

While 'biomass' is generally cited in documents regarding energy generation, this is a broad-encompassing term that often includes the direct use of wood and forestry products for energy and heat generation. Depending on the source of information, the biomass and biofuels are also not necessarily classified as being sustainable sourced. Biofuels are not innately sustainable, so the production methods of the initial biomass are key to the carbon neutrality [4-7]. In addition, it is essential that biofuels do not compete with food supply or contribute to deforestation to develop additional agricultural land [8-11].

To reduce the competition with food supply, products can be produced from lignocellulosic biomass. These sources range from plants such as switchgrass, *Miscanthus*, hardwood and softwood stems, and corn stover [12]. All of these sources range in the availability of cellulose,

hemicellulose and lignin [13], which affects the pre-treatment required to extract the fermentable sugars. While the cost of raw lignocellulosic feedstock is lower than other feedstocks such as corn or sugarcane [12], this pretreatment process adds to the cost and difficulty to producing biofuels from this source. Common pretreatment processes such as steam explosion and acid hydrolysis also result in fermentation inhibitors such as furfural and acetic acid mixed in the final solution [14-17]. In addition, lignocellulosic sources release a large proportion of xylose in addition to glucose, which many common industrial yeast strains cannot utilize natively [18-20].

In order for lignocellulosic sources to be a viable option for biofuel or biochemical production, it is necessary to engineer specialized industrial microbes. These industrial strains must be resistant to fermentation inhibitors while being able to fully utilize xylose as a fermentation feedstock [15, 16, 21, 22].

Engineering metabolism of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a commonly used strain for industrial production. Part of this is due to the natural resistant to ethanol stress [22, 23] and phage contamination [18]. *S. cerevisiae* also has a wide variety of genetic tools available. For example, *S. cerevisiae* has metabolic models [24, 25], simple transformation protocols [26], and a fairly well characterized genetic background [27-29]. In addition to the ease of use, *S. cerevisiae* has many genetic and phenotypic characteristics that make it ideal for studies to improve the production of a wide variety of products. For example, some strains are able to flocculate, forming flocs of clumped cells, separating the yeast easily from the fermentation broth [30-33].

S. cerevisiae also has well characterized organelles, which make it possible to compartmentalize groups of reactions. Compartmentalization has been used to improve local availability of precursors, increasing the overall titer of the product produced [34-37]. In particular, two organelles have been considered for improved biofuel production: the mitochondria [37] and the peroxisome [38]. The peroxisome houses β-oxidation, making it an ideal location for producing fatty-acid derived chemicals. A wide variety of fatty-acid derived chemicals can be produced for use in a variety of products ranging from deodorant to biodiesel. Manipulating fatty acid biosynthesis and β-oxidation provides a solid route for the production of fatty alcohols, a common fatty acid derivative.

Medium chain fatty alcohols

Medium chain fatty alcohols (MCFAs) range from 8-12 carbon chains. These chemicals are commonly used in a variety of personal care products including deodorants, detergents, and cosmetics [39]. While some products contain free fatty alcohols, generally fatty alcohols are converted to alcohol ethoxylates, ethoxysulphates, or sulfates [40]. These common household products can contain between 3% to 30% fatty alcohol (or derivative) [39, 41]. Some MCFAs may be used as drop-in biofuels [42] while related compounds could serve as substitutes for diesel [43].

Currently MCFA production is based on petroleum in the US, or is extracted from sources such as palm oil [44]. Oil extraction from Sunflower or Palm leads to very different fatty acid compositions which impact physical properties of the biodiesel [45]. Microbial production allows both for more control over the product specificity, as well as the feedstock that is initially used. The bio-based production of MCFAs provides the opportunity to sustainably source these

chemicals found in common household products, while also furthering our understanding of lignocellulosic fermentation.

Scope of Research

The aims of this research are to improve the fatty alcohol production using lignocellulosic biomass, targeting two of the key issues for lignocellulosic production: inhibitor resistance and xylose-based fermentation.

This thesis is split into the three main sections. First is a review on some of the less commonly studied fatty-acid derivatives that are relevant to industrial chemical production. Second, the use of flocculation is evaluated in the context of lignocellulosic fermentation. Third, a strain capable of co-utilizing glucose and xylose is evaluated using ^{13}C-metabolic flux analysis.

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Advances and Challenges in the Microbial Production of Nonconventional Fatty Acid Derivatives

Bland, K., Sheng, J., Feng, X*.

Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

* To whom correspondence should be addressed. Phone: (540) 231-2974. E-mail: xueyang@vt.edu.

Abstract

The effects of climate change have made the development of sustainable energy sources and production practices necessary. The use of fatty acids and a variety of derivative compounds provides a solid candidate to serve as biofuels or other chemical precursors. However, while the production of fatty acids and derivatives such as isobutanol have been well studied, there are a variety of derivative compounds that could also be of use to sustainable development. Medium chain derivatives are often difficult to produce since they have limited production in the common cellular hosts such as *Escherichia coli* and *Saccharomyces cerevisiae*. This is also a common issue with the production of alkanes, odd-chain fatty acids, branched chain fatty acids, and hydroxy-fatty acids. These derivatives are less commonly studied for bio-based production although these chemicals are commonly used in surfactants, detergents, biofuels, and more. The microbial production of these compounds faces several unique challenges. Fortunately, there have been several recent advances in the microbial production of these compounds. In addition to recent advancements, further research into these unique challenges is required for the production to become economically viable.

Key Words

Metabolic engineering; fatty acid; alkane; branched chain; biofuel; Escherichia coli; *Saccharomyces cerevisiae*;

Introduction

As the effects of climate change become more evident, the pressures for environmentally friendly and sustainable fuel sources increases. Biofuels are thought of as a potential replacement for fossil fuels due to the ability to sustainable source biomass and achieve a 'net neutral' carbon emission [1-3]. While fatty acids cannot be directly used as biofuels, non-ionic derivatives can [4]. Fuels such as bioethanol, butanol, isobutanol, and biodiesel have to potential to provide energy security by replacing or supplementing fossil fuels. Production of these biofuels using microorganisms varies from using glucose-based starches to second generation biofuel production using lignocellulosic biomass which contains a mixture of glucose and xylose. In addition to fossil fuels, several important chemicals and precursors are derived from petroleum. Biosynthetic production of these chemical precursors could aid in the development of 'green chemistry' and sustainable production practices. The use of bio-based methods also allows for improved specificity of the products produced that is unobtainable otherwise. While there are several species used to produce chemically relevant fatty acids and derivatives, this review will focus on microbial production in *Escherichia coli* and yeasts such as *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*.

Fatty acids serve as an important precursor to a wide variety of biofuels and industrially relevant chemicals. There has been significant work regarding the production of common products such as free fatty acids [5] and small-chain compounds such as isobutanol [6]. However, there are several other fatty acid derivatives that also serve as important biofuels and chemical precursors. There has been less of a focus on these non-conventional fatty acids (NCFAs) than for the common FFAs and derivatives due to a variety of key bottlenecks. These NCFAs are used in a wide variety of products, such as detergents, cosmetics, surfactants and food additives. In many cases, these

fatty-acid derived compounds are expensive and environmentally damaging to produce from petroleum or natural gas. However, by improving microbial production of fatty acids and the nonconventional fatty acid derivatives, there will be a wide range of sustainably sourced chemical precursors and biofuels available to the market.

Overview of Fatty Acid Metabolism

In *S. cerevisiae* and other yeasts, fatty acid biosynthesis is initiated by a type I FAS, a single multi-part complex. As described in Figure 1.1, initially Acetyl-CoA is transferred to the acyl carrier protein (ACP) by the ACP acetyl transferase (AT). Malonyl-CoA is synthesized from Acetyl-CoA by acetyl-CoA Carboxylase (ACC). The chain is then extended using malonyl-CoA as building blocks in the repetitive process of (i) β-ketoacyl-ACP synthesis, (ii) β-ketoacyl-ACP reduction, (iii) β-hydroxyacyl-ACP dehydration, (iv) enoyl-ACP reduction. This cycle repeats, and the termination is generally determined by the ketoacyl synthase domain [7]. In *E. coli*, fatty acid biosynthesis is carried out by type II FAS, which is a system of several distinct enzymes that carry out the condensation process of fatty acid synthesis rather than the single complex in yeast. Like in *S. cerevisiae*, ACC catalyzes the formation of malonyl-CoA to acetyl-CoA. Malonyl-CoA is then transferred to the fatty-acyl-carrier proteins to produce malonyl-ACP. The similar condensation cycle is then carried out by FabH, HAbG, FabZ/FabZ, and FabI to form a fatty acyl-ACP, and the cycle repeats.

The process of β-oxidation is also key to the production of several of the fatty acid derivatives. Although β-oxidation is the degradation of fatty acids, this process supplies valuable precursors when steps can be effectively disrupted. In eukaryotes such as *S. cerevisiae*, β-oxidation

occurs primarily in the peroxisome. The peroxisomal membrane is impermeable to most metabolites, including NADP(H) [8] and acetyl-CoA [9], which results in an isolated supply. Fatty acids are transported through the peroxisomal membrane either as free fatty acids or as activated CoA esters [10]. A variety of *pex* genes are related to the transport of these fatty acids and tagged enzymes though the peroxisomal membrane. Once in the peroxisome, the cyclic process of β-oxidation begins. An acyl-CoA oxidase activates the fatty acid via introduction of a double bond between the alpha and beta carbon. The next two steps are generally carried out by a multifunctional enzyme that involves 2-enoyl-CoA hydratase and a 3-hydroxyacyl-CoA dehydrogenase to produce 3-ketoacyl-CoA. A 3-ketoacyl-CoA thiolase catalyzes the final step which cleaves an acetyl-CoA, a shorted acyl-CoA remaining.

Production of fatty alcohols, a common derivative of fatty acids, involves the reduction of a fatty acyl-CoA to directly form an alcohol. This generally involves heterologous expression of enzymes such as carboxylic acid reductase (CAR) in *E. coli* [11], and fatty acyl-CoA reductase (FAR) in *E. coli* [12, 13] and yeast [14, 15]. Fatty alcohols can also be produced by first forming a fatty aldehyde from fatty acyl-ACPs, then then reducing to a fatty alcohol by expression of both a fatty acyl-CoA reductase (ACR) and aldehyde reductases (AR) [16, 17].

For both *S. cerevisiae* and *E. coli*, several additional steps can then be used to develop a wide variety of derivative compounds. While these additional steps face unique challenges, there are several bottlenecks related to FAB that impact all related derivatives. The bottlenecks of FAB have been reviewed [18, 19], and will only be summarized briefly. In general, the primary bottlenecks of FAB include (i) limited availability of precursor substrates such as acetyl-CoA, (ii) limited cofactor supply, (iii) product toxicity, and (iv) tight regulation of fatty acid metabolism

[18, 19]. Improvement of FAB will also improve the production of derivative products. Currently, one of the highest titers for fatty acid production is 39.1 g/L in *Y. lipolytica* [20]. However, the variety of fatty acid derivatives also have unique production methods, resulting in bottlenecks specific to the compound produced.

Medium Chain Fatty Acids and Alcohols

Medium chain fatty acids and alcohols can be directly used as biofuels and can also serve as precursors to the development of other useful compounds. These medium chain lengths are commonly used in the production of detergents, lubricants, cosmetics, and emulsifiers [21]. However, most biological hosts do not produce medium chain fatty acids and alcohols in high quantities. Rather, C14-C18 fatty acids are produced for incorporation into the membrane and other cellular components [22]. Medium chain fatty alcohols are commonly produced though petroleum-based chemical synthesis or are extracted from plant oils [23]. In biological hosts, these shorter chain lengths are generally produced by disrupting β-oxidation. Production of medium chain lengths faces similar problems to all fatty acid production, however there are several unique challenges. Because these chain lengths are not produced abundantly, high quantities can be toxic [24], and it can be difficult to control the precise chain length output from fatty acid biosynthesis. These challenges have been addressed by (i) improving the cell's tolerance to medium chain fatty acids, (ii) using a heterologous FAS with an alternate chain specificity, and (iii) compartmentalization into the peroxisome to aid in the overproduction of specific chain lengths.

The production of octanoic acid is the most common of the fatty acids and derivatives that will be discussed. Octanoic acid is noteworthy due to the toxicity issues that arise with the

production of large quantities. Productions of these shorter chain lengths has been shown to cause membrane leakage in *E. coli* [24]. Several methods have been used to increase the tolerance of *E. coli* and *S. cerevisiae* to these toxic chain lengths. By expressing a cis-trans-isomerase from *Pseudomonas aeruginosa* to produce trans-unsaturated acids, these acids can be incorporated into the membrane, conferring a higher resilience to the shorter chains. Improvement of the tolerance improves the overall growth and a total of 730 mg/L octanoic acid and 930 mg/L total medium chain fatty acids were produced [25]. Short chain resilience has also been examined by activating the glutamic acid dependent resilience (GADR) system in *E. coli*. The GADR system exists in *E. coli*, but is generally inactive, even when producing high concentrations of MCFAs. By overexpressing the genes *resB* and *dsrA*, the GADR system was activated, which improved growth and resulted in 10mM yield of heptanoic acid (converted from ricinoleic acid) [26]. While *S. cerevisiae* is generally more tolerant to high concentrations of fatty acids, octanoic acid still presents toxicity at higher production levels [27]. By expressing an engineered ACC1, there was an increased production of oleic acid, which led to improved tolerance to shorter lengths FAs such as hexanoic and octanoic acids [27].

By disrupting the β-oxidation pathway rather than altering FAB, shorter chain lengths can be produced. In *S. cerevisiae*, expression of an optimized *H. sapiens* hFAS coupled to β-oxidation disruption via deletion of FAA2, PXA1, and POX1 resulted in a total medium-chain fatty acid titer of 119 mg/L [28]. Of this, octanoic acid comprised about 80% of the total medium-chain fatty acid titer [28]. To investigate the rate limiting steps of β-oxidation in *E. coli*, a gene dosage experiment identified acetyl-CoA synthase and thiolase as key. To alleviate these bottlenecks, CRISPRi gene repression increased MCFA production to 3.8g/L [29]. While disruption of β-oxidation results in

shorter chain lengths, it is still necessary to further improve the specificity of the chain lengths produced in this process.

To improve the specificity of chain lengths for fatty acid and alcohols, there has been research to engineer key enzymes, including the use of heterologous FAS enzymes. Several variations of the *S. cerevisiae* FAS have incorporated both heterologous and engineered portions to the various domains in order to control chain specificity. For example, incorporations of a variant FAS along with the deletion of POX1 to disrupt peroxisome formation resulted in 175mg/L of C6-C12 MCFAs [30]. Hexadecanol (C16) has been produced by incorporating a heterologous fatty acyl-CoA reductase (TaFAR) from an owl, *Tyto alba*, into *S. cerevisiae* [15] and *Y. lipolytica* [31] to produce 655 mg/L and 636.89 mg/L, respectively. By expressing the TaFAR in addition to a xylose utilization pathway, *S. cerevisiae* was also engineered to produce 0.79 g/L hexadecanol in batch fermentation (1.2g/L fed-batch) using xylose as the sole carbon source [32]. As more variations of FAS are identified or engineered and β-oxidation is optimized, it will become much easier to control the quantity and specificity of these C6-C10 MCFAs. While peroxisome formation can be disrupted to move β-oxidation to the cytosol, fatty acid production can also be tagged to compartmentalize these reactions to the peroxisome.

The peroxisome houses β-oxidation in eukaryotes, and acts as an excellent and isolated source of acetyl-coA and NADPH. Compartmentalization has been previously reviewed [33], and will only be discussed in the context of fatty acid/alcohol production. Compartmentalization allows for the isolation of reactions to increase the local availability of enzymes and precursors. The heterologous TaFAR has also been compartmentalized to the peroxisome of *S. cerevisiae*, where β-oxidation primarily takes place, resulting in a pool of available acyl-CoAs [34]. Additional

overexpression of genes encoding PEX7 and ACC1 (acetyl-CoA carboxylase) further improved fatty alcohol production to achieve 1.3 g/L fatty alcohols with hexadecanol as the major product (62.7%) using fed-batch fermentation [34].

While the general bottlenecks of FAB apply, there have been great strides towards improving MCFAs recently. By combining methods to improve specificity and product resilience, the microbial production of MCFAs becomes a much more viable industrial solution. The recent research of engineering FAS enzymes is promising, particularly in *S. cerevisiae*. The multi-part complex FAS is difficult to tune, and with a greater product specificity, the cost of separation can be greatly reduced. However, further work must be done to improve the acetyl-CoA and cofactor availability in addition to the other challenges described above. As the titer and specificity of MCFAs improves, the potential for industrial microbial production increases.

Branched Chain Fatty Acids

Although non-ionic straight chain fatty acid derivatives can be used directly as biofuels, these compounds have limited cold-flow properties [35, 36]. The introduction of methyl groups into straight-chain acids or derivatives can improve the low-temperature viscosity without sacrificing the ability to be used directly as biofuel [37]. Branched chain fatty acids and derivatives are generally produced by initiating fatty acid synthesis using a branched-chain precursor in place of Acetyl-CoA. In general, improvements of the production of branched-chain FAs and derivatives include (i) alternate FabH enzymes that favor branched acyl-coAs are used, and (ii) improvements of the intracellular production of branched chain precursors.

In *E. coli*, the native β-ketoacyl-ACP synthase III (FabH) can only accept acetyl-CoA or propionyl-CoA as a substrate, as opposed to the branched-chain acyl-CoA necessary for branched chain fatty acids and derivatives. However, some Gram-positive organisms such as *Bacillus subtilis* and *Staphylococcus aureus* have an alternate FabH that utilizes branched chain acyl-CoAs, which are synthesized from α-keto acids [38].

The heterologous FabH from *B. subtilis* has been overexpressed in *E. coli* [39-41]. With additional modifications such as the incorporation of branched-chain α-keto acid dehydrogenase complex (BCKD) from *B. subtilis* and some fine-tuning of FAB, the recombinant strain of *E. coli* produced 273 mg/L fatty acid branched-chain esters (FABCEs). These FABCEs represented a total of 93% of the fatty acid esters produced [41] . The same strategy was then used to engineer the yeast *P. pastoris* and the recombinant strain was able to produce 169 mg/L FABCEs [41]. However, the issue remains that the branched-chain substrates required for the heterologous FabH do not readily accumulate in *E. coli* [38]. In addition, the endogenous *E. coli* FabH and the *B. subtilis* FabH compete for the same malonyl-ACP supply. To avoid competition for the same malonyl-ACP supply, a strain of *E. coli* was developed in which the heterologous *B. subtilis* FabH completely replaced the endogenous FabH. With additional tuning to increase the supply of branched-chain acyl-CoAs, the recombinant *E. coli* strain was able to produce 126 mg/L branched chain fatty acids (BCFAs) as 52% of the total free fatty acids produced [40].

As stated previously, accumulation of the branched chain precursors is a consistent issue with the formation of branched chain fatty acids. In addition, the alternate FAB enzymes that are introduced compete with the endogenous counterparts and often have a lower activity. Overall, these issues result in an undesirable blend of branched chain fatty acids and straight chain fatty

acids, which are difficult to separate. Knocking out the endogenous FabH enzymes could provide a solution to limit the competition with heterologous FabH enzymes. However, this solution is not enough. Enzyme engineering to improve the specificity for the branched chain substrates and increasing the availability of these substrates would improve productivity. This is a difficult task because these pathways are often involved in amino acids biosynthesis and knocking out these pathways could potentially hinder growth.

Odd Chain Aldehydes and Alcohols

The straight chain fatty acids and derivatives used in detergents, pharmaceuticals, industrial chemicals, and flavor/fragrance intermediates are very diverse. This includes odd straight chain fatty acids (or even chain alkanes) and derivatives which are not synthesized in high amounts in *E. coli* or *S. cerevisiae*. To produce odd chain fatty aldehydes and alcohols in these hosts, two strategies have been utilized: (i) use of propionyl-CoA rather than acetyl-CoA as the initial substrate, resulting in the building from an odd chain base rather than an even chain [42] and (ii) post-FAB alteration of an even chain fatty acid to form a C_{n-1} fatty aldehyde or alcohol [43, 44].

In *E. coli*, FABH can utilize both propionyl-CoA and acetyl-CoA as substrates. Supplementation of propionate into the media leads to the production of propionyl-CoA and then to odd chain fatty acids, but generally this results in shorter chain lengths [45, 46]. To produce longer odd-chain fatty acids in *E. coli*, heterologous acyl-ACP thioesterase (*Ricinus communis*), propionyl-CoA synthase (*Salmonella enterica*), and β-ketoacyl-acyl carrier protein synthase III were overexpressed to increase specificity towards propionyl-CoA. These modifications resulted in the production of 1205 mg/L total odd straight chain free fatty acids [42]. The highest percentage

off odd-chain achieved by a variant strain was the production of 83.25% of the total free fatty acids [42]. Although this resulted in high titer and percentage of odd chain fatty acids, propionate had to be extraneously added to allow for internal accumulation of propionyl-CoA [42]. Propionate is a compound that is produced naturally in *E. coli* [47] in small amounts, but must be supplemented in order to produce odd-chain fatty acids. Rather than supplementing propionate, there have been examples of manipulations to increase the propionate levels in the cell [46, 48]. Propionate was increased to around 804 mg/l, and with some additional modifications, resulted in 1317 mg/L pentanol [46, 48].

The second strategy to improve odd-chain production involves the use of an α-dioxygenase (α-DOX) to oxidize fatty acids. The α-DOX functions to cleave a carbon from the fatty acid, resulting in a C_{n-1} fatty aldehyde. Using an α-dioxygenase from *Oryza sativa*, a type of rice, long and medium chain C_n fatty acids are oxidized and then converted to C_{n-1} fatty aldehydes [43]. This α-DOX was expressed in *E. coli* and, along with some additional modifications, the recombinant strain was able to produce 101.5 mg/L in tube cultivation and 1.95 g/L using high cell density fed-batch fermentation [43]. The α-DOX has also been incorporated into *S. cerevisiae* and without major metabolic tinkering, was able to produce 19.8 mg/L fatty aldehydes after 48 hours and 20.3 mg/mL fatty alcohols after 60 hours [44].

While the use of the α-DOX lowers the NAD(P)H demand, requiring only 1 NAD(P)H unit per unit of fatty alcohol, there is also a carbon cost from the cleavage to form the C_{n-1} unit. It has been addressed that this carbon cost could potentially inhibit higher yields [43]. Utilization of propionate may be more ideal for higher yields. Manipulations of the propionate pathway could circumvent the need to supply propionate exogenously, and thus reduce the potential carbon cost

of the utilizing the α-DOX. Because the introduction of the α-DOX is a fairly new technique, it is also unclear which technique may result in greater product specificity. In addition, it is difficult to determine the cost/benefit of the carbon cost of α-DOX and the internal production of propionate. As yields improve with improvements to both of these methods, it may result in these two methods remaining competitive with one another, and may exhibit production of different chain lengths.

Hydroxy fatty acids & Dicarboxylic Acids

Hydroxy fatty acids (HFAs) and dicarboxylic acids (DCAs) are useful precursor compounds for the production of plastics [49] as well as other various value-added products [7]. Chemical synthesis of HFAs and DCAs is an expensive and energy demanding process [50]; whereas certain HFAs and DCAs are naturally occurring in a variety of organisms. The most popular method to produce HFAs is to utilize a cytochrome P450 monooxygenase (CYP). These cytochromes are capable of selective oxidation of C-H bonds to form the corresponding hydroxyl (C-OH) products [51]. DCAs are then generally synthesized from the corresponding HFA using an alcohol or aldehyde dehydrogenase. While organisms have been used to produce popular short chain HFAs and DCAs such as adipic acid [52], this review will primarily focus on less common medium and long-chain products.

Hydroxy fatty acids have been synthesized either by (i) exogenously adding fatty acids or (ii) engineering the FAB pathway to provide precursors. The specific chain length of the product is determined by the specific monooxygenase used. To produce ω-hydroxy octanoic acid, the monooxygenase $CYP153A_{Mag}$(G307A)-CPR_{BM3} was expressed in a strain of *E. coli* with modifications to increase octanoic acid including knocking out *fadD*, and the expression of the

plant thioesterase *fatB2* from *Cuphea hookeriana*, which has a preference towards C8 and C1-fatty acid-ACPS [53]. A final concentration of 55μM ω-hydroxy octanoic acid was achieved, although the concentration of octanoic acid was 437 μM [53]. While the titer is lower for this work than others reported, this work focused on a single product, rather than a mixture of fatty acids/hydroxyl fatty acids.

Higher titers have been achieved, such as 548 mg/L of HFAS, using fed-batch fermentation in an engineered *E. coli* strain expressing CYP102A1 from *Bacillus megaterium.* The final product mixture consisted mostly of 9-hydroxydecanoic acid (9-OH-C10), 11-hydroxydodecanoic acid (11-OH-C12), 10-hydroxyhexadecanoic acid (10-OH-C16), and 12-hydroxyoctandecanoic acid (12-OH-C18) [54]. Cytochrome P450 monooxygenases have also been engineered into *S. cerevisiae*. Two CYPs from *Fusarium oxysporum* (CYP539A7 and CYP655C2) were introduced into *S. cerevisiae* and coupled to a heterologous or homologous reductase system. Overall, the highest yield was the CYP539A7 coupled to a heterologous reductase system also from *Fusarium oxysporum*, which produced 73.8 mg/L 10-hydroxydecanoic acid, 72.2 mg/L 12-hydroxydodecanoic acid, and 45.1 mg/L of 8-hydroxyoctanoic acid [55].

To produce dicarboxylic acids in *E. coli*, an engineered CYP from *Marionobacter aquaeolei* was expressed to produce HFAS and an overexpressed alcohol dehydrogenase from *Pseudomonas putida* (*alkJ*) which converted the formed HFAS to the corresponding DCA [56]. Along with additional expression of a TE from *Umbellularia californica* (preference for C12 FFAS) and an overexpressed native γ-glutamyl-γ-aminobutyraldehyde dehydrogenase (puuC), 567 mg/L of C12 α,ω-DCA were produced using a fed-batch fermentation [56].

Overall, the production of hydroxyl fatty acids and dicarboxylic acids faces several unique bottlenecks. Production levels of these compounds are notoriously low, particularly when producing these compounds from internal supplies of FFAs. By exogenously adding FFAs, the production has reached about 6 g/L dodecanedioic acid [57] and 2.6 g/L ω-hydroxy palmitic acid [58]. A particular bottleneck is in the efficiency of the CYP. While some selected CYPs achieved a maximum 79% conversion of the acid into the corresponding HFA [55], most other CYPs had much lower conversion rates. CYP enzymes have been engineered to have higher specificity and efficiency [59, 60], although the number of instances is low.

Alkanes

Long and medium chain alkanes (ranging from C6-C16) are of particular interest for use as drop-in fuels in place of kerosene and diesel fuels [61]. Although the pathways for the microbial synthesis of alkanes and alkenes has been known for several years [62], until recently the productivity has remained very low and limited to *E. coli*. A decarbonylase (ADC) or a fatty aldehyde deformylating oxygenase (FADO) is used to produce alkanes from fatty aldehydes of varying length. Alkenes are synthesized using a similar route as alkanes, but generally a fatty acid decarboxylase is used in place of the ADC or FADO used in alkane synthesis. Alkenes have been produced recently in organisms including *E. coli*, *S. cerevisiae*, and *Y. lipolytica.* A more detailed review of alkanes is available [63], which provides a history of alkanes production from microbes. However, a briefer review of recent improvements of alkanes is discussed below.

E. coli was engineered to produce long chain alkanes with the introduction of an FAR and a fatty aldehyde deformylating oxygenase (FADO) [64]. Further modifications have been made to

enhance the base fatty acid synthesis. Modifications include the deletion of *fadR* (related to upregulation of unsaturated fatty acid biosynthesis) and *fadE* (related to degradation of fatty acyl-CoAs) in *E. coli*. With subsequent expression of fatty acyl-CoA reductase from *Clostridium acetobutylicum* and fatty aldehyde decarbonylase from *Arabidopsis thaliana* 580.8 mg/l short chain alkanes were produced, with major products ranging from C9 to C13 [65]. To produce a slightly longer range of alkanes, a thioesterase from *Umbelularia californica* was expressed in *E. coli* with some additional modifications of a fatty acyl-CoA reductase from *Acinetobacter* sp M-1 (ACR) (which prefers lauroyl-CoA and myristoyl-CoA) and a decarbonylase (ADC) from *Nostoc punciforme*. The best strain was able to produce undecane (C11) at 2.21 m/g and tridecane (C13) at 1.81 mg/g and pentadecane (C15) at 4.01 mg/g [66].

Alkanes have also been produced recently in the host *S. cerevisiae*. By deleting the native *hfd1* (which is thought to convert fatty aldehydes into fatty acids, preventing fatty alcohol production) and by expressing an *E. coli* F/FNR reducing system to supply a fatty aldehyde deformylating oxygenase (FADO) with electrons, tridecane, pentadecane, and heptadecane were produced, with total alkane production of 22.0 µg/gDW [67]. While the titer is much lower than in *E. coli*, this represents one of the first examples of medium/long chain alkane production in *S. cerevisiae*. However, an issue with the *S. cerevisiae* production was that no extracellular alkanes were detected, indicating that there was an accumulation with the cell [67]. Without transport systems (out of the cell), higher production titers could lead to acute toxicity.

Product toxicity is a severe complication of alkane synthesis. In yeast, alkanes do not appear to be exported out of the cell readily [67], leading to dangerous intracellular concentrations. As mentioned previously, there has been research towards improving membrane resilience to fatty

acids and derivatives [25]. However, in addition to these alterations, transport mechanisms to export alkanes may also be necessary. Another obstacle to alkane production is the efficiency of the conversion from aldehydes to alkanes. As discussed above, a strain that produced 4.01 mg/g pentadecane produced 29.06 mg/g fatty aldehyde [66]. The catalytic efficiency of ADO is far too low to result in high production of alkanes. Further research using an array of heterologous ADO enzymes as well as enzyme engineering could lead to a more efficient conversion from aldehydes to alkanes.

Similar to the production of alkanes, the alkene 8-heptadecene was produced in a strain expressing a heterologous acyl-acyl carrier protein (ACP), reductase (AAR), and aldehyde deformlyating oxygenase (ADO) from *Synechococcus elongates* along with the detection of *yqhD* and overexpression of the native *fadR*, resulting in 255.6 mg/L total alkanes. Although the intention was to produce n-alkanes, 8-heptadecene was the major product representing about 90% of the total alkanes produced [68].

In *S. cerevisiae*, a one-step fatty acid decarboxylation pathway was expressed to produce terminal alkenes. A fatty acid decarboxylase from *Jeotgalicoccus* sp. ATCC8456 (*oleT*) was expressed in yeast. Additionally, other more general modifications were made to boost precursor availability as well as cofactor accumulation and tuning of the expression levels. Overall, the final strain was able to produce 3.7 mg/L alkenes, with C17 (58.6 µg/L) and C19 (34.5 µg/L) terminal alkenes being produced as the major products [69]. As expected from the similarity of production mechanism, alkenes face similar bottlenecks as alkane production (see above).

Alkanes and alkenes both face the familiar bottlenecks of limited precursor supply and cofactor availability. Both also face difficulties with controlling the product specificity.

Improvements in product specificity would significantly reduce cost of separation. Alkanes and alkenes serve as a unique potential to supplement or replace petroleum-based diesel fuels. The improvements of microbial alkane production will demonstrate an important lead towards developing more sustainable biofuels.

Conclusions

In general, improvements of fatty acid biosynthesis will also lead to improvements of the derivative compounds. As mentioned previously, improvements of the acetyl-CoA (or other precursor) supply, redox cofactor balancing, and other commonly known bottlenecks will lead to a greater free fatty acid or fatty acyl-CoA supply from which many of the derivatives are directly synthesized. However, improvements of the overall fatty acid supply will not improve the product specificity. Most production of fatty acids and the derivatives results in solutions that are heavily mixed between either the desired and undesired products or between variable chain lengths. Separation of these closely related, but undesired products is often a very expensive and time-consuming process. Improvements are needed in (1) the specificity of chain length of fatty alcohols and acids and (2) the specificity towards the desired substrate in the case of odd and branched chain derivatives.

While improvements for fatty acid synthesis will aid in the production of some of the derivatives, several still face particularly unique bottlenecks that will likely need to be addressed as fatty acid synthesis improves. Particularly for dicarboxylic and hydroxyl fatty acids, the CYPs that are used are highly inefficient, but engineering these proteins has been shown to improve their effectiveness [59, 60]. While the microbial production of these nonconventional fatty acids and

derivatives faces several challenges, there is also significant potential to improve either the availability of the precursors (e.g., dicarboxylic acids), which are innately difficult to synthesize, or to improve the production practices towards being more sustainable and less costly.

Table 1.1 Recent advancements in the production of nonconventional fatty acids.

Table 1.1 Recent advancements in the production of nonconventional fatty acids

Type	Target	Host	Genetic Manipulation	Titer/Achievement	Ref.
Medium Chain	1-Hexadecanol	*S. cerevisiae*	Overexpression of TaFar (Tyto alba), overexpression of ACC1, and heterologous ATP-dependent citrate lyase	1100 mg/L*	[15]
	1-Hexadecanol (From Xylose)	*S. cerevisiae*	Expression of xylose utilization pathway in addition to modifications above.	1200 mg/L*	[32]
	Fatty Alcohols	*S. cerevisiae*	Expression of TaFAR in peroxisome, overexpression of PEX7 and ACC1	1.3 g/L* (62.7% 1-hexadecanol)	[34]
	Fatty Alcohols	*Y. lipolytica*	Overexpression of TaFAR, deletion of FAO1 (limit FA degradation), and DGA1 (increase acyl-CoA)	636.89 mg/L	[31]
	Medium chain fatty acids (C6,8,10)	*S. cerevisiae*	Expression of optimized hFAS (*H. sapiens*), disruption of B-oxidation pathway (deletion of FAA2, ANT1, and PEX11)	119 mg/L	[70]
	Medium Chain fatty acids	*E. coli*	Expression of Cti from *P. aeruginosa* to increase membrane tolerance	930 mg/L (78% Octanoic Acid)	[25]
Branched Chain	Branched chain fatty esters	*E. coli*	Combination of FAB to valine and leucine biosytnehsis. FabHB and BCKD from B. subtilis in addition of expression of aro1C, ws/dgat, adh2, fadD, alsS, ilvC, ilvD, and tesA	273 mg/L	[41]
	Branched chain fatty esters	*P. pastoris*	Chromosomal integration of aro10, adh2, and ws/dat	169mg/L	[41]
	Branched chain fatty acids	*E. coli*	Complete replacement of E. coli FabH with S. aureus FabH, expression of BSalsS, ECilvCD, ECtesA, BSbkd	126 mg/L**	[40]
Odd chain	Odd chain fatty acids	*E. coli*	*ilvG⁻ rfb⁻ rph⁻ fadD⁻*, prpE, B-ketoacyl-ACP synthase II from *S. aureus*, acyl-ACP thioesterase from *R.communis.*	1205 mg/L	[42]
	Odd chain fatty alchols	*E. coli*	Optimized expression of tesA', Expression of a-DOX(O. staiva) and yjgB, and fadR	1.95 g/L (HCD fed batch)	[43]
	Odd chain fatty alcohols	*S. cerevisiae*	Expression of a-DOX(O. staiva), ΔFAA1, ΔFAA4	20 mg/L	[44]
Dicarboxylic acids	Hydroxy fatty acids	*E. coli*	E.coli 'tesA and B. megaterium CYP102A1, ΔfadD, *E.coli* ACCase	548 mg/L*	[54]
	α,ω-dicarobxylic acids	*E. coli*	ΔfadE, UcFatB2 (chain length specific acul-ACP thioesterase), $CPR2_{mut}$ (cytochrome p450 monooxygenase), puuC, alkJ	567 mg/L	[56]
	Cis,cis-muconic acid	*E. coli – E. coli coculture*	ΔptsH, ΔptsI, Δcrr, enhancement of shikimate pathway (mutated alpha subunit of RNA polymerase) & ΔxylA, aroE, aroL, aroA, aroC, and ubiC, shiA gene	0.35 g/g	[71]
Alkanes	Alkanes	*E. coli*	ΔfadE, ΔfadR, Km^R (aldehyde decaronylase from A. thaliana), fadD, Ap^R (fatty acyl-CoA from C. acetobutylicum), tesA	580 mg/L*	[65]
	Long chain alkanes	*S. cerevisiae*	Expression of FAR and FADO from S. engates, Fdx and Fpr (ferrodoxin reductase) from *E. coli*, and knockout of HFD1	22.0 mg/g DW	[67]
	Long chain alkane	*E. coli*	dc from *N. punctiforme*, acrM from A. sp.M-1, fadD from *E. coli*,	8.05 ± 0.37 mg·g–	[66]

Alkenes	Terminal alkenes	*S. cerevisiae*	Δfaa1 Δfaa4 Δctt1 Δcta1 Δccp1 Expression of OleT from *Jeotgalicoccus* sp. ATCC 8456	3.7 mg/L	[69]
	8-heptadecene Alkenes	*E. coli*	Expression of, AAR, and ADO from *S. elongates*, deletion of yqhD, overexpression of native fadR	255.6 mg/L	[68]

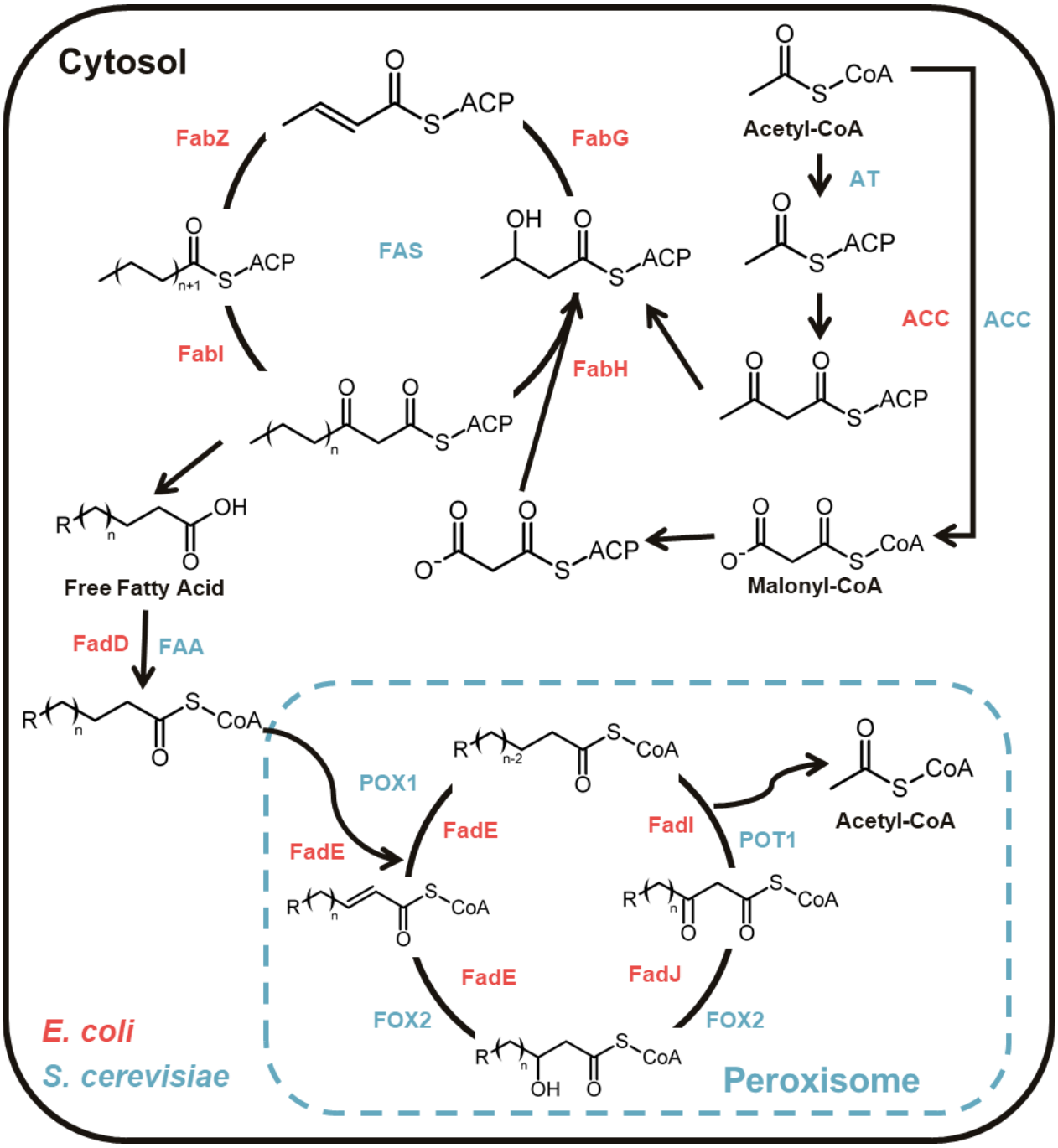

Figure 1.1 Fatty acid biosynthesis and β-oxidation of fatty acids in E. coli and S. cerevisiae.

Fig. 1. Fatty acid biosynthesis and β-oxidation of fatty acids in both E. coli and S. cerevisiae. The enzymes listed is red are the enzymes in E. coli, and generally other bacteria. The enzymes listed

in blue are enzymes of S. cerevisiae, and generally other yeasts. Note, the peroxisome is only present in Eukaryotes such as the yeasts, and in E. coli, β-oxidation takes place in the cytosol.

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Investigation of Biochemical Production Relevance and Uses of Flocculation

Bland, K., Sheng, J., Feng, X*

Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

* To whom correspondence should be addressed. Phone: (540) 231-2974. E-mail: xueyang@vt.edu.

Abstract

Yeast is commonly used as the microbe of choice for industrial biofuel and chemical production. This choice is generally due to the ease of manipulation and resistance to ethanol, but there is another key aspect of yeast that may be understudied: flocculation. The formation of flocs in a solution can aid in the final separation of yeast from a fermentation broth, but there may be other uses as well. It has been proposed that flocculation may be able to pull other unrelated microbes out of solution in addition to the yeast. It has also been proposed that flocculation may confer some resistance to fermentation inhibitors that are common in lignocellulosic biofuel production.

This study aims to evaluate some of these proposed uses of flocculation. First, a flocculant strain of *S. cerevisiae* is grown in a co-culture with *E. coli* to evaluate whether the yeast can significantly pull the *E. coli* from the fermentation broth. Overall, it did not appear that the flocculant yeast was able to interact significantly with *E. coli* to effectively reduce the *E. coli* suspension in the media. A second study evaluated the claim that flocculation may confer some resistance to fermentation inhibitors such as furfural and acetic acid. High concentrations of these inhibitory compounds generally inhibited flocculation in addition to severely reducing the fermentation capacity of the strain. While it does not appear that flocculation can be used to resist inhibitors or separate co-cultures, there are still potential uses regarding the ease of separation from a fermentation broth.

Key Words

Flocculation; *Saccharomyces cerevisiae*; lignocellulosic fermentation; co-cultures;

Background

Saccharomyces cerevisiae is used widely for investigating the production of biofuels and value-added products by industrial yeast. Ease of genetic manipulations and osmotolerance are often cited as yeast's major advantages [1]. An important, but sometimes neglected, characteristic of various strains of yeasts is the ability to flocculate. Flocculation is the reversible, and nonsexual aggregation of individual cells into multicellular 'flocs'. These flocs can either sediment or become suspended on top of (or within) the fermentation broth. Flocculation is used commonly in the brewing industry as a way to reduce the cost and effort of separating out yeast [2]. There has been recent attention to the possibility of flocculation being used as an auto-immobilization process to remove heavy metals and other materials from a solution [3-6]. The possibility of using flocculation to easily separate yeast from a fermentation broth has also been considered for biochemical fermentation as well [7-9].

Flocculation is a surface characteristic of the cell. Even heat-killed cells [3, 4] and the separated cell walls of yeasts can also continue to flocculate [10]. While the exact mechanism is unknown, the most popular explanation for the mechanism of flocculation is the lectin theory [11, 12]. A lectin-like protein present on flocculent cells is believed to interact with α-mannans on neighboring cells. These α-mannan receptors are on both flocculent cells and non-flocculent cells, which make it possible for a flocculant strain to interact with a non-flocculant one. The *flo* genes have been identified as encoding the flocculation lectins responsible for the flocculation characteristic. Various *flo* genes induce a variety of adhesion and flocculation characteristics [13], but the gene *flo1* is known to encode a cell wall protein that causes strong flocculation [14]. Being a surface characteristic of the cell, there are several factors that influence the quality of flocculation. Ca^{2+} is known to strongly promote flocculation while several other common ions

such as Rb^{+}, Fe^{2+}, Co^{2+}, Ni^{2+}, and Al^{2+} have a lesser impact [11]. Metal ions such as Ba^{2+}, Sr^{2+}, and Pb^{2+} inhibit flocculation [11]. Other common ions such as Na^{+} and K^{+} are known to inhibit flocculation at high concentrations, but induce flocculation at lower concentrations [11]. Temperature and pH are also commonly used to easily (and reversibly) inhibit or suspend flocculation in a solution [15]. The presence of fermentable sugars seems to prevent some flocculation in early growth, but this does not seem to be a factor in the later stages of growth [16, 17]. Ethanol seems to positively affect yeast flocculation, which is what has led to the idea that flocculation could potentially be used in the separation process of ethanol-producing industrial strains. The physical interaction of the yeast cells has led many to wonder if flocculation could confer some resistance to fermentation inhibitors [9].

Co-flocculation (also called mutual flocculation) involves the aggregation of a non-flocculating strain and a flocculating strain, so the mixture can still form flocs. There have been examples of co-flocculation using two strains of yeast [18, 19], as well as examples of co-flocculation of yeast with *E. coli* [20, 21]. Co-flocculation with *E. coli* could potentially be used to simplify separation from the fermented product, particularly for co-cultures and other mixed cultures. In addition, flocculation has been shown to have a somewhat "protective nature" due to the physical structure of the floc. While this protective nature is generally used to re-pitch to yeast, it has been proposed that flocculation could also be used as a physical barrier for resistance against the fermentation inhibitors that occur in the pretreatment of lignocellulosic materials [22, 23].

Thus, two potential uses of flocculation for industrial chemical production have been proposed: (i) the use of co-flocculation to pull non-flocculating suspensions out of solution, and (ii) as a strategy to resist fermentation inhibitors. To test these potential uses of flocculation for

industrial use, *flo1* was transformed to develop two strains of *S. cerevisiae*, one with constitutive *flo1* expression, while the other was galactose-inducible. To test co-flocculation, equivalent OD values of yeast and *E. coli* expressing green fluorescent protein (GFP) were added to a galactose solution to induce flocculation. After allowing time for the flocs to settle, fluorescence was measured to determine the relative amount of *E. coli* remaining in the solution.

To test inhibitor resistance, mixed inhibitors were tested, including osmotic stress, furfural, acetic acid, and a mixed inhibitor including both furfural and acetic acids. Overall, it was observed that *S. cerevisiae* expressing *flo1* did not co-flocculate with *E. coli*, contrary to previous reports. In addition, the fermentation inhibitors also demonstrated some mild flocculation inhibition, and there was no strong evidence that the flocculant phenotype conferred any significant resistance to inhibitors compared to the non-flocculant strain. The flocculating characteristic is often included as a potential protective mechanism, or as a way to improve separation. However, this evidence sufficiently demonstrates that this phenotype does not confer these characteristics that may be beneficial for industrial production and that either significant research is required to make flocculation a useful characteristic beyond product separation.

Materials and Methods

Strains, media, and transformations

The yeast strains used in this study were derived from D452-2 (Table 2.1). *E. coli* DH5α was used to maintain and amplify plasmids. The fluorescent *E. coli* is *DH5α* expressing the EGFP-pBAD plasmid, a gift from Michael Davidson (Addgene plasmid # 54762). *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) broth while yeast strains were cultured in a synthetic

complete (SC) media with the appropriate amino acid dropout mix (MP Biomedicals, Solon, OH) at 30°C. SC media contained 0.17 % yeast nitrogen base, 0.5 % ammonium sulfate, and the appropriate amino acids dropout mix (MP Biomedicals, Solon, OH). *S. cerevisiae* was transformed using the LiAc/PEG method [24]. A single colony was picked and cultured in SC media containing 20 g/L glucose.

Co-flocculation Assay

To test co-flocculation, yeast were grown in SC-ura (synthetic complete media without uracil) and *E. coli* in LB with arabinose to induce fluorescence. Cells we washed two times with double distilled water and re-suspended to 1 ml. Constitutively flocculant cells required an EDTA wash in order to re-suspend the cells. Care was taken during these initial wash processes to prevent agitation, as it would induce flocculation. Equivalent volumes of *E. coli* and *S. cerevisiae* cultures were added to a 2% galactose solution to induce flocculation (double distilled water used as the control). Initial optical density at 600 nm (OD) values were read, and the cultures were placed onto a shaker table at 30°C. To collect samples, agitations was stopped and the flocs were allowed to settle to the bottom for 10 minutes. Samples were taken from the top of the fermentation broth. OD values were collected at various time points for both the constitutive and inducible strains. Fluorescence was characterized using an excitation wavelength of488 nm and emission wavelength of 525 nma using a Biotek Synergy 2 Multi-Mode Microplate Reader (Winooski, VT). Following these initial experiments, various *E. coli*/*S. cerevisiae* ratios were attempted as well as 0.1M $CaCl_2$ being added to see if Ca^{2+} was necessary to induce flocculation.

Inhibitor Resistance Assay

Pre-cultures were inoculated into SC-ura-leu with glucose. Culture flasks contained 20 ml SC-ura-leu with xylose. Each culture was grown in duplicate. To inoculate, the 2 ml pre-cultures were centrifuged and re-suspended in 1ml double distilled water. It is necessary to note that the flocculant cells did not completely re-suspend, but that the flocs were reduced in size to the point where it was possible to pipette. For osmotic stress, concentrations of 0.5M and 1.0M NaCl were tested. For fermentation inhibitor resistance, the conditions consisted of: 2g/L of furfural, 2g/L acetic acid, and 2g/L furfural and 2g/L acetic acid. To take measurements, 200ul samples were taken from the shake flasks at various time points and sugar and ethanol concentrations were analyzed via HPLC.

Results

The co-flocculation assay involved measuring the fluorescence of the *E. coli* suspended in the solution after the flocs were allowed 10 minutes to settle. While there was a decrease in fluorescence and OD for the cells with the flocculating yeast, there was not a significant difference when interacting *E. coli* with flocculating and non-flocculating yeast (data not shown). Although the yeast appeared to be flocculating, we further attempted to induce flocculation by adding 0.1M $CaCl_2$ to the media; however, there was still no significant difference between the *E. coli* with the flocculating and non-flocculating yeast (Figure 2.1). Several variations of the *E. coli*: *S. cerevisiae* ratio were used in order ensure that the *E. coli* cells did not completely flood the flocculating *S. cerevisiae (data not shown).* Once again, no significant difference was seen between the flocculating and non-flocculating yeast. Rather than relying on fluorescence alone, CFU counts

for *E. coli* were also recorded, but the results for these counts were repeatedly inconclusive. While there have been suggestions that a flocculating strain could be used to separate *E. coli* from a solution, this set of experiments has demonstrated no additional evidence towards this idea. While the presence of various bacteria in a solution have been shown to induce flocculation, there is not strong evidence that the yeast are actually interacting with the bacteria in the solution. It is possible that some genetic engineering may provide the surface α-mannans required for the two species to interact, the exact mechanism of flocculation is still too unclear to efficiently make these modifications. As the data currently represents, the use of flocculating yeast to separate other bacteria from a solution does not appear to be a valid option to reduce the cost of product separation for industrial fermentation.

Flocculation has also been viewed as a characteristic that could potentially confer some resistance to fermentation inhibitors that arise from the pretreatment of lignocellulosic fuels. To test the feasibility of using flocculation as a resistance mechanism, several assays were used to determine the resistance of a xylose-consuming strain of yeast engineered to constitutively flocculate. Sodium chloride, furfural, acetic acid, and a mixed inhibitor were all evaluated. Under variable NaCl concentrations, the flocculant strains consistently had a lower ethanol production and lower glucose consumption, including the no salt control condition (Figure 2.2). As expected, at high concentrations of 1M NaCl, not only was fermentation significantly affected, flocculation was also inhibited. For 0.5M NaCl, the ethanol produced was similar to that of the control strain, however this was not demonstrated to be significant. While this may indicate a minor resistance to NaCl, it is unclear whether this resistance is usable due to the repression of flocculation at higher concentrations.

Resistance to acetic acid, furfural, and mixed acetic acid and furfural yielded similar results (Figure 2.3). Each respective strain was added to 20 g/l Xylose SC-Ura/Leu media with: no inhibitor; 2 g/l acetic acid; 2 g/l furfural; or 2 g/l of both acetic acid and furfural. Under all three conditions, consumption of xylose and ethanol production were essentially negligible (Figure 2.3). While the flocculant yeast have a slightly lower xylose consumption regardless, the presence of furfural and acetic acid did also mildly affect flocculation, however distinct flocs were still visible in the solution, unlike in the 1M NaCl condition. While it is still possible that flocculation can be used as a separation mechanism, overall, flocculation during fermentation appears to have a more detrimental effect rather than a useful one.

Discussion

The characteristics of flocculation have been shown to be highly variable. In some cases, flocculant strains are resistant to osmotic stress, heat stress [15], fermentation inhibitors [23, 25], acetic acid [26], and low temperatures [27]. In addition, the physical interaction of flocculant yeasts also varies, ranging both in how the flocs suspend in the culture to the physical size of the flocs [11, 28]. The inconsistency and variation of these strains is often cited as a difference in the genetic background of the host [9]. In part, this is because the exact mechanism of flocculation and the regulation of flocculation is not entirely known. In order for flocculation to be used effectively for industrial production for more than just product separation, it is necessary to better characterize flocculant phenotypes and the proteins involved.

Part of the characterization of the mechanism of flocculation is to determine whether or not it is possible for yeast to form flocs with various bacteria. While it has been shown for yeast

to flocculate with other yeast species [19, 29, 30], the evidence of co-flocculation with non-yeasts is minimal and is mostly related to interactions with *Lactobacillus*, a common bioethanol contaminant [31-33]. There have been many studies where flocculation was found to be induced by the presence of various bacteria, but this is thought to be advantageous to the bacteria rather than the opposite being true. Bacteria are thought to induce yeast flocculation in order to reduce yeast consumption of nutrients, leaving more available for the bacteria to consume [34]. In fact, bacterial contamination inducing flocculation is seen as a common problem of industrial fermentation [34, 35]. It may be possible to engineer bacteria to contain the α-mannans necessary for the yeast to interact, and some research has indicated genes that influence mannan structure [36]. Although mixed co-cultures are becoming more common, there is a lack of evidence that flocculation will be a solution to separate all species from the solution. However, there may be use of utilizing reversible yeast flocculation in order to better control culture concentrations. The process could potentially be used as a self-regulatory mechanism to maintain cycling of a co-culture solution. Yeast may flocculate, allowing the bacterial cultures to bloom, then yeast may resuspend and bloom while the bacterial culture dies down. This use is highly theoretical, and requires significant further research in order to be considered for industrial applications.

The relationship between flocculation and inhibitor resistance is also necessary to further characterize. Without these characterizations, it is extremely difficult to identify a causal relationship between a flocculant phenotype and the resistance to a variety of stressors. In these cases, it is possible that flocculation is an associative property that is linked to other alterations in the genetic background in the host. It is also necessary to clearly define the difference between stress tolerance, and stress survival. Flocculation may aid more in stress survival. This could be why heat-killed cells remain flocculant [3, 4], which could aid in protecting cells within the floc

from outside stressors. The inner cells would be protected from the external stressors, but the available nutrients would also be limited, reducing fermentation. Cell survival is still a very valuable characteristic to re-pitch yeast following fermentation and separation [37-39], but may not be as useful during fermentation.

Using flocculation as part of the fermentation process does have potential to reduce costs and improve separation, however the use for co-flocculation with non-yeast and inhibitor resistance do not appear to have the same benefits. Flocculation may help to re-pitch yeast that has been exposed to inhibitors, and inducible or reversible flocculation may be useful to separate yeast from a fermentation broth. In addition, flocculation may be useful for techniques that involve accumulation within the cell, such as the removal of heavy metals from a solution [3-6]. A better understanding of the regulation of flocculation in the presence of certain bacteria may also allow for some control over co-culture concentrations. However, significant work is still necessary in order to make flocculation an effective part of the industrial fermentation process.

Table 2.1. Plasmids and strains used in this study.

Name	Properties	Reference
Escherichia coli		
DH5α	*F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ-thi-1 gyrA96 relA1*	Invitrogen
AEGFP	*DH5alpha:: EGFP-pBAD*	Addgene plasmid # 54762
Saccharomyces cerevisiae		
INVSc1	*MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52*	Invitrogen
ML01	*INVSc1::pML01*	This study
ML02	*INVSc1::pML02*	This study
ML03	*INVSc1::pML03*	This study
ML04	*INVSc1::pML04*	This study
KBF1XP	*INVSc1::pXP, pKBF1*	This study
KBE5XP	*INVSc1::pRS416, pKBF1*	This study
Plasmids		
pRS416	*Ura3*	ATCC
pRS415	*Leu2, ampR*	ATCC
pXF3X07	pRS416-PDC1p-csXR-ADH1t-TEF1p-ctXDH-CYC1t-ENO2p-ppXKS-ADH2t	[40]
pML01	pRS416- GAL1p-FLO1-CYC1t	This study
pML02	pRS416- GAL1p-FLO1-CYCt-GAL1p-FLO8-PCD1t	This study
pML03	pRS416- TEF1p-FLO1-CYC1t	This study
pML04	pRS416- TEF1p-FLO1-CYC1t-PCD1p-FLO8-PCD1t	This study
pKBF1	pRS415- TEF1p-FLO1-CYC1t	This study
EGFP-pBAD	pBAD- *Egfp*	Addgene plasmid # 54762

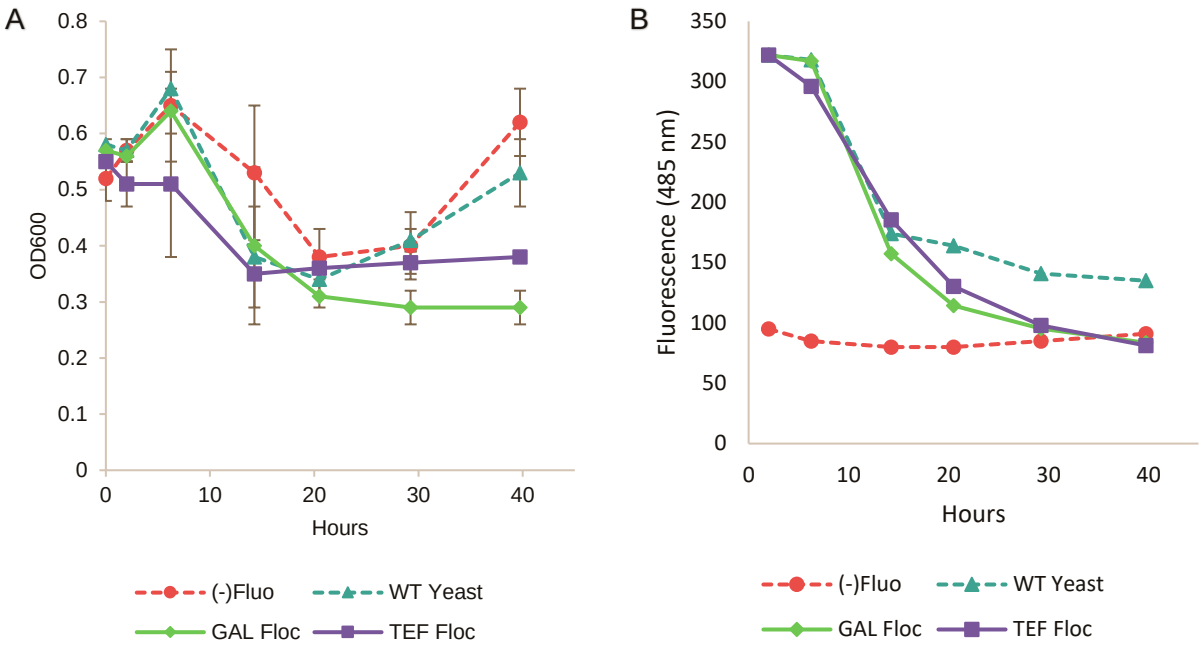

Figure 2.1. E. coli fluorescence with flocculant yeast.

Figure 2.1. (-) Fluo indicates the *E. coli :S. cerevisiae* mixture with uninduced fluorescence and no flocculation. WT Yeast indicates the co-culture with non-flocculating yeast. GAL Floc indicates the galactose-induced flocculation and TEF Floc indicates the constitutively expressed flocculation. (A) The growth profile, note that OD_{600} is used for both yeast and *E. coli*, so this just represents the density of microbes suspended in the solution. (B) Measurements of *E. coli* fluorescence over time. Data was normalized to the 6 hour mark to account for initial differences in fluorescence measurements while the OD values were similar.

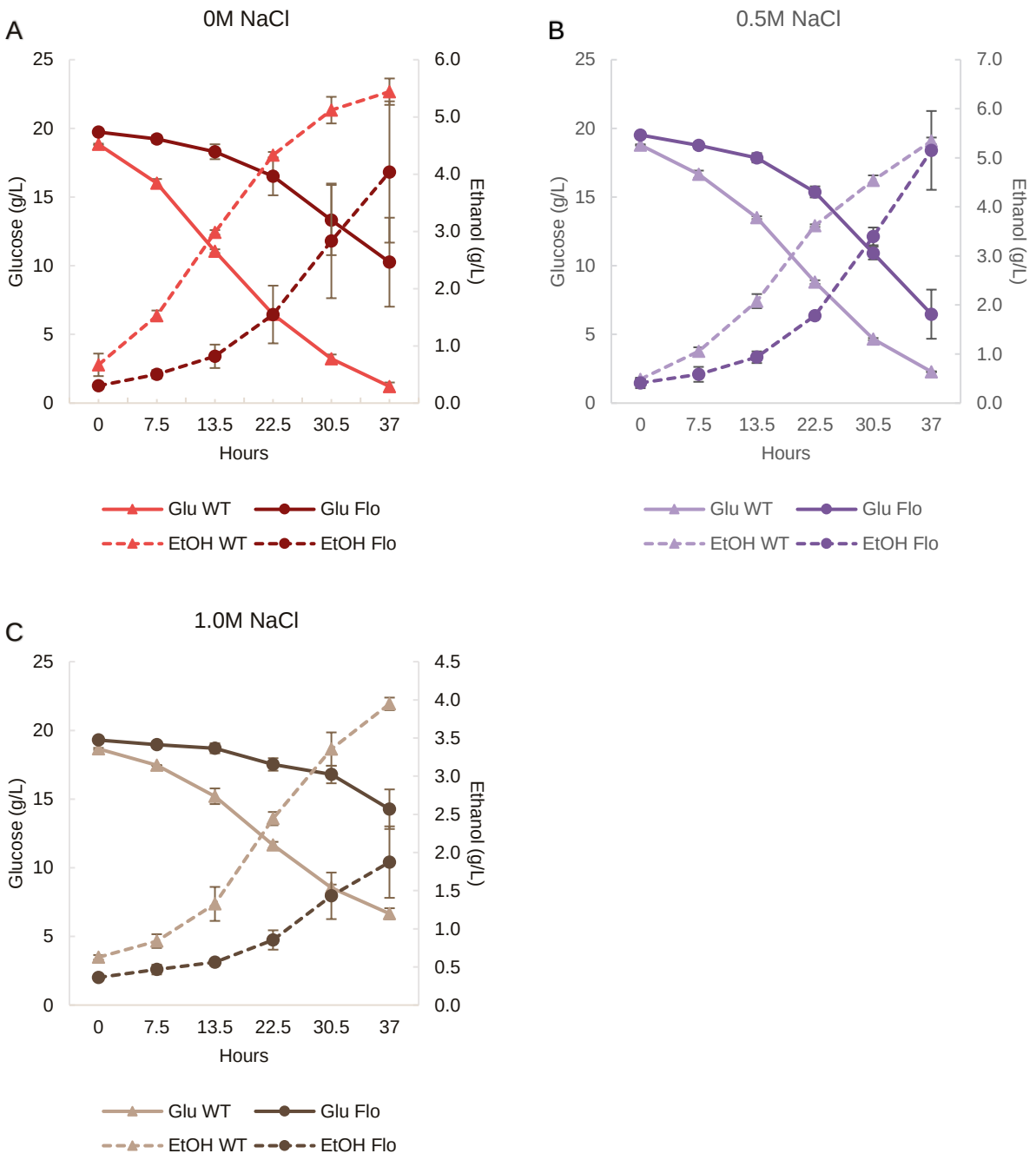

Figure 2.2 Osmotic stress tolerance of flocculant S. cerevisiae

Figure 2.2. Osmotic stress tolerance of constitutively flocculant *S. cerevisiae*. 'WT' indicates the non-flocculant strain, and 'Flo' indicates the constitutively expressed flocculant strain. 'Glu' indicates the glucose consumption and EtOH the ethanol production. (A) shows the 0M NaCl control sample, (B) 0.5 M NaCl, and (C) 1.0M NaCl.

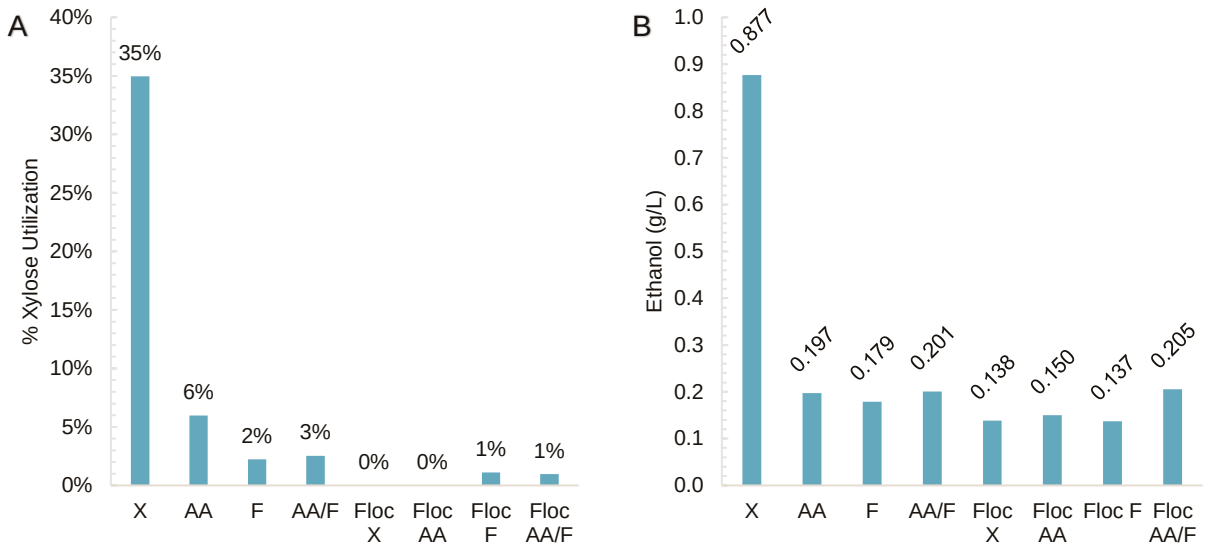

Figure 2.3. Acetic acid and furfural resistance of flocculant yeast.

Figure 2.3. Acetic acid and furfural resistance of flocculant yeast. X indicates the non-flocculant yeast with no inhibitors and Floc X indicates flocculant yeast with no inhibitors. AA – 2 g/L acetic acid, F – 2 g/L furfural, AA/F – 2g/L acetic acid and 2 g/L furfural. Data was collected at 120 hours. (A) The percent of xylose that was utilized (from 20g/L xylose) throughout the fermentation. (B) The ethanol produced throughout the fermentation.

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Metabolic Engineering of *Saccharomyces cerevisiae* to Produce Long Chain and Medium Chain Fatty Alcohols from Xylose

Bland, K., Sheng, J., Senger, R.S., Feng, X*.

Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

* To whom correspondence should be addressed. Phone: (540) 231-2974. E-mail: xueyang@vt.edu.

Abstract

Long chain fatty alcohols (>C12) are commonly used in surfactants, detergents and cosmetics; however, they are difficult to produce chemically. The production of fatty alcohols from renewable lignocellulosic sources is preferred; although, the extraction methods result in mixtures of xylose and glucose. Overexpression of a xylose utilization pathway, combined with a peroxisome-targeted fatty acyl-CoA reductase (TaFAR) yielded a *S. cerevisiae* strain capable of high levels of fatty alcohol production from xylose. It was found that xylose feedstocks, as opposed to glucose, result in higher yield (g/g) fatty alcohol production. In this study, we specifically characterized the differences between the metabolism of glucose and xylose based fatty alcohol production. By analyzing conventional engineering methods with ^{13}C-based metabolic flux analysis (^{13}C-MFA), we are able to determine key pathway shifts that occur. When using xylose as a carbon source, the flux of the pentose phosphate pathway increased, while the flux towards the byproduct ethanol decreased significantly. Fermentation in minimal media, allowing for ^{13}C-MFA studies, resulted in 0.033±0.003 g/g fatty alcohol in glucose media, while fermentation of xylose yielded 0.076±0.003 g/g fatty alcohol. However, using high cell density batch fermentation in YPAX media produced 608.52±9.66 mg/L long chain fatty alcohols.

Key Words

metabolic flux analysis; *S. cerevisiae*, lignocellulosic fermentation, xylose, fatty alcohols

Introduction

Interest remains to develop renewable sources of fuels and chemicals through the use of engineered microbes and fermentation. Currently, most bio-based chemical production is derived from corn or sugar cane [1]. These are less than ideal sources, largely due to the food vs. fuel debate. However, perennial lignocellulosic sources such as *Miscanthus*, switchgrass, and willow have the advantage of more simplistic farming and the ability to grow continually in low-nutrient soils in which food crops are unable to grow [2, 3]. However, there are key challenges associated with the use of lignocellulosic biomass as a carbon source for fermentation. For example, lignocellulosic material requires pretreatment to release sugars, which can lead to accumulation of microbial growth inhibitory chemicals such as furfural and acetic acid [4, 5]. In addition, sugars released from lignocellulose are a mixture of hexoses and pentoses, with the most common pentose being xylose. While common industrial yeast strains, such as *Saccharomyces cerevisiae*, do not utilize xylose natively, there has been significant work to improve the xylose utilization capacity [6-9]. While significant strides have been made, xylose fermentation still continues to suffer slower growth than typical glucose fermentation [10].

While *S. cerevisiae* does not utilize xylose natively, this has become an engineered trait [11-13]. However, it is essential that these strains are capable of mixed-sugar fermentation, as this is a more realistic representation of lignocellulosic feedstocks. Recently, the strain EJ4 was engineered to contain a xylose reductase (XR), xylitol dehydrogenase (XDH), and xylulose kinase (XK) [14]. Additional evolutionary engineering was used to further optimize the strain to co-utilize cellobiose and xylose to produce ethanol [14]. However, xylose fermentation to produce ethanol is generally much lower than fermentation using glucose [15-18]. While this is expected to a degree, this same deficiency is not seen for the production of other bio-based chemicals [19-21].

This is thought to potentially be related to the Crabtree effect, where yeast will preferentially undergo fermentation as opposed to the respiration pathway [22-24]. There has been speculation that xylose fermentation may be more favorable to non-ethanol products, such as fatty acid derived products.

Medium chain fatty alcohols (C8-C12) are commonly used in a variety of personal care products including deodorants, detergents, and cosmetics [25]. These common household products can contain between 3% to 30% fatty alcohol (or derivative) [25, 26]. MCFAs also have the potential to be used as drop-in biofuels [27]. Currently MCFA production is based on petroleum in the United States, or is extracted from sources such as palm oil elsewhere [28]. The bio-based production of MCFAs provides an opportunity to more sustainably develop these common household chemicals, as well as investigate the use of lignocellulosic fermentation.

To evaluate the xylose utilization strain EJ4 for fatty alcohol production, additional modifications were made as described previously [29]. A fatty alcohol reductase (FAR) from the barn owl (*Tyto alba*) was localized to the peroxisome, along with overexpression of native PEX7 and ACC1. In preliminary work it was found that a higher yield of fatty alcohol was achieved by using xylose as a feedstock rather than glucose. This indicates a variation in metabolic pathway utilization for these two sugars that is not well understood.

To elucidate the difference between the metabolism of glucose and xylose fermentation, ^{13}C-based metabolic flux analysis (^{13}C-MFA) was used. ^{13}C-MFA can be used to trace the labeled carbon from the sugar source as it is distributed throughout the cell by measuring the fractionation patterns of labeled amino acids [30]. This process quantifies metabolic flux through pathways involved in central metabolism [31-33]. By comparing ^{13}C-MFA results of both glucose and xylose

fermentation, we were able to determine the key differences in how the cell utilizes these two sugars, which can be used to guide further metabolic engineering strategies to improve the fatty alcohol production of these strains. While the use of ^{13}C-MFA is informative, a minimal media was required of these experiments which also reduced the production of medium chain fatty alcohols. In addition to the ^{13}C-MFA experiments, various medium formulations were tested to stimulate higher production of fatty alcohols. In addition, these formulations were also used to validate some of the metabolic bottlenecks identified by ^{13}C-MFA results. As a result, a final fatty alcohol titer of 0.6 g/L was achieved.

Materials and Methods

Yeast strains, plasmids, media, and transformations

The yeast strains used in this study were derived from D452-2 (Table 3.1). *E. coli* DH5α was used to maintain and amplify plasmids. *E. coli* strains were cultured at 37°C in Luria-Bertani (LB) broth, and yeast strains were cultured in a synthetic complete (SC) media with the appropriate amino acid dropout mix (MP Biomedicals, Solon, OH) at 30°C. SC media contained 0.17 % yeast nitrogen base, 0.5 % ammonium sulfate, and the appropriate amino acids dropout mix (MP Biomedicals, Solon, OH). Three media were used for fermentations: minimal media, YP media, and the SC media. The minimal media formulation has been reported previously [34]. YPAD or YPAX media contains (1% yeast extract, 2% peptone, 0.01% adenine, and 2% dextrose or xylose). *S. cerevisiae* was transformed using the LiAc/PEG method [35]. A single colony was picked and cultured in SC containing 20 g/L glucose.

Fermentation Methods

For the ^{13}C-MFA fermentation, the yeast strains were first grown in 100 mL SC medium, including all the appropriate nucleotides and amino acids, with 20 g/L glucose for 2 days. Then, cells from 5 mL of culture were centrifuged, washed twice with double-distilled water, and inoculated into 5 mL of the appropriate media in disposable tubes overlaid with 0.5 mL dodecane for batch fermentation. Samples were taken at various time points to measure the 1-hexadecanol concentration, OD_{600}, and xylose concentration. At each time point, the yeast cultures were allowed to sit for 2 min until the organic layer could be visualized clearly. To measure the 1-hexadecanol concentration, 3 μL of dodecane was withdrawn from the organic layer and then diluted by 100 times using ethyl acetate followed by analysis using the GC–MS protocol described below. To monitor OD_{600}, 20 μL of yeast culture was taken from the water layer and mixed with 180 μL of double-distilled water, followed by measuring the absorbance at 600 nm using a Biotek Synergy 2 Multi-Mode Microplate Reader (Winooski, VT). To measure the concentration of xylose, 100 μL of yeast culture was taken from the water layer and mixed with 900 μL of double-distilled water, which was then centrifuged at 13,000 rpm for 5 min. The supernatant was taken and analyzed by Shimadzu HPLC (Columbia, MD) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) and Shimadzu RID-10A refractive index detector. The column was kept at 50 °C, and 5 mM sulfuric acid solution was used as a mobile phase with a constant flow rate of 0.6 mL/min. The biological triplicates were implemented in both batch and fed-batch fermentation for all strains.

^{13}C-MFA Methods

The isotopic labeling experiments followed a previously developed protocol [31, 36]. In

general, EJ4XF was cultured under oxygen limited conditions in minimal media containing either 10 g/L ^{13}C- xylose [1-^{13}C] or 10 g/L ^{13}C-glucose (a mixture of 80% [1-^{13}C] and 20% [U-^{13}C] glucose) as the sole carbon source. The cell growth was monitored by $OD_{600,}$ and high performance liquid chromatography (HPLC) was used to assess levels of glucose, xylose, ethanol, glycerol, and acetic acid. The ^{13}C-labeled culture was harvested mid-exponential phase and hydrolyzed with 6M HCl. Tetrahydrofuran and N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich) were used to derivatize amino acids to form tert-butyl dimethylsilyl (TBDMS) derivatives. The derivatized amino acids were then analyzed using gas chromatography-mass spectrometry (GC-MS) (Shimadzu GC2010 GC with a SH-Rxi-5Sil column and a Shimadzu QP2010 MS). Three charged fragments are detected by GC-MS: (i) the [M-57] + group (containing unfragmented amino acids); and the (ii) [M-159] + or (iii) [M-85] + group (containing amino acids that had lost an α-carboxyl group). The labeling pattern (mass distribution vectors, MDVs) were represented by fractions of non-labeled, singly-labeled, and doubly-labeled amino acids. A previously reported algorithm [37] was used to correct for the effect of natural isotopes on the labeling pattern.

The MDVs were used to calculate the summed fractional labeling (SFL) values, which were directly used to calculate the metabolic fluxes with the Biomet Toolbox 2.0 in MATLAB® (MathWorks, Inc., Natick, MA) [38] The central carbon metabolic model used for ^{13}C-MFA calculations was developed previously [36] and based on the KEGG database (http://www.genome.jp/kegg/) [39]. The flux estimation had at least 50 repetitions with variable initial values generated by a genetic algorithm in order to find a likely global solution. The ATP, NADH, and NADPH production and consumption rates were calculated from the fluxes by following previously established protocols [36, 40]. The unpaired Student's t-test ($\alpha = 0.05$) was

used as the statistical method to determine the significance of the net productions of ATP and cofactors for the different strains/conditions tested.

Fatty Alcohol Measurements

Fatty alcohols were detected using a previously developed method [41], and the protocol is described briefly below. In summary, strains were precultured in 3 mL of appropriate media and the cells were inoculated into 5 mL of fresh media overlaid with 10% dodecane to prevent the evaporation of fatty alcohols and to enrich fatty alcohols in the organic layer. The concentration of fatty alcohols was quantified at 48 hours. The glass tubes of cultures were allowed to sit for 2 minutes until the organic layer was visualized clearly. Then, 2uL of dodecane was withdrawn from the organic layer and diluted (idk how many times) using ethyl acetate. The solution was then analyzed by GC-MS (Shimadzu GC-MS-QP2010) with a DB-Wax column with 0.25 um film thickness, 0.25 mm diameter, and 30m length (Agilent Inc., Palo Alto, CA). Tridecane at 2mg/L was used as the internal standard. The GC program was as follows: an initial temperature of 50 °C was maintained for 1.5 min, followed by ramping to 180 °C at a rate of 25 °C/min. The temperature was then ramped to 250 °C at a rate of 10 °C/min, where the temperature was held for 3 min.

Results

^{13}C-MFA Fermentation Yields

The use of ^{13}C-MFA allows the direct comparison of metabolic activity from xylose and glucose fermentation. The appropriate media was supplemented with either 10 g/L ^{13}C- xylose [1-

13C] or 10 g/L ^{13}C-glucose (a mixture of 80% [1-13C] and 20% [U-13C] glucose) as the sole carbon source. Cells were harvested mid-exponential phase and the labeling pattern of the amino acids was used to calculate metabolic fluxes. The growth rates on both glucose (0.084 ± 0.008 h^{-1}) and xylose (0.083 ± 0.008 h^{-1}) were measured by OD_{600} and were approximately the same at the point of cell harvesting (Table 3.2). While these growth rates were similar, the final biomass was higher for glucose fermentation, 5.20 ± 0.02 g/L, than for xylose fermentation 3.27 ± 0.92 g/L. The same pattern was found for the total yield of biomass (g/g substrate) as well, with glucose fermentation yielding 0.20 ± 0.02 g/g glucose and xylose fermentation yielding 0.12 ± 0.00 g/g xylose. Interestingly, although the biomass yield was higher for glucose fermentation, the total fatty alcohol yield was lower, yielding only 0.033 ± 0.003 g/g glucose compared to the 0.076 ± 0.003g/g xylose yield. This is consistent with previous data [21] demonstrating that xylose utilization in *S. cerevisiae* leads to a higher fatty alcohol production than glucose fermentation. This increased fatty alcohol production with xylose fermentation is also coupled with severely reduced ethanol production. Glucose fermentation resulted in a total ethanol yield of 0.49 ± 0.01 g/g glucose while xylose resulted in insignificant values (Table 3.2) .

^{13}C-MFA Results

^{13}C-MFA was used to explain the differences in fermentation yields described above. This is reflected in the calculation of the flux values, with the flux towards ethanol decreasing from 104.98 for glucose fermentation to 0.01 for xylose fermentation. The severe reduction of the flux towards the byproduct ethanol allows for increased flux towards acetyl-CoA and the desired fatty alcohols. Interestingly, this shift away from ethanol production using xylose has also been demonstrated for products such as lactic acid [20, 42] and glycerol [43]. While this is the most

striking difference between xylose and glucose fermentation, the shift in flux patterns help to elucidate possible causes of this shift. While 13C-MFA cannot necessarily provide a mechanism for the flux changes, the variations of flux values can be used to develop possible explanations.

Other key pathway changes between xylose and glucose fermentation are (i) the increased flux towards the pentose phosphate pathway, (ii) increased flux towards TCA. The increased flux towards the pentose phosphate pathway partially explains the high production of NADPH (Figure 3.1). The shift towards a higher NADPH:NADP+ ratio could help to explain the increased flux towards the fatty acid biosynthesis pathways, due to the high need of NADPH to form fatty acids. However, the buildup of NADH in the cell could also be due to the decreased flux towards ethanol, which requires 1 NADH per ethanol molecule produced. It would seem that the high availability of NADH would result in an increased flux towards ethanol, but this is not the case. Xylose fermentation results in a nearly equal consumption and production of ATP, so the increased TCA cycle would be expected in order to maintain the demands of cell growth. This is also demonstrated in Table 3.2 by the reduced biomass resulting from xylose fermentation (3.27 g/L) and glucose fermentation (5.2 g/L).

^{13}C-MFA calculations surrounding cofactor availability partially reveal why xylose may lead to a higher production of fatty alcohols, despite the lower biomass and growth rate. For glucose fermentation, there is a much higher net production of NADPH (Figure 3.2). Each 2-carbon building cycle of a fatty acid molecule requires 2 NADPH molecules, so a high net production of NADPH is expected if the flux towards fatty acid production is low. This is mirrored in the net production of NADH for glucose versus xylose fermentation. There is a much higher net production of NADH for xylose fermentation, which is what is expected due to the differences in

the flux of the TCA cycle. Interestingly, there is also a roughly equivalent consumption and production of ATP using xylose compared to the positive production of ATP for glucose fermentation. Fatty acid production is an energetically consumptive process, but the net negative ATP production indicates that xylose fermentation struggles with achieving a proper cell maintenance energy, which could explain the lower growth rate and biomass.

The energy discrepancies between xylose and glucose fermentation are also reflected in the flux calculations, as shown in Figure 3.1. The fluxes of the production of pyruvate in the mitochondria are much higher in xylose than glucose, indicating a greater need for these central metabolism reactions under xylose fermentation. Because ^{13}C-MFA requires the use of minimal media, some metabolic burden is expected, but the xylose fermentation still demonstrates a much greater need than glucose fermentation. While an "inefficiency" of ATP use is expected due to the overproduction of fatty alcohols, it is necessary to aid in the alleviation of this burden using a more optimal media to improve overall fatty acid and alcohol production.

Culture Media Screening

A disadvantage of the ^{13}C-MFA approach is the requirement to use minimal media, which limits the internal metabolic network efficiency [44]. The ^{13}C-MFA results indicated that the cell maintenance energy is a significant limiting factor for growth and the production of fatty alcohols. To alleviate this bottleneck, a basic culture medium optimization survey was used to identify how the availability of nonessential nutrients would impact xylose utilization and fatty alcohol production. Minimal media with variable mixtures of glucose and xylose were used to determine whether mixing sugars impacted the xylose utilization and the fermentation profile (Figure 3.3).

There was no significant difference in the xylose consumption of the 5 g xylose / 5 g glucose and the 7 g xylose / 3 g glucose mixtures. There was also very little difference between the fatty alcohol production of the 3 minimal media mixtures. This helped indicate that this strain is able to utilize xylose to produce fatty alcohols efficiently, even in the presence of glucose, which is used preferentially to xylose. Only high cell density growth was able to significantly impact the percentage of xylose consumed. However, the use of SC and YPAX media with 10 g/L xylose also improved the xylose consumption in addition to improving the total fatty alcohol production. These results were expected, as SC and YPAX media subsequently contain a larger portion of nonessential nutrients that can help reduce the metabolic burden within the cell. These results also indicate that the provision of nonessential nutrients, as well as improved cell density positively impact both xylose consumption and fatty alcohol production rather than reverting to a more glucose-like metabolism with a large shift towards ethanol production.

Fed Batch Fermentation

With evidence that addition of nonessential nutrients results in increased in fatty alcohol production, instead of ethanol production, a fed batch fermentation was designed to determine how this mode of growth and nutrient addition would contribute to fatty acids production. In addition, it is also necessary to identify whether the severe diversion of flux away from ethanol was due to minimal media conditions, or whether this metabolic flux distribution is also likely under more ideal fermentation conditions. Fermentation was done using YPAX media with 10 g/L xylose, and xylose was replenished at 33, 73, and 97 hours. Cells were inoculated as a high cell density batch in test tube conditions.

The use of ideal growth conditions in fed batch configuration significantly increased the growth and fatty alcohol production of the strain (Figure 3.4). While ethanol production did increase, it was still much lower than is typically seen using glucose fermentation [29]. Overall, the strain was able to produce a total of 608.6 ± 9.7 mg/L long chain fatty alcohols. Approximately 32% was represented by 12C and 68% C16.

Discussion

^{13}C-MFA is a powerful tool that can elucidate complex metabolic activity, particularly in genetically modified organisms. Seemingly small environmental changes, such as the use of xylose over glucose, have a significant impact on the degree to which different metabolic pathways are used. ^{13}C-MFA can guide researchers to identify key bottlenecks that can potentially be alleviated in order to achieve these higher production levels [35, 44-46]. However, it is also essential to understand the limitations of this particular technique. ^{13}C-MFA requires the use of minimal media, so it can be difficult to determine influences of nonessential (and sometimes undefined) nutrients, as opposed to differences related to the use of a traceable sugar substrate.

In this study, the use of xylose significantly reduced the flux towards ethanol while increasing the fatty alcohol production. This result is ideal, particularly due to the lower cost and improved land use implications of lignocellulosic biomass. However, there are still challenges associated with lignocellulosic production. As demonstrated in this study and others [9, 36] the use of xylose does result in a lower growth rate. In addition, the production of fatty alcohols demonstrated in this study is still below the theoretical maximum.

Only a combination of many techniques and genetic modifications will lead to organisms

capable of producing fatty alcohols at industrially profitable levels. Techniques such as localization of fatty alcohol production pathways within the cell, fine tuning the expression of xylose related genes, and proper identification of bottlenecks are all necessary to build upon to generate an industrially-relevant strain.

This study helps to identify that the use of xylose results in a diversion of flux from ethanol flux towards the respiratory pathways, also leading to higher production of fatty alcohols. The use of xylose seems to bypass the regulatory mechanisms in yeast that favor ethanol production when glucose is readily available. However, the exact mechanism of bypassing the Crabtree effect is still unclear. Father research to understand the mechanism of glucose and xylose regulation may allow for a more controlled engineering to produce products of interest.

Conclusions

This work represents the first time that ^{13}C-MFA has been used for xylose-based fatty alcohol production. The ^{13}C analysis demonstrates the differences in flux that lead to xylose producing lower quantities of ethanol, and higher quantities of fatty alcohol per gram of sugar. However, xylose fermentation does impair growth compared to glucose fermentation, and ^{13}C-MFA indicates that the cell maintenance energy is still a limiting factor for the production of fatty alcohols. With improving living conditions conferred by more ideal media and fermentation conditions, the xylose fermentation still results in a much lower ethanol production, indicating that the flux in minimal media is likely similar to that in a more ideal growth condition.

The use of ^{13}C flux analysis also indicates the limitation of cofactor availability which results in a limited FAOH production. While the xylose fermentation results in a greater overall

consumption of NADPH, the production is similar between glucose and xylose, indicating that there is a need of NADPH not being met to further improve fatty alcohol production. Overall, with more ideal growth conditions, we were able to produce a total of 608.6±9.7 mg/L medium chain fatty alcohols from xylose.

Table 3.1. Strains and plasmids used in this study

Strain	Properties	Reference
E. coli		
DH5α	*F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ-thi-1 gyrA96 relA1*	Invitrogen
S. cerevisiae		
D452-2	*MATa, leu2, his3, ura3, can1*	[45]
SR8	D452-2 expressing *XYL1*, *XYL2*, and *XKS1* through integration (two copies for each gene), evolutionary engineering in xylose-containing media, and *ALD6* deletion; point mutation Gly253Asp in *PHO13* gene	[46]
EJ4	SR8 leu2::LEU2 pRS405-gh1-1ura3::URA3 pRS406-cdt-1, evolved by repeated transferring in cellobiose-containing media	[14]
XFEJ4	EJ4::p10FAOH	This study
Plasmids		
pRS410	neoR (select with G418)	Addgene plasmid #11258
P10FAOH	pRS410 - ENO2p-TaFAR-SKL-KI-QL-ENO2t-TPI1p-PEX7-CYC1t-TEF1p-ACC1-TEF1t	This study

Table 3.2. Fermentation profile of glucose and xylose fermentation.

		Final titer (g/L)			Yield (g/g substrate)		
Sugar	Growth rate (1/h)	Biomass	Ethanol	Total fatty alcohol	Biomass	Ethanol	Total fatty alcohol
Glucose	0.084±0.008	5.20±0.02	6.74±0.11	0.54±0.02	0.20±0.02	0.49±0.01	0.033±0.003
Xylose	0.083±0.008	3.27±0.92	0.00±0.00	0.55±0.05	0.12±0.00	0.00±0.00	0.076±0.003

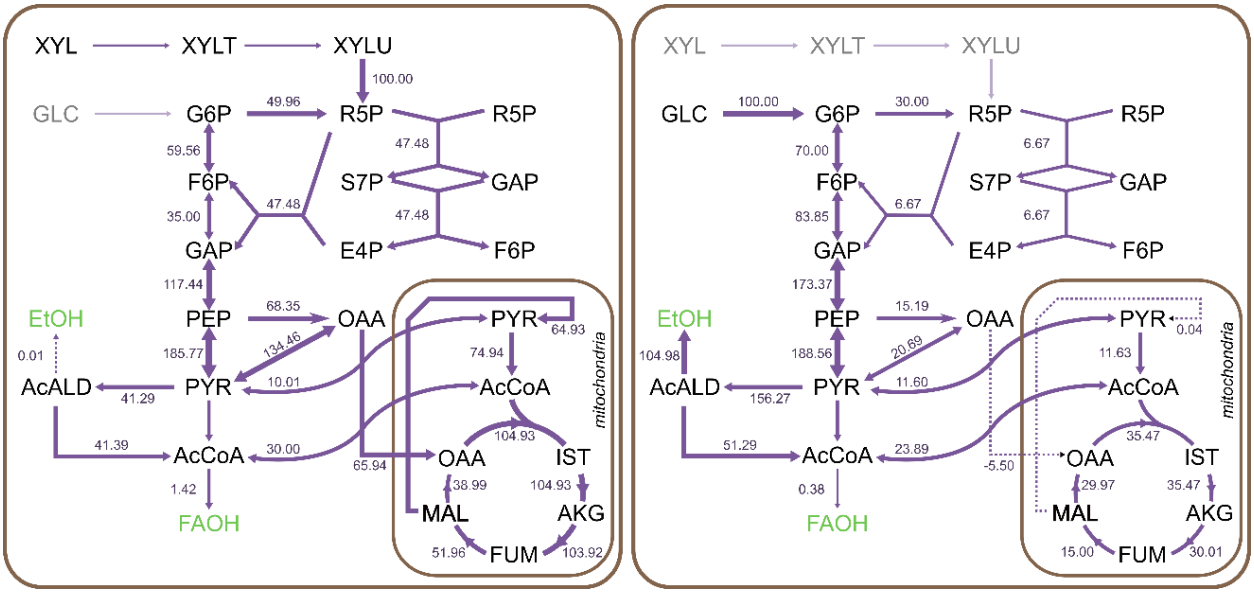

Figure 3.1. 13C Metabolic flux of xylose and glucose fermentation.

Figure 3.1. Metabolic fluxes during xylose and glucose fermentations, as determined by ^{13}C-MFA. (Left) Xylose fermentation and (Right) Glucose fermentation. Ethanol and fatty alcohols are highlighted in green to represent the key end products. The GLC or XYL pathways are faded to show that these pathways were present, but not utilized due to the sugar present.

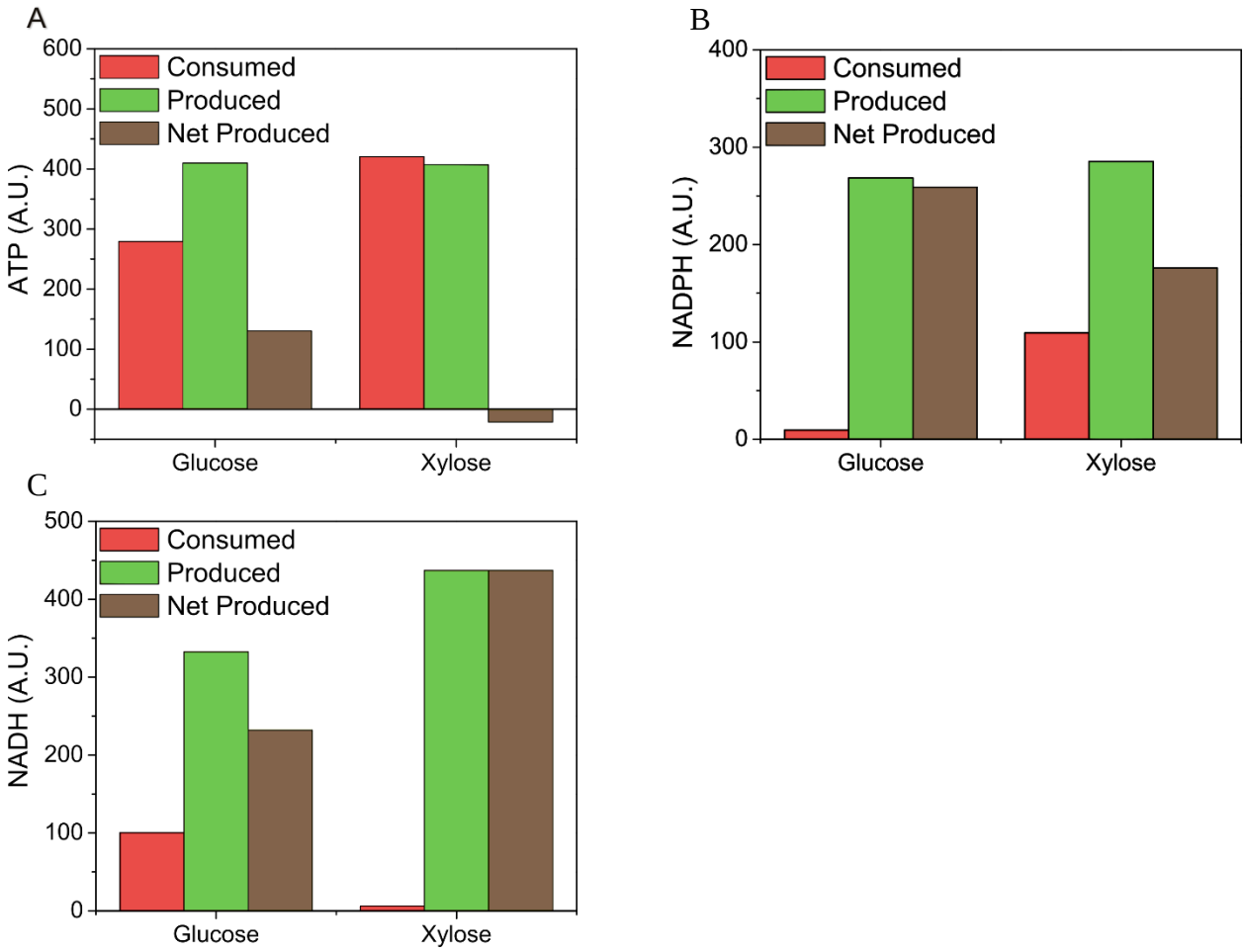

Figure 3.2 Calculated cofactor availability for glucose and xylose fermentation.

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Figure 3.2. ^{13}C-MFA calculated cofactor availability for glucose and xylose fermentations. (A) Net ATP (B) net NADPH and (C) net NADH.

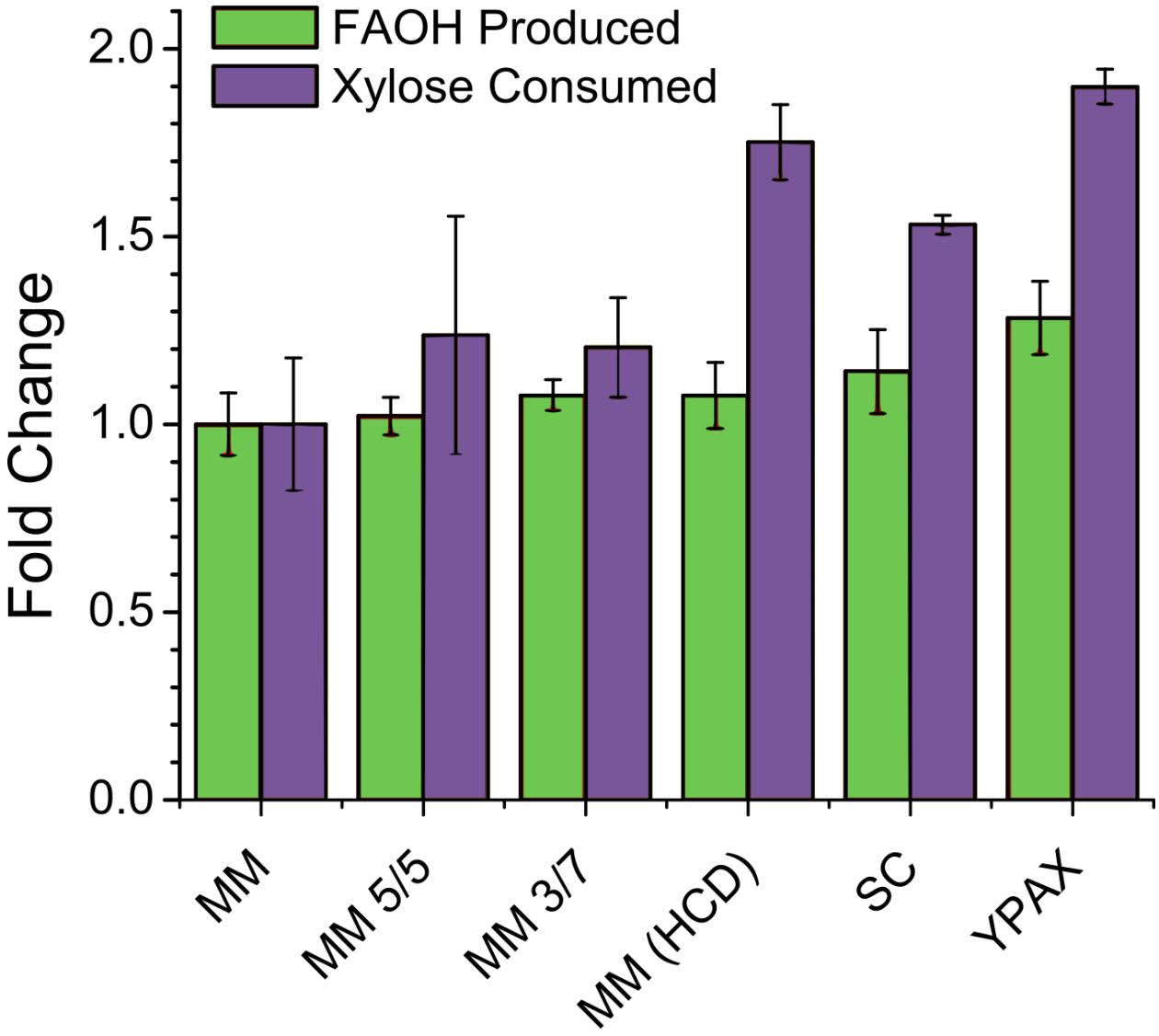

Figure 3.3 Effect of variable media conditions on FAOH production.

Figure 3.3. Effect of variable media conditions on FAOH production. MM indicates minimal media with 10g/L xylose. MM 5/5: minimal media with 5g/L glucose and 5 g/L xylose. MM 3/7: minimal media with 3 g/L glucose and 7 g/L xylose. MM(HCD): Minimal media with 10g/L xylose, grown as a high cell density culture. SC: Synthetic complete media with 10g/L xylose. YPAX: YP media with 10g/L xylose.

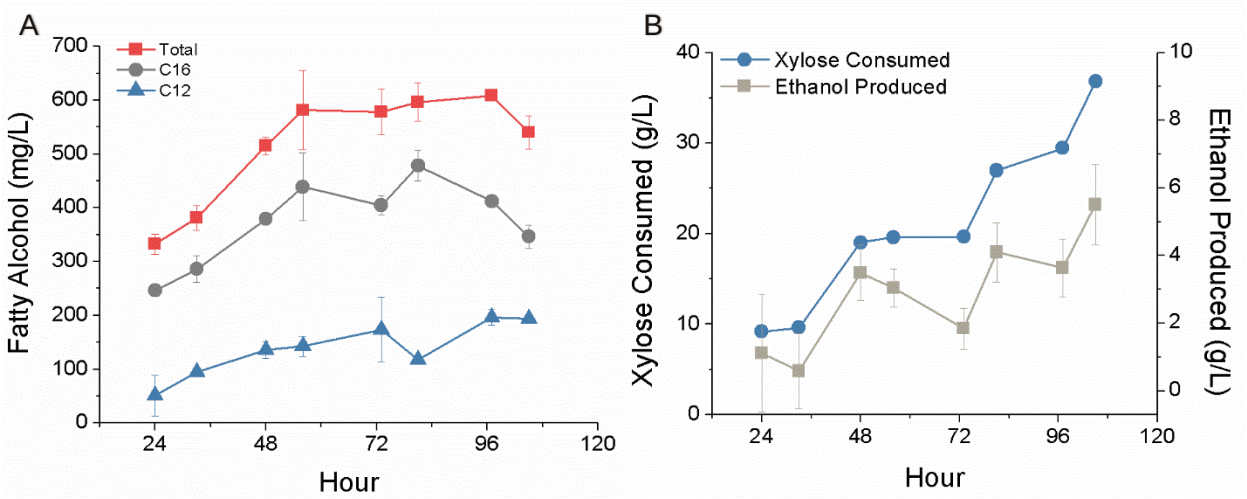

Figure 3.4 FAOH production profile of xylose fermentation.

Figure 3.4. FAOH production profile of fed-batch xylose fermentation. 10g/L xylose was added every 12 hours. (A) The fatty alcohol production profile of the fermentation. C16 and C12 fatty alcohols were the only fatty alcohols present in significant amounts. (B) Xylose consumption and ethanol production.

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General Conclusions and Future Research

Microbial production of biofuels and other important chemical precursors could serve as a way to sustainably produce these compounds. However, bio-based production is not innately sustainable so it is essential that further research improves the variety of feedstocks that can be used for fermentation. The use of lignocellulosic feedstocks would justify the sustainability of biofuels and reduce cost; however, significant work is still needed to make lignocellulosic biomass a competitive feedstock.

Because pretreatment is essential for lignocellulosic biomass, it is necessary to improve the resistance of microbes to the byproducts of this pretreatment process. While flocculation has been shown to improve cell survival, it is still unclear whether this characteristic of yeast can be used to improve tolerance to fermentation inhibitors. However, improved cell survival and decreasing the cost of separation are also key aspects of the fermentation process, and flocculation has been shown to improve both of these characteristics.

In addition to the resistance to pre-treatment byproducts, it is also essential that the microbes of choice are able to effectively utilize xylose. By using ^{13}C-MFA, we were able to identify key differences between xylose and glucose fermentation of a xylose utilizing strain of *S. cerevisiae*. The use of xylose fermentation resulted in a lower flux towards the byproduct ethanol when producing medium chain fatty alcohols. This indicates that the cofactor balance of xylose fermentation is more favorable for FAB pathways than ethanol production. While ^{13}C-MFA requires the use of minimal media, similar results were still found using a rich medium. While ethanol production increased, overall the ethanol production was much lower than would be expected under glucose fermentation.

In order to make production of MCFAs and related products a profitable and sustainable business, much more work is essential. However, research into all aspects of biofuel production are necessary. Improved pretreatment processes, genetic engineering of the feedstock crops to reduce lignin, and improved fermentation processes will all aid in the reduction of cost. In order to improve fermentation, a better understanding of the balance of cofactors for various products is necessary. By controlling the NADP/NADPH ratio in the cells, we are able to exhibit more control over the final product produced and the overall titer. Compartmentalization can also aid in improving this balance by creating local cofactor balances that are less likely to interfere with the overall cell metabolism and growth.

Future work will involve a screening of NADPH-producing enzymes to alter the balances of cofactor availability in the cell. This will include some enzymes that are localized to the peroxisome in order to test the effect of local balances as opposed to the whole cell balance. This screening would provide valuable information as to how different NADP/NADPH balances affect the overall cell growth and product formation.