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Evaluation of Fermentation at 40°C and 30°C for Cost Effective Lignocellulose to Lipid Conversion

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EVALUATION OF FERMENTATION AT 40°C AND 30°C FOR COST EFFECTIVE LIGNOCELLULOSE TO LIPID CONVERSION

A Thesis Presented

by

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ABSTRACT

EVALUATION OF FERMENTATION AT 40°C AND 30°C FOR COST EFFECTIVE LIGNOCELLULOSE TO LIPID CONVERSION

December 2018

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As the world population continues to grow, the demand for energy will continue to rise. Biofuels have become an attractive alternative to replace fossil fuels as a clean and renewable source of energy. The six- and five-carbon sugars contained in lignocellulosic plant biomass is the largest carbohydrate source in the world, and a key feedstock for sustainable biofuel production. The conversion of lignocellulose to lipids is done by using oleaginous yeast as a biocatalyst. Recently, *Arxula adeninivorans* has become a yeast of interest because of its unique properties. These include its unusual metabolic flexibility which allows it to utilize a wide range of carbon and nitrogen sources. *Arxula adeninivorans* is xerotolerant, osmotolerant, thermotolerant, and able to accumulate lipid to over 20% of its dry weight. In particular, *Arxula adeninivorans* has the ability to grow at higher temperatures than most other types of oleaginous yeast. A major cost in an aerobic industrial fermentation is heat removal from the fermenter. At higher temperatures, heat transfer from the fermenter to the external environment is

efficiently performed via evaporative heat loss in cooling towers. In comparison, lower temperature fermentation requires an electricity demanding refrigeration cycle to transfer heat using industrial chillers. By performing experiments with *Arxula adeninivorans*, I sought to evaluate whether higher temperature fermentation is more cost effective. The results suggest that biomass growth is faster at 40°C, but lipid production is better at 30°C. Even with the slight reduction in lipid production at 40°C, lignocellulosic conversion still may be cheaper at 40°C because of the many advantages of higher temperature fermentation, including lower cost heat removal, higher activity of cellulase enzymes required to break down lignocellulose into sugars, and the potential reduction in contamination risk at 40°C as well. In order to improve lipid production at 40°C with *Arxula adeninivorans*, more strain engineering may be necessary to increase lipid productivity and reduce the tendency to form hyphal cell bodies at elevated temperature.

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CHAPTER 1
INTRODUCTION

Renewable fuels and chemicals from lignocellulosic material

As the world population continues to grow, the demand for liquid fuels and chemicals will continue to rise. In 2017, the Unites States alone consumed a total of 7.26 billion barrels of petroleum products (EIA 2018). This averages to about 19.88 million barrels a day (EIA 2018). Energy consumption in China increased by 3.1% from 2016 to 2017. Global oil consumption growth averaged 1.8%, or 1.7 million barrels per day in that same time frame (British Petroleum 2017). By 2040, the world population is expected to reach 9 billion people and world energy use is projected to increase by 28% (EIA 2017).

The main sources of energy in the world today include: petroleum, natural gas, coal, nuclear and renewable energy (EIA 2017). Fossil fuels remain to be the biggest source of energy today. Fossil fuels provide energy-dense liquid fuels, such as jet fuel, and diesel for long-haul trucking, and organic building blocks for chemicals (EIA 2018). Biofuels are one of only a few alternatives to replace fossil fuels for these needs. The burning of fossil fuels has a negative impact on our environment because of the

greenhouse gases (GHG) they emit. These gases include carbon dioxide, methane, and nitrous oxide. The main GHG emitted from fossil fuel usage is carbon dioxide. In 2016, carbon dioxide emissions from the burning of fossil fuels for energy contributed to 76% of total GHG emissions (EIA 2017). Global warming is continually increasing due to GHG emissions, which is leading to severe climate change (Mohajan. 2011). To help reduce the risks of climate change, more than 100 countries have adopted policies to reduce GHG emission for limiting global warming to 2°C or below (relative to pre-industrial levels) by 2050 (Meinshausen et al. 2009). Other problems with petroleum include fluctuating oil prices and possible future supply constraints (Sims et al. 2010)

Biofuels have become an attractive alternative to replace fossil fuels as a source of energy. In particular, the conversion of lignocellulose to produce fuel and renewable chemicals is of interest (Davis et al. 2013). There are two different classifications of biofuels: first-generation biofuels and second-generation biofuels. First-generation biofuels have been around for decades and are produced primarily from food crops such as cereals, sugarcane, corn and oil seeds (Sims et al. 2010). Ethanol is a first-generation biofuel that is made from corn in the United States and from sugarcane in Brazil. Some drawbacks for first-generation biofuels include high production costs and competition for water and land usage. These limitations led to interest in second-generation biofuels. Second generation biofuels are manufactured from non-food biomass. This allows for a broader range of feedstock and production at a greater proportion for future energy demands (Schuck, 2017). Types of second-generation biomass include cereal straw, sugarcane bagasse and forest residues. Different types of waste (such as municipal) and

dedicated energy crops can also be utilized (Sims et al. 2010). Second-generation biofuels have their challenges as well. Because of its robust structure, lignocellulosic biomass must be pretreated in order to maximize the enzymatic convertibility, minimize sugar loss, maximize the production of other valuable by-products (e.g. lignin), and reduce inhibitors that are toxic to the fermenting microorganisms (Jorgensen et al. 2007) . Even with these challenges, second-generation biofuels are a promising alternative as a renewable energy source.

The six- and five-carbon sugars contained in lignocellulosic plant biomass is the largest carbohydrate source in the world, and a key feedstock for sustainable biofuel production. Lignocellulose consist of mainly three polymers; cellulose, hemicellulose and lignin, and also can contain smaller amounts of pectin, protein, extractives and ash (Bajpai, 2016). Cellulose is the most abundant constituent of the plant cell wall and is a homo-polysaccharide composed of D-glucose linked together by β-1,4-glucosidic bonds (Jorgensen et al. 2007). Hemicelluloses are complex heterogeneous polysaccharides composed of monomeric residues: D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid and 4-*O*-methyl-D-glucuronic acid (Jorgensen et al. 2007). Lignin is a complex organic polymer that cannot be readily fermented. Lignin is still valuable and can be used for other purposes such as incorporation into coatings, composites, polymers, fuel and heating, and as a reducing agent (Khitrin, 2010).

Before lignocellulose can be fermented into lipids, the lignocellulosic biomass must first be pretreated to break apart the matrix of polymeric compounds that are

physically and chemically bonded within the biomass (Davis et al. 2013). This pretreatment is typically done at 100°C in the presence of dilute sulfuric acid, where the lignocellulose is broken down into polymers such as cellulose, hemicellulose, and lignin (Davis et al. 2013). Lignin is removed after pretreatment because it cannot be readily fermented (Sun et al. 2002). The next step is to break down the polymers into fermentable sugars by hydrolysis. During hydrolysis, cellulase enzymes break down cellulose fibers into cellobiose and gluco-oligomers and eventually into glucose monomers (Davis et al. 2013). Xylanase enzymes hydrolyze hemicellulose into xylan and ultimately into xylose monomers (Davis et al. 2013).

Commercial cellulase enzymes can be expensive and are commonly derived from the fungus *Trichoderma reesei* (Banerjee et al. 2010). The optimal temperature for cellulase enzymes to break down cellulose is 48°C (Davis et al. 2013). This temperature is important because depending on what temperature the fermentation is operated can determine the type of process that is run. When glucose is hydrolyzed from cellulose, it can inhibit the cellulase enzyme activity if the concentration rises too high (Sun et al. 2002). One way to avoid this inhibition is to run a simultaneous saccharification and fermentation where the biocatalyst ferments the sugars as they are being hydrolyzed (Sun et al. 2002). This can only be done if the biocatalyst used for lignocellulose to lipid conversion can ferment at higher temperatures. If fermentations have to be operated at lower temperatures, the hydrolysis of cellulose must be completed before fermentation in a separate step (Davis et al. 2013).

Oleaginous yeast as a biocatalyst

The concept of producing single cell oil (SCO) from microorganisms has a long history dating back to the 1870s (Ratledge and Wynn, 2002). Microbial lipid production has been studied extensively for synthesis of specialty chemicals and for the production of fuels from low-cost carbon feedstocks (Friedlander et al. 2016). Oleaginous microorganisms are defined as having the ability to accumulate lipid over 20% of their dry weight (Meng et al. 2009). Oils accumulate inside the yeast under nitrogen or other non-carbon limiting conditions into subcellular compartments called lipid droplets or lipid bodies (Koch et al. 2014). These storage lipids serve as energy storage molecules and membrane precursors and usually consist of triacylglycerols (TAGs), with a certain percentage being saturated and unsaturated fatty acids (Friedlander et al. 2016). Biodiesel is made by transesterification of TAGS into fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs) with methanol or ethanol replacing the glycerol backbone of the TAG molecule (Meng et al. 2009). Some characterized species of oleaginous yeast include C*ryptococcus curvatus, Rhodosporidium glutinis, Lipomyces starkeyi,* and *Yarrowia lipolytica* (Ratledge and Wynn, 2002). *Y. lipolytica* is one of the most well-known oleaginous microorganisms and is extensively studied. Because of its genetic toolbox and genome sequence availability, *Y. lipolytica* has become suitable to be metabolically engineered (Wei et al. 2014). An engineered *Y. lipolytica* has been shown to reach a lipid content of 77% and a lipid per gram of glucose yield of 0.21 (Friedlander et al. 2016). By engineering cytosolic redox metabolism in *Y. lipolytica*, a process yield

of 0.27 g-fatty acid methyl esters/g-glucose was achieved, which constitutes a 25% improvement over previously engineered strains (Qiao et al. 2017).

Many high value lipids have been produced by metabolically engineering *Y. lipolytica*. Ricinoleic acid is an unusual hydroxylated fatty acid that can be used in several industrial applications (Beopoulos et al. 2013). Ricinoleic acid production was increased by engineering *Y. lipolytica* to accumulate ricinoleic acid to 43% of its total lipids and over 60 mg/g of cell dry weight (Beopoulos et al. 2013). Itaconic acid is another high value organic acid with diverse applications. An engineered *Y. lipolytica* has been shown to produce 4.6 g/L of itaconic acid, which is a 140-fold increase over the initial strain (Blazeck et al. 2015). Another high value product that can be made using *Y. lipolytica* is omega-3 eicosapentaenoic acid (C20:5; EPA). EPA has a wide range of benefits for improving heart health, immune system, and mental health (Xie et al. 2017). DuPont was able to engineer *Y. lipolytica* to produce EPA to levels as high as 25% of its dry weight (Xie et al. 2017).

Another yeast that has become of biotechnological interest is *Arxula adeninivorans.* This yeast is a non-pathogenic, dimorphic, haploid, ascomycetous yeast that exhibits some unique qualities (Wartmann and Kunze, 2000). Its unusual metabolic flexibility allows it to use a wide range of carbon and nitrogen sources, while also being xerotolerant and osmotolerant (Kunze et al. 2014). *A. adeninivorans* can assimilate both 5-carbon and 6-carbon sugars, which gives it an advantage over other oleaginous yeast that can't ferment both sugars. This flexibility in sugar utilization makes *A.*

adeninivorans an intriguing biocatalyst for lignocellulosic to lipids conversion. One of the interesting qualities of *A. adeninivorans* is its thermotolerance. *A. adeninivorans* has been shown to grow at temperatures up to 48° C without previous adaptation (Malak et al. 2016). At higher temperatures, an *A. adeninivorans* strain has been reported to go through a morphological transition from budding to mycelial form, which effects its gene expression and protein accumulation (Wartman at al. 2000). This ability to grow at high temperatures gives it many biotechnological advantages and potential to be a suitable biocatalyst for renewable fuel and chemical production.

Advantages of fermentation at 40°C vs. 30°C

There are many advantages to running a large-scale industrial fermentation at 40°C vs. 30°C. The biggest is the reduction in cooling cost. During an aerobic fermentation with oleaginous yeast, there is a certain amount of metabolic heat that is released because of the consumption of oxygen during catabolism of sugars (Crater et al. 2016). This exothermic reaction, estimated to be Q ($kJ/m^3/hr$) = 460 * oxygen uptake rate ($mol/ m^3/hr$), releases heat into the fermentation broth and must be removed in order to maintain constant temperature and an efficient fermentation (Crater et al. 2016). If heat isn't removed and broth temperature rises, enzymes can degrade, cells can die, and there can be significant production loss (Crater et al. 2016). Heat transfer away from a fermenter requires a contacting surface, a cooling medium, and an environment to discharge the transfer heat into (Crater et al. 2016). The three most common methods for heat contacting surfaces in a fermenter are wall cooling via vessel jacket, internal cooling

via coils, and external loop cooling via pumping broth through a heat exchanger (Crater et al. 2016). An external loop is the most efficient at heat removal design because its higher heat transfer coefficients and more surface area (Crater et al. 2016). Depending on the fermentation temperature, environmental discharge temperature, and heat contacting surface efficiently, the cooling medium can either be chilled water maintained at low temperature by a chiller, or process water transferring heat through a cooling tower (Crater et al. 2016). Use of a cooling tower can greatly lower capital and operating cost (Crater et al. 2016).

Cost reduction in the saccharification of lignocellulosic material can also be achieved at higher temperature. The activity of saccharification enzymes is optimal at 40°C-50°C and could lead to a cost savings in the amount of enzyme needed as well as the overall hydrolysis process (Suryawati et al. 2008). Some lignocellulosic fermentations use a process called separate hydrolysis and fermentation (SHF) where the saccharification stage is separate from the fermentation stage because of the higher hydrolysis temperatures needed. In a simultaneous saccharification and fermentation (SSF), the lignocellulosic material is hydrolyzed at the same time its being fermented. Fermentations at higher temperature can use the SSF process, which would reduce time and require less equipment and capital cost, while also limiting monomeric sugar inhibition of the hydrolysis enzymes (Babiker et al. 2009).

Fermentations that can operate at higher temperatures are often considered more operationally robust than fermentations at 30°C. At higher temperatures, microbial

contaminants are less likely to grow. When a reactor becomes contaminated, product is lost and sometimes the entire reactor must be restarted. This can lead to losses exceeding tens of thousands of dollars (Babiker et al. 2009). Another advantage of fermentations at 40°C is the potential for faster growth and lipid production. If growth and lipid production are faster at higher temperatures, the capital and operating cost can be lower because a smaller reactor volume is needed. The broth temperature in a reactor will have a lower viscosity at 40°C vs. 30°C. Lower viscosity is beneficial because there is better mixing and mass transfer (Crater et al. 2016). This is important for oxygen transfer because yeast cells consume a large amount of oxygen and need an oxygen transfer rate (OTR) of 100-150 mol/m^3/hour in a stirred tank bioreactor for it to be an efficient process (Crater et al. 2016). Although oxygen solubility decreases at higher temperature, the lower viscosity off-sets this, maintaining a constant OTR at increased temperature (Vogelaar et al. 2000).

Maintenance energy

During yeast growth, cells utilize sugar substrate to meet three major requirements; metabolic intermediates to form cellular material, redox potential, and energy (Lagunas, 1986). A certain consumption of energy that is independent of the specific growth rate is needed by the cells in order to maintain an active and proper physiological state (Pirt, 1982). This energy is referred to as maintenance energy. A certain amount of substrate goes towards the maintenance of the cell for things such as shifts in metabolic pathways, cell motility, defense against oxygen stress, proteomic

balance and turnover of macromolecular compounds, osmoregulation, and regulation proton gradient across cellular membranes (Bodegom, 2007). During cultivation of oleaginous yeast, cell growth is accompanied by lipid production. It is very important to define the kinetics of lipid production in order to scale-up a microbial process (Shen et al. 2013). A common technique used to study yeast kinetics is a continuous culture (chemostat) which allows for control of growth rate and cell yield (Russell and Baldwin, 1979). By continuously cultivating yeast on glucose at various dilution rates under carbon or nitrogen limitation, cell maintenance coefficient, cell mass yield, lipid yield and specific lipid formation information can be obtained (Shen et al. 2013). The maintenance coefficient is used to describe the specific rate of substrate uptake for cellular maintenance. It is important to understand the maintenance energy of cells in a particular industrial process. It's ideal for the energy to be low because maintenance energy utilizes carbon which can take away from carbon used for lipid production.

Arabinose utilization

Arxula adeninivorans can consume a number of different sugars such as glucose, mannose, xylose and arabinose (Jorgensen et al. 2007). By performing shake flasks with *A. adeninivorans*, it was observed that growth on arabinose was slower and lagging compared to other sugars. There are a couple different ways to try to improve arabinose utilization in *A. adeninivorans*. One of these methods is adaptive laboratory evolution. Adaptive laboratory evolution (ALE) is a common and effective tool for scientific discovery and addressing biotechnological needs (LaCroix et al. 2015). Using ALE, a

microorganism can be cultivated under clearly defined conditions for prolonged periods of time, which allows for it to be selected for improved phenotypes (Dragosits and Mattanovich, 2013). During an ALE experiment, mutations arise, and those that are beneficial to the selection pressure are fixed over time in the population (LaCroix et al. 2015). Improved xylose fermentation was achieved using ALE with *Saccharomyces cerevisiae* in a continuous culture in the presence of lignocellulosic inhibitors (Mans et al. 2018). By running an anaerobic chemostat on non-detoxified straw hydrolysate with 20 g/L xylose for 100 generations, a 7.5 reduction in lag phase was observed with the mutagenized *S. cerevisiae* (Mans et al. 2018).

Another way to improve arabinose utilization is by genetic engineering *A. adeninivorans* . Two key genes have been identified during arabinose catabolism in the fungus *Aspergillus niger*. The first gene *larA,* is a L-arabinose reductase enzyme that reduces L-arabinose to L-arabitol (Mojzita et al. 2010). The *larA* gene is transcribed in the presence of L-arabinose, and when *larA* is deleted in *A. niger*, reduced growth on L-arabinose is observed (Mojzita et al. 2010). The second gene *ladA*, is an L-arabitol dehydrogenase enzyme that oxidizes L-arabitol to L-xylulose (Witteveen et al. 1989). When *A. niger* was grown on L-arabinose as the sole carbon source, the enzyme activity of *ladA* was higher compared to other carbon substrates (Witteveen et al. 1989). *A. niger* converts L-arabinose into L-xylulose by two enzymes and then converts L-xylulose to D-xylulose-5-phosphate as part of the pentose phosphate pathway (Mojzita et al. 2010). The pathway can be seen in figure 1. By engineering the L-arabinose reductase and L-arabitol

dehydrogenase enzymes from *A. niger* into wild-type *A. adeninivorans*, an increase in arabinose catabolism could potentially be observed.

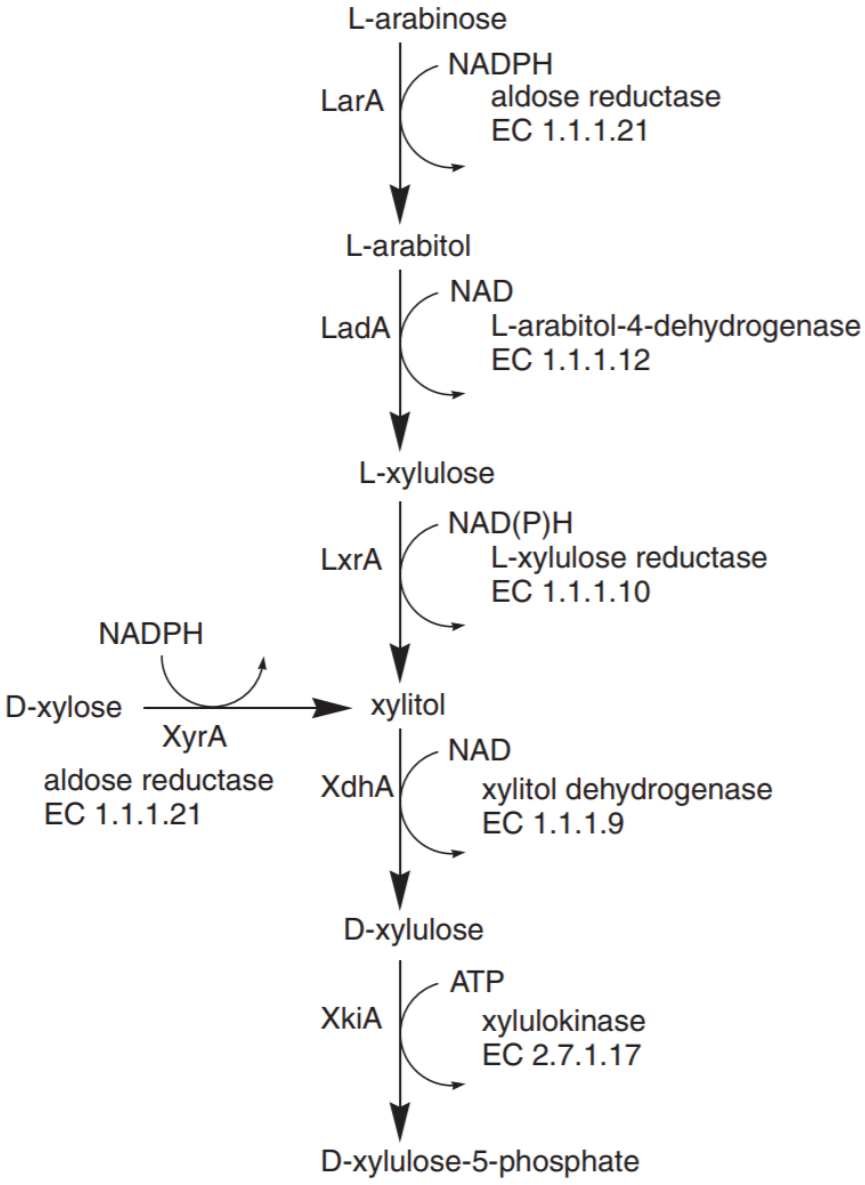


Figure 1. The *A. niger* pathway for L-arabinose and D-xylose catabolism. The first three steps of L-arabinose pathway are catalyzed by three L-arabinose specific

enzymes (LarA, LadA, and LxrA). The last two steps are shared with the D-xylose utilization pathway. D-xylulose-5-phosphate enters the central metabolism via the pentose phosphate pathway (Mojzita et al. 2010).

Thesis objective

By performing experiments with *Arxula adeninivorans*, I sought to evaluate whether higher temperature fermentation is more cost effective. Batch fermentations with *A. adeninivorans* were executed at both 30°C and 40°C on various carbon substrates. Both growth and lipid productivity were evaluated at each temperature. Continuous fermentations were performed in order to measure the maintenance of *A. adeninivorans* at both 30°C and 40°C. Strain engineering and adaptive laboratory evolution (ALE) with *A. adeninivorans* was done to try to improve arabinose utilization. Finally, a techno-economic analysis was done using a base case lignocellulosic to lipids conversion process in order to evaluate the cost at lower and higher temperature fermentations.

CHAPTER 2

MATERIALS & METHODS

List of Abbreviations

ALE- adaptive laboratory evolution

AMP- ampicillin

CER- carbon dioxide evolution rate

CTR- carbon dioxide transfer rate

DCW- dry cell weight

DGA2- diacylglycerol acyltransferase type 2

DNA- deoxyribonucleic acid

FAEE- fatty acid ethyl ester

FAME- fatty acid methyl ester

GHG- greenhouse gas

HPLC- high performance liquid chromatography

HYG- hygromycin

LadA- L-arabitol dehydrogenase enzyme

LarA- L-arabinose reductase enzyme

LFDCW- lipid free dry cell weight

NAT- nourseothricin N-acetyl transferase

NREL- National Renewable Energy Laboratory

OTR- oxygen transfer rate

RNA- ribonucleic acid

SCO- single cell oil

SHF- separate hydrolysis and fermentation

SSF- simultaneous saccharification and fermentation

TAG- triacylglycerol

Strains

The strains used in these experiments are from Novogy Inc. and include NS252, NS496, NS844 and NS1083. NS252 is a wild-type *Arxula adeninivorans (*also known as *Blastobotrys adeninivorans*) from ATCC® 76597 (Kurtzman et al. 2016). The other three strains (NS496, NS844, and NS1083) are daughter strains derived from NS252. NS496 contains an overexpression cassette of an endogenous diacylglycerol acyltransferase type 2 gene (DGA2) from *A. adeninivorans*. The DGA2 enzyme has been recognized as a key component of the lipid pathway, performing the final step of triacylglycerol (TAG) synthesis, incorporation of the third acyl-CoA onto the diacylglycerol backbone and transport into the lipid droplet (Friedlander at al. 2016). Both NS844 and NS1083 contain an overexpression cassette of the heterologous DGA2 gene from the fungus *Chaetomium globosum.* NS1083 also contains a Δ12 desaturase deletion which prevents it from making linoleic acid (C18:2).

Initial shake flask screening

All strains were cultured onto YPD plates (10 g/L yeast extract, 20 g/L bacto peptone, 20 g/L glucose, and 20 g/L agar) and grown overnight at 40°C. A 10 µL loopful of these cells was used to inoculate a 250 mL Erlenmeyer flask with 50 mL of a modified Verduyn medium consisting of glucose (150 g/L), $(NH_4)_2SO_4$ (1.8 g/L), KH_2PO_4 (3 g/L),

$MgSO_4 \cdot 7H_2O$ (0.5 g/L), potassium hydrogen phthalate (1 g/L), disodium phthalate (4.25 g/L), D-biotin (0.05 mg/L), Ca-D-pantothenate (1 mg/L), Nicotonic acid (1 mg/L), Myo-inositol (25 mg/L), Thiamine hydrochloride (1 mg/L), Pyridoxal hydrochloride (1 mg/L), p-aminobenzoic acid (0.2 mg/L), Na_2EDTA (1.5 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.45 mg/L), $MnCl_2 \cdot 2H_2O$ (0.1 mg/L), $CoCl_2 \cdot 6H_2O$ (0.03 mg/L), $CuSO_4 \cdot 5H_2O$ (0.03 mg/L),$Na_2MoO_4 \cdot 2H_2O$ (0.04 mg/L), $CaCl_2 \cdot 2H_2O$ (0.45 mg/L), at pH 5.0. The flasks were run in duplicate and cultured in a New Brunswick shaker for 72 hours at 40°C and 200 rpm. A 500 µL sample was taken from each flask 1-2 times daily and used to determine DCW (dry cell weight) and for HPLC analysis to determine sugar concentration. The average DCW and sugar concentration was determined for each duplicate flask as well as the standard deviation for each timepoint. NS844 was chosen to perform the rest of the shake flasks and fermentation experiments. NS844 shake flasks were run in the same conditions as above with either glucose/ xylose/ mannose/ arabinose (50 g/L) used as the carbon source. Mixed sugar shake flasks were also run consisting of glucose (25.2 g/L), xylose (18.2 g/L) and arabinose (6.6 g/L). (repeated above)

1-L fermentation on glucose/xylose

Flask cultures of NS844 were grown on modified Verduyn medium overnight until an OD_{600} of ~20 was reached. All 50 mL of the flask grown culture was then centrifuged and the supernatant was discarded. The cultures were then brought back up to a volume of 50 mL by adding diH_2O. The entire 50 mL washed culture was used to inoculate a 1-L volume medium in a Dasgip bioreactor consisting of either glucose or

xylose (50 or 150 g/L), $(NH_4)_2SO_4$ (1.8 g/L), KH_2PO_4 (3 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), D-biotin (0.05 mg/L), Ca-D-pantothenate (1 mg/L), Nicotonic acid (1 mg/L), Myo-inositol (25 mg/L), Thiamine hydrochloride (1 mg/L), Pyridoxal hydrochloride (1 mg/L), p-aminobenzoic acid (0.2 mg/L), Na_2EDTA (1.5 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.45 mg/L), $MnCl_2 \cdot 2H_2O$ (0.1 mg/L), $CoCl_2 \cdot 6H_2O$ (0.03 mg/L), $CuSO_4 \cdot 5H_2O$ (0.03 mg/L),$Na_2MoO_4 \cdot 2H_2O$ (0.04 mg/L), and $CaCl_2 \cdot 2H_2O$ (0.45 mg/L).The process parameters for the fermentation were pH 3.5 (controlled automatically by addition of 10 N NaOH), a temperature of either 30°C or 40°C, aeration of 1 vvm, and agitation controlled at 1000 rpm. Fermentations were run for 150 hours and 7 mL samples were taken daily. These samples were used to analyze the fermentation broth by HPLC, determine DCW, and determine lipid content by extraction. For some of the fermentations, off-gas was measured using gas sensors by BlueSens. The off-gas measurements where used to track the carbon dioxide evolution rate (CER) and oxygen transfer rate (OTR) over the course of the fermentation.

High resolution 5-liter fermentations on glucose/xylose

Flask cultures of NS844 were grown on modified Verduyn medium overnight until an OD_{600} of ~20 was reached. All 50 mL of the flask grown culture was then centrifuged and the supernatant was discarded. The cultures were then brought back up to a volume of 50 mL by adding diH_2O. The entire 50 mL washed culture was used to inoculate a 1-L volume seed fermenter in a Dasgip bioreactor. The medium for the seed reactor consisted of glucose (150 g/L), $(NH_4)_2SO_4$ (8.1 g/L), KH_2PO_4 (3 g/L),

$MgSO_4 \cdot 7H_2O$ (0.5 g/L), D-biotin (0.05 mg/L), Ca-D-pantothenate (1 mg/L), Nicotonic acid (1 mg/L), Myo-inositol (25 mg/L), Thiamine hydrochloride (1 mg/L), Pyridoxal hydrochloride (1 mg/L), p-aminobenzoic acid (0.2 mg/L), Na_2EDTA (1.5 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.45 mg/L), $MnCl_2 \cdot 2H_2O$ (0.1 mg/L), $CoCl_2 \cdot 6H_2O$ (0.03 mg/L), $CuSO_4 \cdot 5H_2O$ (0.03 mg/L),$Na_2MoO_4 \cdot 2H_2O$ (0.04 mg/L), and $CaCl_2 \cdot 2H_2O$ (0.45 mg/L). The process parameters for the fermentation were pH 3.5 (controlled automatically by addition of 10 N NaOH), a temperature of 30°C, aeration of 1 vvm, and agitation controlled at 1000 rpm. This reactor was run for 20-24 hours until the cells were growing exponentially in growth phase. Inoculum from the seed reactor was centrifuged and washed with diH_2O. The washed inoculum was used to inoculate a 5-liter New Brunswick bioreactor with a starting optical density of 1.0. The sugar concentration for the single substrate 5-liter fermentations consisted of either glucose or xylose (50 g/L). The sugar concentrations for the multiple substrate mixed sugar fermentations consisted of glucose (38 g/L), xylose (23 g/L), and arabinose (4 g/L). The rest of the medium for all reactors included $(NH_4)_2SO_4$ (1.8 g/L), KH_2PO_4 (3 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), D-biotin (0.05 mg/L), Ca-D-pantothenate (1 mg/L), Nicotonic acid (1 mg/L), Myo-inositol (25 mg/L), Thiamine hydrochloride (1 mg/L), Pyridoxal hydrochloride (1 mg/L), p-aminobenzoic acid (0.2 mg/L), Na_2EDTA (1.5 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.45 mg/L), $MnCl_2 \cdot 2H_2O$ (0.1 mg/L), $CoCl_2 \cdot 6H_2O$ (0.03 mg/L), $CuSO_4 \cdot 5H_2O$ (0.03 mg/L),$Na_2MoO_4 \cdot 2H_2O$ (0.04 mg/L), and $CaCl_2 \cdot 2H_2O$ (0.45 mg/L). The process parameters for the fermentation were pH 3.5 (controlled automatically by addition of 10 N NaOH), a temperature of either 30°C or 40°C, aeration of 1 vvm, and agitation

controlled at 600 rpm. These fermentations were run for ~70 hours and 5 mL samples were taken every 4 hours using a Seg-Flow® 1200 Automated On-line Sampling System. These samples were used to analyze the fermentation broth by HPLC, determine DCW, and determine lipid content by extraction.

1-liter chemostat fermentation

Flask cultures of NS844 were grown on modified Verduyn medium overnight until an OD_{600} of ~20 was reached. All 50 mL of the flask grown culture was then centrifuged and the supernatant was discarded. The cultures were then brought back up to a volume of 50 mL by adding diH_2O. This inoculum was used to inoculate a 1-liter continuous New Brunswick bioreactor with a working volume of 1-L medium consisting of glucose (13.5 g/L), $(NH_4)_2SO_4$ (5 g/L), KH_2PO_4 (3 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), D-biotin (0.05 mg/L), Ca-D-pantothenate (1 mg/L), Nicotonic acid (1 mg/L), Myo-inositol (25 mg/L), Thiamine hydrochloride (1 mg/L), Pyridoxal hydrochloride (1 mg/L), p-aminobenzoic acid (0.2 mg/L), Na_2EDTA (1.5 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.45 mg/L), $MnCl_2 \cdot 2H_2O$ (0.1 mg/L), $CoCl_2 \cdot 6H_2O$ (0.03 mg/L), $CuSO_4 \cdot 5H_2O$ (0.03 mg/L),$Na_2MoO_4 \cdot 2H_2O$ (0.04 mg/L), and $CaCl_2 \cdot 2H_2O$ (0.45 mg/L). The process parameters for the fermentation were pH 3.5 (controlled automatically by addition of 10 N NaOH), a temperature of either 30°C or 40°C, aeration of 1 vvm, and agitation controlled at 600 rpm A 20-liter feed consisting of the same medium was fed into the chemostat at dilution rates of 0.02, 0.04, 0.06, 0.08, 0.13, 0.15 and 0.19/hr. A waste line removed broth from the reactor to maintain a working volume of 1-liter. Enough time

was given for the reactors to go through three volume changes for each dilution before sampling. After the three volume changes the reactor was considered to be at steady state and 5 mL samples were taken every two hours over an eight-hour period. Samples were used to determine the average DCW for each dilution rate.

Analysis of fermentation broth

Samples from bioreactors and flasks were stored at 4°C until the end of each fermentation. The samples were then processed together. Centrifuge tubes were pre-weighed, and 1 mL of fermentation broth was added to each tube for each timepoint. The tubes were then centrifuged at 15,000 rpm for 2 minutes. The supernatant was used for HPLC analysis. The centrifuged cell pellet was washed with diH_2O and the tubes were centrifuged again. A hole was then punctured through the top of the tubes and they were placed into an -80°C freezer for 30 minutes. Next, the tubes were lyophilized overnight and then weighed again to determine DCW. The supernatant from the original centrifuged sample was filtered through 0.22 µM PES 13 mm disc filter. For some samples, the filtered broth was used to determine ammonium concentration using a YSI 7100 MBS. The filtered broth was then analyzed by a Dionex Ultimate 3000 containing an HPX-87H ion exclusion column (Bio Rad, Catalogue number 145-0140) with a Shodex R1-101 refractive index detector. Method conditions included a temperature of 45°C and pump speed of 0.6 mL/min with a running buffer of 5 mM sulfuric acid in HPLC grade diH_2O. Analytes that were measured include glucose, xylose, arabinose, mannose, mannitol, arabitol, erythritol, glycerol, ethanol, citrate, and acetic acid.

Lipid extraction

Samples from bioreactors and flasks were stored at 4°C until the end of each fermentation. The samples were then processed together. Dried biomass was prepared by lyophilization of 1 mL fermentation samples which were washed with diH_2O. The lyophilized cells were bead-beaten in a microfuge tube with glass beads in the presence of chloroform:methanol (1:2) (Folch et al., 1957). The entire mixture was filtered through a 0.22 µM nylon 25 mm disc into a pre-weighed glass vial. The filter was washed twice more with addition chloroform:methanol (1:2). The eluate was then blown down by air on an evaporator until all that remained in the vial was extracted oil. The oil was lyophilized to remove any residual chloroform:methanol. The resulting vial with sample was weighed, and the glass vial weight was subtracted to give a gravimetric measurement of lipid content.

Microscope and images

Cells were visualized using an Olympus BX51 microscope at 100X amplification of 1 µL of cell broth on a glass slide. Images were obtained by using a SPOT Insight Firewire camera and SPOT Advanced computer program.

Adapted laboratory evolution with *Arxula adeninivorans*

NS844 was cultured onto a YPD plate (10 g/L yeast extract, 20 g/L bacto peptone, 20 g/L glucose, and 20 g/L agar) and grown overnight at 40°C. A 10 µL loopful of these cells was used to inoculate a 250 mL Erlenmeyer flask with 50 mL of a modified

Verduyn medium consisting of arabinose (50 g/L), $(NH_4)_2SO_4$ (1.8 g/L), KH_2PO_4 (3 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), D-biotin (0.05 mg/L), Ca-D-pantothenate (1 mg/L), Nicotonic acid (1 mg/L), Myo-inositol (25 mg/L), Thiamine hydrochloride (1 mg/L), Pyridoxal hydrochloride (1 mg/L), p-aminobenzoic acid (0.2 mg/L), Na_2EDTA (1.5 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.45 mg/L), $MnCl_2 \cdot 2H_2O$ (0.1 mg/L), $CoCl_2 \cdot 6H_2O$ (0.03 mg/L), $CuSO_4 \cdot 5H_2O$ (0.03 mg/L),$Na_2MoO_4 \cdot 2H_2O$ (0.04 mg/L), $CaCl_2 \cdot 2H_2O$ (0.45 mg/L), and pH 5.0. The flasks were cultured in a New Brunswick shaker for 24-48 hours at 40°C and 200 rpm. Once the flask was grown up to an optical density of 7-10, a new flask was inoculated from the previous flask. Serial transfers were done until the cells were grown to over 100 generations. The cells were then grown on a plate consisting of 10 g/L yeast extract, 20 g/L bacto peptone, 20 g/L arabinose, and 20 g/L agar. A single colony was picked from the plate and used to inoculate another plate consisting of the same medium. NS844 was also grown on a plate and used to compare growth of the adapted NS844 strain.

A flask culture of NS844 was grown on a modified Verduyn medium (20 g/L glucose as sugar substrate) and the NS844 adapted strain was grown on modified Verduyn medium (20 g/L arabinose as sugar substrate) overnight until an OD_{600} of ~20 was reached. All 50 mL of the flask grown culture was then centrifuged and the supernatant was discarded. The cultures were then brought back up to a volume of 50 mL by adding diH_2O. The entire 50 mL washed culture was used to inoculate a 1-L volume medium in a Dasgip bioreactor consisting of arabinose (50 g/L), $(NH_4)_2SO_4$ (1.8 g/L), KH_2PO_4 (3 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), D-biotin (0.05 mg/L), Ca-D-pantothenate (1

mg/L), Nicotonic acid (1 mg/L), Myo-inositol (25 mg/L), Thiamine hydrochloride (1 mg/L), Pyridoxal hydrochloride (1 mg/L), p-aminobenzoic acid (0.2 mg/L), Na_2EDTA (1.5 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.45 mg/L), $MnCl_2 \cdot 2H_2O$ (0.1 mg/L), $CoCl_2 \cdot 6H_2O$ (0.03 mg/L), $CuSO_4 \cdot 5H_2O$ (0.03 mg/L),$Na_2MoO_4 \cdot 2H_2O$ (0.04 mg/L), and $CaCl_2 \cdot 2H_2O$ (0.45 mg/L).The process parameters for the fermentation were pH 3.5 (controlled automatically by addition of 10 N NaOH), a temperature of 40°C, aeration of 1 vvm, and agitation controlled at 1000 rpm. These fermentations were run for ~100 hours and 3 mL samples were taken every 4 hours using a Seg-Flow® 1200 Automated On-line Sampling System. These samples were used to analyze the fermentation broth by HPLC, determine DCW, and determine lipid content by extraction.

Engineering wild-type *Arxula adeninivorans* for arabinose utilization

Wild-type *A. adeninivorans* strain NS252 was chosen to be genetically engineered for improved arabinose utilization. The two separate synthetic DNA genes (*ladA* and *larA*) from *Aspergillus niger* were ordered from Life Technologies™. The genes were codon optimized to *Saccharomyces cerevisiae* by Life Technologies™ . Lar A was inserted into a Novogy Inc. plasmid which has a NAT (nourseothricin N-acetyl transferase) resistance marker. Lad A was inserted into a Novogy Inc. plasmid which has a HYG (hygromycin) resistance marker. NEBuilder® HiFi DNA Assembly Master Mix was used to assemble the DNA fragments into the vectors. A vector only reaction was run for each plasmid. Once assemblies were made, they were transformed into electrocompetent *E. coli*. This transformation was done by electroporation (shock at 1.8

kV). The *E. coli* cells were then resuspended in LB media to recover for 1 hour at 37°C. The cells were then plated on LB plates with AMP (ampicillin) and incubated overnight at 37°V. The vectors used earlier also contain AMP resistance so only *E. coli* strains with the inserted plasmid should grow on the plates. The following day, colonies were picked from each plate (5 from each insert) and grown in 5 mL of LB-AMP media. Internal primers were ordered to sequence the inserts. Next, the DNA was purified from the *E. coli* strains using the QIAprep® miniprep protocol from Qiagen®**.** The digested DNA was run on a gel for confirmation. Gel Electrophoresis was done to compare DNA inserts vs. the vector only reactions. Vector insert sequence was confirmed by Sanger Sequencing. The DNA was transformed into the wild type *A. adeninivorans* strain NS252 by electroporation. The cells were then allowed to recover by growing on YPD media. They were then plated on NAT or HYG plates and grown over night. The next day, colonies were picked and re-plated on either a YPD-NAT plate (Lar A expression, pNC1322) or a YPD-HYG plate (Lad A expression, pNC1323). From these plates, the different colonies were run in a 96 deep-well plate containing 350 µL medium per 2 mL well to compare growth on arabinose vs. the NS252 wild type strain. The colonies were compared based on optical density and the colonies with the highest optical density were chosen to run in reactors.

The chosen cultures were used to inoculate a 1-L volume medium in a Dasgip bioreactor consisting of arabinose (50 g/L), $(NH_4)_2SO_4$ (1.8 g/L), KH_2PO_4 (3 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), D-biotin (0.05 mg/L), Ca-D-pantothenate (1 mg/L), Nicotonic acid (1 mg/L), Myo-inositol (25 mg/L), Thiamine hydrochloride (1 mg/L), Pyridoxal

hydrochloride (1 mg/L), p-aminobenzoic acid (0.2 mg/L), Na_2EDTA (1.5 mg/L), $ZnSO_4 \cdot 7H_2O$ (0.45 mg/L), $MnCl_2 \cdot 2H_2O$ (0.1 mg/L), $CoCl_2 \cdot 6H_2O$ (0.03 mg/L), $CuSO_4 \cdot 5H_2O$ (0.03 mg/L),$Na_2MoO_4 \cdot 2H_2O$ (0.04 mg/L), and $CaCl_2 \cdot 2H_2O$ (0.45 mg/L).The process parameters for the fermentation were pH 3.5 (controlled automatically by addition of 10 N NaOH), a temperature of 40°C, aeration of 1 vvm, and agitation controlled at 1000 rpm. These fermentations were run for ~100 hours and 3 mL samples were taken every 4 hours using a Seg-Flow® 1200 Automated On-line Sampling System. These samples were used to analyze the fermentation broth by HPLC, determine DCW, and determine lipid content by extraction.

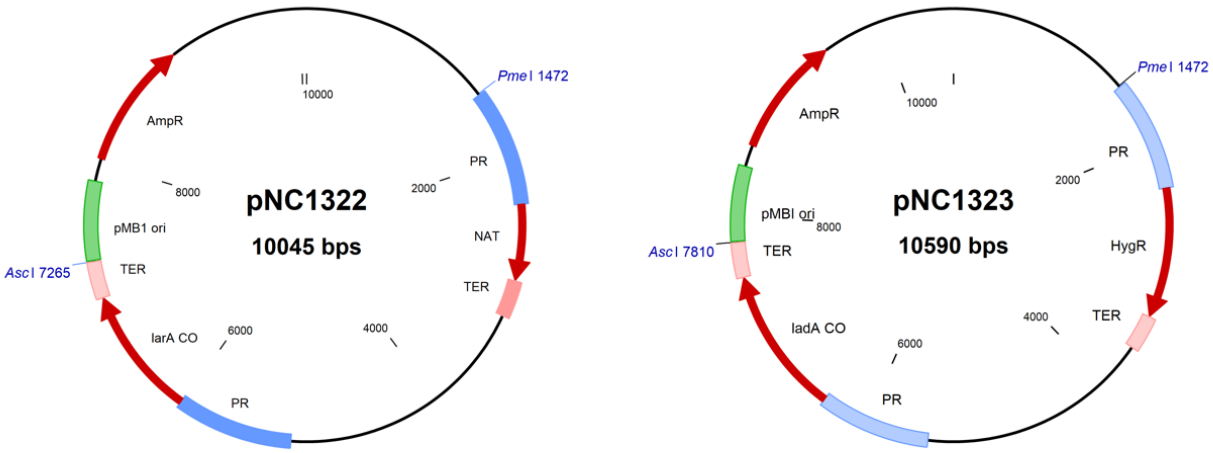


Figure 2. Plasmid maps for pNC1322 and pNC1323. Plasmid pNC1322 contains the larA codon optimized gene and an ampicillin resistant marker (AmpR) for *E. coli* and a nourseothricin N-acetyl transferase resistant marker (NAT) for yeast. Plasmid pNC1323 contains the ladA codon optimized gene and an ampicillin resistant marker (AmpR) for *E. coli* and a hygromycin resistant marker (HygR) for yeast.

A) **LarA Gene Sequence (972 bp)**

ATGAGCCTGCCATCTCATTTCACCATTAACACTGGTGCTAAAATTCCAGCTGTTGGTTTTGGTACTTGGCAAGCTAAACC
ATTGGAAGTTGAAAACGCTGTTGAAGTTGCTTTGAGAGAAGGTTACAGACATATTGATTGTGCTGCCATCTACAGAAAC
GAAACTGAAGTTGGTAACGGCATTAGAAAATCTGGTGTTCCAAGAGAAGAAATCTTCATCACTGGTAAGCTGTGGAAT
ACCAAACATGCTCCAGAAGATGTTGAACCAGCTTTGGATAAGACATTGCAAGATTTGGGTGTTGCTTACTTGGACTTGT
ATTTGATGCATTGGCCATGTGCTTTTAAAGGTGGTGATAAGTGGTTTCCATTGAACGATGATGGTGTTTTCGATTTGGCC
AACATTGATTACATTACCACCTACAGAGCTATGGAAAAGTTGTTGGCTACTGGTAAAGTTAGAGCCATTGGTGTTTCCA
ATTTCAACATCAGAAGATTGGAAGAGTTGCTGGGTCAAGTTTCTGTTGTTCCAGCAGTTAATCAAATTGAAGCCCATCC
ATACTTGCAACAACCTGATTTGTTGCAATTCTGCCAATCCAAGGGTATTTTGATTGAAGCTTATTCCCCATTGGGTAACA
ATCAAACTGGTGAACCTAGAACTGTTGATGATCCATTGGTTCATAGAGTTGCCGGTGAATTGTCTTTGGATCCAGGTCC
TTTGTTGGCTTCTTGGGGTGTTCAAAGAGGTACTGTTGTTTTGTCTAAGTCTGTTACCCCAGCTAGAATTGCTGCTAATTT
GAGAGTTAGAGCTTTGCCAGAAGGTGCTTTTGCTCAATTGAATTCATTGGAAAGACACAAGAGGTTTAACTTCCCAGGT
CATTGGGGTTACGATATTTTTGAAGAAGTTGGCGAAGAAGCCGTTAGACAAACTGCTTTGGCTGCTGGTCCATCTAACA
AAGTTAAGTTCACTGTCTAA

B) **LadA Gene Sequence (1161 bp)**

ATGGCTACCGCTACCGTTTTGGAAAAGGCTAATATTGGTGTTTTCACCAACACCAAACACGATTTGTGGGTTGCTGATG
CTAAACCTACTTTGGAAGAAGTTAAGAACGGTCAAGGTTTACAACCAGGTGAAGTTACCATTGAAGTTAGATCTACTGG
TATCTGCGGTTCCGATGTTCATTTTTGGCATGCTGGTTGTATTGGTCCAATGATAGTTACCGGTGATCATATCTTGGGTC
ATGAATCTGCTGGTCAAGTTGTTGCTGTTGCTCCAGATGTTACTTCTTTGAAACCTGGTGATAGAGTTGCAGTTGAACCT
AACATTATCTGTAACGCTTGTGAACCATGTTTGACCGGTAGATACAATGGTTGTGAAAACGTCCAATTCTTGTCTACTCC
ACCAGTTGATGGTTTGTTGAGAAGATACGTTAACCATCCAGCTATTTGGTGCCATAAGATTGGTGATATGTCTTACGAA
GATGGTGCTTTGTTGGAACCATTGTCTGTTTCTTTGGCTGGTATTGAAAGATCCGGTTTGAGATTGGGTGATCCTTGTTT
GGTTACTGGTGCTGGTCCAATTGGTTTGATTACTTTGTTGTCTGCTAGAGCTGCTGGTGCTTCTCCAATAGTTATTACCG
ATATTGACGAAGGCAGATTGGAATTCGCTAAATCTTTGGTTCCTGATGTCAGAACCTACAAGGTTCAAATTGGTTTATC
CGCTGAACAAAACGCCGAAGGTATTATCAATGTTTTCAACGATGGTCAAGGTAGTGGTCCAGGTGCTTTAAGACCAAG
AATTGCTATGGAATGTACCGGTGTTGAATCTTCTGTTGCTTCTGCAATTTGGTCTGTTAAGTTTGGTGGTAAGGTTTTCG
TTATCGGTGTTGGTAAGAACGAAATGACTGTTCCATTCATGAGATTGTCCACTTGGGAAATTGACTTGCAATACCAGTA
CAGATACTGTAATACTTGGCCTAGAGCTATCAGATTGGTTAGAAACGGTGTTATCGACTTGAAGAAGTTGGTTACCCAT
AGGTTCTTGTTGGAAGATGCTATTAAGGCTTTTGAAACTGCTGCTAATCCAAAGACTGGTGCCATCAAAGTTCAAATCA
TGTCCTCTGAAGATGATGTTAAGGCAGCTTCAGCTGGTCAAAAGATTTGA

Figure 3. Gene sequences for LarA and LadA. The LarA has a gene sequence of 972 base pairs and the LadA has a gene sequence of 1161 base pairs.

Plasmid	Novogy Primer ID	Sequence
pNC1322	NP2526	AGCCTATGGAAAGGCGCGCC
	NP5085	GATCCAAAGACAATTCACCGGCAAC
	NP5086	TGGCAAGCTAAACCATTGGAAG
pNC1323	NP2526	AGCCTATGGAAAGGCGCGCC
	NP5083	CACCAGCAGCTCTAGCAGAC
	NP5084	GAACGGTCAAGGTTTACAACCAG

Table 1. Primers used for sequencing. Three Novogy primers (NP2526, NP5085, and NP5086) were used to sequence LarA in plasmid pNC1322 and three Novogy primers (NP2526, NP5083, and NP5084) were used to sequence LadA in plasmid pNC1323.

CHAPTER 3

RESULTS

Initial shake flask screening shows NS844 to be the optimal strain for these studies

Novogy strains NS252, NS496, NS844 and NS1083 were screened in shake flasks to determine the optimal strain to use for these studies. Their performance was evaluated on glucose. Growth for all the strains can be seen in figure 4A. NS844 reached the highest DCW of 14.9 g/L in 67 hours. The final DCWs for NS252, NS496 and NS1083 were 13.9 g/L, 11.5 g/L and 11.9 g/L respectively. The total amount of glucose consumed for each strain after 67 hours can be seen in figure 4B. NS496 consumed 58 g/L of glucose, which was the most amount of the four strains. The total glucose consumed for NS252, NS844 and NS1083 was 39 g/L, 53 g/L and 45 g/L respectively. NS844 was chosen to evaluate for the rest of the experiments due to its favorable growth and glucose consumption.

Next, shake flask experiments were executed with NS844 to observe its performance on glucose, xylose, mannose, and arabinose. The sugar consumption and growth for each substrate can be seen in figure 5. All 50 g/L of sugar was consumed in ~68 hours for glucose, xylose, and mannose (figure 5A, 5B, and 5C). Sugar consumption

for arabinose lagged and there was still 19.1 g/L of arabinose at 120 hours into the fermentation (figure 4D). Growth on xylose reached the highest DCW of 8.2 g/L at 68 hours while growth on glucose and mannose reached a DCW of 6.8 and 6.7 g/L respectively. NS844 only reached a DCW of 5.3 g/L on arabinose after 120 hours.

A mixed sugar shake flask fermentation was also done to observe sugar utilization on multiple substrates. These mixed sugar flasks represent a mock lignocellulosic hydrolysate with 50.4% glucose, 36.3% xylose, and 13.3% arabinose (Palmqvist and Hahn-Hägerdal, 2000). Growth on mixed substrates can be seen in figure 5E. Diauxic growth with NS844 was observed. Glucose was totally consumed ~35 hours into fermentation. As soon as glucose was exhausted, NS844 started to utilize the xylose. All of the xylose was consumed by 68 hours. NS844 did not utilize the arabinose efficiently. By 120 hours, there was still 4.2 g/L left of the starting 7.2 g/L arabinose. Overall, NS844 ferments xylose and mannose comparably to glucose. NS844 ferments xylose and glucose simultaneously in a mixed sugar fermentation, although glucose utilization is faster. NS844 does not utilize arabinose efficiently in either the individual sugar fermentation or the mixed sugar fermentation.

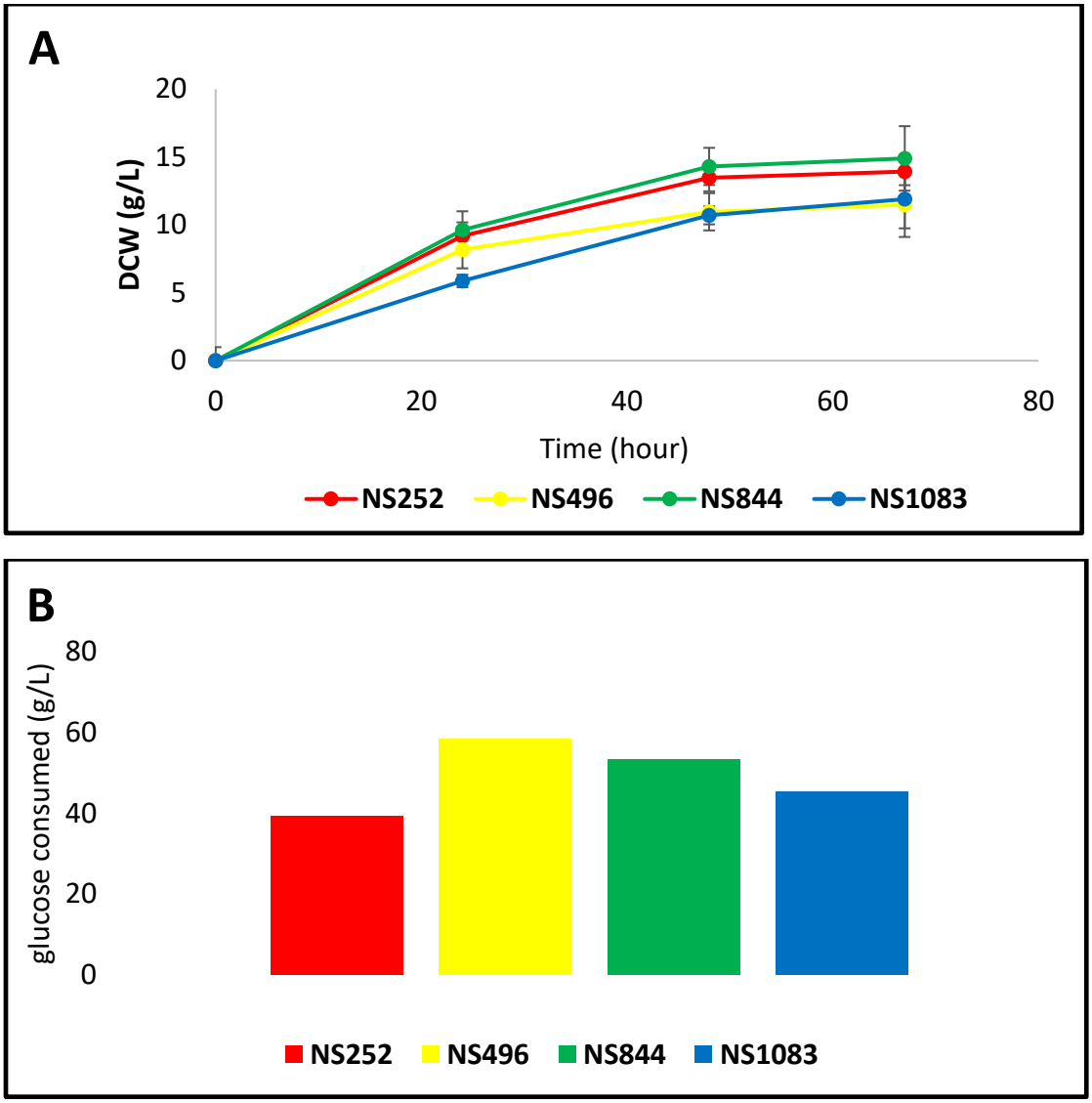
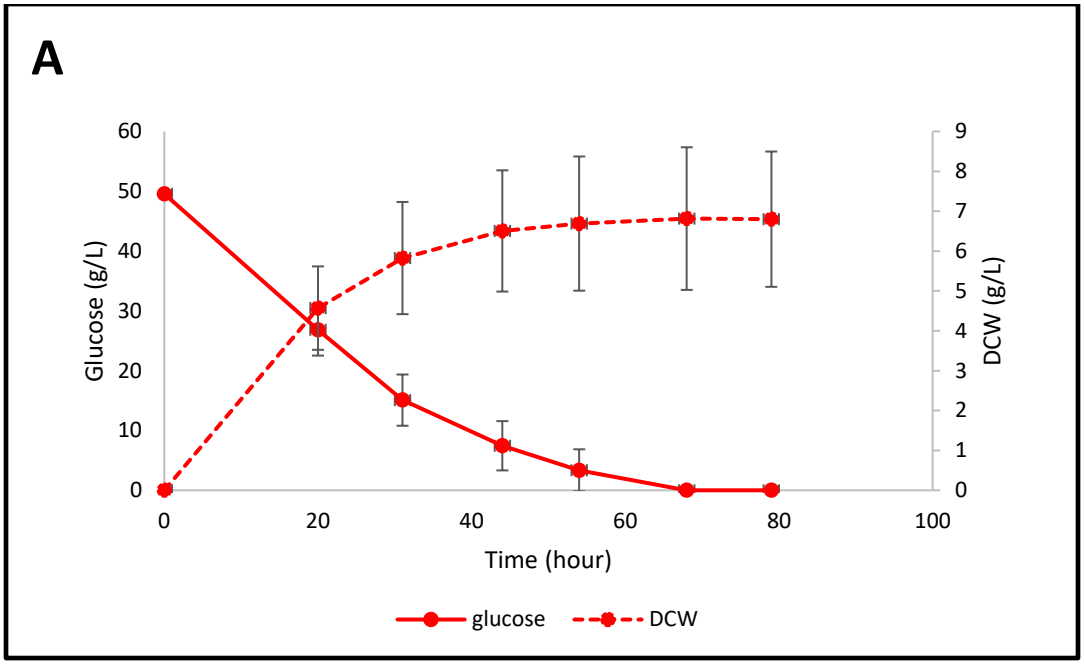
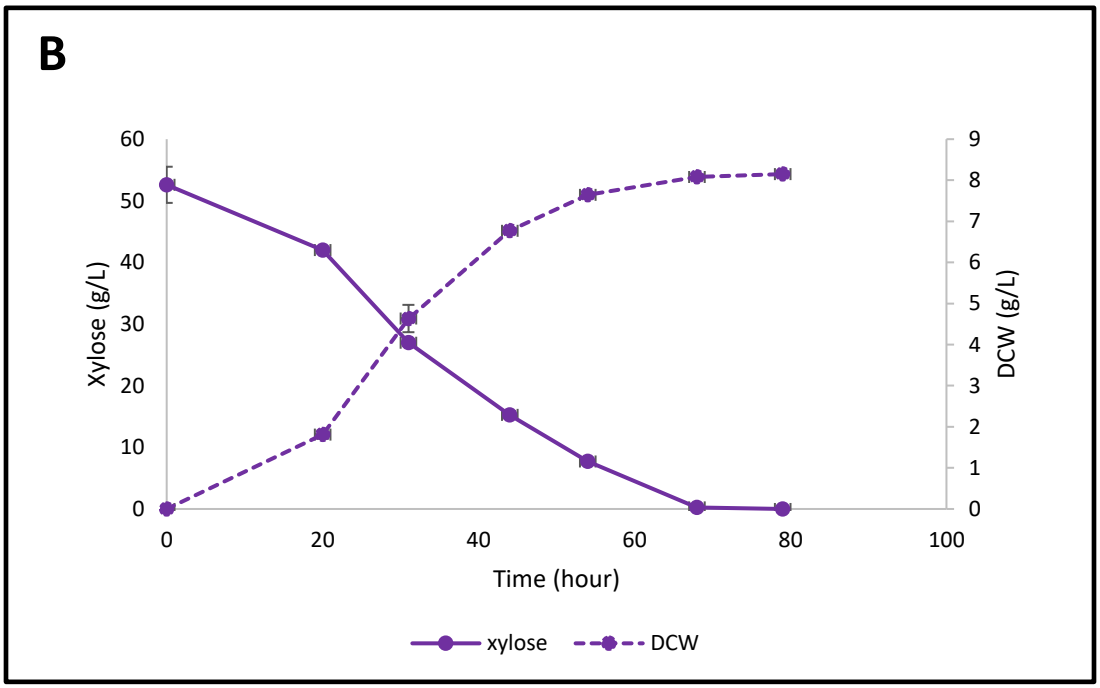
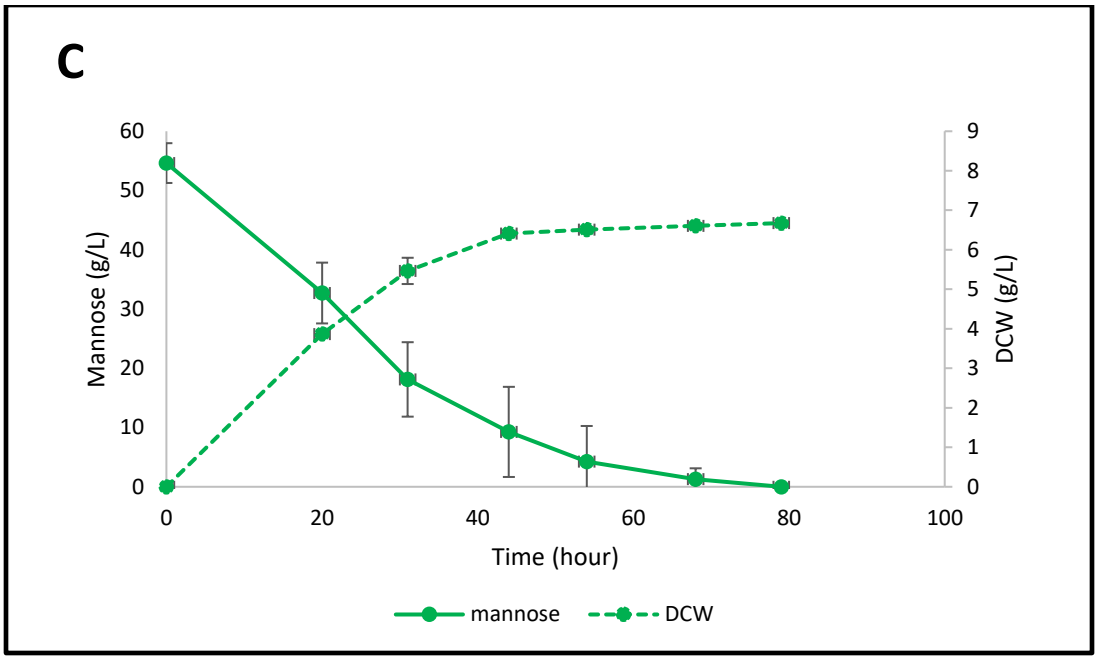
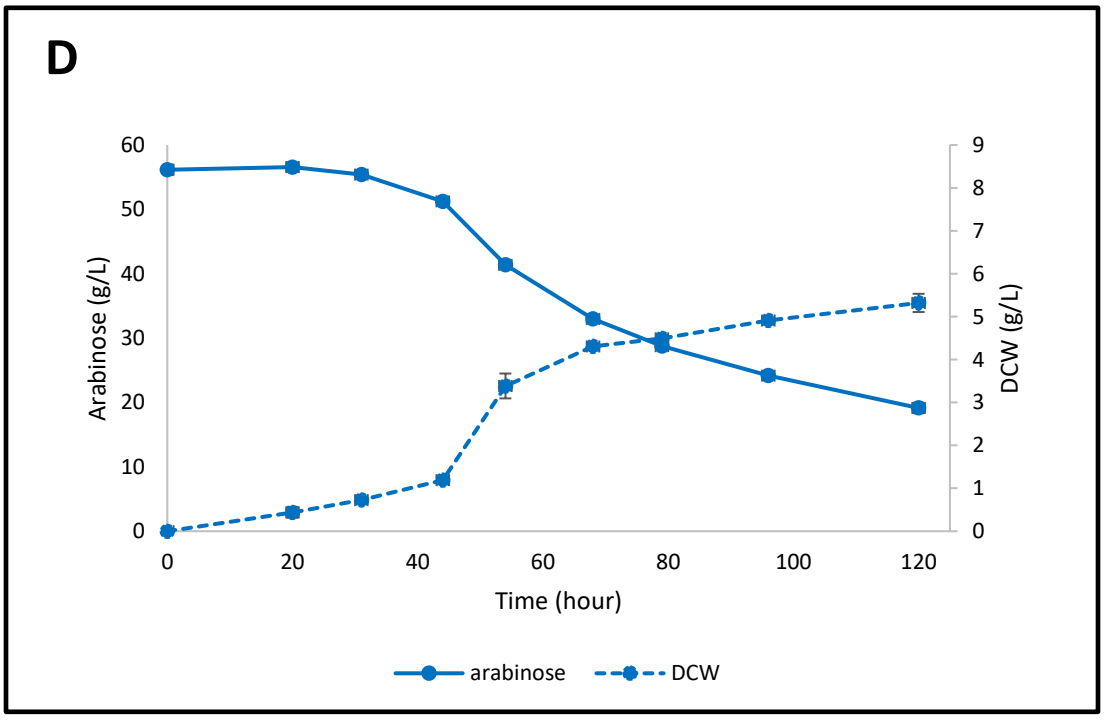


Figure 4. Initial shake flask screening: NS252, NS496, NS844 and NS1083 were run in shake flask on 150 g/L glucose. Flasks were run in duplicate. **A)** DCWs were calculated to plot growth. **B)** HPLC was used to measure sugar consumption after 67 hours. Initial glucose concentration was 150 g/L.


A
Glucose (g/L)
DCW (g/L)
Time (hour)
glucose
DCW


B
Xylose (g/L)
DCW (g/L)
Time (hour)
xylose
DCW


C
Mannose (g/L)
DCW (g/L)
Time (hour)
mannose
DCW


D
Arabinose (g/L)
DCW (g/L)
Time (hour)
arabinose
DCW

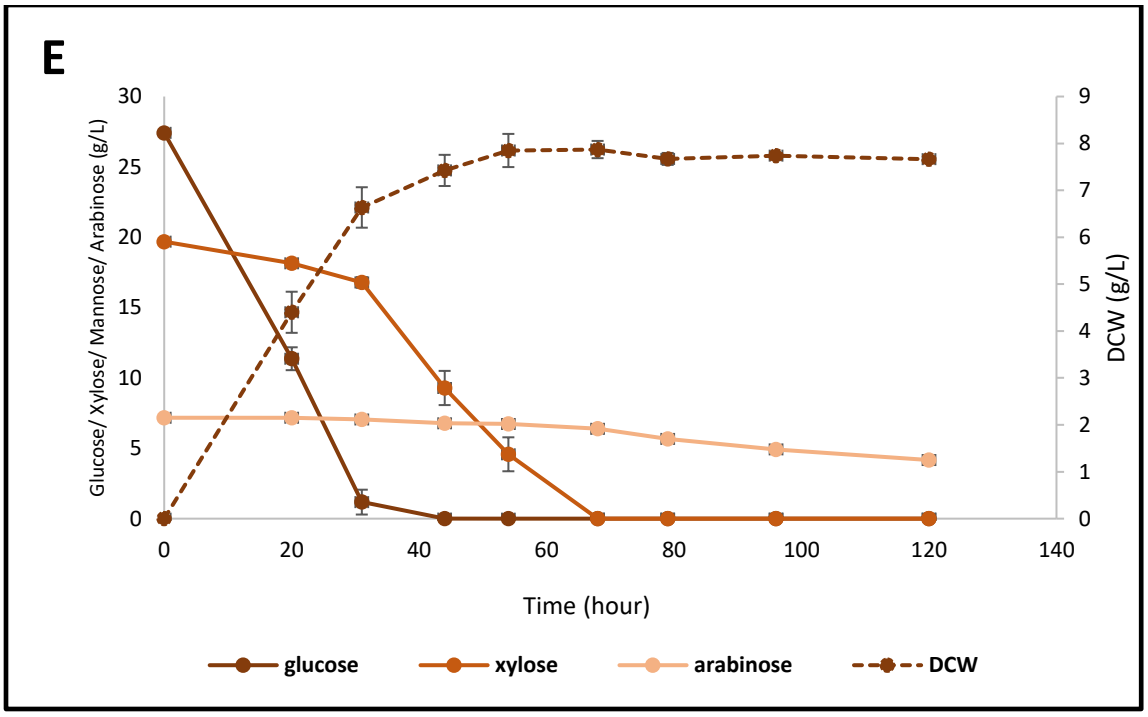
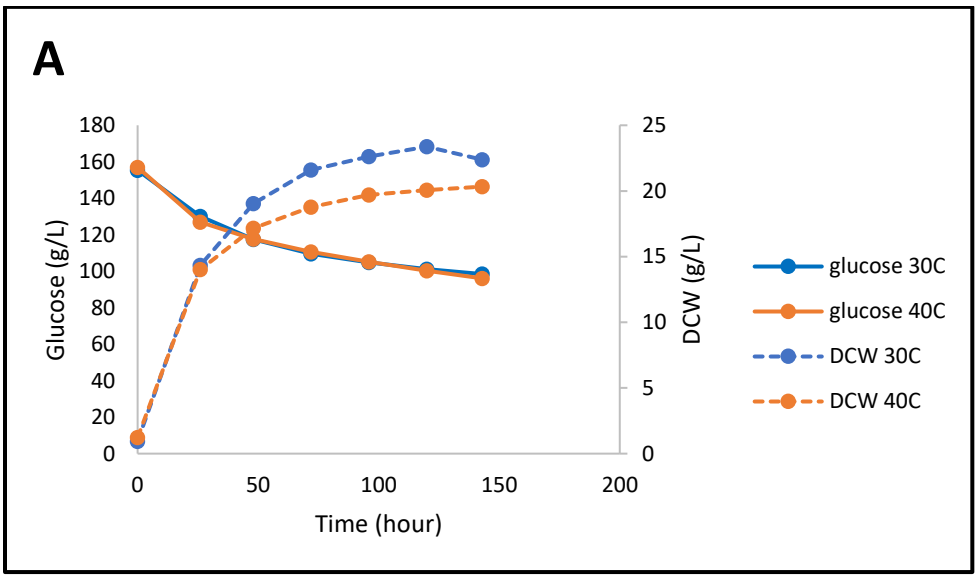
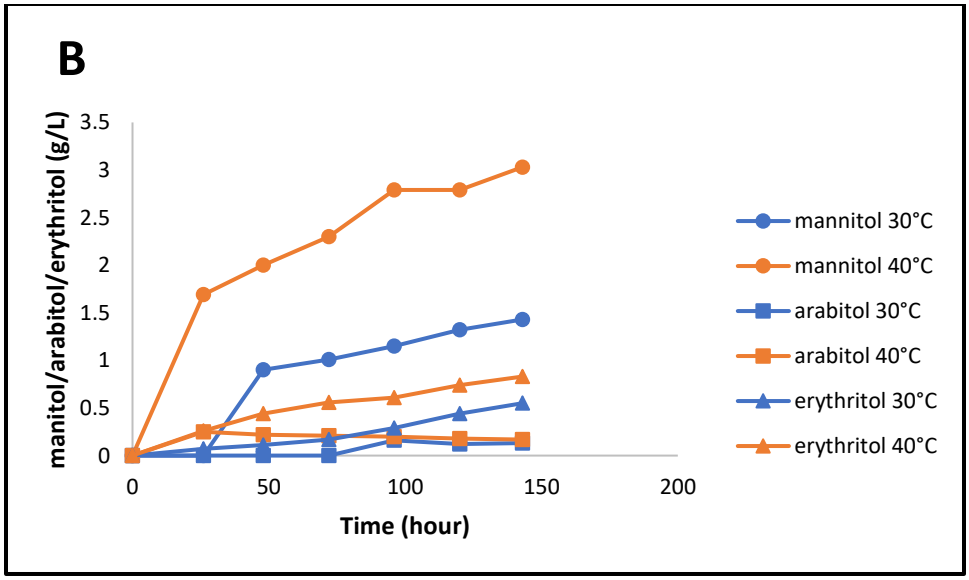


Figure 5. NS844 shake flask fermentations: NS844 was grown in shake flasks at 40°C on 50 g/L **(A)** glucose, **(B)** xylose, **(C)** mannose, and **(D)** arabinose. Flasks were run in duplicate. HPLC was used to measure sugar consumption and DCW were calculated to plot growth. **(E)** NS844 was grown in shake flasks in a mixed sugar fermentation which consisted of 27.4 g/L glucose, 19.7 g/L xylose, and 7.2 g/L arabinose. The flasks were run in duplicate. HPLC was used to measure sugar consumption and DCW were calculated to plot growth.

1-liter fermentations on glucose/xylose to evaluate growth and lipid production at 30°C and 40°C

The growth and lipid production of NS844 was evaluated in 1-liter Dasgip bioreactors on 150 g/L glucose at both 30°C and 40°C. After 140 hours of fermentation, NS844 reached a DCW of 22.4 g/L at 30°C and a DCW of 20.3 g/L at 40°C (figure 6A). Although NS844 reached a slightly higher DCW at 30°C, it actually consumed slightly more glucose at 40°C (56.9 grams at 30°C vs. 60.9 grams at 40°C). This was most likely due to a little extra polyol production at 40°C. NS844 produces mannitol, arabitol, and erythritol as byproduct. NS844 produced a total polyol concentration of 2.1 g/L at 30°C compared to 4.1 g/L at 40°C (figure 6B). It's possible that slightly more carbon is converted to carbon dioxide at 40°C as well. When cells are growing they produce carbon dioxide. The amount of carbon dioxide was measured throughout each fermentation using Bluesens off-gas technology. More carbon dioxide was produced in the first 10 hours of fermentation at 40°C vs. 30°C (figure 6C). This is an indication that during growth phase, when there is still nitrogen being utilized, NS844 grows faster at 40°C vs. 30°C. The amount of carbon dioxide produced was used to calculate the specific growth rate (μ_{max}) for each temperature during growth phase. The maximum specific growth rate was calculated to be 0.418 at 30°C vs. 0.563 at 40°C. The lipid content of the cells at 143 hours was determined via extraction of lipid by chloroform:methanol (figure 6D). The cells had a lipid content of 41.5% at 30°C and a content of 35.2% at 40°C. This gives a lipid yield 0.16 grams lipid per gram glucose at 30°C and 0.12 grams lipid per gram glucose at 40°C.


A
Glucose (g/L)
DCW (g/L)
Time (hour)
glucose 30C
glucose 40C
DCW 30C
DCW 40C


B
manitol/arabitol/erythritol (g/L)
Time (hour)
mannitol 30°C
mannitol 40°C
arabitol 30°C
arabitol 40°C
erythritol 30°C
erythritol 40°C

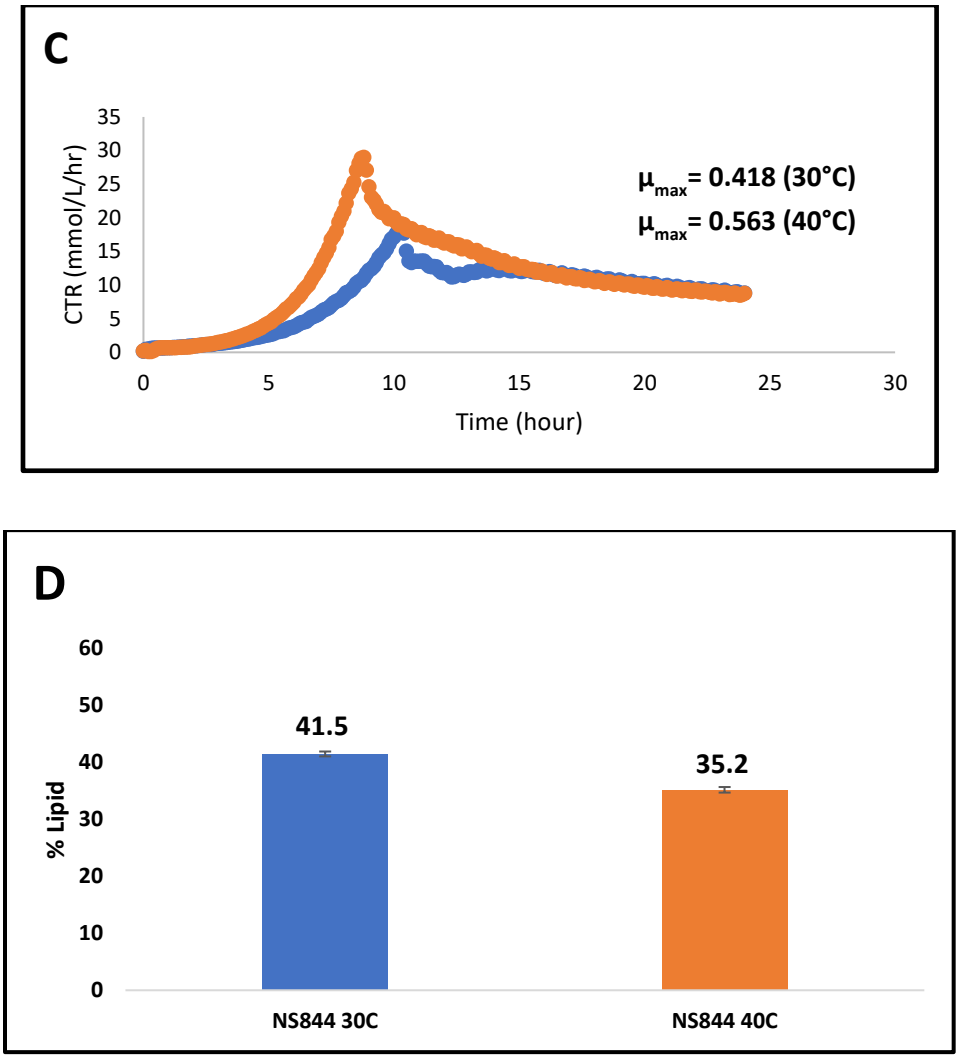
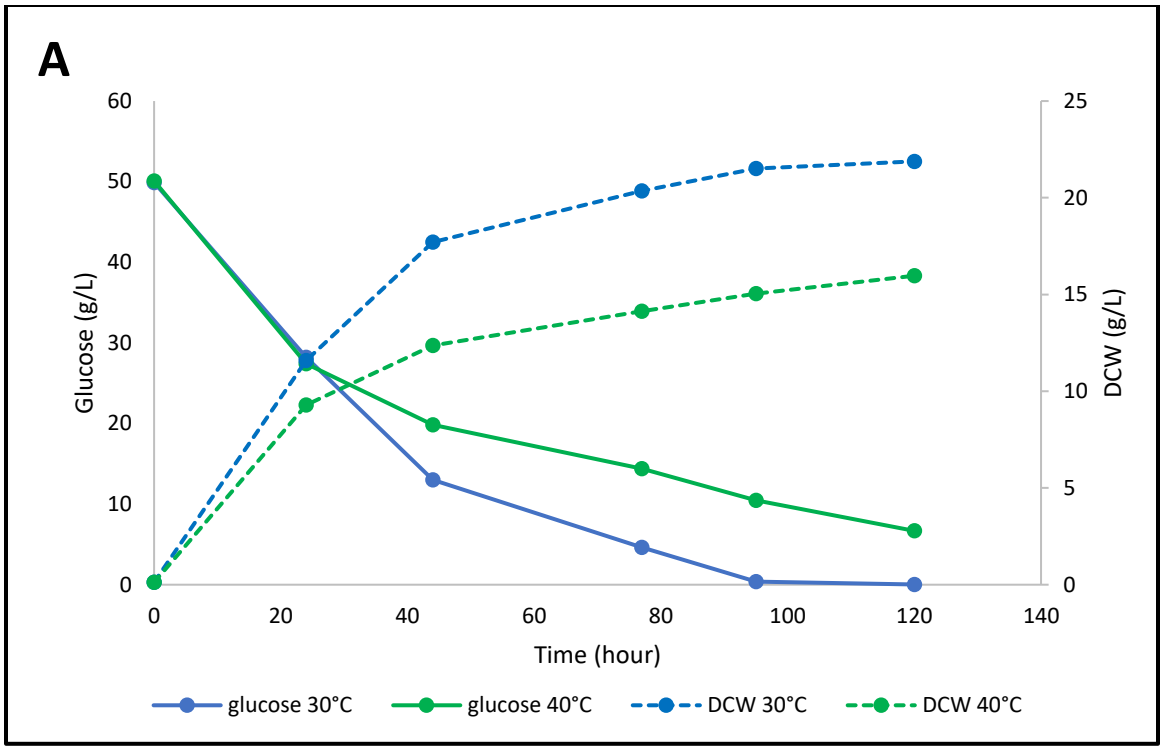
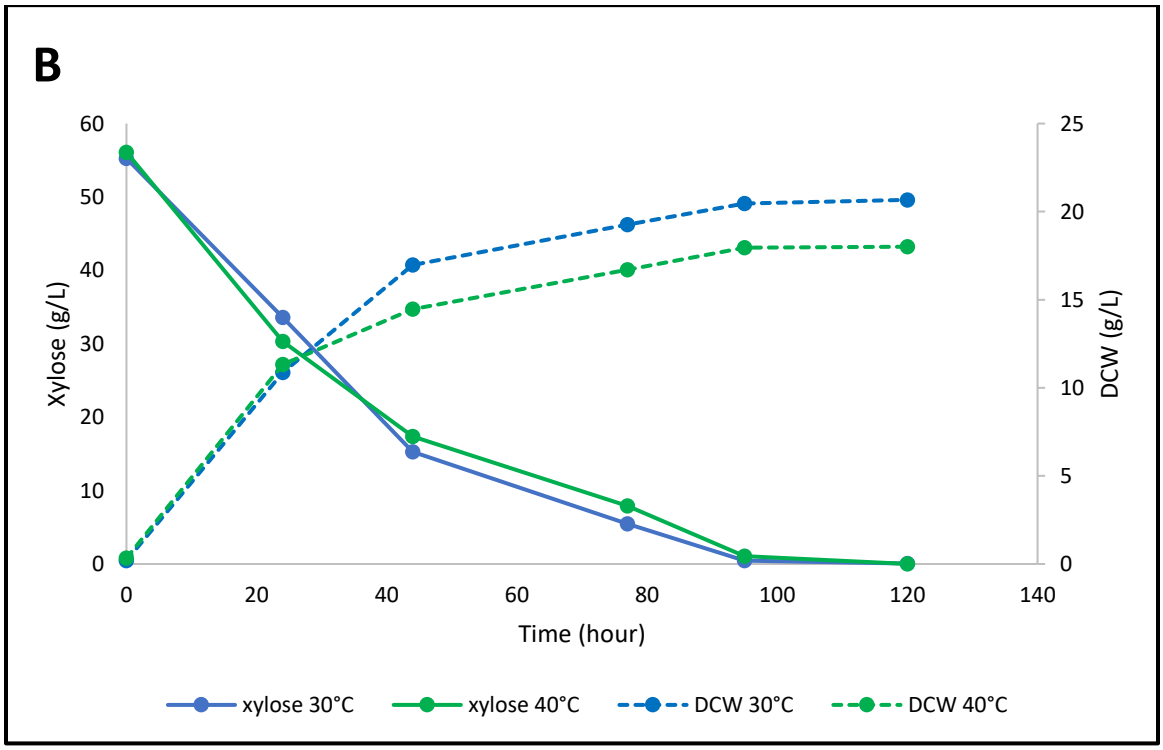


Figure 6. 1-liter glucose batch fermentations: NS844 was fermented in 1-liter Dasgip bioreactors on 150 g/L glucose at 30°C and 40°C. **(A)** HPLC was used to measure glucose consumption and DCW was used to plot growth. **(B)** HPLC was used to measure polyol production at 30°C and 40°C. Polyols measured include mannitol, arabitol, and erythritol. **(C)** Bluesens off-gas sensors were used to track carbon dioxide evolution through the first 24 hours of fermentation. **(D)** Lipid content was determined by chloroform:methanol extraction of the final cells. Cells were extracted in triplicate.

Next, NS844 was fermented in 1-liter bioreactors on either 50 g/L glucose or xylose at both 30°C and 40°C. From the previous 1-liter fermentation, which showed NS844 only consumed 50-60 g/L of sugar at a nitrogen loading of 1.8 g/L ammonium sulfate, it was decided to reduce the starting sugar concentration from 150 g/L to 50 g/L. NS844 reached a final DCW of 21.8 g/L on glucose at 30°C and a DCW of 16.0 at 40°C (figure 7A). After 120 hours of fermentation, 49.9 grams of glucose were consumed at 30°C and 43.4 grams of glucose were consumed at 40°C. Glucose was exhausted by 95 hours at 30°C and still had 6.7 g/L left at 120 hours for the 40°C fermentation. The lipid content of the cells at 95 hours was determined via extraction of lipid by chloroform:methanol (figure 7C). NS844 grown on glucose reached a lipid content of 40.4% at 30°C and a content of 34.4% at 40°C. This gave lipid yields of 0.17 grams of lipid per gram glucose at 30°C and 0.13 grams of lipid per gram glucose at 40°C.

NS844 reached a final DCW of 20.5 g/L on xylose at 30°C and a DCW of 18.0 at 40°C at 95 hours (figure 7B). All of the starting xylose was consumed by 95 hours for both temperatures. The lipid content of the cells at 95 hours was determined via extraction of lipid by chloroform:methanol (figure 7C).). NS844 grown on xylose reached a lipid content of 39.3% at 30°C and a content of 33.8% at 40°C. This gave lipid yields of 0.15 grams of lipid per gram xylose at 30°C and 0.11 grams of lipid per gram xylose at 40°C. The initial growth of NS844 was faster at 40°C, but the lipid production of NS844 was better at 30°C when fermented on glucose.


A
Glucose (g/L)
DCW (g/L)
Time (hour)
glucose 30°C
glucose 40°C
DCW 30°C
DCW 40°C


B
Xylose (g/L)
DCW (g/L)
Time (hour)
xylose 30°C
xylose 40°C
DCW 30°C
DCW 40°C

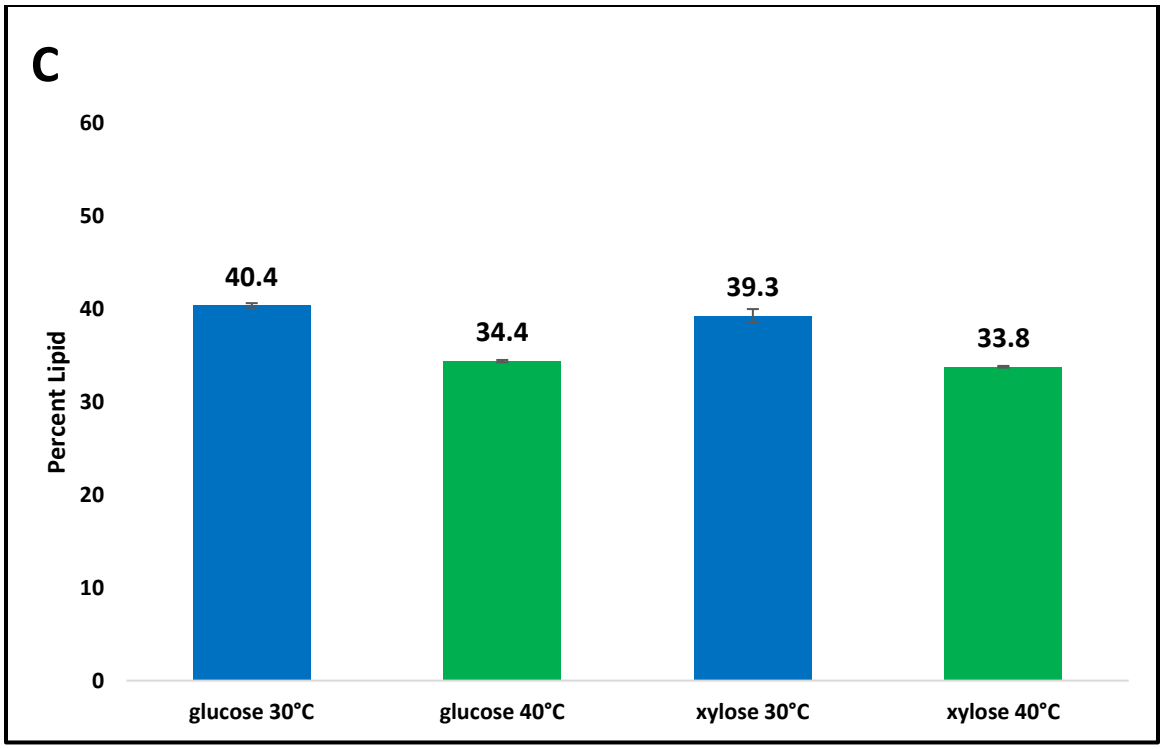


Figure 7. 1-liter batch glucose/xylose fermentation: NS844 was fermented in 1-liter Dasgip bioreactors on either 50 g/L glucose or 50 g/L xylose at 30°C and 40°C. **(A)** HPLC was used to measure glucose consumption and DCW was used to plot growth on glucose. **(B)** HPLC was used to measure xylose consumption and DCW was used to plot growth on xylose. (**C)** Lipid content was determined by chloroform:methanol extraction of the DCW at 95 hours grown on glucose and xylose at 30°C and 40°C. Cells were extracted in triplicate.

High resolution 5-liter fermentations

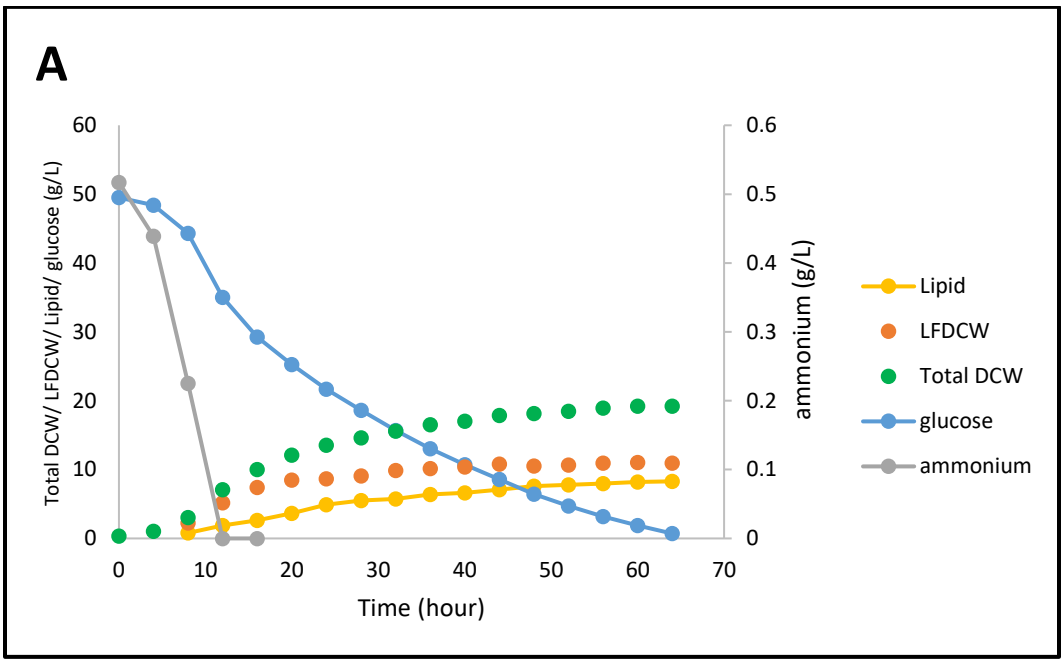
In order to get higher resolution fermentations, the previous 1-liter fermentations were repeated in 5-liter New Brunswick bioreactors. The growth and lipid production of NS844 grown on either glucose or xylose were evaluated at both 30°C and 40°C. A Seg-Flow® 1200 Automated On-line Sampling System was used to sample the reactors every 4 hours. Growth rates and sugar consumption rates can be calculated with more precision with more time points. There also is better mixing at 5-liters which leads to better oxygen transfer and mass transfer.

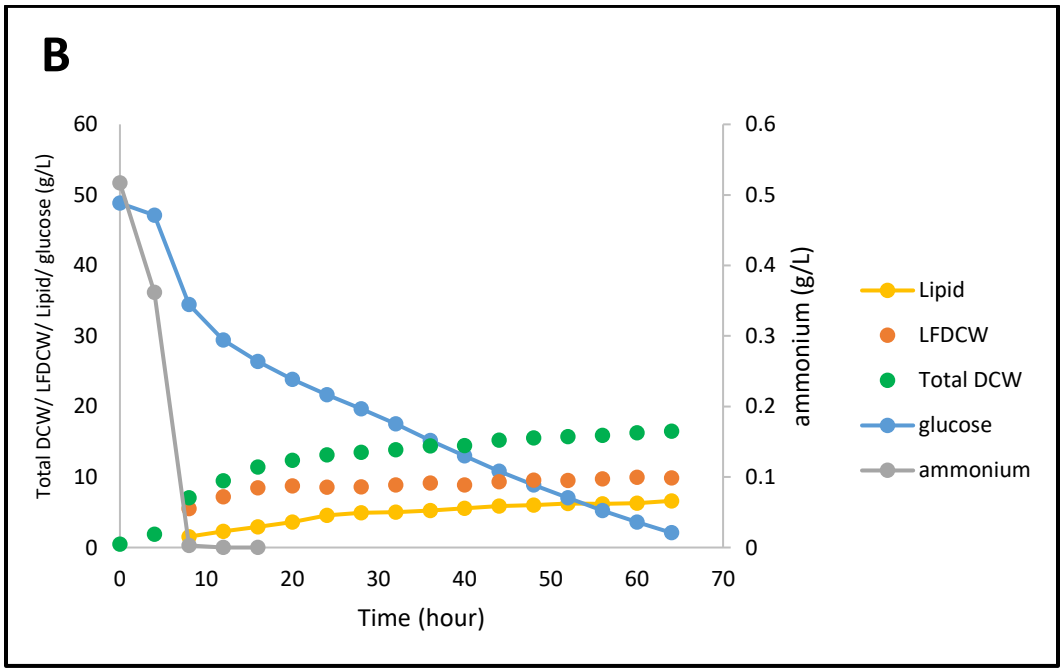
NS844 reached a final DCW of 19.2 g/L (8.3 g/L lipid and 10.9 g/L lipid free DCW) at 64 hours of fermentation time and 30°C on glucose (figure 8A). The final DCW at 64 hours for the 40°C run was 16.5 g/L (6.6 g/L lipid and 9.9 g/L lipid free DCW) (figure 8B). After 64 hours there was 0.7 g/L glucose left at 30°C and 2.1 g/L left at 40°C. Ammonium concentration was measured using a YS1 7100 MBS. The ammonium concentration at 8 hours was 0.225 g/L for the 30°C run and 0 g/L for the 40°C run. In nitrogen limited fermentation, NS844 utilizes nitrogen during the growth phase. The nitrogen was used up quicker at 40°C, indicating a faster initial growth at 40°C. The maximum specific growth rate (μ_{max}) for 30°C and 40°C was calculated to be 0.29 1/hr and 0.35 1/hr respectively. Although initial growth was faster at 40°C, lipid production was better at 30°C (figure 8E). At 30°C, the lipid yield was calculated to be 0.17 grams lipid per gram glucose and the cell specific productivity was calculated to be 0.012 grams lipid per gram cell mass per hour. At 40°C, the lipid yield was calculated to be 0.14 grams lipid per gram glucose and the cell specific productivity was calculated to

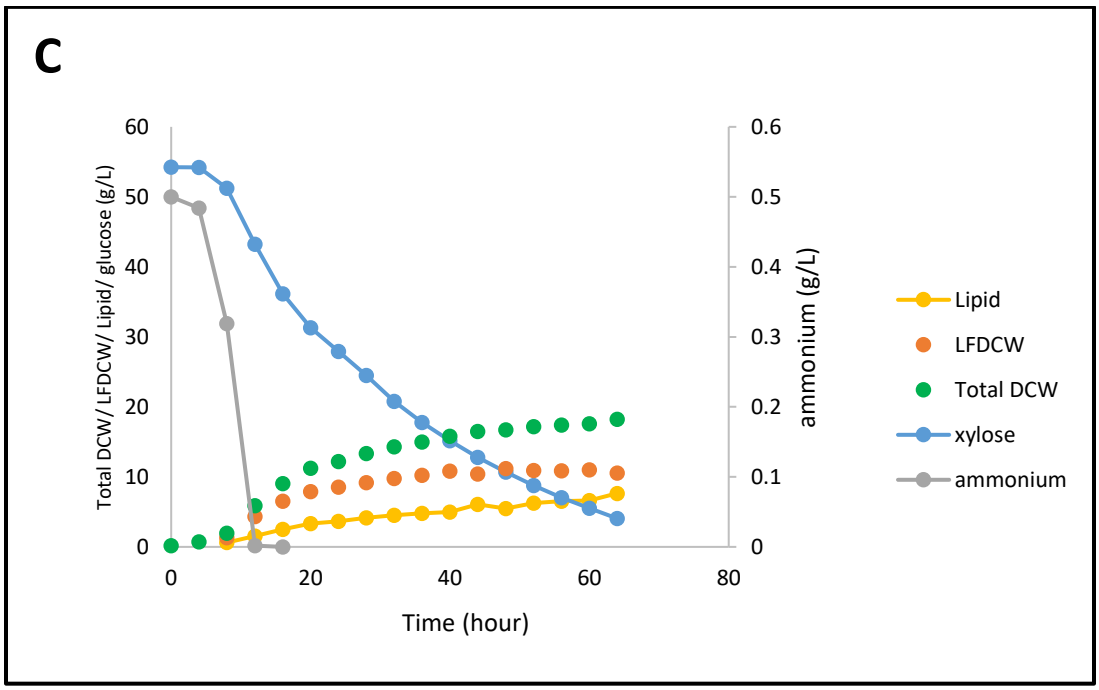
be 0.011 grams lipid per gram cell mass per hour. Images of the cells were taken at both temperatures on glucose (figure 8F).

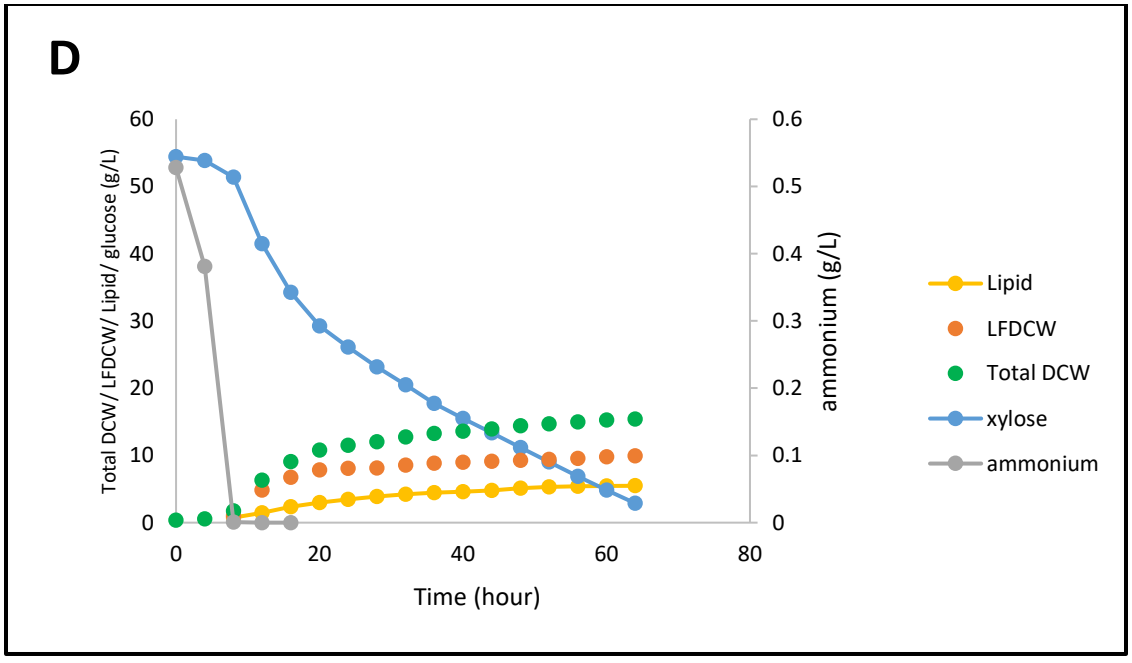
NS844 reached a final DCW of 18.2 g/L (7.7 g/L lipid and 10.5 g/L lipid free DCW) at 64 hours of fermentation time and 30°C on xylose (figure 8C). The final DCW at 64 hours for the 40°C run was 15.4 g/L (5.5 g/L lipid and 9.9 g/L lipid free DCW). After 64 hours there was 4.1 g/L xylose left at 30°C and 2.9 g/L left at 40°C (figure 8D). The ammonium concentration at 8 hours was 0.319 for the 30°C run and 0 g/L for the 40°C run. The maximum specific growth rate (μ_{max}) for 30°C and 40°C was calculated to be 0.372 hr^{-1} and 0.32 hr^{-1} respectively. Lipid production was lower at 40°C compared to 30°C, just like it was lower at the higher temperature on glucose (figure 8E). At 30°C, the lipid yield was calculated to be 0.14 grams lipid per gram glucose and the cell specific productivity was calculated to be 0.010 grams lipid per gram cell mass per hour.). At 40°C, the lipid yield was calculated to be 0.11 grams lipid per gram glucose and the cell specific productivity was calculated to be 0.009 grams lipid per gram cell mass per hour. Although lipid production was lower at the higher temperature, it is promising that NS844 seemed to utilize xylose and grow just as fast as it did on glucose. The cells appear to have a budding yeast morphology at 30°C but take on a mycelial form at 40°C (figure 8F).

In conclusion, NS844 ferments xylose comparably to glucose. The initial growth of NS844 was faster at 40°C on both sugars. The lipid production of NS844 was higher at 30°C for both sugars. The mycelial formation at 40°C seems to have a negative impact on the lipid productivity at 40°C.


A
Total DCW/ LFDCW/ Lipid/ glucose (g/L)
ammonium (g/L)
Time (hour)
Lipid
LFDCW
Total DCW
glucose
ammonium


B
Total DCW/ LFDCW/ Lipid/ glucose (g/L)
ammonium (g/L)
Time (hour)
Lipid
LFDCW
Total DCW
glucose
ammonium


C
Total DCW/ LFDCW/ Lipid/ glucose (g/L)
ammonium (g/L)
Time (hour)
Lipid
LFDCW
Total DCW
xylose
ammonium


D
Total DCW/ LFDCW/ Lipid/ glucose (g/L)
ammonium (g/L)
Time (hour)
Lipid
LFDCW
Total DCW
xylose
ammonium

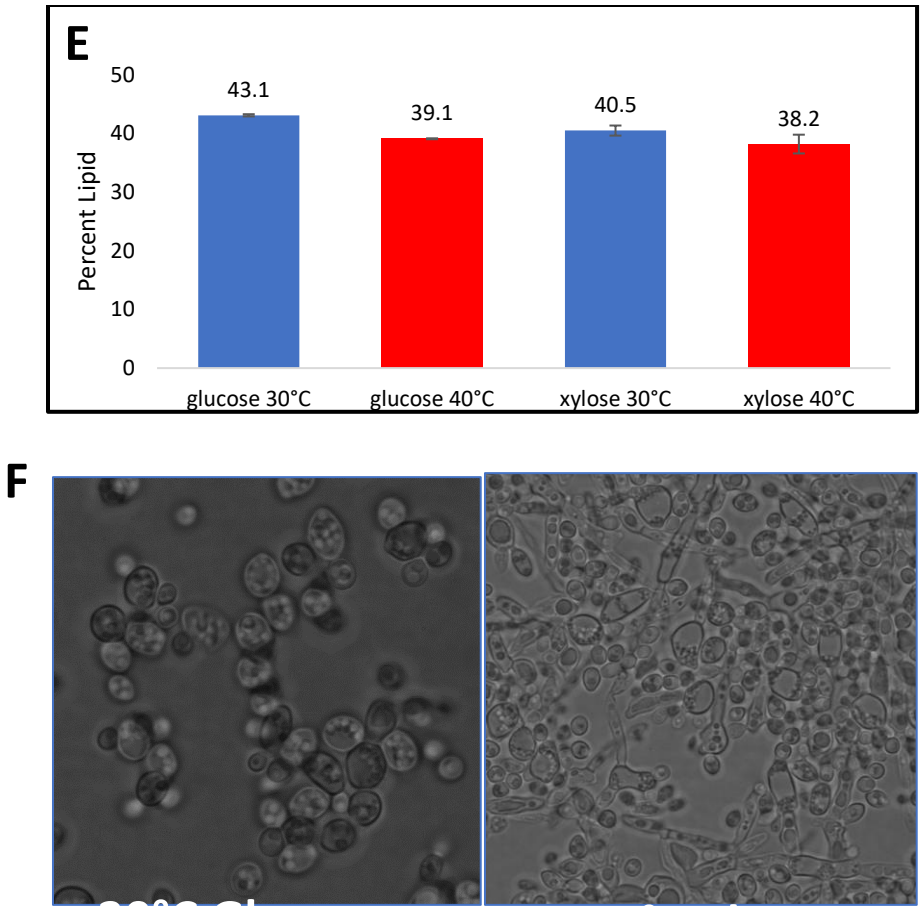


Figure 8. High resolution 5-liter fermentations on glucose/xylose. NS844 was fermented in 5-liter New Brunswick bioreactors on either 50 g/L glucose or 50 g/L xylose at 30°C and 40°C. HPLC was used to measure sugar consumption, DCW was used to plot growth, YSI was used to measure ammonium concentration, lipid and lipid free dry cell weight (LFDCW, the non-lipid mass of the cell consisting of protein and carbohydrates) were determine by extractions and then plotted for fermentations on **(A)** 50 g/L glucose at 30°C, **(B)** 50 g/L glucose at 40°C, **(C)** 50 g/L xylose at 30°C, and **(D)** 50 g/L xylose at 40°C. (**E)** Lipid content was determined by chloroform:methanol extraction of the DCW at 60 hours grown on glucose and xylose at 30°C and 40°C. Cells were extracted in triplicate. **(F)** Images of cells grown at 30°C and 40°C on glucose.

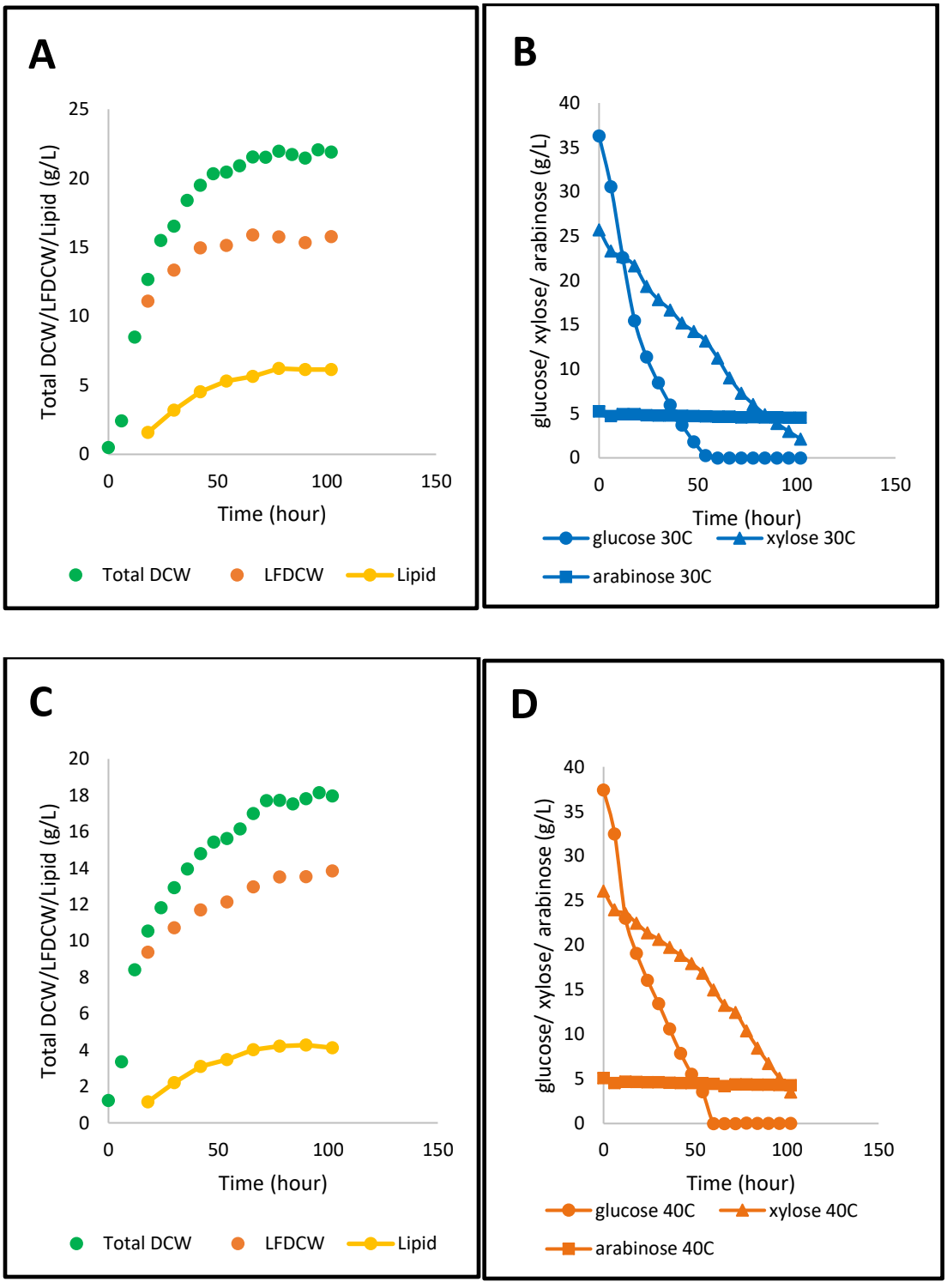
Next, NS844 was fermented on a mixed sugar substrate at 30°C and 40°C. This mixed sugar substrate would be representative of a lignocellulosic hydrolysate used in industrial fermentations (Palmqvist and Hahn-Hägerdal, 2000). Lignocellulosic material is often pre-treated into cellulose and hemicellulose and then hydrolyzed to glucose, xylose, arabinose and mannose. The mixed sugar concentrations for these fermentations consisted of 38 g/L glucose, 23 g/L xylose, and 4 g/L arabinose.

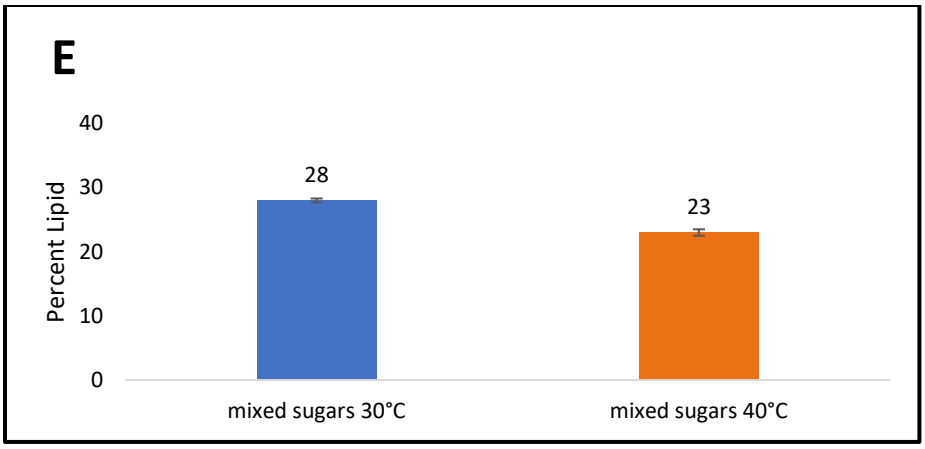
For the 30°C fermentation, NS844 reached a DCW of 21.9 g/L (15.8 g/L LFDCW and 6.1 g/L lipid) in 102 hours (figure 9A). All of the glucose was consumed during the first 54 hours of the fermentation at a rate of 0.67 grams per hour (figure 9B). The remaining xylose concentration was at 2.1 g/L by the end of the fermentation (102 hours). There was little to no arabinose consumed after 102 hours. Unlike the previous mixed sugar shake flasks experiment, there does not appear to be a diauxic growth on glucose and xylose. Xylose is consumed at a constant rate 0.23 grams per hour throughout the fermentation regardless of the glucose concentration in the fermenter. The final lipid content at 30°C was 28 percent (figure 9E). This gives a lipid yield of 0.10 grams of lipid per a gram of sugar and a cell specific productivity of 0.004 grams of lipid per a gram of sugar per hour. Both lipid yield and productivity were slower on mixed sugars compared to either batch glucose or xylose fermentations.

For the 40°C fermentation, NS844 reached a DCW of 18.0 g/L (13.8 g/L LFDCW and 4.2 g/L lipid) in 102 hours (figure 9C). All of the glucose was consumed during the first 60 hours of the fermentation at a rate of 0.62 grams per hour (figure 9D). The remaining xylose concentration was at 3.5 g/L by the end of the fermentation (102

hours). There was little to no arabinose consumed after 102 hours. As seen in the 30°C fermentation, there does not appear to be a diauxic growth on glucose and xylose. Xylose is consumed at a rate of 0.22 grams of sugar per hour throughout the fermentation. Interestingly, xylose consumption increases from 0.19 grams per hour to 0.27 grams per hour after glucose is exhausted at 60 hours. The final lipid content at 40°C was 23 percent (figure 9E). This gives a lipid yield of 0.07 grams of lipid per a gram of sugar and a cell specific productivity of 0.003 grams of lipid per a gram of sugar per hour. Lipid yields and productivity were lower on mixed sugars compared to just glucose or xylose for both 30°C and 40°C fermentations. Cells at 30°C appear to have a budding morphology while cells at 40°C appear to take on a mycelial form (figure 9F).

NS844 ferments xylose and glucose simultaneously in a mixed sugar fermentation, although glucose use is faster. Once glucose is completely consumed, NS844 seems to increase its rate of xylose utilization. NS844 does not utilize arabinose at all in a mixed sugar fermentation. The lipid yields for NS844 at both 30°C and 40°C on mixed sugars are much lower than the yields on solely glucose or xylose as the carbon source. NS844 does not convert mixed sugars to lipids as efficiently as it does with glucose or xylose. Further strain engineering may be necessary to improve performance on mixed sugars.


A
Total DCW/LFDCW/Lipid (g/L)
Time (hour)
Total DCW
LFDCW
Lipid
B
glucose/ xylose/ arabinose (g/L)
Time (hour)
glucose 30C
xylose 30C
arabinose 30C
C
Total DCW/LFDCW/Lipid (g/L)
Time (hour)
Total DCW
LFDCW
Lipid
D
glucose/ xylose/ arabinose (g/L)
Time (hour)
glucose 40C
xylose 40C
arabinose 40C



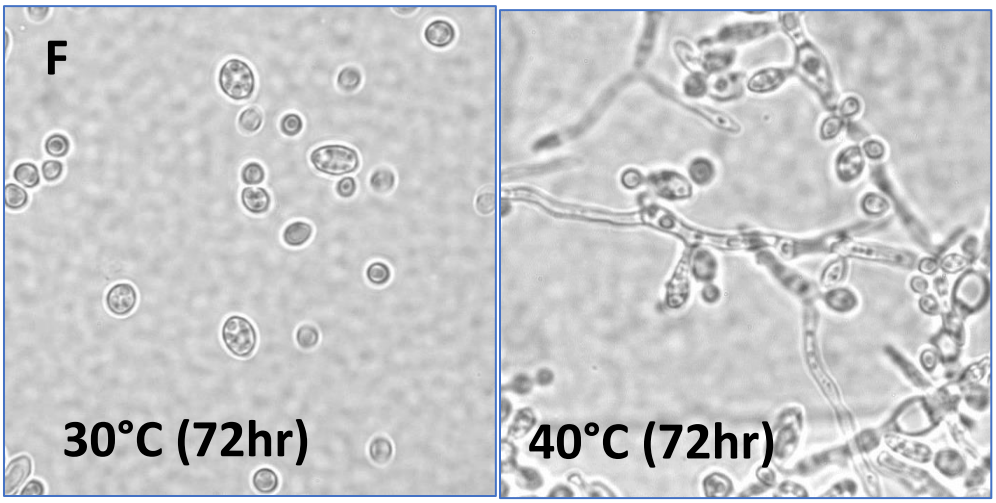


Figure 9. High resolution 5-liter fermentations on mixed sugars. NS844 was fermented in 5-liter New Brunswick bioreactors on 38 g/L glucose, 23 g/L xylose, and 4 g/L arabinose at 30°C and 40°C. . HPLC was used to measure sugar consumption for fermentations at **(A)** 30°C and **(C)** 40°C. Lipid and lipid free DCW was determine by extractions and then plotted with total DCW for fermentations at **(B)** 30°C and **(D)** 40°C. Cells were extracted in triplicate **(F)** Images of cells grown at 30°C and 40°C on mixed sugars.

1-liter chemostat fermentations were carried out to determine the maintenance energy

Continuous cultures of NS844 were run in a 1-liter New Brunswick chemostat reactor at 30°C and 40°C at different dilution rates in order to calculate the maintenance energy at each temperature. By changing the dilution rate of the feed, the specific growth rate of the yeast can be controlled. When the dilution rate is increased, the cell mass and cell yield on glucose increases slightly as well up until the dilution rate exceeds the maximum growth rate (Shen et al. 2013). The specific glucose uptake rate (q_s, g glucose/ g cell mass/ hr) was determined by using the equation in figure 10A. The glucose concentration being fed-into reactor (13.5 g/L), the glucose concentration in reactor (0 g/L), the cell DCW, and the dilution rates are known (0.04, 0.08, 0.13 , 0.15, and 0.19 ($hour^{-1}$). No carbon based by products (citrate and polyols) were detected by HPLC either. This allows calculation of the specific glucose uptake rate at each dilution. By using the Herbert-Pirt equation (figure 10B), the glucose specific uptake rate can be plotted against the dilution rates (Shen et al. 2013). The maximum cell mass yield ($Y_{x/s}^{max}$) can be determined from the slope of q_s vs. dilution rate, and at steady state the specific growth rate (μ) is equal to the dilution rate. When plotted, the y-intercept will also give the maintenance coefficient (m_s) at each temperature. The maintenance coefficient is expressed in mg of glucose per gram of cell per hour. This determines how much glucose NS844 utilizes for maintenance at each temperature. The maintenance coefficient was determined to be 5.4 mg of glucose per gram cell mass per hour at 30°C (figure 10C) and 7.8 mg of glucose per gram cell mass per hour at 40°C (figure 10D).

A

Specific substrate uptake rate

$$q_S = \frac{C_{S_0} - C_S}{C_X} D$$

C_{s0}= concentration of glucose fed into reactor
C_s= concentration of glucose in reactor (0 g/L)
C_x= concentration of cell mas (g/L)
D= dilution rate , h^{-1}

B

Herbert-Pirt Equation

$$q_S = \frac{1}{Y_{X/S}^{\max}} \mu + m_S$$

$Y_{x/s}^{max}$ =maximum cell mass yield on glucose
μ= specific growth rate (steady state μ=D)
m_s= maintenance coefficient (g/ g cell mass/h)

*Shen et al. 2013. Kinetics of continuous cultivation of the oleaginous yeast *Rhodosporidium toruloides.* Journal of Biotechnology. 168 (2013) 85-89

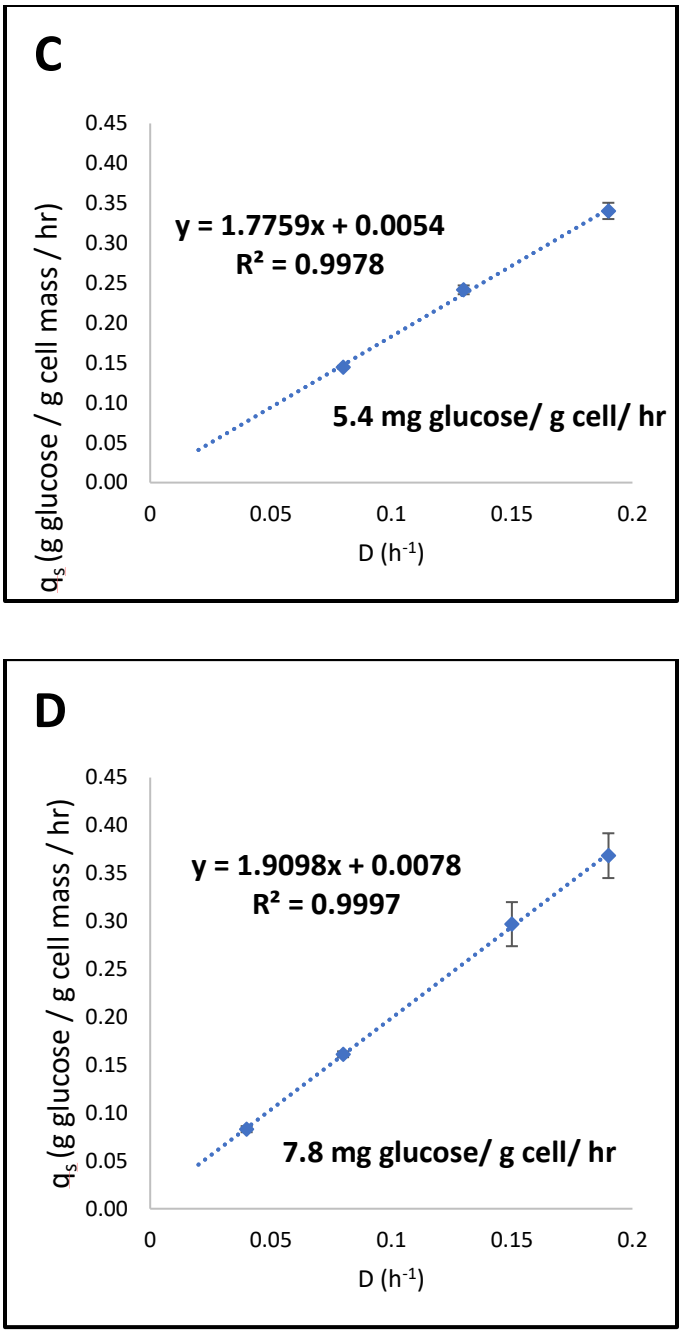


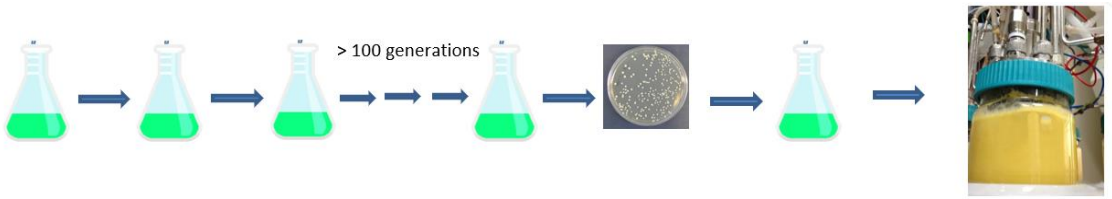
Figure 10. 1-liter chemostat fermentations. The specific substrate uptake rate **(A)** and Herbert-Pirt Equation **(B)** were used to determine the maintenance coefficient for NS844 at 30°C **(C)** and 40°C **(D)** based on chemostat fermentations with dilution rates of 0.04, 0.08, 0.13, 0.15 and 0.19 ($hour^{-1}$).

Adaptive laboratory evolution to isolate strains more suited for growth on arabinose

Previous fermentations showed that NS844 does not utilizes arabinose as efficiently as other sugars. It's important that *A. adeninivorans* is capable of utilizing different 5-carbon and 6-carbon substrates because lignocellulose material consist of various sugars. To have an efficient lignocellulose to lipid conversion process, the yeast biocatalyst must be able to utilize all of the different sugars. In this experiment, adaptive laboratory evolution was performed in order to increase arabinose utilization in NS844. Batch fermentations on 50 g/L arabinose were executed with NS844 and NS844 (adapted). The adapted strain was conditioned on arabinose for over 100 generations by serial transfer in shake flasks. The adaptive laboratory evolution steps can be seen in figure 11A. A shake flask was inoculated with NS844 and allowed to grow to exponential growth phase (optical density 7-10), which took ~ 48 hours. A portion of these cells were then transferred to another flask and allowed to grow to exponential growth phase. This process was repeated 12 times until over 100 generations were achieved. It was observed that it took NS844 less time (< 48 hours) to reach exponential growth phase after more transfers were done. This indicates that through beneficial mutations, NS844 able to utilize arabinose faster when under selective pressure. After the serial transfers were completed, the adapted strain was cultured on a plate with arabinose as the carbon source. A single colony was then picked and grown in a shake flask with arabinose. This shake flask was used to inoculate a 1-L bioreactor. The NS844 adapted strain was run in comparison to the NS844 (not adapted) strain in bioreactors. The growth and arabinose consumption can be seen in figure 11B. NS844 appears to lag in growth for the first 12-16 hours of fermentation while the NS844 adapted strain only has a 4-hour lag. Although

NS844 lags more than the adapted strain, they both consume all of the arabinose by 100 hours. The maximum specific growth rate was similar for both strains as well. The maximum specific growth rate was 0.35 hr^{-1} for NS844 and 0.36 hr^{-1} for the adapted strain. Both strains reached a maximum cell density of ~20 g/L. The strain adaptation on arabinose appeared to only reduce the lag phase for growth on arabinose. Thus, we have isolated two strains that reduce the lag phase for growth on arabinose.

A



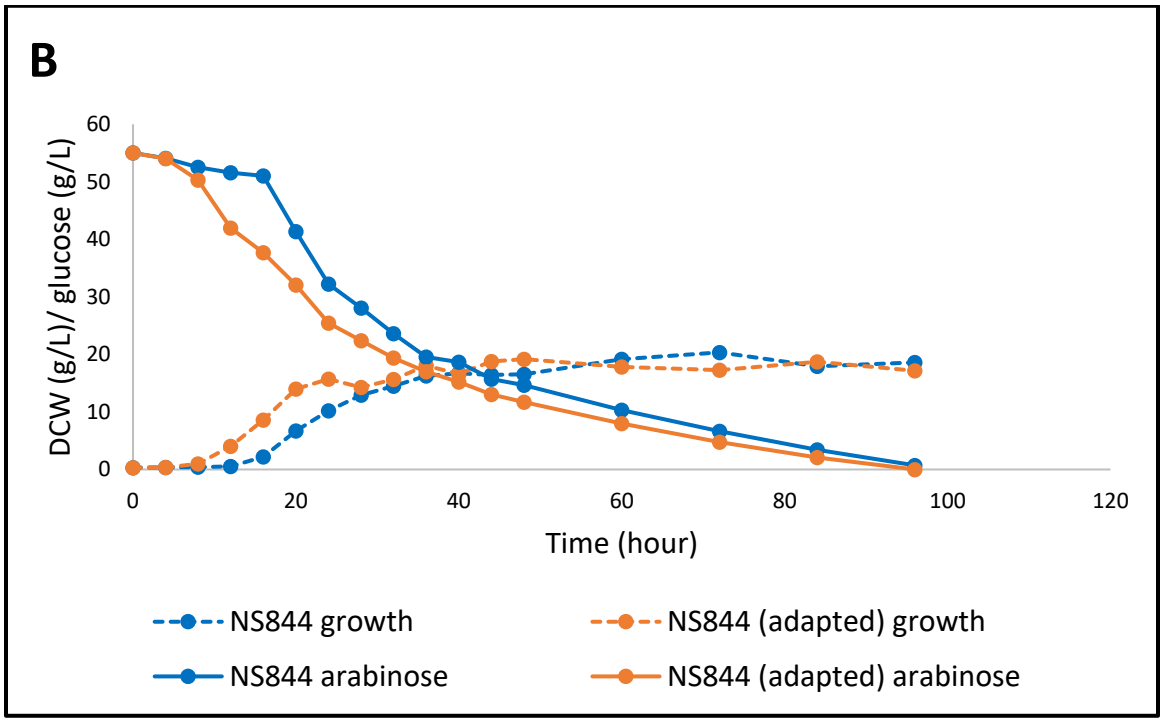


Figure 11. Adaptive laboratory evolution on arabinose. **A)** NS844 was conditioned on arabinose by serial transfer in shake flasks for over 100 generations. NS844 was then cultured on plates with arabinose as the carbon source. A single colony was picked and grown in shake flask and then used to inoculate a 1-L bioreactor. **B)** NS844 and NS844 (adapted on arabinose) were fermented in 1-liter Dasgip bioreactors on 50 g/L arabinose. HPLC was used to plot sugar consumption and DCW was used to plot growth

Strain engineering for arabinose utilization

Since adaptive laboratory evolution did not increase arabinose utilization with NS844, strain engineering was performed with *A. adeninivorans* to try to increase utilization. Batch fermentations on 50 g/L arabinose were executed with the wild-type strain NS252, engineered NS252 (LAR A), and engineered NS252 (LAD A). The growth and arabinose consumption can be seen in figure 12. The growth is similar for all three strains and they all reach a maximum DCW ~17 g/L. Arabinose consumption was also similar for all three strains. NS252 consumed 49.8 g/L in 85 hours. NS252 (LAR A) consumed 47.9 g/L and NS252 (LAD A) consumed 52.7 g/L of arabinose in 85 hours. The maximum specific growth rate for NS252 was 0.42 hr^{-1}. This was slightly higher than NS252 (LAR A) and NS252 (LAD A) which had maximum specific growth rates of 0.37 hr^{-1} and 0.34 hr^{-1} respectively. One interesting observation was that when the engineered strains were grown on glucose in shake flasks, they were unable to grow on arabinose afterward in the reactor. However, the wild-type strain was able to grow on arabinose after pre-culturing on glucose in shake flasks. All strains were pre-cultured on arabinose because of this glucose to arabinose problem.

By engineering the wild-type NS252 with either the L-arabinose reductase gene (*larA)* or the L-arabitol dehydrogenase gene (*ladA)*, the arabinose utilization did not improve. The arabinose consumption was actually slightly lower in the engineered strains compared to the wild-type. To further troubleshoot the metabolic engineering of the arabinose utilization pathway, several steps will need to be taken in future studies. One possible explanation could be that there was a change in the sequence of the gene when it

was inserted into yeast. The genes were first synthesized in bacteria and then inserted into yeast. This bacteria to yeast insertion could have caused a problem with the correct sequence being inserted. Perhaps the genes were either transcribed inefficiently or they were transcribed but not enough protein was synthesized. This could have negatively impacted arabinose utilization. Another possible explanation for the poor performance of the engineered strains is that both *ladA* and *larA* need to be co-expressed. If, for example a metabolic intermediate accumulates resulting in negative feedback on the rest of the arabinose utilization pathway. If both genes are co-expressed, the conversion from L-arabinose to L-arabitol to L-xylulose might be more efficient. It's also possible that the *ladA* and *larA* genes from *A. niger* don't work as well in *A. adeninivorans*. More strain engineering using heterologous genes from other microorganisms that utilize arabinose should be done in *A. adeninivorans*. Additionally, enzymatic assays to test for functional *ladA* and *larA* expression is recommended. Studies of pathway-wide RNA, protein, and metabolite (transcriptomics, proteomics, and metabolomics) levels could additionally inform further arabinose pathway engineering.

The results from various fermentations suggest that biomass production is faster at 40°C, but lipid production is better at 30°C. *A. adeninivorans* was shown to have similar maintenance energies at 30°C (5.4 mg glucose/ g cell/ hour) and 40°C (7.8 mg glucose/ g cell/ hour). The maintenance energies of *A. adeninivorans* are similar to other oleaginous microorganisms and lower than bacteria. Fermentation experiments here have shown that *A. adeninivorans* can ferment xylose and mannose comparably to glucose. More work needs to be done to increase arabinose utilization in *A. adeninivorans*.

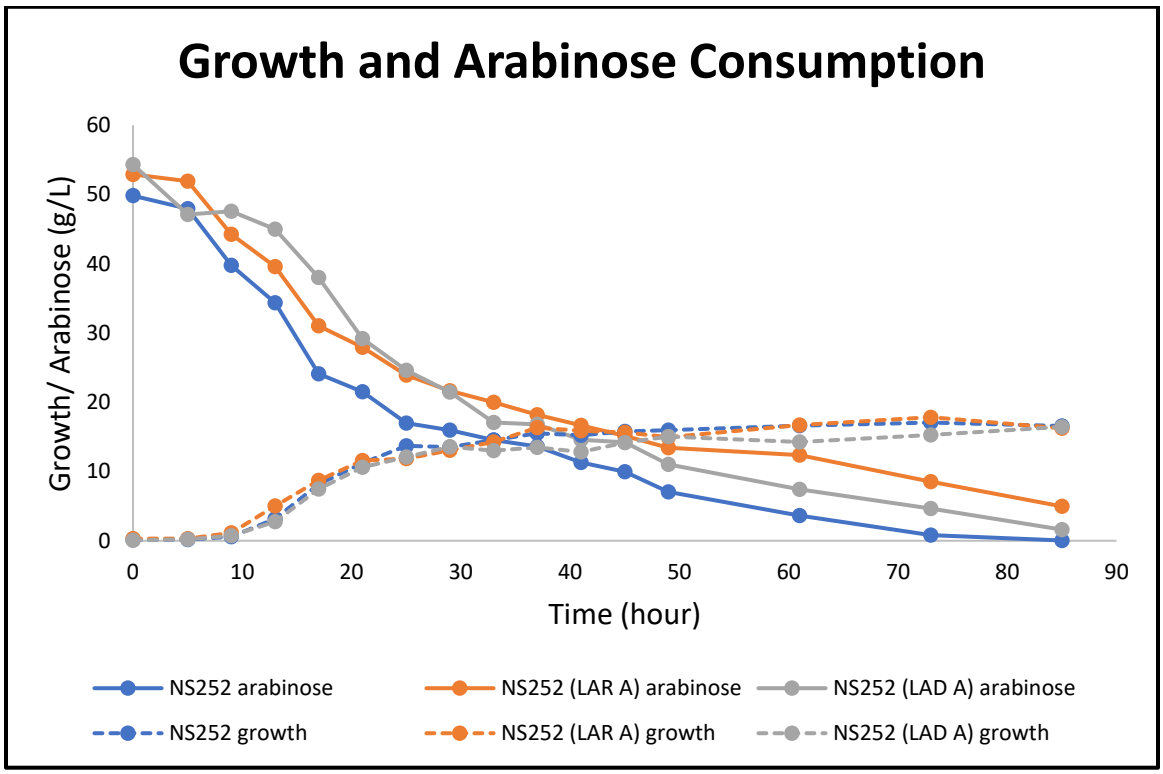


Figure 12. Strain engineering for arabinose utilization. Wild-type NS252, engineered NS252 (LAR A), and engineered NS252 (LAD A) were fermented in 1-liter Dasgip bioreactors on 50 g/L arabinose. HPLC was used to plot sugar consumption and DCW was used to plot growth.

CHAPTER 4

TECHNO-ECONOMIC ANALYSIS

Introduction/ purpose

A major cost in an aerobic industrial fermentation is heat removal from the fermenter. At higher temperatures, heat transfer from the fermenter to the external environment is efficiently performed via evaporative heat loss in cooling towers. In comparison, lower temperature fermentation requires an electricity demanding refrigeration cycle to transfer heat using industrial chillers. The transition from low cost to high cost cooling also depends on the environmental temperature that heat is discharged into. As a design criteria, a 35°C fermentation if often considered at the edge of this transition for United States based facilities (Crater et al. 2016). Therefore, it is important to compare the operating and capital cost at 30°C vs. 40°C.

Assumptions and model

In order to estimate the cost of running an industrial chiller to transfer heat in a 30°C oleaginous yeast fermentation , a process design and economics report from the National Renewable Energy Laboratory (NREL) was used as a base case for some of the calculations (Davis et al. 2013). The base case process for the conversion of lignocellulose to lipids can be seen in figure 13. In the first step, lignocellulosic biomass is pretreated at 100°C in the presence of dilute sulfuric acid (Davis et al. 2013). Here,

lignocellulose is broken down into polymers which include cellulose and hemicellulose. These polymers are then broken down by hydrolysis in the next step. During hydrolysis, cellulase enzymes are added to the slurry at 48°C. This is the optimal temperature for optimal hydrolysis of the polymers (Davis et al. 2013). The cellulase enzymes break down the cellulose fibers into cellobiose and gluco-oligomers and eventually into glucose monomers (Davis et al. 2013). Xylanase enzymes hydrolyze hemicellulose into xylan and ultimately into xylose monomers (Davis et al. 2013). After the hydrolysis step, the fermentable sugars are transferred into the fermentation reactor. This is where the oleaginous yeast biocatalyst ferments the sugars into lipids. The final steps of the process include downstream recovery and eventually a clean oil. In these steps, yeast cells must be broken up to access the lipid bodies inside of them.

For this techno-economic analysis, the cost of heat removal from the fermentation using a chiller will be determined. Most oleaginous yeast fermentations are performed at 30°C. It is important to maintain this temperature in order to keep the yeast in an optimal metabolic state. During an aerobic fermentation with oleaginous yeast, a certain amount of metabolic heat that is released because of the consumption of oxygen during catabolism of sugars (Crater et al. 2016). In order to maintain a temperature of 30°C, both a chiller and cooling tower are used to remove heat from the fermenter. For these calculations, a 1,000,000 L stainless steel bioreactor with a working volume of 750,000 L will be used with an oxygen transfer rate (OTR) of 100 mmol/L/hour. The OTR can be set stoichiometrically from the production rate of free fatty acids or triacylglyceride (Davis et al. 2013). The general stoichiometry of the conversion of glucose and oxygen to oleic acid-containing triacylglyceride, carbon dioxide and water can be seen in figure 14.

At an OTR of 100 mmol/L/hour, a productivity of 1.53 g/L/hour of lipid can be produced following the stoichiometry in figure 14. This 1.53 g/L/hour volumetric productivity is when cells are in the stationary phase. Cell biomass is produced in the growth phase for the first ~24 hours. Cells then transition to the stationary phase where they produce lipids. The biomass is typically made in a seed train like in the NREL model where cells are grown in five consecutive seed fermenters (Davis et al. 2013). First, a 75 L seed reactor is inoculated, and cells are grown to exponential phase. From this inoculum, another larger seed reactor is inoculated. The process is repeated, scaling up the seed reactor volume each time, until cells are grown in a 750,000 L seed reactor (Davis et al. 2103). The inoculum grown in the final 750,000 L seed reactor is typically used to inoculate multiple production fermenters. In these production fermenters, cells are in the stationary phase converting lignocellulosic sugar to lipid. Each production fermentation will run for 50 hours

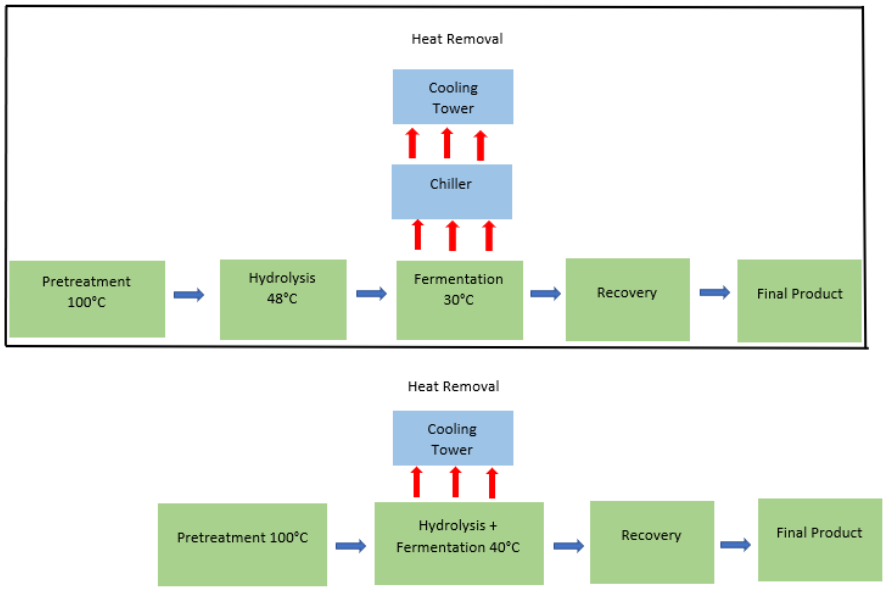


Figure 13. National Renewable Energy Laboratory (NREL) lignocellulosic conversion base case process. Shown is the process for the conversion of lignocellulosic biomass to lipid (NREL base case process outlined in black box). These steps include pretreatment of biomass, hydrolysis of polysaccharides into sugars, fermentation, downstream recovery, and final product. Fermentation at 30°C requires both a chiller and cooling tower. Fermentation at 40°C requires only a cooling tower and the hydrolysis step could potentially be done at the same time.

$$\mathbf{23\ C_6H_{12}O_6 + 58\ O_2 \longrightarrow 1\ C_{57}H_{104}O_6\ (triolein) + 81\ CO_2 + 86\ H_2O}$$

Figure 14. General lipid stoichiometry. The conversion of glucose and oxygen produces triolein (lipid), carbon dioxide and water.

Results

A standard fermentation for converting lignocellulosic sugars to lipids could take 50 hours to run with an oxygen transfer rate (OTR) of 100 mmol/L/hr. Over a 50-hour fermentation with a working volume of 750,000 L, 3,750,000 moles of oxygen would be utilized for the conversion of sugar to lipid. Aerobic fermentations are exothermic and produce heat. For an oleaginous yeast fermentation, 110 kcal of heat are produced per mole of oxygen consumed (Davis et al. 2013). Since 1 kcal is equal to 4.1868 kJ (Mohanty, 1994), the amount of heat produced over a 50-hour fermentation is 1.725×10^9 kJ. This would give a rate of 9,583 kJ/second of heat produced over the course of the fermentation. This is equal to 9,583 kW for a 50-hour fermentation in a 750,000 L fermenter.

According to the NREL report, the compressor electricity demand for a 2,350-ton Trane chiller was estimated to be 0.56 kW/ton of refrigeration (Davis et al. 2013). One ton of refrigeration is equal to 3.5 kW of energy (Arora, 2000). Therefore, the 9,583 kW produced during the fermentation would lead to 2,738 tons of refrigeration needed for cooling. This gives an energy demand of 1,533 kW of electricity . The average price of electricity in the United States is 13.30 cents per kilowatt hour (kWh) (Choose Energy, 2018). By taking the total electricity demand, the average price of electricity and the 50-hour fermentation run time, the total cost of electricity for running a chiller is estimated to be $10,327. If a fermentation plant was operating at 80% capacity for the year, then 146 fermentations could be run. The electricity demand of the chillers for the entire year would cost $1,507,808.

According to the NREL report, the minimum fuel selling price for renewable oil would be $5.35/ gallon (Davis et al. 2013). Since there are 3.78 L in a gallon, the minimum fuel selling price would be $1.42 / L. The density of oil is 0.91 kg/L (Broaddus, 2014), therefore, the selling price of oil would be $1.56/ kg. At a volumetric productivity of 1.53 g/L/hour of oil in a 750,000 working volume fermenter for 50 hours, there would be 57,375 kg of oil produced per a fermentation. The electricity cost of running a chiller for one fermentation was estimated to be $10,327. This would lead to a cost of $0.18/ kg of oil. Since the NREL base case selling price of oil is $1.56/ kg, the total cost for the electricity demand of the chillers constitutes 11% of the total oil selling price.

Conclusions

A typical aerobic fermentation process at 30°C would use both a chiller and a cooling tower to remove heat from the fermenter. According to the NREL report, the purchasing price and installation cost of a cooling tower would be $2,887,345 (Davis et al. 2013). The purchasing price and installation cost of two 2350-ton Trane chillers would be $2,411,264 (Davis et al. 2013). The total cost for both the cooling tower and chillers would be $5,298,609. When a fermentation is operated 40°C, heat can be removed by just using a cooling tower. This would allow for a savings of $2,411,264 toward purchasing and installation costs. Assuming a 10-year capital depreciation , there would also be a savings of $0.18/kg of oil produced by operating at higher temperatures and avoiding the use of chillers.

There are also other areas in the process which allow for possible cost savings when operating at 40°C. At higher temperatures cellulase enzymes perform better. Previous studies have shown that the activity of cellulase enzymes is twice as much at 40°C compared to 30°C (Ortega et al. 2000). This would allow for the possibility of eliminating a separate reactor for the hydrolysis step. The hydrolysis step and the fermentation could be run at the same time in a simultaneous saccharification and fermentation (figure 11). Eliminating the hydrolysis reactor would save $ 8,524,578 in purchasing and installation costs (Davis et al. 2013). *A. adeninivorans* also has the potential to produce its own cellulase and xylanase enzymes. This would eliminate the need for the addition of commercial enzymes which can often be expensive. Many types of oleaginous yeast can't ferment both 5-carbon and 6-carbon sugars. A separation of the sugars after pretreatment and hydrolysis is needed in this case Experiments in this paper have shown that *A. adeninivorans* is capable of fermenting glucose and xylose simultaneously. Therefore, the separation of sugars is not needed resulting in more cost savings. Another advantage of 40°C fermentation is the reduced risk of contamination. Bacteria and other microorganisms can often contaminate large scale industrial fermentations. Contamination can be extremely costly leading to loss of product or product quality, lost batches and production time and the cost of decontamination. . Altogether, there is great potential in cost savings while operating at 40°C and using *A. adeninivorans* as a biocatalyst.

CHAPTER 5

DISCUSSION

The use of *A. adeninivorans* as a biocatalyst for lignocellulose to lipids conversion is a promising technology. The results from various fermentations suggest that biomass production is faster at 40°C, but lipid production is better at 30°C. One of the reasons for this reduced lipid production at 40°C is thought to be because of the morphology of the yeast. At 40°C the yeast cells form a hyphal morphology . This may impact its ability to convert sugars into lipid. Perhaps there is an optimal temperature between 30°C and 40°C where the cells can grow and produce lipid at a reasonable rate while not going into hyphal form. Another solution is to genetically engineer *A. adeninivorans* to reduce the tendency of it to form hyphal cell bodies. Further strain engineering may be needed to increase lipid productivity as well.

A. adeninivorans was shown to have similar maintenance energies at 30°C (5.4 mg glucose/ g cell/ hour) and 40°C (7.8 mg glucose/ g cell/ hour). The maintenance energies of *A. adeninivorans* are similar to other oleaginous microorganisms and lower than bacteria. When converted to µmol glucose/ g cell/ hour, the maintenance energies of *A. adeninivorans* at 30°C and 40°C are 30 and 43 µmol glucose/ g cell/ hour respectively. This is comparable to the yeast *Candida parapsilosis*; (50 µmol glucose/ g cell/ hour) and

Saccharomyces cerevisiae; 70 µmol glucose/ g cell/ hour (Ykema et al. 1989). The maintenance energy of some bacteria, such as *Klebsiella aerogenes* or *Bacillus licheniformis*, can be as high as 240-300 µmol glucose/ g cell/ hour. It is important that the maintenance energy of *A. adeninivorans* is not large because less substrate is utilized for cell maintenance and more is used for lipid production.

Another advantage of *A. adeninivorans* is its ability to ferment multiple sugar substrates. Fermentation experiments here have shown that *A. adeninivorans* can ferment xylose and mannose comparably to glucose. *A. adeninivorans* also ferments xylose and glucose simultaneously in a mixed sugar fermentation, although glucose use is faster than xylose. This is important because lignocellulosic biomass is composed of various amount of different 5-carbon and 6-carbon sugars (Jorgensen et al. 2007). The ability to ferment different sugars into lipids simultaneously will save money in an industrial fermentation process. This separation adds time and money to the overall process. The potential to run a simultaneous saccharification and fermentation process (SSF) with *A. adeninivorans* eliminates the need for a separate hydrolysis reactor (Babiker et al. 2009) It's ability to ferment arabinose is still not optimal. Adaptative laboratory evolution for selective pressure on arabinose seemed to reduce the lag phase but not increase the rate of arabinose utilization of *A. adeninivorans*. Two genes from *A. niger* (*larA* and *ladA*), which are involved in the arabinose utilization pathway, were genetically engineered into wild type *A. adeninivorans*. Both of these engineered strains did not show an improvement in arabinose utilization. More strain engineering with *A.* adeninivorans, and further characterization of the arabinose metabolic pathway, will be necessary in order to improve arabinose utilization in this yeast.

Calculations from the techno-economic analysis show that the electricity cost of running a chiller adds $10,327 to each 750,000 L fermentation. This constitutes up to 11% ($0.18/ kg of oil) of the final selling price of $1.56/ kg of oil. There is also an extra $2,411,264 needed for purchasing and installing cost of chillers for a 30°C fermentation. Chillers also require a refrigeration cycle for cooling. This can lead to greenhouse gas emissions which have a negative impact on our environment. While not directly studied in this thesis, other cost savings with higher temperature fermentation include; lower enzyme loading, SSF vs. SHF process, and less risk for contamination. By engineering *A. adeninivorans* for higher lipid production, it's use as a biocatalyst in higher temperature fermentations could become a more cost-effective process for lignocellulose to lipid conversion.

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