

ENGINEERING AND EVALUATION OF YEAST STRAINS FOR THE PRODUCTION OF
LACTIC ACID FROM CELLULOSIC SUGARS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Food Science and Human Nutrition
with a concentration in Food Science
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2016

Urbana, Illinois

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ABSTRACT

Terrestrial biomass consists largely of lignocellulosic materials. Abundant in nature, lignocellulosic biomass can be cultivated easily on land otherwise unsuitable for traditional crops or be harvested from crop waste, such as corn stover. After pretreatment and hydrolysis, the lignocellulosic biomass will yield several sugars including glucose (6C), xylose (5C) and cellobiose (a glucose dimer). Complete conversion of each of these sugars is necessary to efficiently produce a target fermentation product from lignocellulosic hydrolysates. Until now, biofuels, such as ethanol, have been the only major fermentation product produced at an industrial-scale from lignocellulosic biomass. The primary goal of my dissertation research is to utilize metabolic engineering to construct a recombinant microbe capable of rapidly and efficiently producing value-added non-ethanol products from these lignocellulosic sugars. With this in mind, lactic acid has been selected as the target product to develop this lignocellulosic chemical production platform. Lactic acid has many industrial uses including as a feedstock for surgical implants, 3D printing, and renewable polyesters. The production of lactic acid from xylose was first achieved by cloning and introduction of a heterologous lactate dehydrogenase gene (*ldhA*) from the filamentous fungus *Rhizopus oryzae* into an engineered, xylose-fermenting *Saccharomyces cerevisiae* yeast strain. Simultaneous co-fermentation of xylose and cellobiose for the production of lactic acid by yeast was achieved by the introduction of the *ldhA* cassette into an engineered, xylose- and cellobiose-fermenting *S. cerevisiae*. Through screening on a variety of fermentation conditions and carbon sources, it was determined that non-repressing sugars, such as xylose and cellobiose, resulted in high lactic acid yields and negligible ethanol yields despite no genotypic disruption of the native yeast ethanol pathways. Similarly, a high

lactic acid yield was also achieved from lactose and cheese whey by the *ldhA*-expressing yeast strain. Production from lignocellulosic feedstocks was scaled-up to a one-liter bioreactor as a proof-of-concept, resulting in lactic acid yields exceeding 70 g lactic acid/g sugar with titers greater than 120 g/L of lactic acid. To further improve lactic acid yield by the engineered strains, a variety of industrial *S. cerevisiae* yeast strains were screened for their ability to tolerate organic acids, low pH conditions, and common fermentation inhibitors such as furfural. The industrial strain screening provided insight towards the future development of a highly lactic acid-resistant *Saccharomyces spp.* strain. In addition to attempting to improve lactic acid production by selecting an ideal parental yeast strain, several metabolic engineering approaches were implemented to elucidate ideal genetic characteristics of an engineered *ldhA*-expressing strain. Primarily, it was discovered that deletion of *JEN1* and/or *ADY2*, genes which code for monocarboxylate transporters, either reduced lactic acid uptake ($\Delta ADY2\Delta JEN1$ or $\Delta JEN1$) or reduced lactic acid yield by at least 25 % ($\Delta ADY2\Delta JEN1$). Collectively, this research has demonstrated a viable platform for the production of non-fuel chemicals from lignocellulosic feedstocks by engineered yeast and generated new understanding for the molecular basis of lactic acid production by engineered microbes.

ACKNOWLEDGEMENTS

Though only one name appears on the cover of this dissertation, the science and discussions contained within are the combined efforts of many hard-working individuals.

I would like to thank the following groups and individuals:

- Dr. Yong-Su Jin for his guidance and training
- The members of Dr. Yong-Su Jin's laboratory for assisting throughout my graduate program
- The members of Dr. Michael Miller's laboratory for general discussion and camaraderie, especially Jeni Hoeflinger and Max Van Tassell
- My committee members including Drs. Hans Blaschek, Nicki Engeseth, Hao Feng, Michael Miller, Christopher Rao, and Huimin Zhao
- Victoria Nguyen, Dr. Marjorie A. Jones, and Dr. Craig C. McLauchlan from the Illinois State University Department of Chemistry for training me during my undergraduate program
- The Department of Food Science & Human Nutrition Heads of Department Faye Dong and Sharon Nickols-Richardson
- Dr. Graciela Padua for checking the formatting of this dissertation
- The USDA National Institute of Food and Agriculture, the Jonathan Baldwin Turner Graduate Fellowship, the American Institute of Chemical Engineers, and Abbott Nutrition for financial support throughout my graduate program
- The numerous staff and faculty at the Carl R. Woese Institute for Genomic Biology, especially Janessa Gentry and Nikki Gentry, and the Animal Sciences Laboratory who shared their materials, instruments, space, and time
- The Department of Food Science & Human Nutrition staff, including Leslie Alexander, Terri Cummings, Amanda Floyd, Sanna Frazier, Betsy Lancaster, David Lopez, Audra Martin, Janice Trudell, Barb Vandeventer, and many others for their excellent support
- The Department of Animal Sciences staff, especially April Wellman
- The staff at the Division of Research Safety for helping us maintain a safe work environment
- My undergraduate "army" who assisted with numerous experiments and provided me excellent mentoring opportunities: Andrew Adiputra, Whi Yeon Cho, Zhengkai Gong, Chang-Hoon Hwang, Eunbee Kim, David Steffen, Vijay Subramaniam, Vimal Subramaniam, and Viridiana Viveros
- My friends and family who were all integral to completion of this work

Finally, I would like to thank my wife, Emily, who entered this stage of my life as my girlfriend, became my fiancée, and ended up my partner for life.

TABLE OF CONTENTS

CHAPTER I	INTRODUCTION TO YEAST METABOLIC ENGINEERING FOR PRODUCTION OF RENEWABLE CHEMICALS	1
CHAPTER II	LACTIC ACID PRODUCTION FROM XYLOSE BY ENGINEERED <i>SACCHAROMYCES CEREVISIAE</i> WITHOUT PDC OR ADH DELETION	18
CHAPTER III	LACTIC ACID PRODUCTION FROM CELLOBIOSE AND XYLOSE BY ENGINEERED <i>SACCHAROMYCES CEREVISIAE</i>	44
CHAPTER IV	CONVERSION OF LACTOSE, BOVINE MILK, AND WHEY TO LACTIC ACID BY ENGINEERED <i>SACCHAROMYCES CEREVISIAE</i>	69
CHAPTER V	CHARACTERIZATION AND SCREENING OF INDUSTRIAL YEAST STRAINS FOR IMPROVED LACTIC ACID TOLERANCE AND PRODUCTION	80
CHAPTER VI	PHENOTYPIC AND GENOTYPIC ANALYSIS OF ENGINEERED LACTIC ACID-PRODUCING YEAST TO ELUCIDATE UNDERLYING MECHANISMS	104
CHAPTER VII	SUMMARY AND FUTURE STUDIES	135
	REFERENCES	139

CHAPTER I INTRODUCTION TO YEAST METABOLIC ENGINEERING FOR PRODUCTION OF RENEWABLE CHEMICALS

1.1 General background

As human society has grown and developed, our demand for fuels and commodity chemicals has accelerated. This demand has manifested as many different outputs for both fuels and chemicals. For fuels, we have two major categories: transportation fuels and non-transportation fuels. Here, we will mainly discuss transportation fuels, which are currently primarily derived from non-renewable fossil fuels. These hydrocarbons, such as coal, petroleum, or natural gas, are processed into gasoline, ethanol, jet fuel, or other specialized products [1]. Approximately 80 % of energy use by humans is derived from fossil fuels, with up to 58 % consumed for transportation [2, 3]. Because the rate of natural production of fossil fuels has been increasingly outpaced by humanity's usage for decades, renewable alternatives for transportation fuels are considered a societal necessity [1].

As with fuels, many non-fuel chemicals are produced using unsustainable fossil fuel feedstocks. This petrochemical-based system is non-renewable and, as with fuels, an alternative method of production is needed to allow for continued advancement of human society. In particular, the petrochemical industry produces chemicals used in nearly every industry on earth. Many bulk chemicals, such as ethylene and propylene, are produced in the 1 – 100 million annual tons range, relying majorly if not entirely on fossil fuels as a feedstock for chemical catalysis [4].

Portions of the content of this chapter are currently under peer-review and revision with authorship by Timothy L. Turner, Heejin Kim, In Iok Kong, Jing-Jing Liu, Guo-Chang Zhang, and Dr. Yong-Su Jin, who was the director of the research.

Although fossil fuels and the petrochemical industry have been instrumental in the advancement of human society, as mentioned, humanity's use of fossil fuels far outpaces its rate of natural replenishment [5, 6]. The overall process by which fossil fuels are collected and processed has been described as the "peak oil" theory. M.K. Hubbert first presented the "peak oil" theory in 1956, stating that oil production in any geographical region would raise rapidly, level off ("peak"), and then decline rapidly, following a standard bell curve [1]. Recently, "peak oil" concerns in the short-term have been somewhat alleviated due to significant advances in fossil fuel harvesting technologies such as hydraulic fracturing or "fracking" [7, 8]. These advanced drilling technologies have also caused M. Hubbert's "peak oil" predictions for the United States to become inaccurate, with available oil exceeding the original prediction [9]. The fracking process requires the injection of high-pressure fluids deep into the earth to break large rock formations, providing easier access to buried fossil fuels [10]. Despite the economic benefits of fracking, there is a significant concern for financial, health, and environmental risks associated with the technique, including links to increased seismic activity [11-14].

Beyond the risks associated with the harvesting and processing of petrochemical-based fuels and chemicals, there also exists a significant environmental risk for the usage of these products. Namely, anthropogenic climate change is the process by which sequestered carbon is released into the Earth's atmosphere, in part by the usage of chemicals or burning of fuels, resulting in a net increase in atmospheric greenhouse gases [15]. As atmospheric greenhouse gas concentrations increase, the global climate is expected to warm and diminished living conditions are expected [16, 17]. Because carbon dioxide makes up the majority of anthropogenic greenhouse gas emissions, it is often the target compound for research seeking to reduce manmade climate change [18]. The production of fuels and chemicals from renewable, plant-

based biomass, is expected to decrease the rate of anthropogenic climate change, given appropriate land-use and other necessary considerations [19, 20].

The finite supply of fossil fuels, the risks associated with harvesting hard-to-obtain fossil fuels, and the concerns about manmade climate change related to fossil fuel use has collectively pushed researchers and governments towards producing fuels and chemicals from renewable biomass by engineered microbes [21, 22]. In particular, yeasts have served as major platform microbes for many of these studies.

Yeasts, specifically the species *Saccharomyces cerevisiae*, are well-studied organisms, even beyond their traditional use for the production of beer and other fermented foods and beverages [23]. Extensive tools exist for the manipulation and engineering of yeasts [24-27]. These tools have allowed for harnessing the *S. cerevisiae* native ability to grow in minimal medium, their generally recognized as safe (GRAS) designation, and their tolerance towards low pH and acidic conditions [28, 29]. With these tools and inherent physiological advantages, scientific advances for the production of fuels and chemicals from biomass by *S. cerevisiae* have improved dramatically in recent years. In this dissertation and the associated studies, I have aimed to further improve our knowledge of yeast metabolism and physiology with a specific goal of better understanding the production of lactic acid from lignocellulosic sugars by engineered *Saccharomyces cerevisiae*.

1.2 Yeast research processes

With modern metabolic engineering techniques improved since their advent in the 1970s and the more recent development of synthetic biology procedures, yeast engineering technologies have grown dramatically [21]. Many yeast engineering approaches follow a scheme

known as the “Design, Build, Test, and Learn” cycle [30, 31]. This scheme first requires a target outcome or goal. For example, a target goal could be to produce ethanol from the pentose sugar xylose by engineered *S. cerevisiae*, which natively are unable to ferment xylose.

Once the desired outcome is determined, a parental yeast strain, often a wild-type strain, is selected as the target organism to be engineered. The steps for engineering the parental strain are as follow: 1) **Designing** the specific yeast engineering steps, including plasmids and transformation protocols, 2) **Building** the engineered strain by introduction of target genetic perturbations, 3) **Testing** the newly-developed strain, often being fermentation and sampling, and 4) **Learning** from the new strain (**Fig. 1.1**). The new knowledge obtained from this process can then be factored into the design of the next strain and the cycle will repeat until the target outcome is reached. This systematic approach has led to significant advances in the development of engineered *S. cerevisiae* capable of fermenting novel substrates for the production of fuels and chemicals. Although not all studies explicitly state this four-step process, the general concept is applied in many cases and has been used for nearly all experiments within this dissertation.

1.3 Chemical production by engineered or evolved yeast

There has been an intensive effort for the engineering of *S. cerevisiae* to produce non-fuel, value-added chemicals. Historically, *S. cerevisiae* has been used for ethanol production by the food or fuel industries, but scientific advances for the purpose of ethanol production by yeast can often easily be applied to non-fuel production. As previously mentioned, *S. cerevisiae* has GRAS status and their genetic system has been studied heavily. Thus, many genetic tools are available [26, 32, 33] which ease the engineering of this host organism to produce

nonconventional target products. These products include food additives, pharmaceuticals, advanced biofuels, and valuable chemicals for industrial applications.

Natively, *S. cerevisiae* produces numerous minor and major intermediates and metabolites, especially those throughout the glycolytic pathway, the pentose phosphate pathway, and the tricarboxylic acid pathway [35]. However, to accumulate a significant concentration of these intermediates (or other, non-native compounds) for industrial purposes, considerable engineering or evolution of *S. cerevisiae* is often necessary. Methods, such as the Design, Build, Test, and Learn approach (**Fig. 1.1**) or tools, such as CRISPR/Cas9 [32], have been largely applied for the purpose of producing ethanol by yeast fermentations, but can and have been easily re-tooled for constructing yeast capable of producing many other chemicals. These chemicals cover many broad categories including isoprenoids, fatty acids, organic acids, rare sugars, sugar alcohols, and others. A recent tour de force of *S. cerevisiae* engineering came from Galanie et al., in which the group required 23 enzymes from bacteria, mammals, plants, and yeast to produce a tiny amount of opioids, albeit at roughly five orders of magnitude below what would be necessary for industrial scale-up [36]. However, this demonstrates a future for yeast biotechnology in which a single biosynthetic pathway can create downstream products that may otherwise take multiple chemical catalysis steps. Many of these chemicals and their associated studies are listed in **Table 1.2**.

1.3.1 Categories of chemicals produced by *S. cerevisiae*

1.3.1.1 Isoprenoids

Isoprenoids, also known as terpenes, are a diverse group of chemical compounds

typically utilized as medicine, cosmetics, nutritional supplements, food additives, or even as a potential future biofuel [37]. *S. cerevisiae* harbor natural metabolic pathways to produce certain isoprenoids, however, yields and productivities are very poor [38]. Despite the poor natural production, isoprenoids have great interests due to their diverse structures and a wide range of potential uses. Monoterpenes (C10) and sesquiterpenes (C15) are two of the main candidates for jet fuels and biodiesel alternatives, due to their low freezing temperature and high ignition stability properties. To produce isoprenoids, acetyl-CoA production is of a high importance since all isoprenoids share the mevalonate metabolic pathway starting from acetyl-CoA [39-41]. Either the bacterial 1-deoxyl-D-xylulose 5-phosphate (DXP) pathway or the eukaryote/archaea mevalonate (MVA) pathway is essential for the biosynthesis of isoprenoids. Both pathways end with the formation of five-carbon monomers dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). DMAPP and IPP are then condensed and modified by prenyltransferases to form isoprenoid precursors such as geranyl pyrophosphate (GPP, C10) and farnesyl pyrophosphate (FPP, C15) [42].

Monoterpenes (C10) are derived from GPP by monoterpene synthases. Fischer et al. is the first group able to produce geraniol, a monoterpene and alcohol, with a titer of up to 5 mg/L in *S. cerevisiae* by a mutation of *ERG20* (farnesyl pyrophosphate synthase) and the overexpression of heterologous geraniol synthase (monoterpene synthases) from *Ocimum basilicum* [40]. To improve the monoterpene biosynthesis, Ignea et al. applied the yeast sterol biosynthesis pathway genes, *HMG2*, *ERG20*, and *IDI1*, and also co-expression of two terpene synthase enzymes (cineole synthase) from *Salvia fruticosa* and *Salvia pomifera*. The final titer of cineole was up to 1 g/L [43].

Sesquiterpenes (C₁₅) are another isoprenoid-derived potential fuel source which has recently gained interest for several industrial applications. Bisabolene, a precursor of bisabolane, was produced at a titer of over 900 mg/L in engineered *S. cerevisiae* by Peralta-Yahya et al. [44]. The yeast was first engineered by overexpression of acetyl-CoA acetyltransferase (*ERG10*), isoprenyl diphosphate isomerase (*IDI1*), and farnesyl pyrophosphate synthase (*ERG20*), truncated HMG-CoA reductase (*tHMGR*), and the transcriptional regulator of the sterol pathway (Upc2-1). Then researchers examined six different bisabolene synthases isolated from *Arabidopsis thaliana*, *Picea abies*, *Pseudotsuga menziesii*, and *Abies grandis*. At last, they developed the highest titer with the codon-optimized bisabolene synthase (BIS) from *A. grandis* [44]. Recently, Özaydın et al. screened the *S. cerevisiae* deletion collection for carotenoid production and constructed a strain producing the highest titer of up to 5.2 g/L of bisabolene through double deletion of YJL064W and YPL062W [45].

1.3.1.2 Organic acids

Organic acids are widely used for many applications including usage as food additives. However, organic acids also serve as building blocks of many larger polymers by undergoing several steps of chemical catalysis. For example, lactic acid is produced by engineered *S. cerevisiae* by introducing lactate dehydrogenase (*ldh*). Through catalysis, polylactic acid (also known as polylactide; PLA) can be produced [46]. PLA is a renewable and biodegradable polyester used for many purposes including as a filament for 3D printing, for producing medical screws/implants, and for producing plastic diningware. Numerous studies have been conducted for producing lactic acid from engineered *S. cerevisiae* from a variety of feedstocks including glucose [47, 48], xylose [49], and cellobiose [50]. Currently, no study using engineered yeast has

been able to achieve the theoretical maximum of lactic acid production from glucose, xylose, cellobiose, or a mixture of these carbon sources, so work is on-going to further improve these fermentation processes.

Succinic acid is value-added organic acid which can be overproduced by engineered yeast [51-53]. Like lactic acid, succinic acid can be used as a precursor to several polyesters [54]. Furthermore, succinic acid is designated as GRAS by the U.S. Food and Drug Administration, which has allowed its use in the food industry as an acidity regulator. As an intermediate of the citric acid cycle (or tricarboxylic acid cycle), yeast will natively produce succinic acid if provided an aerobic environment, but overproduction of succinic acid requires multiple genetic perturbations. For example, Otero et al. constructed an engineered *S. cerevisiae* with deletions of *SDH3*, *SER3*, and *SER33* to reduce primary succinate consuming reactions and to interrupt glycolysis-derived serine [53]. The resulting engineered yeast displayed a 30-fold improvement in succinic acid titer and a 43-fold improvement in succinic acid yield as compared to the control strain.

Beyond succinic acid, glycolic acid, a C2 hydroxy acid, has gained attention in recent years. The global glycolic acid production in 2011 was approximately 40 million kg with expected value to more than double by 2018 [55]. Glycolic acid is often used as a building block of a polyglycolate. The polyglycolate polymer is used as a packaging material due to its high gas-permeability and mechanical strength. However, most glycolic acid is produced in a chemical process which relies on non-renewable fossil resources [55]. As an alternative, a biological route for the production of glycolic acid exists by converting glyoxylate through glyoxylate reductase into glycolic acid. In order to successfully overproduce glycolic acid, efficient glyoxylate reductase activity in an engineered *S. cerevisiae* is required. A further

improvement, up to approximately 1 g/L glycolic acid, can be achieved by deletions of the malate synthase (*MLS1*) and the cytosolic form of isocitrate dehydrogenase (*IDP2*) genes [55]. As the current generation of organic acids produced by *S. cerevisiae* continues to improve and develop, it is likely that more new, rare, or hard-to-obtain organic acids will begin to be produced in laboratories by engineered *S. cerevisiae* strains.

1.3.1.3 Rare sugars and sugar alcohols

Sugars such as L-ribose, D-allose, D-tagatose, and D-psicose are classified as rare sugars. As the name implies, these sugars are rarely found in nature, but also have beneficial health properties. L-ribose, for example, is considered as a very important intermediate to produce chemicals for pharmaceutical and food products [56, 57]. While D-ribose is very common in nature, L-ribose is not found in nature based on current knowledge. The driving demand of L-ribose production is due to its potential building block for L-nucleoside-based pharmaceutical compounds. L-nucleoside-based compounds or analogs play an important role in treating viral infections and cancers [58]. Currently, research regarding rare sugar production by engineered yeast is very limited.

Sugar alcohols, such as erythritol, xylitol, or sorbitol, have a high demand in the food industry due to their sweetening properties without causing dental caries [59]. In general, sugar alcohols are not fermentable by *S. cerevisiae*, which limits reuptake by engineered yeast designed to overproduce target sugar alcohols. As one primary example, xylitol shares similar sweetening power with sucrose, yet it does not contribute to dental caries and has a cooling effect when eaten. A chemical hydrogenation process to produce xylitol has existed for decades [60], but more recently, several groups have produced high xylitol titers and yields from

biological, engineered yeast systems [61-63]. Oh et al. were able to produce xylitol rapidly and efficiently using an engineered *S. cerevisiae* expressing xylose reductase (*XYL1*), a cellodextrin transporter (*cdt-1*), and an intracellular β -glucosidase (*gh1-1*) via simultaneous utilization of xylose and cellobiose [62]. As a result, the engineered *S. cerevisiae* was able to produce xylitol at the maximum theoretical yield by co-utilization of xylose and cellobiose.

1.4 Current scope and future outlook of industrial chemical production by yeast

These recent developments for producing non-fuel chemicals from yeast have generated *S. cerevisiae* strains which can overproduce native yeast compounds or, through expression of heterologous genes, produce non-native compounds. As with biofuel production, only a small portion of laboratory-scale yeast chemical production platforms have made the transition to the industrial-scale. Largely, this transition is made difficult by low yields and productivities.

Many non-*S. cerevisiae* microbes are employed for industrial chemical production due to the wide-range of target chemicals produced by the biobased chemical industry. Although *S. cerevisiae* is extremely hardy and can be easily engineered, there are still instances where other microbes are preferred for a target product. Perhaps the most notable example is the use of engineered *E. coli* for the production of recombinant insulin [64], and over 150 recombinant therapeutics have been approved by the European Medicines Agency [65]. However, only approximately one third of approved therapeutics utilize engineered *E. coli*, with *S. cerevisiae* and other yeast also account for a significant portion of industrial therapeutics, fuels, and chemicals [65].

Currently, there are dozens of companies worldwide which employ microbial fermentation for the production of renewable chemicals. In many cases, the exact specifications

of the species of microbe used or the precise metabolic pathway engineering protocol are not entirely disclosed. However, some of the more notable companies using a yeast-based fermentation platform include DSM, Verdezyne, BioAmber, Amyris, and NatureWorks, which produce, respectively, succinic acid [66], adipic acid [67], 1,4-butanediol [68], farnesene [69], and lactic acid [70].

As previously discussed, the United States Environmental Protection Agency's Renewable Fuel Standard mandates a certain amount of fuel must be derived from renewable resources [71]. However, there is no equivalent legislation in the U.S. or worldwide which forces or encourages industry-wide production of renewable chemicals from non-petroleum sources. A global effort to limit global warming to 2 °C by reducing greenhouse gas emissions provides a minor incentive for biobased chemical production [72]. The influence this legislation has on biobased chemicals is small due to less than 10 % of total fossil fuels being employed for chemical catalysis, with the vast majority going towards the energy and transportation fuel industries [73, 74].

1.5 Motivations and research objectives

Equipped with rapid advances in metabolic engineering, synthetic biology, and genomics, the production of fuels and non-fuel chemicals by engineered *S. cerevisiae* has developed tremendously. Several of these advances have transitioned to industrial-scale fermentation processes, allowing for the sustainable production of many valuable chemicals from renewable biomass. Despite these advances and growing number of industrial examples, many barriers still exist which can hinder the further adoption of *S. cerevisiae* industrial fermentations.

Currently, global oil prices have reached the lowest levels in approximately a decade [75]. Low oil prices are a major detriment not only to the cost-effective production of renewable fuels and chemicals, but also to consumer and government sentiment regarding the short-term importance of developing a renewable chemical industry infrastructure. Furthermore, reduced oil prices significantly lower the cost of petroleum-based chemicals, which places additional pressure on renewable, fermentation-based biochemical production. Despite these pressures, many industrial biobased processes, such as succinic acid production (from *E. coli*) [76] and bioethanol production (from *S. cerevisiae*) [77, 78], are still considered to be feasible or even preferential to petrochemical production.

The objective of this dissertation is to not only develop optimal yeast strains for the production of lactic acid from lignocellulosic sugars, but also, to elucidate the underlying mechanisms which are necessary for this process to occur. The specific objectives include: 1) produce lactic acid from xylose and cellobiose by engineered yeast, 2) provide evidence of laboratory scale-up of this process, 3) identify industrial yeast, with all strains used in this dissertation listed in **Table 1.3**, which have increased lactic acid tolerance, 4) modify native carboxylate transporters to improve or change lactic acid yields, and 5) uncover the basis by which perturbations of carboxylate transporters impacts lactic acid production at the molecular level. Throughout this process, the “Design, Build, Test, and Learn” cycle (**Fig. 1.1**) will be employed and, ideally, these studies will have a positive impact on future laboratory- and industrial-scale lactic acid production projects.

1.6 Figures

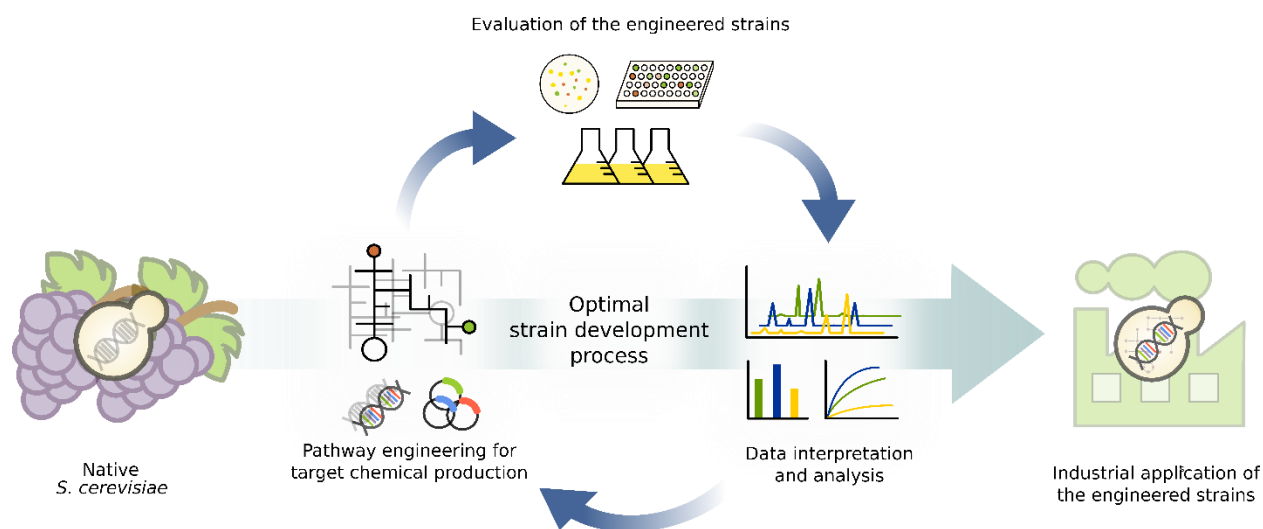


Fig. 1.1 Schematic demonstrating the step-by-step process for the Design, Build, Test, and Learn metabolic engineering/synthetic biology cycle used to develop engineered *Saccharomyces cerevisiae* for industrial-scale production of renewable fuels and chemicals.

Product	Substrate	Result	Genetic Modification(s)	Reference
Hydrocodone	Glucose	~0.3 µg/L titer	Expression of 23 genes encoding for various enzymes, overexpression of two native genes, and inactivation of one native gene	[36]
Geraniol	Glucose	5 mg/L titer	<i>ERG20</i> mutation and <i>O. basilicum</i> monoterpene synthase expression	[79]
Cineole	Galactose	1 g/L titer	Overexpression of <i>HM2</i> , <i>ERG20</i> , and <i>IDI1</i> with expression of two genes encoding for terpene synthases from <i>S. fruticosa</i> and <i>S. pomifera</i>	[43]
Bisabolene	Glucose and galactose	>900 mg/L titer	Overexpression of <i>ERG10</i> , <i>IDI</i> , <i>ERG20</i> , <i>tHMGR</i> , and <i>Upc2-1</i> with <i>A. grandis</i> BIS expression.	[44]
Bisabolene	Glucose or galactose	5.2 g/L titer	Deletion of YJL062W and YPL064W	[45]
Lactic acid	Glucose	44% tm	First use of <i>R. oryzae ldhA</i> in <i>S. cerevisiae</i>	[80]
Lactic acid	Glucose	62.2% tm	Bovine LDH controlled by <i>PDC1</i> promoter	[81]
Lactic acid	Glucose	86-93% tm	Selected cells with high intracellular pH	[82]
Lactic acid	Glucose	60% higher titer	<i>SSB1</i> knockout	[83]
Lactic acid	Glucose	81.5% tm	Bovine LDH and <i>PDC1ΔPDC5Δ</i>	[48]
Lactic acid	Glucose and xylose	69% tm	<i>R. oryzae ldhA</i> with <i>S. stipitis XYL1</i> , <i>XYL2</i> , and <i>XYL3</i> balanced expression and <i>PHO13ΔALD6Δ</i>	[49]
Lactic acid	Glucose, xylose, and cellobiose	66% tm	<i>R. oryzae ldhA</i> with <i>S. stipitis XYL1</i> , <i>XYL2</i> , <i>XYL3</i> , and <i>N. crassa cdt-1</i> and <i>gh1-1</i> balanced expression with <i>PHO13ΔALD6Δ</i>	[50]
Succinic acid	Glucose	12.97 g/L titer	Cytosolic retargeting of <i>MDH3</i> , <i>FRDS1</i> , and <i>E. coli FumC</i> with <i>PYC2</i> overexpression and <i>GPD1ΔFUM1Δ</i>	[51]
Succinic acid	Glucose	43-fold increase	<i>SDH3ΔSER3ΔSER33Δ</i> and directed evolution	[53]
Glycolic acid	Xylose and ethanol	~1 g/L titer	<i>A. thaliana GLYR1</i> and <i>MLS1ΔIDP2Δ</i> with <i>ICL1</i> and XR/XDK/XK xylose utilization pathway expression	[55]
Xylitol	Xylose and cellobiose	~100% tm	<i>S. stipitis XYL1</i> , <i>N. crassa cdt-1</i> and <i>gh1-1</i> expression with <i>ALD6</i> , <i>IDP2</i> , and <i>ZWF1</i> overexpression	[62]
Xylitol	Glucose and xylose	~100% tm	Two <i>XYL1</i> genes, <i>ZWF1</i> , and <i>ACS1</i> expression with fed-batch optimization	[63]

Table 1.2 Biobased chemicals from *Saccharomyces cerevisiae*. tm = theoretical maximum

Strain	Description	Source
<i>S. cerevisiae</i> D452-2	<i>MATa, leu2, his3, ura3, and can1</i>	[84]
D10	D452-2 <i>leu2::LEU2</i> pYS10	[85]
SR6	D10-6 <i>ura3::URA3</i> pSR6-X123	[85]
SR7	SR6 <i>his1::HIS1</i> pSR3-X23	[85]
SR7e3	Evolved strains of SR7 (SR7e1, SR7e2, and SR7e3)	[85]
SR8	SR7e3 <i>ald6::AUR1-C</i> pAUR_d_ALD6	[85]
SR8L	SR8 pITy3- <i>ldhA</i> -G418	Chapter II
SR8HL	SR8 pITy3- <i>ldhA</i> -HygB	Chapter VI
SR8HL-Emp	SR8HL pRS42K	Chapter VI
SR8HL-J1	SR8HL pRS42K-JEN1	Chapter VI
SR8HL-A2	SR8HL pRS42K-ADY2	Chapter VI
EJ3	SR8 <i>leu2::LEU2</i> pRS405-gh1- <i>ura3::URA3</i> pRS406-cdt-1	[86]
EJ4	Evolved strain of EJ3 by repeated transferring in cellobiose-containing media	[86]
EJ4ΔJEN1	EJ4 pRS42H- <i>JEN1Δ</i>	Chapter VI
EJ4ΔADY2	EJ4 pRS42H- <i>ADY2Δ</i>	Chapter VI
EJ4ΔADY2ΔJEN1	EJ4 <i>ADY2Δ</i> pRS42H- <i>JEN1Δ</i>	Chapter VI
EJ4ΔADY2ΔJEN1-empty	EJ4 <i>ADY2ΔJEN1Δ</i> / pRS42N	Chapter VI
EJ4ΔADY2ΔJEN1/ADY2	EJ4 <i>ADY2ΔJEN1Δ</i> / pRS42N-ADY2	Chapter VI
EJ4L	EJ4 pITy3- <i>ldhA</i> -G418	Chapter VI
EJ4LΔJEN1	EJ4L pRS42H- <i>JEN1Δ</i>	Chapter VI
EJ4LΔADY2	EJ4L pRS42H- <i>ADY2Δ</i>	Chapter VI
EJ4LΔADY2ΔJEN1	EJ4L <i>ADY2Δ</i> pRS42H- <i>JEN1Δ</i>	Chapter VI
EJ4LΔADY2ΔJEN1-empty	EJ4L <i>ADY2ΔJEN1Δ</i> / pRS42N	Chapter VI
EJ4LΔADY2ΔJEN1/ADY2	EJ4L <i>ADY2ΔJEN1Δ</i> / pRS42N-ADY2	Chapter VI
ATCC 2360 (JIN 01 or J1)	Wild-type strain	ATCC
ATCC 4098 (JIN 02 or J2)	Wild-type strain	ATCC
ATCC 4124 (JIN 03 or J3)	Wild-type strain	ATCC
ATCC 4126 (JIN 04 or J4)	Wild-type strain	ATCC
ATCC 4127 (JIN 05 or J5)	Wild-type strain	ATCC
ATCC 4921 (JIN 06 or J6)	Wild-type strain	ATCC
ATCC 7754 (JIN 08 or J8)	Wild-type strain	ATCC
ATCC 9763 (JIN 09 or J9)	Wild-type strain	ATCC
ATCC 20597 (JIN 10 or J10)	Wild-type strain	ATCC
ATCC 20598 (JIN 11 or J11)	Wild-type strain	ATCC
ATCC 24855 (JIN 12 or J12)	Wild-type strain	ATCC
ATCC 24858 (JIN 13 or J13)	Wild-type strain	ATCC
ATCC 24860 (JIN 14 or J14)	Wild-type strain	ATCC
ATCC 26422 (JIN 15 or J15)	Wild-type strain	ATCC
ATCC 38554 (JIN 16 or J16)	Wild-type strain	ATCC
ATCC 46523 (JIN 17 or J17)	Wild-type strain	ATCC
ATCC 56069 (JIN 18 or J18)	Wild-type strain	ATCC
ATCC 60222 (JIN 19 or J19)	Wild-type strain	ATCC
ATCC 60223 (JIN 20 or J20)	Wild-type strain	ATCC
ATCC 60493 (JIN 21 or J21)	Wild-type strain	ATCC
ATCC 62914 (JIN 23 or J23)	Wild-type strain	ATCC
ATCC 66348 (JIN 24 or J24)	Wild-type strain	ATCC
ATCC 66349 (JIN 25 or J25)	Wild-type strain	ATCC
ATCC 96581 (JIN 26 or J26)	Wild-type strain	ATCC
J1X	ATCC 2360 pSR6-X123	Chapter V
J2X	ATCC 4098 pSR6-X123	Chapter V

Table 1.3 (cont.)

J3X	ATCC 4124 pSR6-X123	Chapter V
J4X	ATCC 4126 pSR6-X123	Chapter V
J5X	ATCC 4127 pSR6-X123	Chapter V
J6X	ATCC 4921 pSR6-X123	Chapter V
J8X	ATCC 7754 pSR6-X123	Chapter V
J9X	ATCC 9763 pSR6-X123	Chapter V
J10X	ATCC 20597 pSR6-X123	Chapter V
J11X	ATCC 20598 pSR6-X123	Chapter V
J12X	ATCC 24855 pSR6-X123	Chapter V
J13X	ATCC 24858 pSR6-X123	Chapter V
J14X	ATCC 24860 pSR6-X123	Chapter V
J15X	ATCC 26422 pSR6-X123	Chapter V
J16X	ATCC 38554 pSR6-X123	Chapter V
J17X	ATCC 46523 pSR6-X123	Chapter V
J18X	ATCC 56069 pSR6-X123	Chapter V
J19X	ATCC 60222 pSR6-X123	Chapter V
J20X	ATCC 60223 pSR6-X123	Chapter V
J21X	ATCC 60493 pSR6-X123	Chapter V
J23X	ATCC 62914 pSR6-X123	Chapter V
J24X	ATCC 66348 pSR6-X123	Chapter V
J25X	ATCC 66349 pSR6-X123	Chapter V
J26X	ATCC 96581 pSR6-X123	Chapter V
J1C	ATCC 2360 pRS425-BTT	Chapter V
J2C	ATCC 4098 pRS425-BTT	Chapter V
J3C	ATCC 4124 pRS425-BTT	Chapter V
J4C	ATCC 4126 pRS425-BTT	Chapter V
J5C	ATCC 4127 pRS425-BTT	Chapter V
J6C	ATCC 4921 pRS425-BTT	Chapter V
J8C	ATCC 7754 pRS425-BTT	Chapter V
J9C	ATCC 9763 pRS425-BTT	Chapter V
J10C	ATCC 20597 pRS425-BTT	Chapter V
J11C	ATCC 20598 pRS425-BTT	Chapter V
J12C	ATCC 24855 pRS425-BTT	Chapter V
J13C	ATCC 24858 pRS425-BTT	Chapter V
J14C	ATCC 24860 pRS425-BTT	Chapter V
J15C	ATCC 26422 pRS425-BTT	Chapter V
J16C	ATCC 38554 pRS425-BTT	Chapter V
J17C	ATCC 46523 pRS425-BTT	Chapter V
J18C	ATCC 56069 pRS425-BTT	Chapter V
J19C	ATCC 60222 pRS425-BTT	Chapter V
J20C	ATCC 60223 pRS425-BTT	Chapter V
J21C	ATCC 60493 pRS425-BTT	Chapter V
J23C	ATCC 62914 pRS425-BTT	Chapter V
J24C	ATCC 66348 pRS425-BTT	Chapter V
J25C	ATCC 66349 pRS425-BTT	Chapter V
J26C	ATCC 96581 pRS425-BTT	Chapter V
J17XL	J17X pITy3- <i>ldhA</i> -G418	Chapter V
<i>Escherichia coli</i> TOP10	Chemically competent <i>E. coli</i>	Thermo Fisher

Table 1.3 A list of all strains and their sources used throughout this dissertation.

Plasmid	Description	Source
pLDH68X	<i>R. oryzae ldhA</i> template	Provided by Dr. Chris Skory
pITy3- <i>ldhA</i> -G418	pPGK1- <i>ldhA</i> -PGK1t-Kanamycin/G418	Chapter II
pITy3- <i>ldhA</i> -HygB	pPGK1- <i>ldhA</i> -PGK1t-Hygromycin B	Chapter VI
pSR6-X123	<i>XYL1</i> , <i>XYL2</i> , <i>XYL3</i>	[87]
pRS425-BTT	<i>cdt-1</i> and <i>gh1-1</i>	[88]
pAG32	Hygromycin B template	Addgene
pRS42K	2- μ m origin, drug resistance marker (G418)	[89]
pRS42K-JEN1	pRS42K- <i>pGPD1-JEN1-CYCt</i>	Chapter VI
pRS42K-ADY2	pRS42K- <i>pGPD1-ADY2-CYCt</i>	Chapter VI
gRNA-ura-HYB	gRNA URA3 expression plasmid	[90]
pRS42H- Δ JEN1gRNA	pRS42H-JEN1gRNA	Chapter VI
pRS42H- Δ ADY2gRNA	pRS42H-ADY2gRNA	Chapter VI
pRS42N	pRS42-series conferring NAT resistance	Internal/Not published
pRS42N-ADY2	pRS42N-ADY2	Chapter VI
Primers	Description	Source
cLdhA-U	gggcccGGTACCGTCGACatggtattacactcaaggctc	Chapter II
cLdhA-D	gggcccGGTACCGGATCCtcaacagctacttttagaaaagg	Chapter II
Jin419	AGTCACATCAAGATCGTTTATGG	Chapter V
Jin420	GCACGGAATATGGGACTACTTCG	Chapter V
Jin421	ACTCCACTTCAAGTAAGAGTTTG	Chapter V
T102-JdDNA-U	aagttttctctaaagagattaatactgctactgaaaat CTTACGCCCAAGAACGTAGC	Chapter VI
T103-JdDNA-D	atagagaagcgaacacgccctagagagcaatgaaaagtga GCTACGTTCTTGCGGCGTAAG	Chapter VI
T104-AdDNA-U	actaaacaaccacaaaacaactcatatacaacaataat AGCACCTCCACTTGGTCAA	Chapter VI
T105-AdDNA-D	ttttatttcaatagttctcggtattagtagtgctgctc TTGACCAAGTGGAGGGTGCT	Chapter VI
T106-J-Confirm-F	AGCTAATAGCCGACAAACGG	Chapter VI
T107-J-Confirm-R	GCTACGTTCTTGCGGCGTAAG	Chapter VI
T108-A-Confirm-F	CATCTGCAGCGAGATACGAA	Chapter VI
T109-A-Confirm-R	TTGACCAAGTGGAGGGTGCT	Chapter VI
T110-J-gRNA-F	CGGTACGTACACTAGTTCTTTG	Chapter VI
T111-J-gRNA-R	GTACGTACGAGCTCAGACAT	Chapter VI
T112-A-gRNA-F	GCGTACGTACACTAGTTCTTTG	Chapter VI
T113-A-gRNA-R	GTACGTACGAGCTCAGACAT	Chapter VI
Ady2OE-F	gtacgtacggatccACCTTGGGATATCGTTGGA	Chapter VI
Ady2OE-R	gtacgtacccgcggGGCAAACGATAGACCTTTC	Chapter VI

Table 1.4 A list of plasmids and primers used throughout this dissertation.

CHAPTER II LACTIC ACID PRODUCTION FROM XYLOSE BY ENGINEERED *SACCHAROMYCES CEREVISIAE* WITHOUT PDC OR ADH DELETION

2.1 Introduction

Lactic acid is a chemical that has not been produced from fossil fuels currently [91, 92]. Lactic acid is biologically produced in excess of 260,000 tons annually [93] and it is primarily used as a precursor for the production of polylactide [91, 92]. Polylactide is a biodegradable polyester that has numerous applications, including use in plastic cups, bags, packaging materials, and surgical implants [94, 95]. While current production methods for producing lactic acid utilize sugars derived from corn starch or sugarcane as the primary substrate, utilization of cellulosic sugars derived from lignocellulosic biomass will be desirable in terms of availability and sustainability. Moreover, lactic acid from lignocellulosic biomass, such as *Miscanthus*, switchgrass, or corn stover would contribute to reducing carbon emissions [96].

The yeast *Saccharomyces cerevisiae* has been explored for its potential as a candidate for producing fuels and chemicals [97]. As opposed to lactic acid bacteria or *Escherichia coli*, which both suffer from limited growth in harsh fermentation conditions and are susceptible to phage infections, *S. cerevisiae* is a robust and acidic pH-resistant yeast that is preferred for lactic acid production. Natively, *S. cerevisiae* is incapable of producing lactic acid. Therefore, introduction

The content of this chapter was published. The authors, according to appearance in citation include Timothy L. Turner, Guo-Chang Zhang, Soo Rin Kim, Vijay Subramaniam, David Steffen, Christopher D. Skory, Ji Yeon Jang, Byung Jo Yu, and Yong-Su Jin. 2015. Lactic acid production from xylose by engineered *Saccharomyces cerevisiae* without PDC or ADH deletion. Applied Microbiology and Biotechnology 99.19 p.8023-8033. I performed the research with help from the co-authors and Dr. Yong-Su Jin was the director of the research.

of lactate dehydrogenase (LDH) into *S. cerevisiae* is necessary to produce lactic acid. LDH catalyzes the one-step conversion of pyruvate into lactic acid without producing carbon dioxide as a byproduct. Prior studies which produced lactic acid from *S. cerevisiae* introduced bovine LDH [81], *Lactobacillus plantarum* LDH [98], *Rhizopus oryzae* LDH [80], or *Lactobacillus casei* LDH [100] into *S. cerevisiae* to produce lactic acid from glucose. Although high lactic acid titers were achievable, disruption of the ethanol pathway by deletion of pyruvate decarboxylase (PDC) or alcohol dehydrogenase (ADH) was needed to minimize ethanol accumulation from glucose.

In order to produce lactic acid from cellulosic sugars such as xylose by engineered yeast, it is necessary to introduce a xylose metabolic pathway into *S. cerevisiae* as well as LDH. Through a combination of rational and inverse metabolic engineering approaches, we developed an efficient xylose-fermenting *S. cerevisiae* (SR8) [85]. Possessing balanced expression levels of *XYL1*, *XYL2*, and *XYL3* and deletion of *ADL6* and *PHO13*, the SR8 strain was able to ferment xylose rapidly and efficiently. In this study, we introduced *ldhA* from the fungi *Rhizopus oryzae* into the SR8 strain to produce lactic acid from xylose as well as glucose (**Fig. 2.1**). In terms of redox balance, the production of lactic acid from pyruvate requires the conversion of one NADH to an NAD^+ , which is identical to the redox balance from the two enzymatic reactions which allow for ethanol production from pyruvate. Therefore, replacement of ethanol production with lactic acid production by *S. cerevisiae* should cause no change to the overall redox of the cellular system as compared to the wild type *S. cerevisiae* producing ethanol. By introduction of a heterologous xylose assimilation pathway and a heterologous lactate dehydrogenase (LDH), the naturally low pH-tolerant *S. cerevisiae* could be a viable host strain for the conversion of cellulosic sugars into lactic acid.

2.2 Materials and Methods

2.2.1 Media, strains, and plasmids

Yeast cells were cultured in yeast extract peptone medium (10 g/L yeast extract and 20 g/L peptone, YP) containing glucose (YPD) or xylose (YPX). The concentrations of the sugars were displayed as numbers following their initials (e.g., YPD20, YP medium containing 20 g/L of glucose). In order to construct an *S. cerevisiae* strain capable of converting xylose to lactic acid, an engineered yeast strain (SR8) capable of fermenting xylose was transformed with an integrating plasmid which contained *ldhA* from *R. oryzae* under the control of the *PGK1* promoter. The integrating plasmid was based on a multi-copy integration pITy3 plasmid containing a *kanMX* antibiotic marker. The *ldhA*-expression cassette amplified from the pLDH68X plasmid [102] using primers cLdhA-U:

gggcccGGTACCGTCGACatggtattacactcaaaggtc and cLdhA-D:

gggcccGGTACCGGATCCtcaacagctacttttagaaaagg was inserted into SalI and BamHI enzyme sites of the pITy3 plasmid. *Escherichia coli* TOP10 were used for gene cloning and manipulation and were grown in Luria-Bertani medium; 50 µg/mL of kanamycin was added to the medium when required. The plasmid was transformed into the strain using a high-efficiency lithium acetate transformation method [103]. Yeast transformants were selected on YPD20 plates containing 300 µg/mL G418.

2.2.2 Flask fermentation experiments

S. cerevisiae stock cultures were maintained on YPX agar (2% w/v agar, 1% w/v yeast extract, 2% peptone, 2% xylose) plates. Yeast precultures were grown overnight in YPX40

medium in 5 mL total volume and harvested at mid-exponential phase, then washed twice with sterilized water to prepare inoculums for fermentations. Flask fermentations were performed using 25 mL of YP medium containing appropriate amounts of glucose or xylose in 125 mL Erlenmeyer flasks with an initial OD = 1 or 10 (A600 nm) as indicated with dry cell weight (DCW) also listed. For xylose fermentations, no glucose contamination was detected as determined by high-performance liquid chromatography (HPLC). Approximately 35 g/L of calcium carbonate (CaCO_3) was used as a neutralizing agent in specific experiments. All fermentations were held at 30 °C and at 100 RPM in a MaxQ4000 orbital shaker (Thermo Fisher Scientific Inc., USA) unless otherwise specified. Anaerobic fermentations were performed in an anaerobic glove chamber maintained in an $\text{N}_2\text{-CO}_2\text{-H}_2$ (85:10:5) atmosphere (Coy Laboratory Products, USA) at 30 °C and were allowed to sit idly without agitation, although flasks were shaken by hand before each sample was taken. All experiments were repeated independently in duplicate with variations indicated with error bars.

2.2.3 Bioreactor fermentation

Yeast precultures were grown in YPX medium and harvested at mid-exponential phase, then washed twice with sterilized water to prepare inoculums for fermentations. The bioreactor fermentations were conducted in YP medium containing 80 g/L of xylose using a BioFlo/CelliGen 115 bioreactor (New Brunswick Scientific Co., USA). An initial yeast cell concentration of OD =10 (A600 nm) was used. Working volume was set at 1 L inside of a 2 L glass vessel. Aeration was maintained at 1.5 L/min using microfiltered (0.22 μm) ambient air and an impeller rotation of 200 RPM. Temperature was maintained at 30 °C. NaOH (10 N) was added as needed to maintain a pH value of 6.

2.2.4 Lactate dehydrogenase activity assay

Lactate dehydrogenase enzymatic activity was measured by coupling the conversion of pyruvate to lactic acid to the oxidation of NADH to NAD⁺ with each reaction evaluated by a Biomate 3 UV-visible spectrophotometer (Thermo Fisher Scientific Inc., USA) at 340 nm. Glucose- or xylose-grown yeast were harvested at mid-exponential phase and centrifuged for 1 minute at $3200 \times g$ by a 5810R benchtop centrifuge with a swing-bucket rotor (A-4-81, Eppendorf, Germany) and the supernatant was removed. Fresh cell pellets were washed with a cocktail containing one dissolved tablet of cOmplete protease inhibitor (Roche Applied Science, Germany), 1 mM dithiothreitol, and 25 mM Tris-HCl at pH 7.5. The washed cells were transferred to a microcentrifuge tube, centrifuged for 1 minute at $21,000 \times g$ by a 5424R benchtop centrifuge (Eppendorf, Germany), and the supernatant was discarded. The cell pellet was resuspended with 1 mL of the described cocktail and a PCR tube-sized volume of 0.5 mm diameter glass beads was added to the microcentrifuge tube. The tube was alternated between 30 seconds on ice and 30 seconds of vortexing at high speed for 20 minutes. The cells were then centrifuged for 10 minutes at $21,000 \times g$ at 4 °C and the resulting raw cell extract was used for all enzymatic activity assays. Protein concentrations of freshly lysed cell extracts was measured by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., USA) following the supplied protocol using the included bovine serum albumin as the protein standard. Calculated activities were reported in units per milligram of soluble protein (U/mg). One U was defined as the amount of enzyme required to convert 1 μmol of NADH to NAD⁺ per minute. All results were reported as the average of two replicates. Reactions were carried out in 50 mM potassium phosphate buffer at pH 6.7 containing final concentrations of 1 mM pyruvate, 0.4 mM NADH, and an 80 μL aliquot of freshly prepared cell extract. The reaction was conducted at room temperature by

addition of pyruvate into a final volume of 800 μ L. Reactions were also attempted using 100 mM Tris HCl buffer at pH 6.7 containing final concentrations of 1 mM pyruvate, 0.4 mM NADH, and an 80 μ L aliquot of freshly prepared cell extract, although they were unsuccessful as detailed in the **Section 2.3.1**.

2.2.5 Analytical methods

Cell density for fermentations not using CaCO_3 was monitored by optical density (OD) at 600 nm using a Biomate 3 UV-visible spectrophotometer (Thermo Fisher Scientific Inc., USA). Due to the inability of CaCO_3 to fully dissolve in the media, cell density was not measured for experiments containing CaCO_3 . Glucose, xylose, xylitol, glycerol, acetate, ethanol, and lactic acid concentrations were determined by a 1200 Series high-performance liquid chromatography (Agilent Technologies Inc., USA) instrument equipped with a refractive index detector using a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., USA). The column was eluted with 0.005 N of H_2SO_4 at a flow rate of 0.6 mL/min at 50 $^\circ\text{C}$. pH was monitored using a PHM210 pH meter (Radiometer Analytical SAS, France) with attached Accumet 13-620-290 pH probe (Thermo Fisher Scientific Inc., USA).

2.3 Results

2.3.1 Construction of a xylose-fermenting, lactic acid-producing *S. cerevisiae* (SR8L)

We constructed an *S. cerevisiae* strain capable of producing lactic acid from xylose by introducing a fungal *ldhA* from *R. oryzae* into a xylose-fermenting *S. cerevisiae*. We used a Ty-integration vector for introduction of the *ldhA* expression cassette. As the copy number and

location of the Ty-integration vector can vary [104], we assumed that expression levels of *ldhA* in each transformant might be different. Therefore, we picked thirty colonies which grew on a YPD plate containing 300 µg/mL G418 and evaluated the production of lactic acid from xylose by each colony. From these colonies, only mild variation was observed, but the colony with the highest lactic acid yield (g/g) from xylose was named SR8L and used for all further experiments. Successful integration of the *ldhA*-containing plasmid was confirmed by colony PCR.

To further confirm successful expression of *ldhA*, we measured lactate dehydrogenase (LDH) activity from the crude cell extract of the SR8L strain grown on glucose or xylose. When initially measuring the LDH activities in the crude extracts of the SR8 and SR8L strains, we observed overlapping activity between PDC/ADH and LDH when using 100 mM Tris-HCl buffer (pH 6.7) for the reactions. We resolved this issue by replacing the Tris-HCl buffer with 50 mM potassium phosphate buffer (pH 6.7) instead (more explanations in Discussion). Using the potassium phosphate buffer, glucose- or xylose-grown SR8L expressed LDH activity of 1.4 ± 0.3 U/mg protein or 1.0 ± 0.2 U/mg protein, respectively. The parental strain (SR8), grown on glucose or xylose, expressed non-detectable LDH activity (<0.01 U/mg) with either sugar.

2.3.2 Lactic acid production from glucose by engineered *S. cerevisiae* (SR8L) at low initial cell density

We investigated lactic acid production profiles from glucose or xylose by the SR8L yeast strain. We conducted fermentation experiments in YP medium containing glucose, with or without a neutralizing agent (CaCO₃). Under YPD with 4% glucose medium without CaCO₃ (**Fig. 2.2b**) at an initial cell density of OD = 1 (0.47 mg/mL DCW, A600 nm), the engineered strain (SR8L) produced 6.9 g/L of lactic acid from ~40 g/L of glucose within 6.5 hours, but the

pH of the medium drastically reduced to 3.7 from the initial pH of 6.5. The yield and productivity of lactic acid from glucose were 0.17 g lactic acid/g glucose and 1.05 g lactic acid/L/h, respectively. Still, ethanol was a dominant fermentation product and its concentration reached to 15.4 g/L with a yield of 0.38 g ethanol/g glucose and a productivity of 2.4 g ethanol/L/h. Maximum concentrations of acetate and glycerol were 0.4 g/L and 1.1 g/L, respectively. The SR8L cell density increased from ~0.47 to 3.76 mg/mL DCW (A600 nm) by the end of the fermentation. This fermentation indicated that the SR8L strain produced twice as much ethanol as lactic acid from glucose when a neutralizing agent was not used. Under the same condition (YPD40) without CaCO_3 at an initial cell density of OD = 1 (0.47 mg/mL DCW, A600 nm), the parental SR8 strain produced no appreciable lactic acid, but accumulated 18.3 g/L of ethanol, a yield of 0.45 g ethanol/g glucose and a productivity of 2.6 g ethanol/L/h (**Fig. 2.2a**). The pH of the medium reduced from 6.5 to 5.3, the acetate titer was 0.25 g/L, and glycerol titer was 1.0 g/L. The SR8 cell density increased from ~0.47 to ~5.64 mg/mL DCW (A600 nm) by the end of the fermentation, a final cell density 50% greater than the SR8L strain in the same conditions.

To examine if controlling pH would improve lactic acid yield by the SR8L strain, we repeated the previous experiments, but added 35 g/L CaCO_3 (**Fig. 2.2d**). At an initial cell density of OD = 1 (0.47 mg/mL DCW, A600 nm) with CaCO_3 , the SR8L strain produced 9.9 g/L of lactic acid from ~40 g/L of glucose within 7.5 hours (**Fig. 2.2d**). This resulted in a yield of 0.22 g lactic acid/g glucose and a productivity of 1.32 g lactic acid/L/h. During the fermentation, the pH of the medium decreased from ~6.6 to 5.5. Ethanol accumulation reached a peak of ~14 g/L, a yield of 0.31 g ethanol/g glucose, and a productivity of 1.9 g ethanol/L/h. Titrers for acetate and glycerol reached a peak of 0.5 g/L or 0.9 g/L, respectively. Presumably, the higher pH

maintained by CaCO_3 addition improved lactic acid production from glucose by the SR8L strain. When grown in YPD with an initial cell density of $\text{OD} = 1$ (0.47 mg/mL DCW, A600 nm) with 35 g/L of CaCO_3 , the parental SR8 strain converted ~40 g/L of glucose into a non-appreciable amount of lactic acid, but accumulated 19.4 g/L of ethanol, a yield of 0.43 g EtOH/g glucose, and a productivity of 2.6 g/L/h (**Fig. 2.2c**). During the fermentation, the pH of the medium increased from 6.5 to 6.8. Acetate and glycerol accumulation reached a maximum of 0.4 g/L or 1.5 g/L, respectively. The fermentation profile was similar to the fermentation without CaCO_3 (**Fig. 2.2a**), suggesting that CaCO_3 had no influence on the parental SR8 strain fermentation profile.

2.3.3 Lactic acid production from xylose by engineered *S. cerevisiae* with low initial cell density

We then tested whether SR8L, which possessed a xylose metabolic pathway, could efficiently produce lactic acid from xylose. With an initial cell density of $\text{OD} = 1$ (0.47 mg/mL DCW, A600 nm), SR8L was unable to completely utilize all of 40 g/L of xylose in YP medium without neutralizing agent (**Fig. 2.3b**). Approximately 15 g/L of xylose remained in the medium after 74 hours, resulting in a total consumption of 27 g/L of xylose. At 45 hours, when lactic acid was at the highest concentration of 10.1 g/L, approximately 21 g/L of xylose had been consumed, resulting in a yield of 0.48 g lactic acid/g xylose and a productivity of 0.22 g lactic acid/L/h. At the same 45 hour time point, only 2 g/L of ethanol were produced, a yield of 0.1 g ethanol/g xylose and a productivity of 0.046 g/L/h. The pH of the medium decreased drastically from 6.2 to 3.6 at 45 hours. At the end of the fermentation, xylitol, acetate, and glycerol titers were 0.1 g/L, 3.45 g/L, and 1.2 g/L respectively. The SR8L cell density increased from ~0.47 to

2.12 mg/mL DCW (A600 nm) by the end of the fermentation, nearly half the final cell density of the glucose fermentation (**Fig. 2.2b**) without CaCO_3 . Although SR8L (containing a heterologous *ldhA* encoding for lactate dehydrogenase) could not utilize all available xylose, lactic acid was a primary fermentation product, with very little ethanol or xylitol production. In contrast, the parental strain (SR8) utilized 40 g/L of xylose completely within 45 hours in the same fermentation conditions (**Fig. 2.3a**). Ethanol production reached 13.6 g/L with a yield of 0.35 g ethanol/g xylose and a productivity of 0.48 g ethanol/L/h. After 28 hours, the pH of the medium decreased to 5.1 from an initial pH of 6.3. Xylitol, acetate, and glycerol accumulations after 28 hours were 1.9 g/L, 0.5 g/L, or 3.5 g/L respectively. The SR8 cell density increased from ~0.47 to 7.52 mg/mL DCW (A600 nm) by the end of the fermentation, a final cell density almost four times greater than the SR8L strain in the same conditions.

We reasoned that the combination of low pH and high lactic acid accumulation was the leading roadblock for the SR8L strain to utilize all available xylose, so we conducted another xylose fermentation after adding CaCO_3 as a neutralizing agent. As expected, the fermentation profile with the neutralizing agent greatly improved. Using YP medium containing 40 g/L of xylose with 35 g/L of CaCO_3 (**Fig. 2.3d**) with an initial cell density of OD = 1 (0.47 mg/mL DCW, A600 nm), the SR8L strain utilized all but 4.3 g/L of xylose within 56 hours. This resulted in a lactic acid accumulation of 28.9 g/L, a yield of 0.69 g lactic acid/g xylose and a productivity of 0.52 g lactic acid/L/h. The ethanol titer was negligible despite the SR8L strain contained an ethanol-producing pathway that was proven to be functional in glucose media or xylose media without addition of CaCO_3 . The initial pH of the medium was 6.6 which decreased to 5.6 within 56 hours. Xylitol, acetate, and glycerol accumulations reached 0.5 g/L, 0.4 g/L, or 3.3 g/L respectively. As with glucose (**Fig. 2.2d**), SR8L produced a significant amount of lactic

acid, especially when pH was maintained by addition of CaCO_3 . The rate of fermentation was roughly half of the parental SR8 in similar conditions (**Fig. 2.3c**), which may be due to the accumulation of lactic acid. Surprisingly, SR8L accumulated very little xylitol (~ 0.012 g xylitol / g xylose), a xylose pathway intermediate that otherwise accounts for up to 10% of the consumed xylose in the parental SR8 strain. To summarize, when a higher pH was maintained by addition of CaCO_3 , the SR8L strain produced nearly three times as much lactic acid and was able to ferment all available xylose.

The parental SR8 strain with an initial cell density $\text{OD} = 1$ (0.47 mg/mL DCW, A600 nm), in YP medium containing ~ 40 g/L of xylose and 35 g/L of CaCO_3 , utilized all but 1.9 g/L of xylose within 25 hours (**Fig. 2.3c**). Lactic acid was not accumulated and ethanol titer reached a peak of 15 g/L with a yield of 0.35 g ethanol/g xylose and a productivity of 0.61 g ethanol/L/h. During the fermentation, the initial pH of 6.6 reduced only slightly to a final pH of 6.5. Xylitol, acetate, and glycerol titers reached maximums of 3.9 g/L, 0.9 g/L, or 3.3 g/L, respectively. Compared to the lactic acid-producing SR8L strain, the parental SR8 strain produced substantially more xylitol (3.9 g/L compared to 0.5 g/L).

2.3.4 Lactic acid production from glucose by engineered *S. cerevisiae* at lower temperatures

Owed to a significantly greater production of lactic acid from xylose-grown SR8L than from glucose-grown SR8L and the slower rate of xylose utilization, we speculated that a slower sugar uptake rate could be the cause of the increased lactic acid titer from xylose. To assess this hypothesis, we conducted flask fermentations of SR8 and SR8L grown in YP medium with 40 g/L of glucose and 35 g/L of CaCO_3 at 30 °C, 23 °C, and 16 °C. With an initial cell density of $\text{OD} = 1$ (0.47 mg/mL DCW, A600 nm), the SR8 and SR8L strains grown at 30 °C were nearly

identical to results shown in **Fig. 2.2c** and **2.2d**, respectively (data not shown). When grown at 23 °C and 16 °C, the titers and yields of each product were nearly identical to that at 30 °C, although unsurprisingly, the rate of glucose uptake and productivity were significantly reduced (data not shown). Specifically, the lactic acid titer from SR8 was still negligible while the lactic acid titer from SR8L was approximately the same as when grown at 30 °C.

2.3.5 Lactic acid production from xylose by engineered *S. cerevisiae* at high initial cell density and high initial xylose concentration

To determine whether the SR8L strain can produce lactic acid even from high concentrations of sugar, a flask fermentation with 100 g/L of xylose and 35 g/L of CaCO₃ was conducted. With an initial cell density of OD = 10 (4.70 mg/mL DCW, A600 nm), the SR8L was able to consume all but 4.5 g/L of xylose within 126 hours (**Fig. 2.4**). At 102 hours, when 90 g/L of xylose had been utilized, lactic acid concentrations reached up to 60 g/L, representing a yield of 0.67 g lactic acid/g xylose and a productivity of 0.59 g/L/h. Compared to the fermentation experiment with a lower xylose concentration (40 g/L) containing CaCO₃ (**Fig. 2.3d**), the yield and productivity were similar, which suggests that SR8L can completely ferment high concentrations of xylose as long as pH is maintained using CaCO₃. Most importantly, at 102 hours, accumulated xylitol, glycerol, acetate, or ethanol by the SR8L strain were less than 1 g/L each. Collectively, this result suggests that efficient production of lactic acid from xylose might be feasible without disruption of the ethanol-producing pathway via deletion of *pdc* or *adh*, which is necessary to produce lactic acid from glucose by engineered yeast.

2.3.6 Lactic acid production from xylose by engineered *S. cerevisiae* under strict anaerobic conditions

Although the SR8L strain was able to produce lactic acid from xylose under oxygen-limited conditions, i.e. a micro-aerobic environment, large-scale industrial fermentations often utilize nearly complete anaerobic conditions. To examine whether the SR8L strain could produce lactic acid from glucose or xylose under strict anaerobic conditions, we conducted several fermentations in an anaerobic chamber with an initial cell density of OD = 1 (0.47 mg/mL DCW, A600 nm). Agitation was only provided when samples were taken by brief manual shaking of the flask. In YP medium containing 40 g/L of glucose and 35 g/L CaCO₃, the SR8L strain utilized nearly all glucose within 20 hours (**Fig. 2.5a**). At 20 hours, lactic acid titer reached to 7.8 g/L with a yield of 0.21 g lactic acid/g glucose and a productivity of 0.39 g/L/h. As with the oxygen-limited fermentation (**Fig. 2.2d**), ethanol was the major product accumulating a titer of ~15 g/L. Other minor byproducts were formed including glycerol and acetate. In YP medium containing ~40 g/L of xylose and 35 g/L CaCO₃, the SR8L strain utilized all xylose within 180 hours (**Fig. 2.5b**). At 140 hours, lactic acid titer reached to 15 g/L with a yield of 0.43 g lactic acid/g xylose and a productivity of 0.10 g/L/h, respectively. In contrast to oxygen-limited conditions where negligible amounts of byproducts (<0.1 g/L of ethanol, 3.3 g/L of glycerol, and 0.5 g/L of xylitol) were accumulated (**Fig. 2.3d**), the strict anaerobic condition resulted in accumulation of substantial amounts of ethanol (5.6 g/L), glycerol (7.3 g/L), and xylitol (1.5 g/L). This result suggests that homo-lactic acid production from xylose by the SR8L strain is at least slightly dependent on respiratory pathways and oxygen availability.

2.3.7 Lactic acid production from xylose by engineered *S. cerevisiae* in a 1 L bioreactor

To assess whether lactic acid production from xylose by the SR8L strain at a larger-scale, a bioreactor experiment with a working volume of 1 L was conducted. YP medium containing 80 g/L of xylose was used with an initial cell density of OD = 10 (4.70 mg/mL DCW, A600 nm). NaOH (10 N) was added as needed in place of CaCO₃ to maintain a pH value of 6 throughout the fermentation. Within 105 hours, nearly all xylose was consumed (**Fig. 2.6**). The lactic acid titer reached 49.1 g/L with a yield of 0.60 g lactic acid/g xylose and a productivity of 0.47 g/L/h. Titrers of ethanol (< 3 g/L), glycerol (< 8 g/L), xylitol (< 1 g/L), and acetate (< 2.5 g/L) all remained relatively low. The cell concentration increased 20% to ~12 (A600 nm). After 105 hours, 50 g /L of xylose was added to the bioreactor and was fermented within an additional 200 hours, resulting in a final lactic acid titer of ~80 g/L (data not shown).

2.4 Discussion

In order to produce lactic acid through microbial fermentation of sugars, prior studies have employed various microorganisms including *Bacillus* sp. [105, 106], *Rhizopus oryzae* [107, 108], and cyanobacteria [109]. Mostly, glucose was used as a carbon source to produce lactic acid. In a study which introduced a heterologous LDH into the natively xylose-fermenting yeast *Pichia stipitis*, xylose had been shown to be an effective carbon source for lactic acid production [110]. Here, we have demonstrated lactic acid production in an engineered yeast (*S. cerevisiae*) that cannot naturally ferment xylose (unlike the naturally xylose-fermenting *P. stipitis*), with a high yield and minimal byproduct formation (**Fig. 2.4**). Besides *Pichia stipitis*, other microbes including *Candida sonorensis*, *Rhizopus oryzae*, and *Candida utilis* have been engineered to produce lactic acid from xylose, but until now, lactic acid production from xylose-fermenting *S.*

cerevisiae has not been reported [108, 111]. In our study, the nearly undetectable production of ethanol was achieved without deletion or disruption of *pdh* or *adh* genes, which has been previously studied for producing lactic acid from glucose by *S. cerevisiae* [81, 112]. The SR8L strain, as shown, produces lactic acid as the primary product when grown on xylose, but produces ethanol as the primary product when grown on glucose. Additionally, the presence of *ldhA* in SR8L does not appear to inhibit glucose utilization even when lactic acid is produced.

The present study has constructed an engineered *S. cerevisiae* strain to produce *L*-lactic acid from xylose as a sole carbon source. This was achieved through introduction of a heterologous *R. oryzae*-sourced *ldhA* gene coding for lactate dehydrogenase into a xylose-fermenting *S. cerevisiae*. Of significant interest, we observed that the engineered strain (SR8L) primarily produced ethanol from glucose and lactic acid from xylose. We named this phenomenon as a substrate-dependent product formation (S-DPF). Similarly, Ilmén and colleagues engineered a *Pichia stipitis* strain capable of producing more lactic acid when fermenting xylose than glucose, although lactic acid was always the major product even when fermenting glucose [110]. From ~100 g/L xylose in CaCO₃-buffered YNB medium, the engineered *P. stipitis* strain accumulated 58 g/L of lactic acid and 4-5 g/L of ethanol; from ~100 g/L glucose in YNB medium, the *P. stipitis* strain accumulated ~40 g/L of lactic acid and 20 g/L of ethanol [110]. While the engineered *P. stipitis* produced a mixture of lactic acid and ethanol from xylose, our engineered *S. cerevisiae* was able to produce only lactic acid from xylose. Putative molecular mechanisms behind this S-DPF are currently not clear but we speculated several underlying mechanisms. First, because the xylose utilization rate of SR8L was much slower than on glucose, we hypothesized that a slower rate of sugar consumption, regardless of which sugar is used, results in a greater lactic acid yield. In order to test this, we grew SR8L in

YPD40 with 35 g/L CaCO₃ at 30 °C, 23 °C, and 16 °C, but despite an unsurprising decrease in glucose uptake rate at the lower temperatures, the yields of all products remained very similar (data not shown). Although the low temperature fermentations did not modify the lactic acid yield, the slower rate of xylose uptake compared to glucose uptake by the SR8L strain may still explain why more lactic acid is produced when fermenting xylose. The K_M of *R. oryzae* LDH on pyruvate is approximately 0.55 mM [113], which is a substantially lower than that of the *S. cerevisiae* PDC which has been measured as high as 3 mM and most recently at 2.29 mM [114]. This suggests that the slower uptake of xylose compared to that of glucose results in a lower intracellular pyruvate concentration, allowing for LDH to rapidly convert pyruvate into lactic acid before the pyruvate concentration is high enough for the lower affinity PDC to become effective.

Because conversion of pyruvate to ethanol or lactic acid both result in regeneration of NAD⁺, the redox balance by either pathway will be the same and therefore redox imbalance is unlikely to be a major factor for the substrate-dependent product formation. While cofactor differences of xylose reductase (XR) and xylitol dehydrogenase (XDH) might cause cofactor imbalance during xylose fermentation, we speculate that the imbalance would not be substantial enough to cause the changes in product formation patterns. Rather, it is possible that the lack of glucose signaling in xylose-fermenting *S. cerevisiae* resulted in weaker fluxes toward ethanol production, allowing the one-step conversion of pyruvate to lactic acid to take priority [115, 116]. In particular, the recognition of extracellular glucose by *SNF3*- or *RGT2*-encoded glucose sensors is known to cause the suppression of *JEN1* coding for lactic acid permease. However, *JEN1* is induced when non-fermentable carbon sources are metabolized [117-119], especially when xylose is used as a sole carbon source [120]. Since the SR8L strain produces ethanol as the

major product from glucose, but produces lactic acid as the major product from xylose, a highly-induced *JEN1* in the xylose condition may be one factor resulting in this phenotype. We can speculate that the up-regulation of *JEN1* might allow SR8L cells to rapidly expel lactic acid when grown on xylose. As the conversion of pyruvate to lactic acid is a reversible reaction and lactic acid can act as an allosteric inhibitor of LDH, efficient elimination of lactic acid from the cytosol might enhance metabolic fluxes toward lactic acid production.

When first measuring the LDH activities in the crude extracts of the SR8 and SR8L strains, we observed overlapping activity between PDC/ADH and LDH. As pyruvate can be a substrate for both PDC and LDH, NADH can be oxidized with the production of lactic acid via LDH, and with the production of ethanol if there is a strong activity of ADH via PDC. Under our initial LDH activity assay conditions using 100 mM Tris-HCl buffer (pH 6.7) and 3 mM pyruvate, crude extracts from the SR8 strain displayed a false-positive LDH activity, suggesting that the oxidation of NADH via PDC and ADH from pyruvate into ethanol was interfering with our LDH activity assay. In order to alleviate this issue, we reduced pyruvate concentrations from 3 mM to 2 mM, or even 1 mM in the assay as LDH has a much lower K_M for pyruvate than PDC. This reduction of the pyruvate concentration in the LDH assay resulted in a significant decrease in false-positive LDH activity in the crude extract of the SR8 strain but failed to completely eliminate NADH oxidation from the PDC/ADH reaction. Therefore, we replaced the 100 mM Tris-HCl buffer with 50 mM potassium phosphate (pH 6.7) buffer, as higher concentrations of phosphate (more than 25 mM) are known to decrease the affinity of PDC for pyruvate [121, 122]. After these modifications, we observed that crude extracts from the SR8 strain did not show any false-positive LDH activity but crude extracts from the SR8L strain showed strong LDH activity.

Serendipitously, we also observed that SR8L not only produced lactic acid at a relatively high yield (0.69 g lactic acid/g xylose), but also accumulated a nearly undetectable amount of ethanol when calcium carbonate (CaCO_3) was used as a neutralizing agent. As shown in **Fig. 2.3d**, ≤ 0.3 g/L of ethanol was detected during the xylose fermentation, a yield of < 0.007 g ethanol/g xylose. This represented nearly complete inactivity of the ethanol pathway naturally present in *S. cerevisiae*, and a 98% reduced ethanol titer (~ 0.3 g/L in contrast to ~ 15 g/L) as compared to the parental strain in the same condition. It is well understood that disruption or deletion of pyruvate decarboxylase genes (*PDC1*, *PDC5*, and *PDC6*) can significantly reduce or eliminate ethanol production, but at the cost of cell health [123, 124]. Similarly, deletion of alcohol dehydrogenase (*ADH*) can impair or eliminate ethanol production, but requires additional resources and may result in only temporary disruption of ethanol production [125]. For our study, no deletion of any *pdc* or *adh* genes was conducted, which resulted in relatively healthy cells that were capable of rapidly utilizing all available xylose, although this is dependent on usage of a neutralizing agent for maintaining pH above ~ 3.5 . Previous attempts to genotypically disrupt ethanol production in lactic acid-producing yeast have resulted in limited productivity [126, 127].

Of additional interest is the relative lack of byproducts in the final fermentation broth, resulting in a nearly homofermentative conversion of xylose to lactic acid. Xylitol accumulation, which results in wasted carbons and a reduced target product yield, is a long-term and yet unsolved problem for xylose utilization by engineered yeast possessing the heterologous xylose reductase (XR)/xylitol dehydrogenase (XDH) pathway [87, 128-130]. Xylitol accumulation has been resolved in engineered yeast which utilize the xylose isomerase (XI) pathway [131]. In this report, SR8L, which possesses the aforementioned XR/XDH xylose-utilization pathway,

converted ~100 g/L of xylose into ≤ 0.5 g/L xylitol, a yield of ~0.005 g xylitol/g xylose (**Fig. 2.4**). The single-step lactic acid pathway, consisting of only the reduction of pyruvate to lactic acid as opposed to two steps for the decarboxylation and reduction of pyruvate to ethanol may be one factor attributing to this phenotype. Importantly, the low accumulation of xylitol can lead to a higher target product yield and minimization of purification steps.

Acetate titer remained at ≤ 2 g/L during the YPX100 with 35 g/L CaCO_3 fermentation (**Fig. 2.4**), a yield of ~0.02 g acetate/g xylose. The glycerol titer reached 1.9 g/L and a yield of ~0.02 g glycerol/g xylose. Collectively, each measured byproduct (ethanol, xylitol, acetate, and glycerol) reached a maximum combined titer of 5.4 g/L, a combined yield of ~0.06 g byproducts/g xylose. Combined with a lactic acid yield of approximately 0.63 g lactic acid/g xylose (at 126 h), ~69% of xylose was converted into various end products, with the remainder likely for cell biomass, cell maintenance (especially export of lactic acid), or other minor metabolites. Although cell biomass, minor metabolites, and unspent medium nutrients would still require removal, the low concentrations of otherwise major byproducts in xylose fermentations (**Fig. 2.4**) may result in cheaper and less complex purification for the purpose of industrial lactic acid production. In general, purification cost, alongside feedstock cost, is a significant hindrance to cost-effective production of organic acids from microbial sources [132]. Industrial-scale production of lactic acid would require fermentation volumes far-exceeding our 1 L bioreactor fermentation (**Fig. 2.6**). In addition, a lower pH with minimal buffer or neutralizing agents and lignocellulosic hydrolysate medium rather than complex YP medium containing purified sugars would be necessary to minimize feedstock and purification costs. Further scale-up of xylose fermentations by SR8L as well as methods to improve lactic acid tolerance are on-going. The efficacy of using lignocellulosic feedstocks for the production of value-added chemicals has been

questioned for many reasons, such as price competitiveness and inefficient fermentation results. However, this study provides initial insight into considerations that non-glucose sugars, i.e. inexpensive and abundant cellulosic sugars, may be preferential for producing chemicals from heterologous pathways in *S. cerevisiae* [133]. Specifically, xylose could be a preferred sugar for the production of lactic acid by engineered yeast, assuming proper considerations for pH and oxygen control, resulting in minimal ethanol production without genetic disruption of the native ethanol pathway.

Here, we have demonstrated successful lactic acid production from xylose by engineered *S. cerevisiae*. This newly engineered yeast, named SR8L, accumulated lactic acid at a yield of ~0.21 g lactic acid/g glucose in CaCO₃-containing YPD medium, whereas the same strain accumulated lactic acid at a yield of ~0.69 g lactic acid/g xylose in CaCO₃-containing YPX medium with a xylitol titer of ≤ 0.5 g/L. Importantly, in YPX40 medium containing 35 g/L CaCO₃, SR8L accumulated a minimal level of ethanol (0.3 g/L) and ~28.8 g/L of lactic acid during the fermentation, representing an ethanol:lactic acid ratio of nearly 1:100 without deletion of *pdc* or *adh*. This suggests that xylose is a preferential carbon source for production of lactic acid from *S. cerevisiae*, and also indicates that direct genotypic disruption of the yeast ethanol pathway is not necessarily needed for production of lactic acid. Moreover, we envision that the substrate-dependent alteration in fermentation products reported here might be exploited for the efficient production of value-added products by engineered *S. cerevisiae*.

2.5 Figures

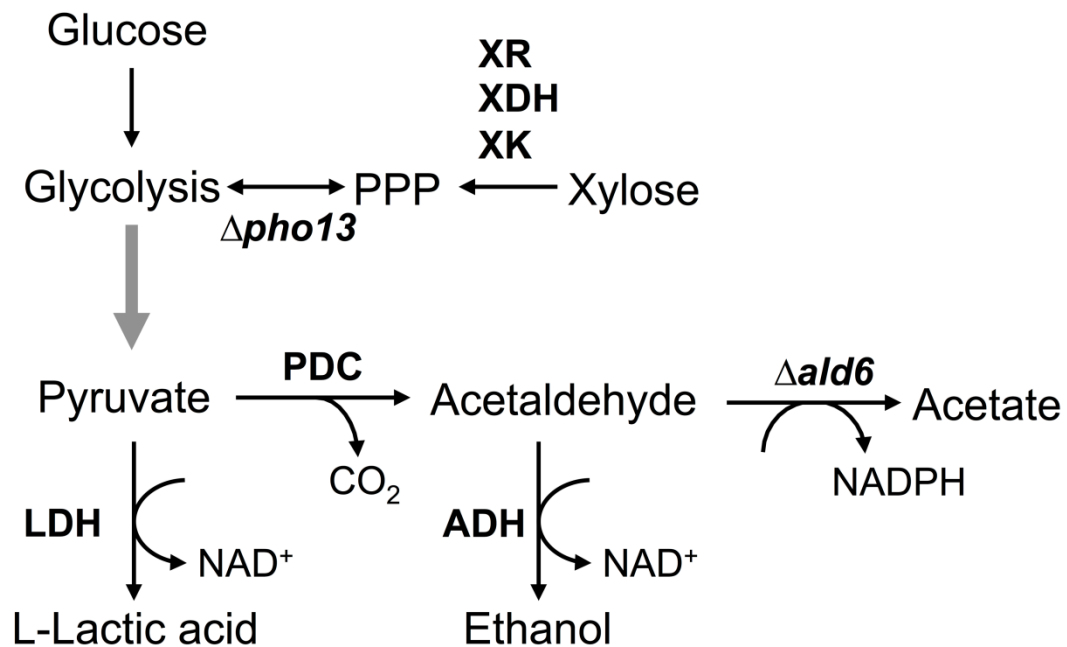


Fig. 2.1 Metabolic pathway for the engineered SR8L xylose-utilizing yeast with a heterologous lactic acid pathway. XR, XDH, and XK were previously expressed and *ald6* and *pho13* were deleted to allow for efficient xylose utilization and minimization of acetate production. In this study, *ldhA*, which encodes for lactate dehydrogenase (LDH) was introduced to allow for lactic acid production from xylose or glucose.

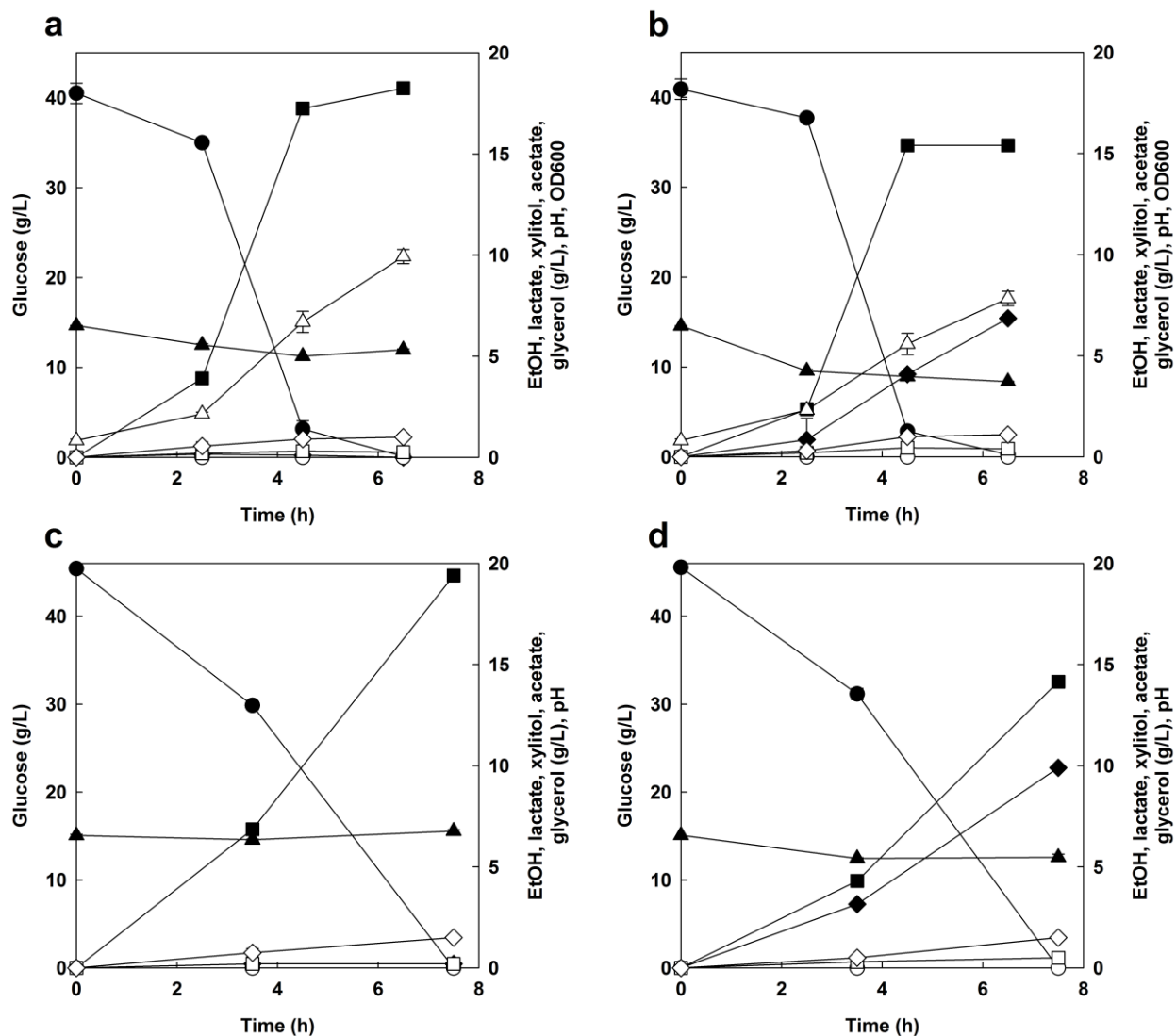


Fig. 2.2 Fermentation profiles for yeast grown in YPD medium. **a** Parental SR8 strain without CaCO_3 . **b** Recombinant SR8L strain without CaCO_3 . **c** Parental SR8 strain with 35 g/L CaCO_3 . **d** Recombinant SR8L strain with 35 g/L CaCO_3 . Glucose (*closed circle*), lactic acid (*closed diamond*), ethanol (*closed square*), pH (*closed triangle*), xylitol (*open circle*), acetate (*open square*), glycerol (*open diamond*), and OD (*open triangle*) are shown. The values are the means of two independent experiments and the *error bars* indicate the standard errors.

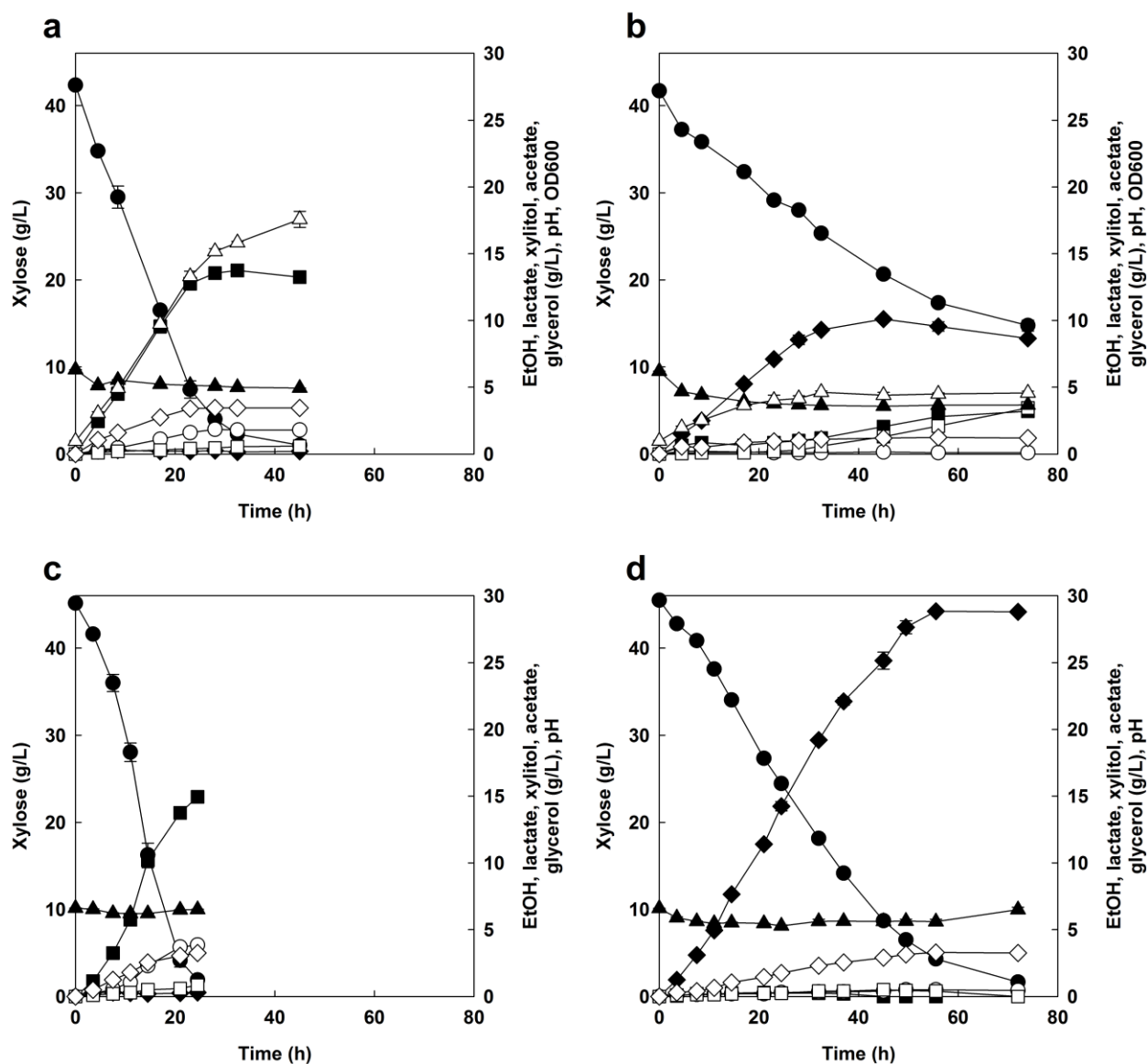


Fig. 2.3 Fermentation profiles for yeast grown in YPX medium. **a** Parental SR8 strain without CaCO_3 . **b** Recombinant SR8L strain without CaCO_3 . **c** Parental SR8 strain with 35 g/L CaCO_3 . **d** Recombinant SR8L strain with 35 g/L CaCO_3 . Xylose (closed circle), lactic acid (closed diamond), ethanol (closed square), pH (closed triangle), xylitol (open circle), acetate (open square), glycerol (open diamond), and OD (open triangle) are shown. The values are the mean of two independent experiments and the *error bars* indicate the standard errors.

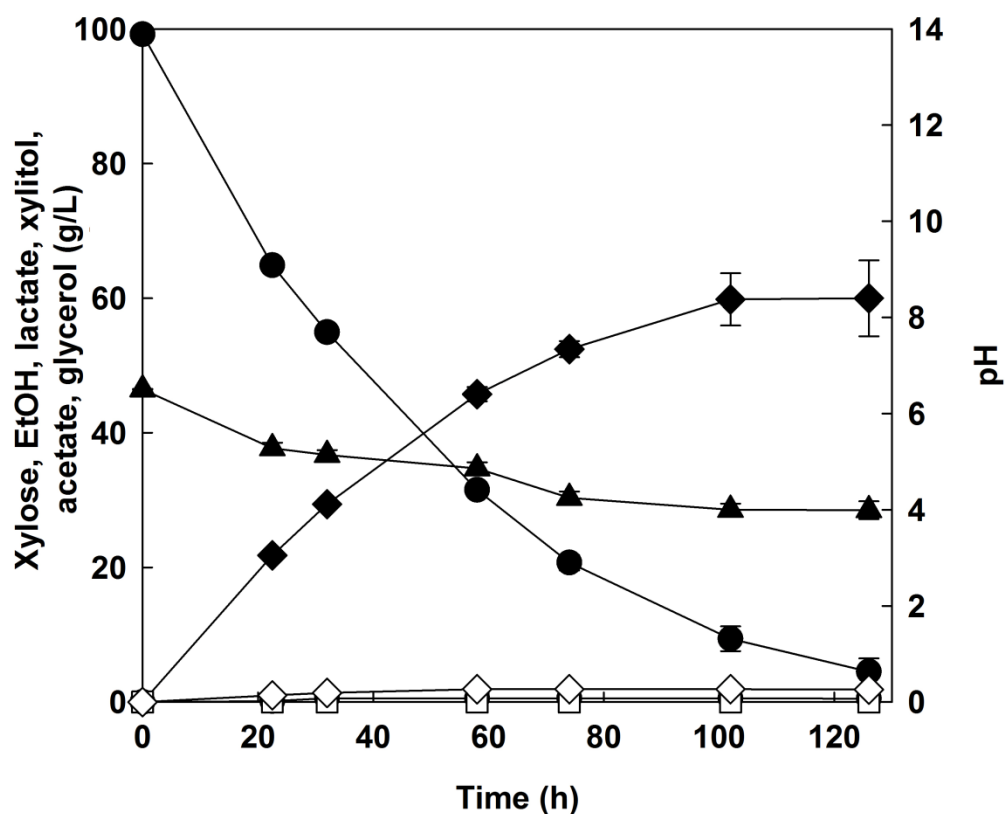


Fig. 2.4 Recombinant strain SR8L displaying a standard fermentation profile for 100 g/L of xylose (*closed circle*) in complex YP medium supplemented with 35 g/L of CaCO_3 . SR8L produced ~60 g/L of lactic acid (*closed diamond*). Ethanol (*closed square*), pH (*closed triangle*), xylitol (*open circle*), acetate (*open square*), and glycerol (*open diamond*) are also shown. The values are the mean of two independent experiments and the *error bars* indicate the standard errors.

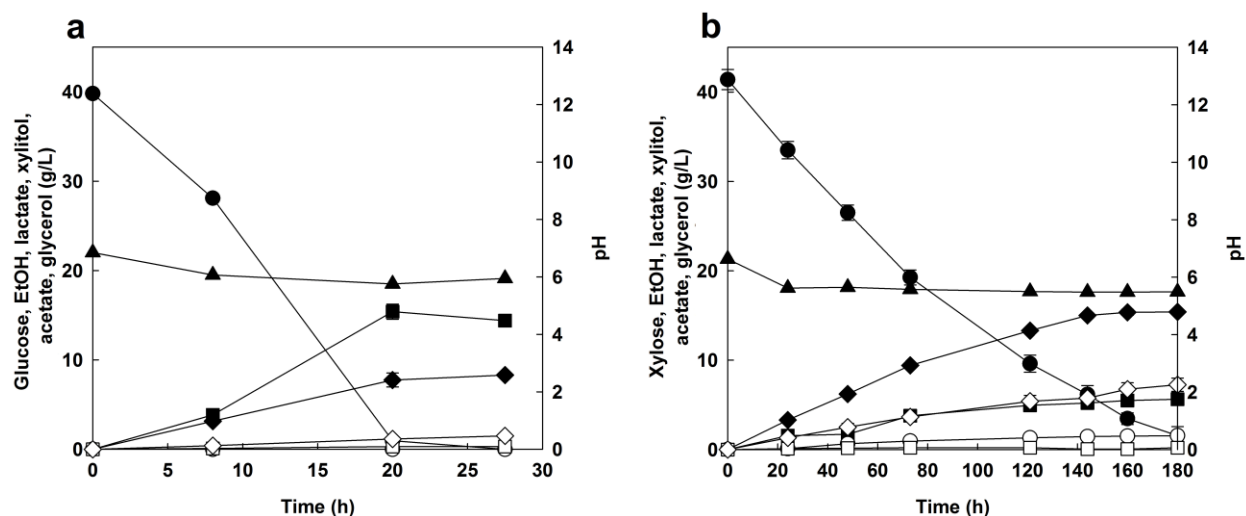


Fig. 2.5 Fermentation profiles for yeast grown in an anaerobic glove box. **a** Recombinant strain SR8L grown in YPD medium containing 35 g/L of CaCO_3 . SR8L fermented ~40 g/L of glucose (*closed circle*) producing 8.3 g/L of lactic acid (*closed diamond*). **b** Recombinant strain SR8L grown in YPX medium containing 35 g/L of CaCO_3 . SR8L converted ~40 g/L of xylose (*closed circle*) into ~15 g/L of lactic acid (*closed diamond*). Ethanol (*closed square*), pH (*closed triangle*), xylitol (*open circle*), acetate (*open square*), and glycerol (*open diamond*) are also shown. The values are the mean of two independent experiments and the *error bars* indicate the standard errors.

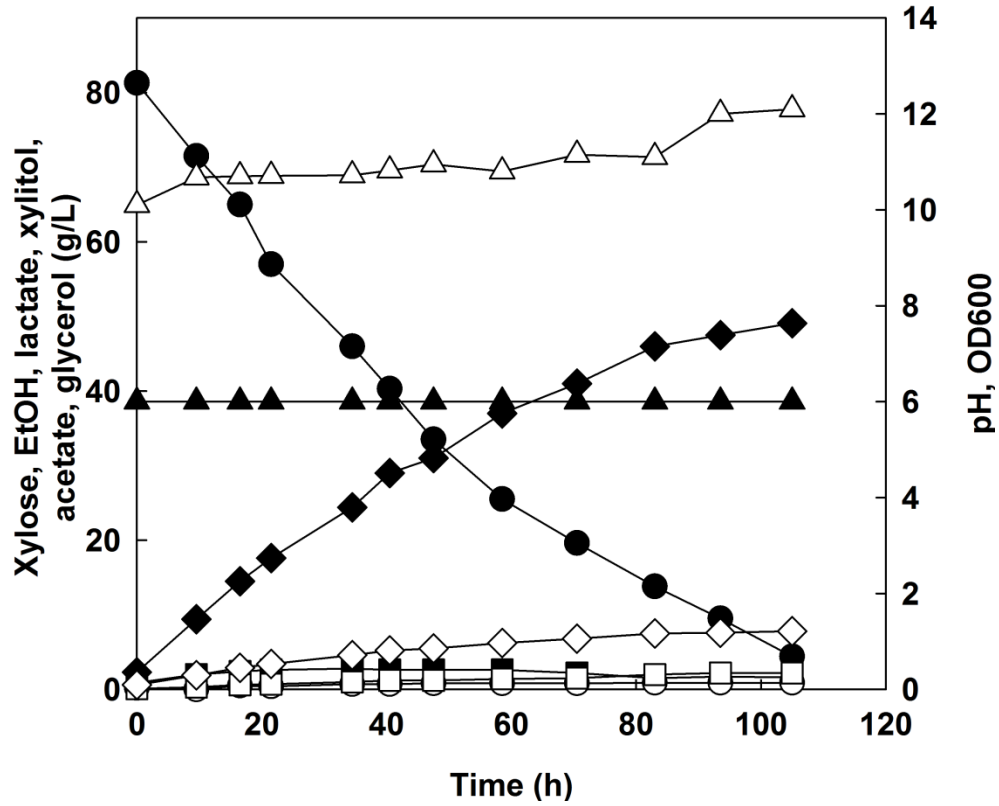


Fig. 2.6 Recombinant strain SR8L displaying a standard fermentation profile in a 1 L working volume bioreactor containing YP medium with 80 g/L of xylose (*closed circle*). SR8L produced ~50 g/L of lactic acid (*closed diamond*). Ethanol (*closed square*), pH (*closed triangle*), xylitol (*open circle*), acetate (*open square*), glycerol (*open diamond*), and OD (*open triangle*) are shown. Initial cell density was established at OD =10 (A600 nm) and a pH value of 6 was maintained by addition of 10 N NaOH as necessary.

CHAPTER III LACTIC ACID PRODUCTION FROM CELLOBIOSE AND XYLOSE BY ENGINEERED *SACCHAROMYCES CEREVISIAE*

3.1 Introduction

Economic production of value-added chemicals from renewable biomass is an important step towards a sustainable economy. Lactic acid is one such value-added chemical which is produced in excess of 260,000 tons annually [93]. Uses for lactic acid have been known for decades. Commercial purposes for lactic acid include use as a mosquito attractant and [134] use as a natural food preservative [135] in part because lactic acid is classified as GRAS (generally recognized as safe) by government agencies worldwide. Although widely used as a natural food preservative before, lactic acid has also found extensive use as a precursor for polylactic acid (PLA) synthesis recently [93]. PLA is a durable thermoplastic polyester which can be used in numerous products including consumer plastic ware and surgical sutures [136]. In the expanding 3D printing market, PLA has been used as a fabrication filament. For these and numerous other reasons, PLA has become one of the most highly-studied renewable polyesters. [46]

Currently, almost all industrially-produced lactic acid is generated by fermentation of glucose by lactic acid bacteria [47, 137]. Percent theoretical yields of lactic acid produced from various carbon sources by engineered *S. cerevisiae* have been reported as high as 81.5 % from glucose [48], 69 % from xylose [49], and 78 % from cellobiose [138]. Many groups have studied

The content of this chapter was published. The authors, according to appearance in citation include Timothy L. Turner, Guo-Chang Zhang, Eun Joong Oh, Vijay Subramaniam, Andrew Adiputra, Vimal Subramaniam, Christopher D. Skory, Ji Yeon Jang, Byung Jo Yu, In Park, and Yong-Su Jin. 2015. Lactic acid production from cellobiose and xylose by engineered *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* 113.5 p.1075-1083. I performed the research with help from the co-authors and Dr. Yong-Su Jin was the director of the research.

the production of lactic acid from glucose by a variety of host organisms including yeast [99, 112], fungi [139], *Escherichia coli* [140], and lactic acid bacteria [141]. As with lactic acid production, corn- or sugarcane-derived glucose has been a major feedstock for the production of biofuel additives or biofuels, such as ethanol. Increasing demand for sugars, including use for biofuel production, has been considered a catalyst for increasing food costs [142]. Because fuel and chemical demands are ever-increasing, utilization of sugars from generally non-edible lignocellulosic materials for the production of these chemicals could alleviate pressure on the already constrained food supply [143]. In addition, a fermentation process to transform lignocellulosic materials into useful products would be relatively carbon-neutral, unlike most fossil fuel-based processes which release significant amounts of once-sequestered carbon into the atmosphere [144]. Disappointingly, there are few commercial examples for converting lignocellulosic materials into value-added fuels or chemicals.

Major sugars in the cellulosic hydrolysates are glucose and xylose, constituting upwards of 40 % and 20 % of plant biomass, respectively [145]. Because xylose makes up a significant portion of terrestrial biomass, efficient fermentation of xylose is necessary for complete utilization of lignocellulosic biomass. Unfortunately, many microorganisms are not able to ferment xylose. However, engineered yeast [146] have been developed in recent years as platform microbes for conversion of xylose into fuels or chemicals. Still, most engineered strains exhibit the glucose repression problem, i.e. xylose cannot be utilized until glucose is depleted, when cellulosic hydrolysates containing both glucose and xylose are fermented. To bypass this problem, simultaneous co-fermentation of xylose and cellobiose has been demonstrated by engineered *Saccharomyces cerevisiae* [147]. Cellobiose is a disaccharide of glucose molecules connected by a β -1,4 linkage that cannot natively be transported into the cell by *S. cerevisiae*.

Through intracellular utilization of cellobiose via cellobiose transport across the cell membrane and an intracellular β -glucosidase, the glucose repression problem was avoided [148]. Even the feasibility of simultaneous co-utilization of cellobiose, xylose, and acetic acid has been demonstrated recently [149]. In order to construct engineered yeast capable of simultaneously fermenting cellobiose and xylose, we have optimized expression of a heterologous *XYL1* (xylose reductase), *XYL2* (xylitol dehydrogenase), and *XYL3* (xylulose kinase) from *Scheffersomyces stipitis*, which allowed for utilization of xylose. Deletion of *ALD6* (acetaldehyde dehydrogenase) and *PHO13* (a general phosphorylase) further improved xylose fermentations [85]. Genome integration of a heterologous cellodextrin transporter (*cdt-1*) and an intracellular β -glucosidase (*gh1-1*) from the cellulolytic fungi *Neurospora crassa* permitted the ability to ferment cellobiose and maintain stable gene expression. Laboratory evolution on medium containing cellobiose as the sole carbon source increased the copy numbers of *cdt-1* and *gh1-1*, resulting in the EJ4 strain being capable of rapid and efficient xylose and cellobiose co-utilization [149].

The objective of this study was to utilize the efficient xylose- and cellobiose-fermenting capabilities of the EJ4 yeast strain to produce lactic acid. To achieve this, we introduced a heterologous lactate dehydrogenase gene (*ldhA*) from *Rhizopus oryzae* using the pITy3 multi-copy integration vector [150]. This vector was chosen to integrate multiple copies of *ldhA*, as previous studies have shown improved lactate dehydrogenase activity from expressing more than one copy of the lactate dehydrogenase gene in engineered yeast [112]. In addition, Ty-integration sites are believed to have few functional genes, so there is a limited chance for disruption of important native genes [151]. Prior to this study, engineered *S. cerevisiae* have not been used to produce lactic acid from a mixture of lignocellulosic sugars. However, using *Corynebacterium glutamicum*, Sasaki et al. were able to convert a mixture of 40 g/L glucose, 20 g/L xylose, and 10

g/L cellobiose into a mixture of lactic, succinic, and acetic acids at a total yield of 0.85 g organic acids/g sugar [152]. Although their sugar mixture was predominantly glucose, their study was one of the earliest demonstrations for converting mixtures of lignocellulosic sugars into value-added products. Their study provided initial insight into the potential for one-pot monocultures capable of producing value-added products from lignocellulosic materials. Here, we use an engineered *S. cerevisiae*, which can convert one mol of pyruvate into one mol of ethanol or one mol of lactic acid resulting in the regeneration of one NAD^+ , providing equal redox balance from producing ethanol or lactic acid. For the purposes of this study, deletion of genes involved in the native yeast ethanol pathway (pyruvate decarboxylase and alcohol dehydrogenase) was not conducted, allowing the EJ4L strain the ability to convert pyruvate to both ethanol and lactic acid.

3.2 Materials and Methods

3.2.1 Media, strains, and plasmids

Yeast were cultured in YP medium (10 g/L yeast extract and 20 g/L peptone) containing glucose (YPD), xylose (YPX), cellobiose (YPC), or glycerol (YPG). The concentrations of the sugars were displayed as numbers following their initials (e.g., YPD20, YP medium containing 20 g/L of glucose). To construct an *S. cerevisiae* strain capable of producing lactic acid from cellobiose and xylose, an engineered strain (EJ4) capable of fermenting cellobiose and xylose was transformed with an integrating plasmid which contains *ldhA* from *R. oryzae* under the control of the constitutive PGK promoter. The integrating plasmid was based on a multi-copy integration pITy3 plasmid containing a *kanMX* antibiotic marker [150]. The *ldhA*-expression

cassette amplified from the pLDH68X plasmid using primers cLdhA-U: gggcccGGTACCGTCGACatggtattacactcaaaggtc and cLdhA-D: gggcccGGTACCGGATCCtcaacagctacttttagaaaagg was inserted into SalI and BamHI enzyme sites of the pITy3 plasmid. *E. coli* TOP10 were used for gene cloning and manipulation and were grown in Luria-Bertani medium; 50 µg/mL of kanamycin was added to the medium when required. A 20 µL reaction containing ~2,000 ng of the plasmid, 10 units of the *Xho*I enzyme (New England Biolabs, USA), and the appropriate buffer was placed in a 37 °C water bath for 90 minutes to linearize the plasmid. A double-enzyme digestion of the plasmid was used for gel electrophoresis to confirm appropriate fragment sizes. The linearized plasmid was transformed into the EJ4 strain using a standard high-efficiency lithium acetate method [103]. Yeast transformants were selected on YPD20 plates containing 300 µg/mL G418. Colony PCR of suspected transformants was conducted to confirm successful *ldhA* integration.

3.2.2 Flask fermentations

S. cerevisiae stock cultures were maintained on YPC (2% w/v agar, 1% w/v yeast extract, 2% peptone, 2% cellobiose) plates. Yeast precultures were grown in YPX40 medium at 300 RPM and harvested at mid-log phase, then washed twice with sterilized water to prepare inoculums for fermentations. For experiments comparing preculture effects of different sugars (see Results and Discussion, 3.2.), yeast cells were precultured in YP medium containing 40 g/L of glucose, xylose, cellobiose, or glycerol with an initial cell inoculum in the fermentation of ~1 (0.47 mg DCW/mL, OD₆₀₀). All flask fermentations were performed using 25 mL of YP medium containing an appropriate concentration of sugar in 125 mL Erlenmeyer flasks with an initial cell concentration of ~1 or ~10 (0.47 or 4.70 mg DCW/mL, OD₆₀₀) as indicated. Approximately 35

g/L or 50 g/L of calcium carbonate (CaCO_3) was used as a neutralizing agent in specific experiments. All flask fermentations were oxygen-limited and were held at 30 °C and at 100 RPM in a MaxQ4000 orbital shaker (Thermo Fisher Scientific Inc., USA). The rate of oxygen transfer for flask fermentations was determined to be 6.2 mmol O_2 /L/h by the sodium sulfite oxidation method [153, 154]. All experiments were repeated independently in duplicate with variations indicated with error bars.

3.2.3 Bioreactor fermentations

Yeast precultures were grown in YPX40 medium and harvested at mid-log phase, then washed twice with sterilized water to prepare inoculums for fermentations. The bioreactor fermentations were conducted in YP medium containing 10 g/L of glucose, 40 g/L of xylose, and 80 g/L of cellobiose using a BioFlo/CelliGen 115 bioreactor (New Brunswick Scientific Co., USA). An initial yeast cell concentration of ~ 1 (0.47 mg DCW/mL , OD_{600}) was used. Working volume was set at 1 L inside of a 2 L glass vessel. Aeration was maintained at a flow rate of 1.5 L/min of microfiltered ($0.22 \text{ }\mu\text{m}$) ambient air and an impeller rotation of 200 RPM. Temperature was maintained at 30 °C. NaOH (10 N) was added as needed to maintain a pH value of 6.

3.2.4 Lactate dehydrogenase activity assay of crude cell extracts

Lactate dehydrogenase enzymatic activity was measured by coupling the conversion of pyruvate to lactic acid to the oxidation of NADH to NAD^+ with each reaction evaluated by a UV-visible spectrophotometer at 340 nm. Glucose-, xylose-, or cellobiose-grown yeast were harvested at mid-log phase and centrifuged for 1 minute at $3200 \times g$ by a 5810R benchtop centrifuge with a swing-bucket rotor (A-4-81, Eppendorf, Germany) and the supernatant was

removed. Fresh cell pellets were washed with a cocktail containing one dissolved tablet of cOmplete protease inhibitor (Roche Applied Science, Germany), 1 mM dithiothreitol, and 25 mM Tris-HCl (pH 7.5). The washed cells were transferred to a microcentrifuge tube, centrifuged for 1 minute at $21,000 \times g$ by a 5424R benchtop centrifuge (Eppendorf, Germany) and the supernatant was discarded. The cell pellet was resuspended with 1 mL of the described cocktail and a PCR tube-sized volume of 0.5 mm glass beads was added to the microcentrifuge tube. The tube was alternated between 30 seconds on ice and 30 seconds of vortexing at high speed for 20 minutes. The cells were then centrifuged for 10 minutes at $21,000 \times g$ at 4 °C and the resulting raw cell extract was used for all enzymatic activity assays. Protein concentrations of freshly lysed cell extracts were measured by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., USA) following the supplied protocol using the included bovine serum albumin as the protein standard. Calculated activities were reported in units per milligram of soluble protein (U/mg). One U was defined as the amount of enzyme required to convert 1 μmol of NADH to NAD^+ per minute. All results were reported as the average of two replicates. Reactions were carried out in 50 mM potassium phosphate buffer at pH 6.7 containing final concentrations of 1 mM pyruvate, 0.4 mM NADH, and an 80 μL aliquot of freshly prepared cell extract. The reaction was conducted at room temperature by addition of pyruvate into a final volume of 800 μL .

3.2.5 Analytical methods

Cell concentration was monitored by optical density (OD) at 600 nm using a Biomate 3 UV-visible spectrophotometer (Thermo Fisher Scientific Inc., USA). Due to the low solubility of CaCO_3 in the fermentation media, cell concentration was not measured for experiments

containing CaCO₃. Glucose, cellobiose, xylose, xylitol, glycerol, acetate, ethanol, and lactic acid concentrations were determined by a 1200 Infinity Series high performance liquid chromatography (HPLC, Agilent Technologies Inc., USA) instrument equipped with a refractive index detector using a Rezex ROA-Organic Acid H⁺ (8%) column (Phenomenex Inc., USA). The column was eluted with 0.005 N of H₂SO₄ at a flow rate of 0.6 mL/min at 50 °C. The pH value was monitored using a PHM210 pH meter (Radiometer Analytical SAS, France) with attached Accumet 13-620-290 pH probe (Thermo Fisher Scientific Inc., USA).

3.3 Results and Discussion

3.3.1 Construction of an engineered *S. cerevisiae* (EJ4L) capable of producing lactic acid from cellobiose and xylose

An engineered *S. cerevisiae* strain with the ability to ferment cellobiose, xylose, and/or glucose into lactic acid was constructed by introduction of a fungal lactate dehydrogenase (*ldhA*) from *R. oryzae* into an engineered yeast (EJ4) which can ferment cellobiose and xylose [149]. Because the plasmid containing *ldhA* was a multi-copy, random-integration plasmid (pITy3, [150]), we reasoned some variations in lactic acid production could occur among transformants. Therefore, we screened 30 transformants for their ability to produce lactic acid from cellobiose. Although the variations of lactic production were minor, we chose the strain that produced the highest lactic acid yield (g lactic acid/g cellobiose) and named the resulting strain as EJ4L. In order to examine the expression levels of *ldhA* in the EJ4L strain grown on different carbon sources, we measured the lactate dehydrogenase (LDH) activity from crude cell extracts of glucose-, xylose-, or cellobiose-grown EJ4L. The LDH activities were 1.2 ± 0.1 , 1.0 ± 0.1 , and

1.35 $\pm$ 0.2 U/mg protein for glucose-, xylose-, and cellobiose- grown cells, respectively. The lactate dehydrogenase activities of 1-1.35 U/mg protein from EJ4L were similar to that reported by a previous study [80]. This consistent lactate dehydrogenase activity in the EJ4L strain provided further confirmation of successful integration of *ldhA* into EJ4. Unsurprisingly, negligible activity was detected when measuring the crude cell extract of the parental EJ4 strain.

3.3.2 Effects of EJ4L precultures containing various carbon sources on lactic acid yield

We conducted experiments to assess whether using different carbon sources for preculturing the EJ4L strain affected the lactic acid yield of the proceeding fermentation. To assess this, we precultured the EJ4L strain on YP medium containing 40 g/L of glucose, xylose, cellobiose, or glycerol before inoculating with an initial cell concentration of ~ 1 (0.47 mg DCW/mL, OD₆₀₀) into YP medium containing 35 g/L of CaCO₃ and 40 g/L of glucose, xylose, or cellobiose. The yields of lactic acid and ethanol from each of the twelve sets of fermentations showed only minor variation between preculture conditions (**Fig. 3.1**). For glucose fermentations, glucose-, xylose-, or cellobiose-precultured EJ4L were similar, but glycerol-precultured cells resulted in a lower lactic acid yield (0.19 g lactic acid/g glucose, 19 % of theoretical maximum). When fermenting xylose, both xylose- and glycerol-precultures resulted in slightly higher lactic acid yields (0.77 g lactic acid/g xylose, 77 % theoretical maximum) as compared to glucose- or cellobiose-precultures. Finally, for fermenting cellobiose, the xylose preculture resulted in the highest lactic acid yield (0.60 g lactic acid/g cellobiose, 60 % theoretical maximum) and no ethanol production. Overall, the variation was relatively minor between the four preculture conditions. To maintain a consistent method going forward, xylose

was used as the preculture carbon source as xylose-precultured EJ4L generally had high lactic acid yields and low ethanol yields.

In general, *S. cerevisiae* is a robust microbe that is easy to handle in a laboratory due to its relatively consistent growth phenotype. The preculture carbon source had only a mild effect on the proceeding fermentation to which the EJ4L strain was transferred, which suggests that the EJ4L strain is robust and predictable. In certain instances, the preculture condition of yeast has been shown to influence yeast physiology in several ways, including gene regulation [155] and ethanol tolerance [156]. For the EJ4L strain, these data (**Fig. 3.1**) suggest that consideration of preculture media might not be overly important when pursuing improved lactic acid yields from lignocellulosic sugars.

3.3.3 Cellobiose- or xylose-fermenting EJ4L produce more lactic acid than glucose-fermenting EJ4L

Using xylose as the preculture sugar, we conducted flask fermentations of EJ4L using YP medium containing 40 g/L glucose, 40 g/L of xylose, or 40 g/L of cellobiose. An initial cell concentration of ~ 1 (0.47 mg DCW/mL , OD_{600}) was used and 35 g/L of CaCO_3 was added as a neutralizing agent. The EJ4L strain consumed all glucose within 10 hours, and produced ethanol (12 g/L) and lactic acid (11.5 g/L) at similar titers (**Fig. 3.2a**). The yields of ethanol and lactic acid were 0.29 and $0.27 \text{ g product/g glucose}$, respectively, with percent theoretical yields of 58% and 27% , respectively. The productivity for lactic acid was 1.17 g/L/h . The titers of acetate and glycerol never exceeded 0.5 g/L . The pH value of the fermentation medium decreased from an initial 6.6 to approximately 5.4 . Importantly, it is apparent that the ethanol pathway, which consists of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), was the primary

metabolic route by which glucose is metabolized. To further improve the yield of lactic acid from glucose by engineered *S. cerevisiae*, disruption of the ethanol pathway might be necessary. As there are at least three known PDC genes (*pdc1*, *pdc5*, and *pdc6*), deletion of one or more of these genes has been shown to improve lactic acid yield from glucose by engineered *S. cerevisiae* containing a heterologous lactate dehydrogenase [157]. However, PDC deletion in *S. cerevisiae* often results in detrimental phenotypes due to redox imbalance and limitation in C2 precursor (acetyl-CoA) supply [158]. While the introduction of LDH can resolve the redox imbalance problem through providing an alternative route to regenerate NAD^+ , growth phenotypes of the lactic acid-producing yeast might not be robust because the LDH-expressing strain still suffers from limited C2 precursor production. In the case of ADH deletion [80] or ADH/PDC double-deletion [159], cell growth on glucose was found to be hindered. In Skory's study, it was speculated that ADH deletion caused accumulation of acetaldehyde in the cell, resulting in a lethal cell condition. In both studies, ethanol production was not completely eliminated which is likely due to the presence of multiple PDC and ADH genes in *S. cerevisiae*, but only *pdc1* and/or *adh1* were deleted in these studies [80, 159]. To maintain rapid cell growth, it might be desirable to not delete the native ethanol-producing pathway for lactic acid production. However, this can result in reduction in the yield of lactic acid due to carbon usage for ethanol production.

The xylose fermentation (**Fig. 3.2b**) by the EJ4L strain completed within 72 hours and primarily produced lactic acid (25.2 g/L). Interestingly, almost no ethanol (0.4 g/L) was accumulated from xylose at the end of the fermentation even though the EJ4L strain has a functional ethanol production pathway. The yields of ethanol and lactic acid were 0.01 and 0.66 g product/g xylose, respectively, and the percent theoretical yields were 0.02 % and 66 %, respectively.

respectively. The productivity for lactic acid from xylose was 0.33 g/L/h. The titers of acetate, glycerol, and xylitol were < 0.5 g/L. The pH value of the fermentation medium decreased to approximately 5.6. Compared to the glucose fermentation, the xylose fermentation by the EJ4L strain produced negligible ethanol and an almost 2.5 times higher lactic acid yield (0.66 vs. 0.27 g lactic acid/g sugar). When fermenting xylose instead of glucose, the EJ4L strain produced almost no ethanol, a phenotype similar to that of Δpdc yeast [28], but the detrimental effects from the deletion of multiple *pdc* genes were not observed.

When the EJ4L strain ferments cellobiose (**Fig. 3.2c**), lactic acid was the major product (21.7 g/L) and ethanol was a minor product (1.3 g/L). All cellobiose was fermented within 48 hours. The yields of ethanol and lactic acid were 0.03 and 0.56 g product/g cellobiose, respectively, and the percent theoretical yields were 0.6 % and 56 %, respectively. The productivity of lactic acid was 0.44 g/L/h. The concentrations of acetate and glycerol were less than 0.5 g/L and the pH value of the medium decreased to approximately 5.1. The average yield of lactic acid by the EJ4L strain from xylose and cellobiose (0.61 g lactic acid/g xylose or cellobiose) is more than double that from glucose (0.27 g lactic acid/g glucose). The average yield of ethanol by the EJ4L strain from xylose and cellobiose (0.02 g ethanol/g xylose or cellobiose) is almost 15 times lower than from glucose (0.29 g ethanol/g glucose).

The production of lactic acid from glucose by engineered yeast is well-studied [80, 112]. The major limitation for using *S. cerevisiae* to produce lactic acid was the reduced lactic acid yield because of the overproduction of ethanol. Moreover, because *S. cerevisiae* cannot ferment cellulosic sugars, such as xylose and cellobiose, engineered *S. cerevisiae* cannot be used for production of lactic acid from inexpensive and abundant cellulosic sugars. The engineered EJ4L

strain has provided initial evidence that high yield lactic production without ethanol production is feasible even without deletion of the *PDC* or *ADH* genes if xylose or cellobiose is used.

3.3.4 Lactic acid production from galactose, glycerol, mannose, and sucrose

Because the EJ4L strain produced different yields of lactic acid and ethanol from glucose, xylose, or cellobiose, we conducted fermentations using other carbon sources which are abundant in nature. EJ4L was grown on YP medium containing 35 g/L CaCO_3 and 40 g/L of one of the following carbon sources: galactose, glycerol, mannose, or sucrose. When the EJ4L strain was grown on glycerol, no ethanol or lactic acid were produced (**Fig. 3.3b**), although the strain was able to consume glycerol.

The EJ4L strain was able to produce lactic acid and ethanol by fermenting galactose, mannose, and sucrose. The ethanol and lactic acid yields from galactose were 0.20 and 0.33 g product/g galactose, respectively (**Fig. 3.3a**), with percent theoretical yields of 40 % and 33 %, respectively. The yield of ethanol and lactic acid from mannose were nearly identical with 0.27 and 0.24 g product/g mannose, respectively (**Fig. 3.3c**), with percent theoretical yields of 54 % and 24 %, respectively. Finally, the sucrose fermentation resulted in low yields for both ethanol and lactic acid of only 0.17 and 0.09 g product/g sucrose, respectively (**Fig. 3.3d**), with percent theoretical yields of 34 % and 9 %, respectively. With these data, an organization based on lactic acid yield from various carbon sources by the EJ4L strain can be determined. In terms of average lactic acid yield, from greatest to least: xylose (0.66), cellobiose (0.56), galactose (0.33), glucose (0.27), mannose (0.24), and sucrose (0.09, g lactic acid/g sugar). This order is based on 25 mL flask fermentations ($n = 2$) of the EJ4L strain in YP medium with 40 g/L of the appropriate sugar and 35 g/L of CaCO_3 acid neutralization.

S. cerevisiae has been known for years to display different phenotypes depending on which carbon source it is utilizing. However, most of those studies have focused on comparing glucose, fructose, or sucrose as *S. cerevisiae* cannot natively ferment xylose or cellobiose. In this study, interesting metabolic shifts are observed when cells are fermenting cellobiose and xylose as compared to glucose.

3.3.5 EJ4L can simultaneously co-ferment cellobiose and xylose to produce lactic acid at high titers

As shown, the EJ4L strain can efficiently ferment xylose or cellobiose as single sugars into lactic acid (**Fig. 3.2b** and **3.2c**). To assess whether these sugars could be simultaneously co-fermented, we conducted a fermentation by the EJ4L strain in YP medium containing 40 g/L of both cellobiose and xylose with 50 g/L of CaCO_3 (**Fig. 3.4**). Initial cell concentration was ~ 10 (4.70 mg DCW/mL, OD_{600}). All sugars were fermented within 115 hours and lactic acid was the major product (62 g/L). Ethanol accumulation was minor, with a peak titer of 2 g/L. The yields of ethanol and lactic acid were 0.02 and 0.73 g product/g sugar, respectively, with percent theoretical yields of 0.04 % and 73 %, respectively. The productivity for lactic acid was 0.54 g/L/h. The cumulative titer of acetate, glycerol, and xylitol was <0.8 g/L. The pH value of the fermentation medium decreased to approximately 4.9.

The EJ4L strain fermented 80 g/L of combined lignocellulosic sugars and produced over 60 g/L of lactic acid. During this process, very little ethanol was produced despite a functional ethanol pathway that is well-expressed when fermenting glucose. To the best of our knowledge, this is the first example that a mixture of xylose and cellobiose, without glucose, has been converted to an organic acid (lactic acid) by engineered *S. cerevisiae*.

3.3.6 EJ4L can efficiently ferment cellulosic sugars into lactic acid

When lignocellulosic materials are depolymerized, the resulting hydrolysate will contain a mixture of sugars. Therefore, effective industrial scale-up of a lignocellulosic fermentation process must be able to ferment high concentrations of mixed sugars. Accordingly, we conducted a flask fermentation of the EJ4L strain in YP medium containing 10 g/L of glucose, 40 g/L of xylose, and 80 g/L of cellobiose with 50 g/L of CaCO_3 (**Fig. 3.5**). A high initial cell concentration of ~ 10 (4.70 mg DCW/mL, OD_{600}) was used. All sugars were fermented within 200 hours and lactic acid was the major product (83 g/L). Ethanol accumulation was higher early in the fermentation with a peak titer of approximately 8.4 g/L in the first 70 hours, but by the end of the fermentation the titer had decreased below 4 g/L. The yields of ethanol and lactic acid were 0.03 and 0.66 g product/g sugar, respectively, with percent theoretical yields of 0.06 % and 66 %, respectively. The productivity for lactic acid was 0.42 g/L/h. The cumulative titer of acetate, glycerol, and xylitol was < 4 g/L. The pH value of the fermentation medium decreased from 6.2 to 5.0.

This fermentation provides evidence that high concentrations of lignocellulosic sugars can be converted to lactic acid with minimal byproduct accumulation even with a functional ethanol pathway. Of specific interest here is that, while glucose was utilized initially, xylose and cellobiose were co-fermented once all glucose was consumed. This simultaneous sugar utilization, as opposed to a sequential utilization, is considered a key aspect for efficient fermentation of lignocellulosic biomass [148]. This sugar-utilization phenotype is consistent with the parental EJ4 strain [149] from which the lactic acid-producing EJ4L strain was derived. A major hindrance to efficient scale-up of the EJ4L strain is the low productivity for lactic acid.

This can likely be resolved by utilizing a significantly increased initial cell concentration, as an OD₆₀₀ of ~10 (4.70 mg DCW/mL) is relatively low by industrial standards.

3.3.7 The EJ4L strain can produce a high titer of lactic acid from lignocellulosic sugars in a bioreactor

To test whether the EJ4L strain could produce lactic acid from lignocellulosic sugars at a larger scale, we conducted a bioreactor fermentation in one-liter of YP medium containing 10 g/L of glucose, 40 g/L of xylose, and 80 g/L of cellobiose (**Fig. 3.6**). A pH value of 6 was maintained throughout the fermentation by addition of 10 N NaOH as needed. Initial cell concentration was ~1 (0.47 mg DCW/mL, OD₆₀₀). To allow for increased cell growth, we sparged ambient, microfiltered (0.22 µm) air into the bioreactor at a flow rate of 1.5 L/min. The flow rate was maintained throughout the fermentation to provide a consistent and clear understanding of the EJ4L phenotype in the given medium.

All sugars were fermented within 170 hours and lactic acid was the major product (81.6 g/L). Ethanol accumulation reached a peak of 2.6 g/L, but decreased to 1.3 g/L by the end of the fermentation. The yields of ethanol and lactic acid were 0.01 and 0.65 g product/g sugar, respectively, with percent theoretical yields of 0.02 % and 65 %, respectively. The productivity for lactic acid was 0.49 g/L/h. The cumulative titer of acetate, glycerol, and xylitol was <6 g/L, with glycerol accounting for 5 g/L. The pH value of the fermentation medium decreased from 6.2 to 6.0 and was maintained at 6.0 ± 0.02 . After 170 hours and all initial sugars were consumed, 40 g of cellobiose and 40 g of xylose were added to the bioreactor and within an additional 48 hours the lactic acid titer had reached over 120 g/L (data not shown), at which point the fermentation was halted.

Initially, we attempted a bioreactor fermentation at pH 4 (data not shown) with all other conditions identical to the above fermentation. Glucose and xylose were fermented at a similar rate as the pH 6 bioreactor fermentation (**Fig. 3.6**), but after 200 hours, less than 10 g/L of cellobiose had been fermented. We then attempted another fermentation at pH 5 (data not shown) with otherwise identical conditions. Again, glucose and xylose were fermented similarly to the pH 6 fermentation (**Fig. 3.6**), but the rate of cellobiose utilization was slow, with only 40 g/L being consumed within 120 hours. We suspect that the heterologous cellodextrin transporter (*cdt1*) and/or intracellular β -glucosidase (*gh1-1*) of EJ4L are highly pH sensitive. Therefore, the ability for the EJ4L strain to produce lactic acid from cellobiose at a low pH is currently limited. Development of low pH-tolerant *cdt1* and *gh1-1* are needed for improving cellobiose utilization by the EJ4L strain at low pH. In general, production of lactic acid at a low pH, without the need for buffers or neutralizing agents such as CaCO_3 , would be economically ideal for industrial-scale fermentations, as purification of lactic acid-salt mixtures can be costly. Many studies discuss avenues towards isolating or developing low-pH or lactic acid-tolerant yeast [82], although a unique approach might be needed to target improvement of the cellobiose pathway in the EJ4L strain. Despite the difficulty for fermenting cellobiose at a low pH, the EJ4L yeast strain provides evidence that high concentrations of lignocellulosic sugars can be converted into lactic acid without the need for deleting *pdc* or *adh* to reduce the production of ethanol. Development of *S. cerevisiae* strains that are able to tolerate the harsh growth inhibitors found in lignocellulosic hydrolysates will be necessary before lactic acid can be produced from lignocellulosic materials at an efficient industrial level.

The molecular mechanisms to explain why lactic acid is produced at a significantly higher yield from xylose and cellobiose rather than glucose by the EJ4L yeast strain are still

unclear. However, there are several possible explanations for this phenomenon. Natively, yeast possess several glucose-sensing proteins, such as SNF3 or RGT2. In the presence of glucose, these sensors have been found to suppress *JEN1*, a gene which codes for lactate permease [115, 116, 118]. However, the presence of non-fermentable carbon sources induces *JEN1* [117, 118]. As a result, there may be an elevated lactate permease activity when fermenting xylose or cellobiose which could improve the capability of the EJ4L strain to efficiently remove otherwise toxic intracellular lactic acid. Because intracellular lactic acid can act as an allosteric inhibitor of LDH, efficient removal of lactic acid by an upregulated *JEN1* in the presence of xylose and/or cellobiose may be the driving force for improved lactic acid production from non-glucose sugars.

The redox balances of glucose, xylose, and cellobiose metabolism for the production of lactic acid are not identical. When converted to ethanol or lactic acid, a mol of glucose, or a half mol of cellobiose will net two moles of ATP. However, one mol of xylose fermented by the xylose reductase, xylitol dehydrogenase, and xylulokinase pathway (as expressed in the EJ4L strain in this study) into ethanol or lactic acid will net 1.67 moles of ATP and surplus NADH [149, 160]. Because glucose and cellobiose have similar redox balances while xylose differs, it is difficult to draw a clear conclusion to explain why xylose and cellobiose appear preferential for lactic acid production based on this information.

3.4 Conclusions

This study discloses fermentation results of an engineered *S. cerevisiae* strain, EJ4L, possessing the ability to ferment cellobiose and xylose into lactic acid. Native *pdc* and *adh* genes were not deleted, but still, almost no ethanol was produced when fermenting a cellobiose and xylose mixture. In a one L bioreactor fermentation, the EJ4L strain converted 10 g/L of glucose,

40 g/L of xylose, and 80 g/L of cellobiose into over 80 g/L of lactic acid and negligible ethanol.

This study provides evidence that lignocellulosic sugars may be preferential for producing lactic acid by engineered *S. cerevisiae*.

3.5 Figures

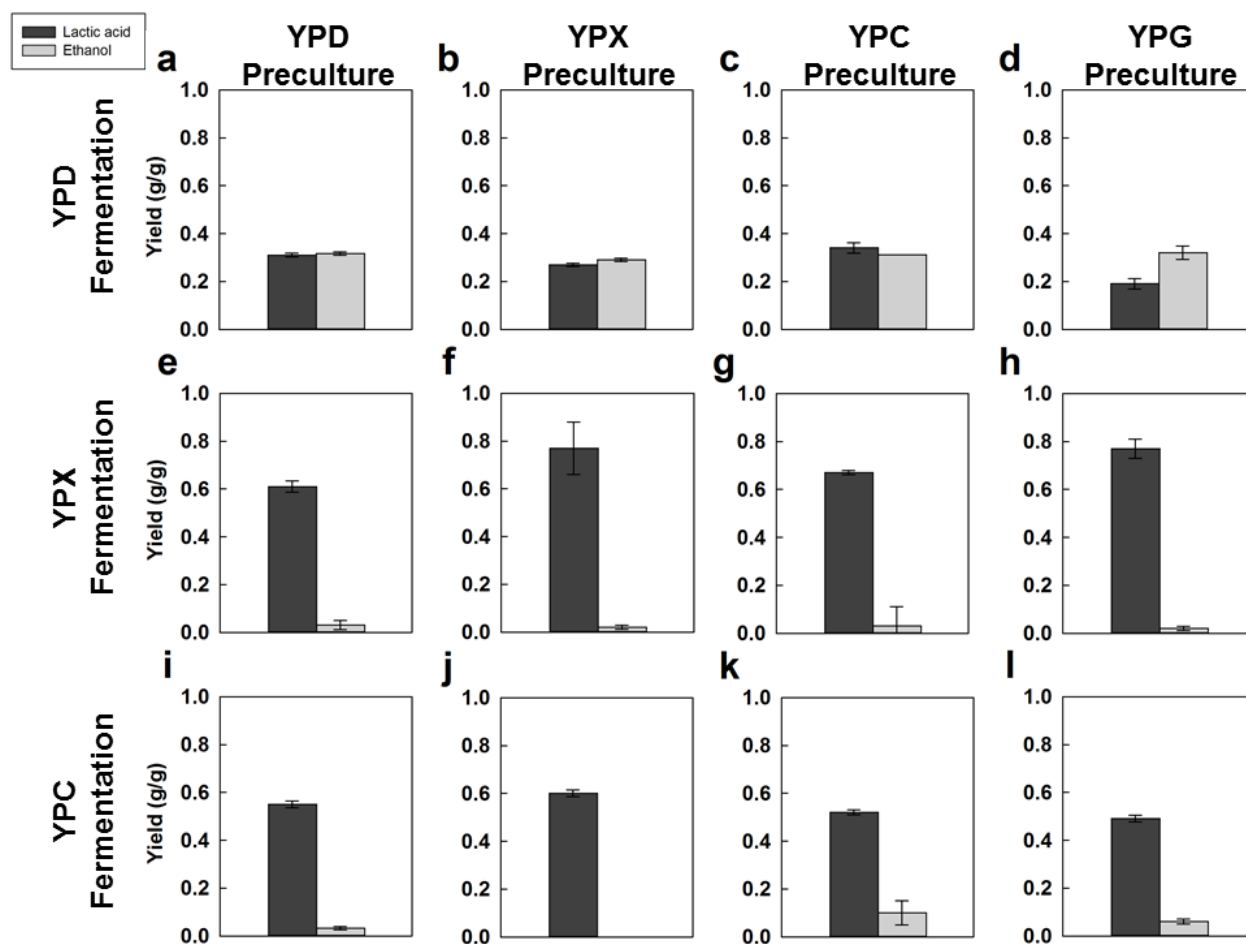


Fig. 3.1 EJ4L grown on four different preculture conditions (YP medium containing 40 g/L of glucose, xylose, cellobiose, or glycerol) were inoculated into flasks containing fresh YP medium containing 40 g/L of glucose, xylose, or cellobiose and 35 g/L CaCO_3 . Minor variation in the yields of lactic acid and ethanol occurred depending on the preculture carbon source. Results are the averages of duplicate experiments with error bars.

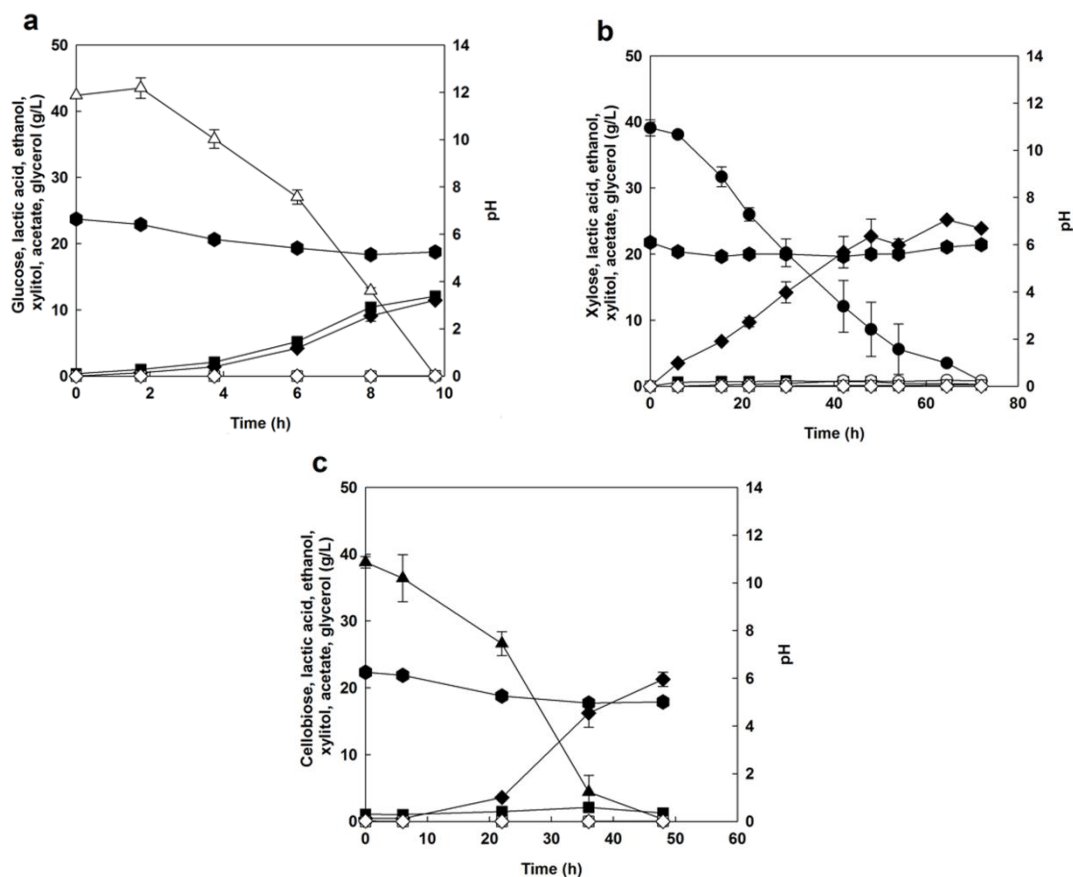


Fig. 3.2 Fermentation profiles of the EJ4L strain grown on YP medium containing 35 g/L of CaCO_3 and 40 g/L of glucose (Fig. 3.2a), xylose (Fig. 3.2b), or cellobiose (Fig 3.2c). The ethanol yields from glucose, xylose, and cellobiose were 0.29, 0.01, and 0.03 g ethanol/g sugar, respectively. The lactic acid yields from glucose, xylose, and cellobiose were 0.27, 0.66, and 0.56 g lactic acid/g sugar, respectively. Despite possessing a fully-functioning ethanol pathway, the EJ4L strain produces primarily lactic acid from both xylose and cellobiose. Symbols: glucose (open triangle), xylose (closed circle), cellobiose (closed triangle), xylitol (open circle), ethanol (closed square), lactic acid (closed diamond), acetate (open square), glycerol (open diamond), and pH (closed hexagon). Results are the averages of duplicate experiments with error bars.

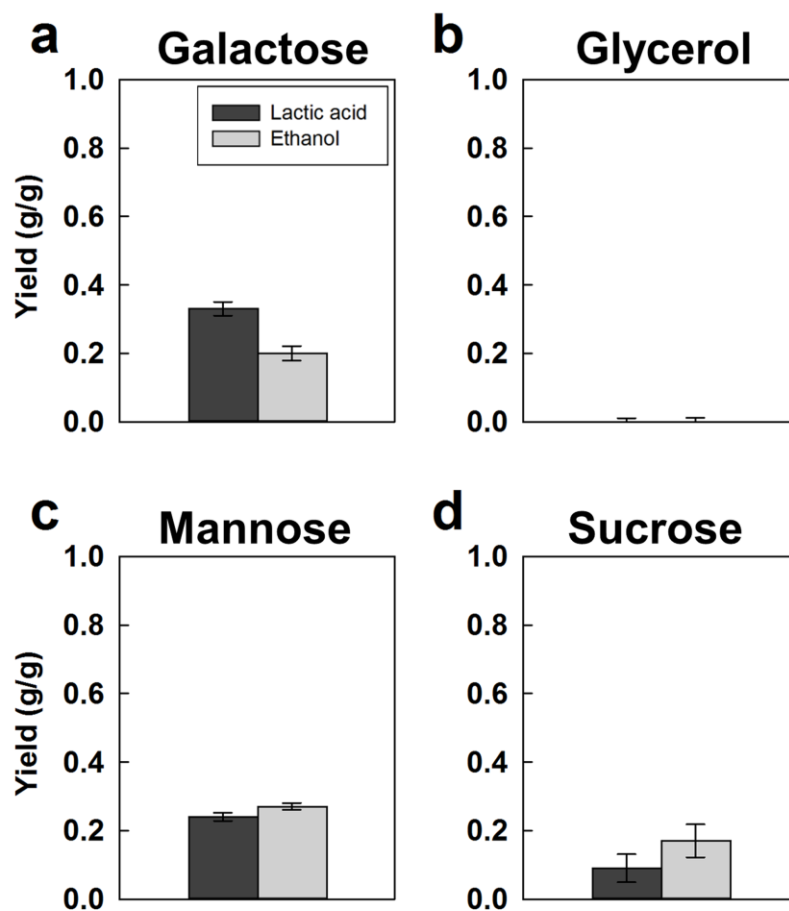


Fig. 3.3 Yields of lactic acid and ethanol by the EJ4L strain grown on YP medium containing 35 g/L of CaCO_3 and 40 g/L of galactose (Fig. 3.3a), glycerol (Fig. 3.3b), maltose (Fig. 3.3c), or sucrose (Fig. 3.3d). No lactic acid or ethanol was produced from glycerol. The yield of lactic acid was the highest from galactose (0.33 g lactic acid/g galactose). Results are the averages of duplicate experiments with error bars.

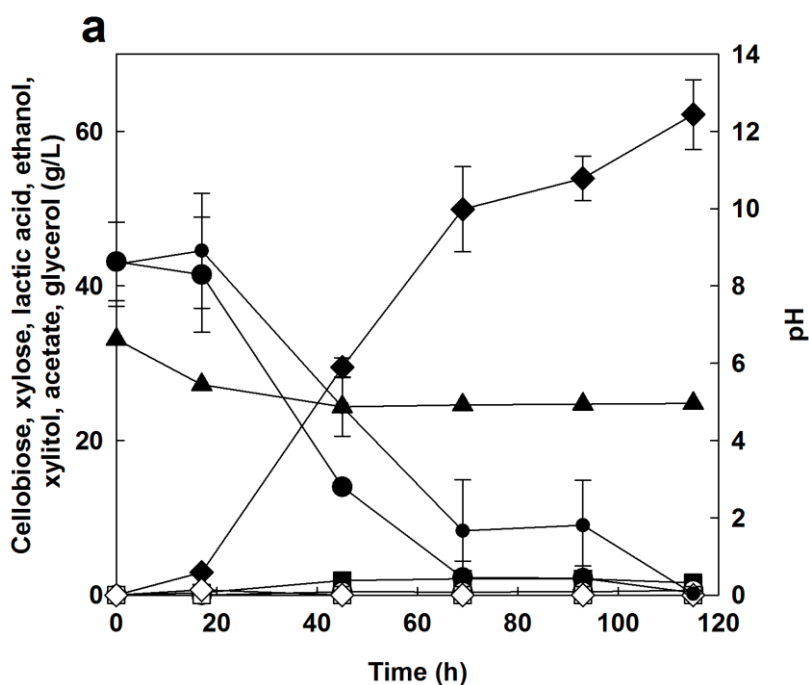


Fig. 3.4 Fermentation profile of the EJ4L strain grown on YP medium containing 50 g/L of CaCO_3 , 40 g/L of xylose, and 40 g/L of cellobiose. The EJ4L strain simultaneously fermented cellobiose and xylose resulting in a lactic acid yield of 0.73 g lactic acid/g sugar and an ethanol yield of 0.02 g ethanol/g sugar. Symbols: xylose (closed circle), cellobiose (closed triangle), xylitol (open circle), ethanol (closed square), lactic acid (closed diamond), acetate (open square), glycerol (open diamond), and pH (closed hexagon). Results are the averages of duplicate experiments with error bars.

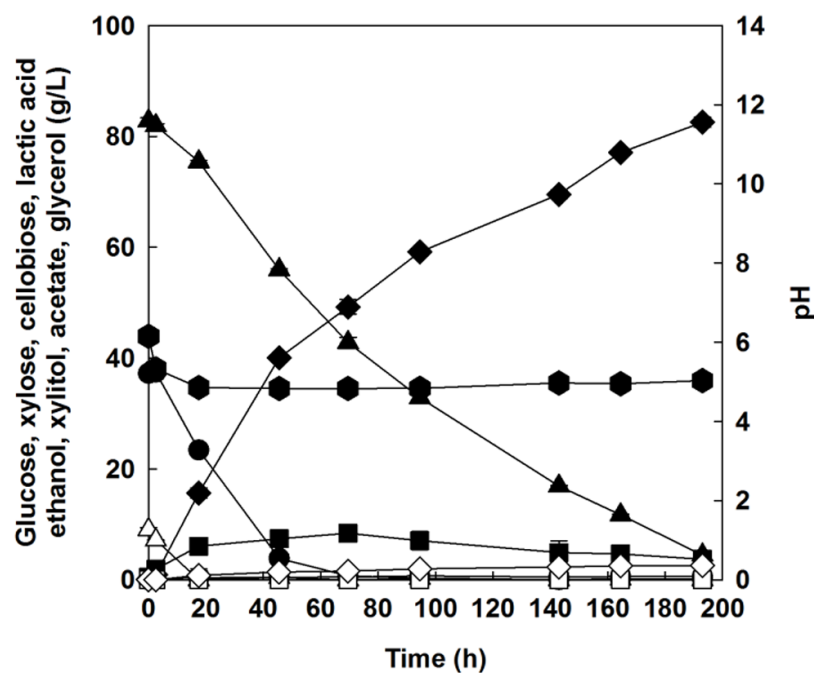


Fig. 3.5 Fermentation profile of the EJ4L strain grown on YP medium containing 50 g/L of CaCO_3 , 10 g/L of glucose, 40 g/L of xylose, and 80 g/L of cellobiose. The EJ4L strain fermented all sugars, resulting in a lactic acid yield of 0.66 g lactic acid/g sugar and an ethanol yield of 0.03 g ethanol/g sugar. Symbols: glucose (open triangle), xylose (closed circle), cellobiose (closed triangle), xylitol (open circle), ethanol (closed square), lactic acid (closed diamond), acetate (open square), glycerol (open diamond), and pH (closed hexagon). Results are the averages of duplicate experiments with error bars.

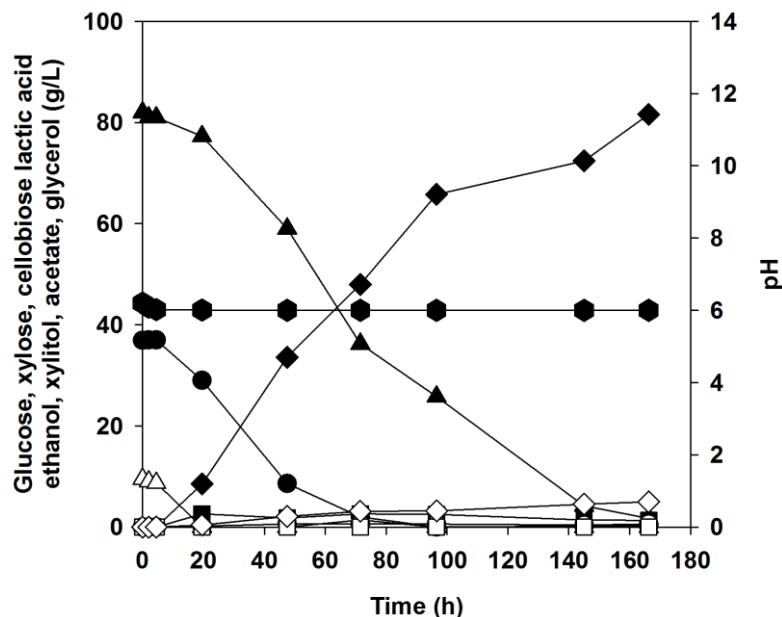


Fig. 3.6 Fermentation profile of the EJ4L strain grown on YP medium containing 10 g/L of glucose, 40 g/L of xylose, and 80 g/L of cellobiose in a one-liter (working volume) bioreactor. The pH value was maintained at 6.0 using 10 N NaOH. The EJ4L strain fermented all sugars, resulting in a lactic acid yield of 0.65 g lactic acid/g sugar. Symbols: glucose (open triangle), xylose (closed circle), cellobiose (closed triangle), xylitol (open circle), ethanol (closed square), lactic acid (closed diamond), acetate (open square), glycerol (open diamond), and pH (closed hexagon).

CHAPTER IV CONVERSION OF LACTOSE, BOVINE MILK, AND WHEY TO LACTIC ACID BY ENGINEERED *SACCHAROMYCES CEREVISIAE*

4.1 Introduction

Whey is a significant and troublesome byproduct of the dairy industry [161]. In particular, cheese production can generate up to 9 kg of whey per 1 kg of cheese, with the disaccharide lactose serving as the major constituent of the permeate after whey filtration [162]. Acid whey is specifically problematic, as it is produced in excess as a byproduct of Greek yogurt production and has proven difficult for the yogurt industry to efficiently reuse or discard in part due to the relatively low pH of acid whey ($\text{pH} \leq 5.1$) [163, 164]. In response, one creative solution for the problem of whey as a dairy industry byproduct is to use engineered microbes to ferment the lactose contained in the whey for the production of value-added fuels and chemicals [162]. However, even this solution is not easy, as it is recommended that the whey concentration used should result in up to 120 g/L lactose in order to minimize downstream purification costs [161, 165]. Therefore, an engineered microbe must not only be able to withstand the acidity of acid whey and osmotic stress induced by a high concentration of lactose, but must also produce a target product at a high yield and productivity.

Previously, our lab has developed an engineered *Saccharomyces cerevisiae* baker's yeast strain which can efficiently produce ethanol at a yield of 0.361 g ethanol/g lactose from whey medium containing 150 g/L of lactose [manuscript under review]. Natively, most yeast,

The content of this chapter will be submitted for peer-review to the *Journal of Dairy Science* as a short communication. The authors, according to appearance in citation include Timothy L. Turner, Eunbee Kim, ChangHoon Hwang, Guo-Chang Zhang, Jing-Jing Liu, and Yong-Su Jin. I performed the research with help from the co-authors and Dr. Yong-Su Jin was the director of the research.

including *S. cerevisiae*, cannot ferment lactose. Our lactose-fermenting strain was created by introduction and expression of two heterologous genes cloned from the fungus *Neurospora crassa*: a lactose transporter (*CDT-1*) to transport lactose into the yeast cell and a β -galactosidase (*GH1-1*) to cleave the lactose disaccharide into glucose and galactose [166, 167].

4.2 Materials, Methods, and Results

Here, we have expanded upon the initial concept of producing a natural fermentation product of *S. cerevisiae*, ethanol, towards producing a heterologous value-added product, lactic acid. As a result, this study provides a proof-of-concept for the conversion of purified lactose, milk, and whey into lactic acid by an engineered *S. cerevisiae* strain. Yeast, especially *S. cerevisiae*, are particularly ideal microbes for industrial chemical production due to their tolerance to high external osmotic stress (such as high sugar concentrations), their tolerance against low pH (essential for overproducing organic acids), their generally recognized as safe (GRAS) status, and for the wide range of genetic perturbation tools available to study, introduce, and delete genes within the yeast genome. Furthermore, the theoretical maximum yield of lactic acid from lactose is 1.00 g lactic acid/g lactose due to the lack of carbon loss throughout the pathway. Perhaps most importantly, no oxygen is theoretically necessary for this process to occur, so a large-scale lactose-to-lactic acid fermentation could be conducted entirely anaerobically, significantly reducing the costs otherwise accrued if oxygen were added. We expressed a heterologous lactate dehydrogenase (*ldhA*), cloned from the fungus *Rhizopus oryzae*, into the *CDT-1* and *GH1-1* expressing *S. cerevisiae* as previously reported [50]. The *ldhA* was integrated into the *S. cerevisiae* chromosome using the pITy3-*ldhA*-G418 plasmid to integrate at Ty δ loci [150] and selected using G418 (geneticin) as an antibiotic selection

pressure. For all experiments, two engineered yeast strains were used, both expressing the *CDT-1* and *GH1-1* lactose-fermenting pathway: EJ4 (no *ldhA*) and EJ4L (expressing *ldhA*). EJ4 served as the control strain which in all fermentations was unable to produce lactic acid. Importantly, the native ethanol production pathway consisting of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) was not disrupted in either the EJ4 or EJ4L strains, allowing the yeast cells to produce ethanol as necessary [50].

S. cerevisiae stock cultures were maintained on yeast extract-peptone dextrose (YPD) agar plates (2% agar, 1% yeast extra, 2% peptone, and 2% glucose) at 4° C. Yeast precultures were grown in YPD40 medium at 300 RPM and harvested at mid-log phase and then washed twice with sterilized water to prepare inoculums for fermentations. All flask fermentations were conducted using 25 mL of media in a 125 mL Erlenmeyer flask maintained at 30 °C on a 100 RPM MaxQ4000 orbital shaker (Thermo Fisher Scientific Inc., MA). Flask fermentations in **Fig. 4.2** were performed using YP medium containing 40 g/L of purified lactose (Sigma-Aldrich, MO) with an initial cell concentration of ~1 (0.47 DCW/mL, OD600); the YP medium and lactose were sterilized separately via autoclave. Flask fermentations in **Fig. 4.3** were performed using Horizon Organic brand, shelf-stable, 1% fat, organic, cow's milk (Whitewave Services, Inc., lot #36-4016 03:37) which was used prior to the printed "best before" date. Because the milk was shelf-stable, we did not autoclave it prior to use. Flask fermentations in **Fig. 4.4** contained YP medium with 250 g/L whey from bovine milk (Sigma-Aldrich, MO) which resulted in ~140 g/L of free lactose. Due to suspected microbial contamination when using non-autoclaved whey (data not shown), all results in **Fig. 4.4** are from autoclaved whey medium. Approximately 35 g/L of calcium carbonate (CaCO₃) was used as a neutralizing agent in specific

experiments. All experiments were repeated independently in duplicate (n=2) with variations indicated with error bars.

Yeast cell concentration was monitored by optical density (OD) at 600 nm using a Biomate 3 UV–visible spectrophotometer (Thermo Fisher Scientific Inc., MA). Lactose, glycerol, acetate, ethanol, and lactic acid concentrations were determined by a 1200 Infinity Series high performance liquid chromatography (HPLC, Agilent Technologies Inc., CA) instrument equipped with a refractive index detector using a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., CA). The column was eluted with 0.005 N of H₂SO₄ at a flow rate of 0.6 mL/min at 50 °C. The pH value was monitored using a PHM210 pH meter (Radiometer Analytical SAS, France) with attached Accumet 13-620-290 pH probe (Thermo Fisher Scientific Inc., MA).

Our initial test of the ability for our engineered *S. cerevisiae* strain (EJ4L) to produce lactic acid from lactose utilizes purified lactose. Previous results indicated that a *CDT1* and *GH1-1* expressing yeast strain without a functional lactate dehydrogenase could produce ethanol at a yield of 0.334 g ethanol/g lactose [manuscript under review]. With the introduction of the *ldhA* gene into our EJ4 yeast strain, the EJ4L strain can efficiently produce lactic acid from lactose. From 41 g/L of lactose, the EJ4L strain could produce 23.77 g/L of lactic acid in 143 hours, resulting in a yield of 0.58 g lactic acid/g lactose and a productivity of 0.17 g/L-h (**Fig. 4.2**). No ethanol production was detected during the fermentation. The pH of the medium maintained above 5.0 throughout the fermentation (data not shown) despite significant lactic acid production due to our addition of 35 g/L CaCO₃ which acted as a neutralizing agent. As a major result, this is the first record of direct conversion of lactose to lactic acid by an engineered *S. cerevisiae* strain.

After confirming the successful conversion of lactose to lactic acid by the engineered EJ4L strain, we assessed whether the lactose in cow's milk could be efficiently converted to lactic acid. One difficulty in the dairy industry is finding an efficient use for waste milk from sick cattle [168]. Obviously, milk from contaminated cows cannot be fed to humans or even other livestock without significant risk for spreading disease. However, using modern biotechnology tools, it may eventually become viable to convert the lactose present in cow's milk from contaminated cows into value-added products, such as lactic acid. Therefore, we tested this concept using shelf-stable cow's milk without any other supplements or modifications, such as the addition YP medium or CaCO_3 , as a proof-of-concept (**Fig. 4.3**). From ~52 g/L of lactose present in the milk, by 121 h, the EJ4L strain consumed ~42 g/L of lactose, producing ~10.3 g/L of lactic acid, resulting in a yield of ~24.4 g lactic acid/g lactose and a productivity of 0.085 g/L-h (**Fig. 4.3**). Through 71 h, no ethanol production was detected, but by 140 h, the rate of ethanol production began to exceed the rate of lactic acid production, suggesting that as the lactic acid concentration increased, the metabolic flux shifted away from lactic acid production towards ethanol production. The parental EJ4 strain, which does not express *ldhA*, produced no lactic acid, as expected (data not shown). As a major result, this experiment serves as an initial proof-of-concept that cow's milk can be converted into other value-added products, which may be a useful process for industrial-scale dairy farms after further scaled-up tests and optimizations.

Finally, we tested whether our EJ4L strain could efficiently ferment whey from bovine milk to produce lactic acid. Initially, we used whey powder which was not autoclaved, but observed bacterial contamination in each of our attempted fermentations as indicated by rapid production of acetic acid (data not shown). To alleviate this contamination issue, we autoclaved

the medium to destroy native bacteria before inoculating with the EJ4L strain. As a result, we were able to conduct our whey fermentations without any microbial contaminants. The EJ4L strain could produce ~15.6 g/L of lactic acid in 110 hours, resulting in a yield of 0.356 g lactic acid/g lactose and a productivity of 0.142 g/L-h (**Fig. 4.4**). No ethanol was detected during the fermentation. The parental EJ4 strain, which does not express *ldhA*, produced no lactic acid, as expected (data not shown). As a major result, this experiment provides initial insight into the potential for using waste whey, such as acid whey, into a value-added product.

Here, we have demonstrated some initial steps necessary for converting dairy farm waste into value-added products. Still, several barriers must be crossed before engineered yeast could be used for industrial-scale production of value-added products, such as lactic acid, from lactose, waste milk, or acid whey. First, the engineered strain would need to have no antibiotic resistance. Large-scale production of fuels and chemicals using any engineered microbe with an inherent antibiotic resistance is generally not supported by the United States' Food and Drug Administration (FDA) due to the risk for spreading antibiotic resistance [169]. In this proof-of-concept study, our engineered EJ4L yeast strain possesses G418 (geneticin) resistance via the integrated pITy3-*ldhA*-G418 cassette. However, using auxotrophic markers or the modern CRISPR/Cas9 [27] engineering protocol, it is possible to develop an *ldhA*-expressing yeast strain without the need for an antibiotic marker.

Second, the lactic acid productivity (g/L-h) would need to be increased to produce an economically viable fermentation process. This could be achieved by improving the lactose fermentation pathway (*CDT-1* and *GH1-1*) through increased copy number of the genes. Perhaps more simply, the fermentation rate could be increased by starting with a significantly higher initial yeast cell density at 10-fold or 100-fold higher than used in this study. By improving the

lactose fermentation pathway and increasing the initial cell density, a higher lactic acid productivity could be achieved.

Finally, a rapid and cost-effective purification process to separate spent whey media and yeast cells from the produced lactic acid is a necessity. At least two separation steps will be needed, including a filtration method to remove solids and then a method to isolate lactic acid from the liquid media. Many purification methods already exist and are sometimes applied to industrial-scale lactic acid fermentations, but these methods may need to be modified specifically for the purification of lactic acid from whey media [170, 171].

4.3 Conclusions

In conclusion, we demonstrate that introduction of a heterologous lactose fermentation pathway consisting of a lactose transporter (*CDT-1*) and an intracellular β -galactosidase (*GH1-1*) and a heterologous lactate dehydrogenase (*ldhA*) into a laboratory *S. cerevisiae* yeast allows for production of lactic acid from lactose, cow's milk, or bovine milk whey with minimal to no ethanol accumulation. The yield of 0.358 g lactic acid/g lactose from a 25 g/L whey medium was achieved with no detectable ethanol production, suggesting that homolactic acid production from lactose can be achieved in engineered *S. cerevisiae* even without deletion of the native ethanol pathway.

4.4 Figures

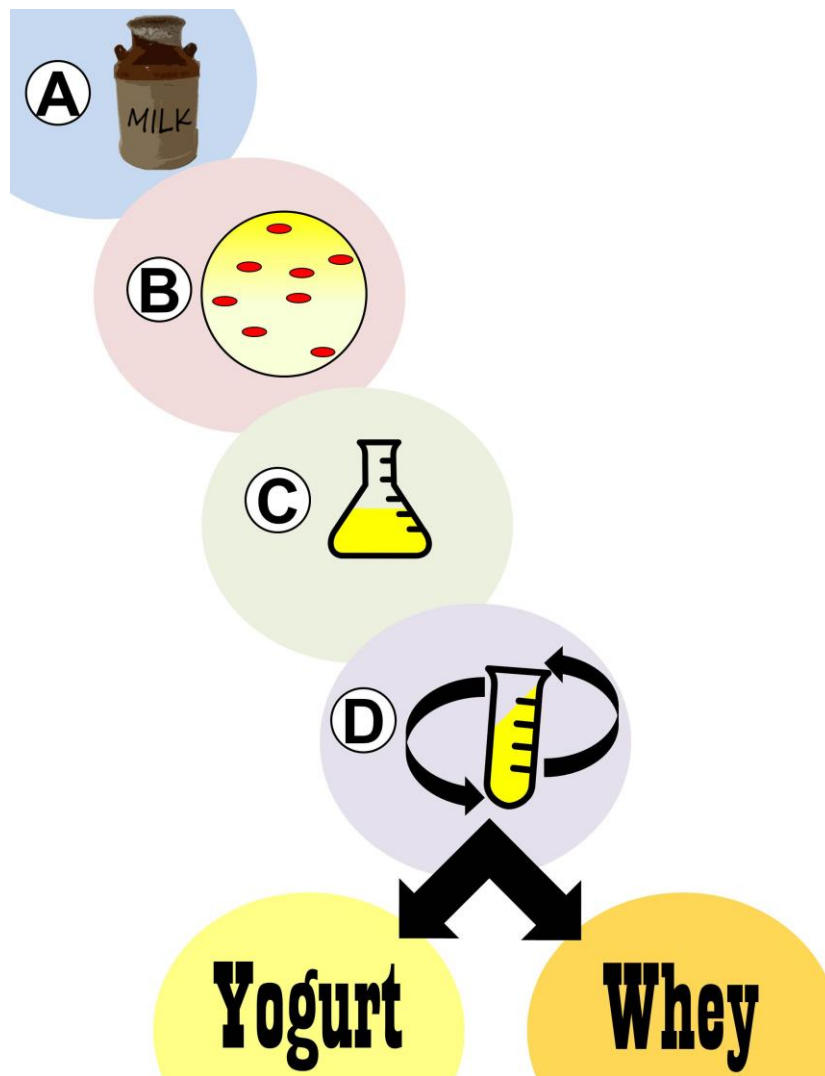


Fig. 4.1 A broad schematic showing the process for the production of yogurt and whey. **a** Milk is harvested from cows and most often pasteurized. **b** A starter culture, often lactic acid bacteria, is inoculated into the milk. **c** The milk is fermented and thickens, a process caused by the lactic acid bacteria consuming lactose naturally present in milk and producing lactic acid, which lowers the pH of the milk causing protein degradation and coagulation. **d** The thickened mixture is centrifuged to separate the solid yogurt (sold as Greek yogurt) and the liquid fraction (whey), which still contains significant lactose and often has an acidic pH.

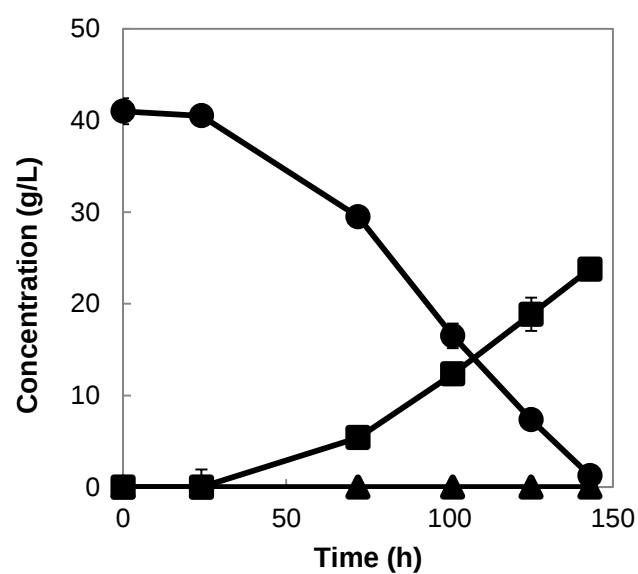


Fig. 4.2 Fermentation profile of the EJ4L strain grown in YP medium containing 40 g/L of lactose and 35 g/L of CaCO_3 as the sole carbon source. Symbols: lactose (*closed circle*), lactic acid (*closed square*), and ethanol (*closed triangle*). Results are the averages of duplicate experiments with standard deviation indicated by error bars.

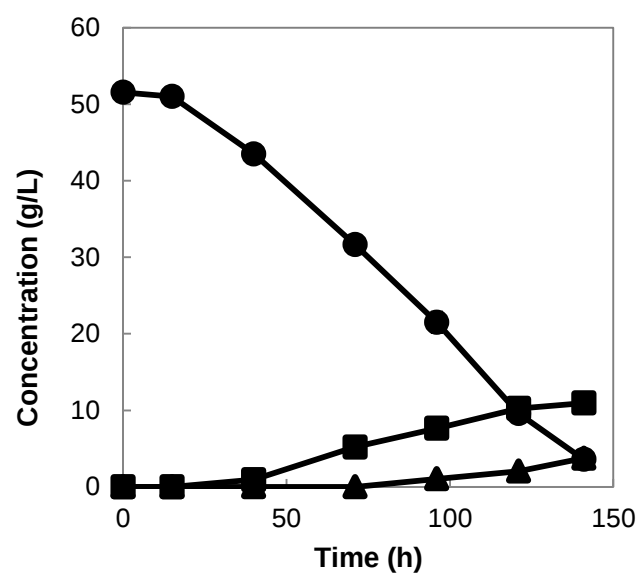


Fig. 4.3 Fermentation profile of the EJ4L strain grown in shelf-stable cow's milk with no additional supplements. Symbols: lactose (*closed circle*), lactic acid (*closed square*), and ethanol (*closed triangle*). Results are the averages of duplicate experiments with standard deviation indicated by error bars.

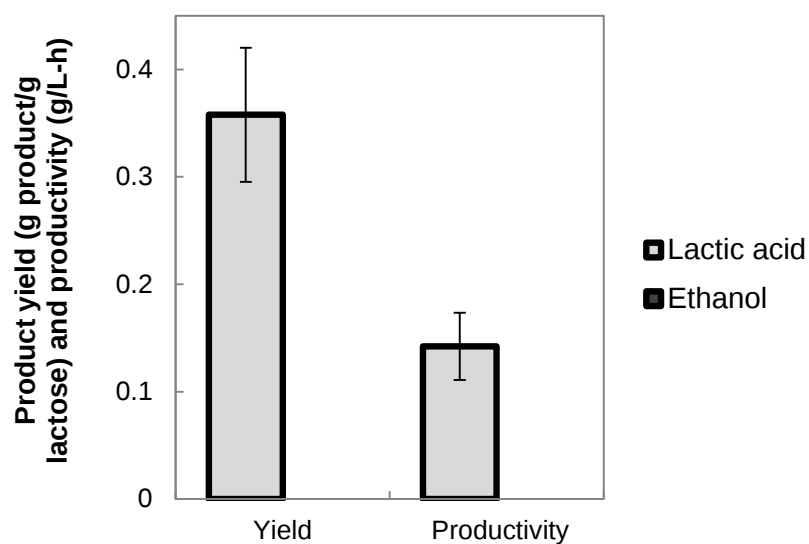


Fig. 4.4 Lactic acid and ethanol yields (g product/g lactose) and productivities (g/L-h) of the EJ4L strain grown for 100 h in YP medium containing 25 g/L of whey. No measureable ethanol was detected. Results are the averages of duplicate experiments with standard deviation indicated by error bars.

CHAPTER V CHARACTERIZATION AND SCREENING OF INDUSTRIAL YEAST STRAINS FOR IMPROVED LACTIC ACID TOLERANCE AND PRODUCTION

5.1 Introduction

Yeast have served many important industrial purposes for humans over thousands of years. Specifically, the *Saccharomyces cerevisiae* species has acted as the primary species for the production of wine, beer, and bread [172]. Although the production of baked goods and fermented foods or beverages has historically been the major industrial application for *S. cerevisiae*, many new avenues by which yeast fermentation capabilities are harnessed have developed in recent decades. These new usages are due in part to rapid and significant advances in our understanding of yeast genetics and physiology [173]. Through our improved understanding of yeast hardiness and functions, we have exploited the natural low pH and acid tolerance of *S. cerevisiae* to produce various organic acids.

Generally, organic acids are defined as organic chemicals containing a carboxylic acid group which does not completely dissociate in water in most circumstances, often being described as “weak acids”. Lactic acid, succinic acid, glycolic acid, and malic acid are perhaps the most notable of organic acids which have been overproduced by engineered *S. cerevisiae*. **Table 5.1** lists several organic acids which have been produced by engineered yeast, the major uses of these acids, and relevant references.

Although several microbial species are used for industrial production of value-added

The content of this chapter is in preparation for submission, with some modification to include additional results specifically related to lactic acid added for the purposes of this dissertation. In Lok Kong and I will be co-first authors, with help from Dr. Guochang Zhang, and Dr. Yong-Su Jin was the director of the research.

products, yeast are perhaps the most well-studied. Yeast can natively ferment glucose for the production of ethanol and this capability has been used for wine production for thousands of years [174, 175]. In recent decades, the yeast *Saccharomyces cerevisiae* has been extensively utilized for biofuel production [176]. The majority of ethanol produced by *S. cerevisiae* is from fermentation of sugarcane- or corn-derived glucose [177]. Although industrial yeast fermentation has resulted in more than 50 billion liters of annual ethanol production in the US alone, the availability of corn and sugarcane is a limiting step in using biofuels as a replacement for fossil fuels [178]. Therefore, abundant lignocellulosic crops may be viable alternative feedstocks for production of bio-based fuels and chemicals [179]. Although *S. cerevisiae* is well-studied with many available genetic manipulation tools available, it does not efficiently produce fuels and chemicals from processed lignocellulosic hydrolysates due to the harsh environment present in the hydrolysate. Specifically, the low pH of lignocellulosic hydrolysates coupled with the presence of many known and unknown fermentation inhibitors act as the major limitations for efficient production of fuels and chemicals by engineered *S. cerevisiae* from lignocellulosic hydrolysates [180, 181]. Although this problem is fairly-well understood, attempts to overcome lignocellulosic hydrolysate toxicity have failed to yield *S. cerevisiae* strains that are fully-resistant to hydrolysate toxicity without otherwise hindered phenotypes [182]. Furthermore, modifying lignocellulosic hydrolysate to remove inhibitors or balance the pH can result in reduced sugar availability [183] or increased production costs [184]. Owing to these hurdles, developing an engineered microbe to efficiently ferment lignocellulosic hydrolysates may be easiest if the parental strain is natively resistant to the many inhibitors present in hydrolysates [185]. In particular, laboratory strains are generally regarded as possessing lowered tolerance to fermentation inhibitors as compared to industrial yeast strains and therefore industrial strains

may be ideal for producing fuels and chemicals from lignocellulosic hydrolysates [186]. Because numerous strains of yeast exist, it is difficult and time-consuming to screen all known strains for their resistances to common fermentation inhibitors present in lignocellulosic hydrolysates such as hydroxymethylfurfural (HMF), furfural, and acetate. With this in mind, we have selected 24 *Saccharomyces spp.* yeast strain which are publically available from the American Type Culture Collection (ATCC) and screened each strain to assess their fermentation phenotypes in a variety of conditions. Here, we report our findings.

5.2 Materials and Methods

5.2.1 Industrial strain backgrounds

All *Saccharomyces spp.* strains were obtained from the American Type Culture Collection (ATCC). In total, 24 strains were selected. A list containing the ATCC nomenclature for each strain, the original isolation source of the strain, and a representative literature citation for each strain is shown in **Table 5.2**.

5.2.2 General flask fermentations

Yeast cells were cultured in YP medium (10 g/L yeast extract and 20 g/L peptone) containing glucose (YPD), xylose (YPX), cellobiose (YPC), maltose, mannose, or sucrose. Concentrations of the sugars were displayed as numbers following their initials (e.g., YPD160, YP medium containing 160 g/L of glucose). Stock cultures were maintained on YPD agar (20 g/L agar) plates in 4 °C. Yeast precultures were grown in YP medium containing 40 g/L of glucose in 5 mL total volume and harvested at mid-exponential phase. Calcium carbonate

(CaCO₃) was used as a neutralizing agent for lactic acid fermentations where indicated. Fermentations were conducted with an initial volume of 50 mL in 250 mL Erlenmeyer Pyrex[®] flasks (Corning, MA). Flasks were shaken at 100 RPM on an Innova 2300 shaker (New Brunswick Scientific, CT) in a 30 °C incubation room. Cell optical density (OD) was measured via NanoDrop 200C (Thermo Fisher Scientific, MA) or BioMate 3 UV-visible spectrophotometer (Thermo Fisher Scientific, MA) and the initial OD was adjusted to ~1. Glucose, xylose, cellobiose, glycerol, acetate, ethanol, and lactic acid concentrations were determined by use of a 1200 Infinity series HPLC system (Agilent Technologies, CA) equipped with a refractive index detector using a Rezex ROA-Organic Acid H⁺ (8%) column (Phenomenex Inc., CA). The column was eluted with 0.005 N H₂SO₄ at a flow rate of 0.6 mL/min at 50 °C.

5.2.3 Xylose-fermenting strain construction and evaluation

The pSR6-X123 plasmid [87] containing *Scheffersomyces stipitis* *XYL1*, *XYL2*, and *XYL3* genes was linearized by XcmI digestion and introduced into the *URA3* locus of all industrial yeast strains to allow for xylose utilization. A standard high-efficiency lithium acetate transformation method was used for chromosomal introduction of the linearized plasmid [103]. Eight transformants of each strain were screened in 5 mL of YP medium containing 40 g/L of xylose at 30 °C. The transformant from each strain with the highest xylose uptake rate and ethanol productivity was selected for further study. Comparisons between selected transformants of each strain were conducted following protocol as listed in **Section 5.2.2** with the exception that xylose was used as the carbon source in place of glucose. Strains were named using the following system: ATCC 2360 (JIN 01) was renamed J1X, ATCC 4098 (JIN 02) was renamed J2X, and so forth.

5.2.4 Cellobiose-fermenting strain construction and evaluation

The pRS425-BTT plasmid [88] containing *Neurospora crassa* β -glucosidase (*gh1-1*) and cellobextrin transporter (*cdt-1*) genes was linearized and introduced into all industrial yeast strains to allow for cellobiose utilization. A standard high-efficiency lithium acetate transformation method was used for chromosomal introduction of the linearized plasmid [103]. Several transformants of each strain were screened in 5 mL of YP medium containing 40 g/L of cellobiose at 30 °C. The transformant from each strain with the highest cellobiose uptake rate and ethanol productivity was selected for further study. The transformant from each strain with the highest cellobiose uptake rate and ethanol productivity was selected for further study. Comparisons between selected transformants of each strain were conducted following protocol as listed in **Section 5.2.2** with the exception that cellobiose was used as the carbon source in place of glucose. Strains were named using the following system: ATCC 2360 (JIN 01) was renamed J1C, ATCC 4098 (JIN 02) was renamed J2C, and so forth.

5.2.5 Gas pressure analysis

Strains were evaluated for their gas pressure production in sealed glass bottles, with a higher gas pressure indicative of higher CO₂ production and in-turn higher ethanol production. Jars with a maximum volume of 100 mL were filled with 20 mL of either YP medium containing 20 g/L of glucose, YP medium containing 20 g/L of glucose and 25% hydrolysate, or Verduyn's [187] medium with 20 g/L of glucose and 0.628 g/L of complete supplement mixture (MP Biomedicals). The initial pH value of each medium was adjusted to 6. The fermentation bottles were shaken at 100 RPM on an Innova 2300 shaker (New Brunswick Scientific, CT) in a 30 °C incubation room. Cell optical density (OD) was measured as discussed in **Section 5.2.2** and the

initial cell density was 1 (OD₆₀₀). Gas pressure was monitored with RF gas production modules (ANKOM Technology, NY) as pounds per square inch every 5 minutes.

5.2.6 Micro well plate fermentations

The cell growth rate and density was determined for growth in YP medium containing 20 g/L of glucose with an initial pH adjusted to 4.5, 5.0, 5.5, 6.0, or 6.5. A 200 µL initial volume of medium was placed into individual wells of Costar 96-well flat-bottom polystyrene plates with lids placed on top (Corning, MA). The initial cell density was 1 (OD₆₀₀) and microplates were incubated at 30 °C and 200 RPM in a Symphony incubating microplate shaker (VWR, PA). An aliquot of 50 µL of mineral oil was placed on top of each well to prevent evaporation.

5.2.7 Inhibitor resistance plating

Precultures of *S. cerevisiae* strains were grown at 30 °C in YP medium containing 20 g/L of glucose. Cells were harvested at mid-exponential phase and adjusted to an initial cell density of 1 (OD₆₀₀) for further use. Prior to plating, 10¹ to 10⁶ dilutions of cells were made in Costar 96-well flat-bottom polystyrene plates (Corning, MA). Cells were picked from the 96-well plate using a sterile metal stamper and inoculated onto the top of sterile agar-containing petri dishes. The agar media included the following: YP medium containing 20 g/L of glucose incubated at 30 °C, 37 °C, 42 °C and 45 °C, YP medium containing 20 g/L of glucose with 2 g/L of hydroxymethylfurfural (HMF) at 30 °C, YP medium containing 20 g/L of glucose with 1 g/L of furfural at 30 °C, and YP medium containing 20 g/L of 1 g/L, 2 g/L, or 3 g/L of acetate at 30 °C.

5.2.8 Flow cytometry

Precultures of *S. cerevisiae* strains were grown at 30 °C in YP medium containing 20 g/L of glucose and harvested at mid-exponential phase. Cell density was adjusted to 1 (OD₆₀₀), then fixed and permeabilized with cold ethanol (70%), which allowed SYTOX green dye to penetrate the cells to stain the nucleic acid. The DNA contents of stained cells were analyzed and detected by the excitation and emission spectra of the SYTOX green/DNA complex using a LSR II Flow Cytometry Analyzer (BD Biosciences, CA). Strain ploidy was determined by comparison with strains that contain 1n, 2n, 3n, or 4n ploidy.

5.2.9 Mating-type test

A halo assay based on yeast pheromone response was used to determine strain mating-type [188]. Mating type tester strains (DBY7730 for MAT α and DBY7442 for MATa) [189] were first spread on YPD 20 g/L agar plates. Each ATCC 4124 spore clone was then spotted on the lawn of each tester strain, and the plates were incubated overnight. The presence of a halo around a spore spot was used to score its mating type. For the mating type PCR method, colony PCR of spore cells was performed with a mixture of three primers, Jin419, Jin420, and Jin421 (**Table 1.4**). Cells with a single band of 0.49 kb (MATa) or 0.37 kb (MAT α) were selected as mating-competent cells with a respective mating type. Cells with two bands, indicating the presence of both MATa and MAT α genes, were excluded.

5.2.10 Sporulation efficiency determination

Single colonies of each strain were obtained from YPD plates during the ascospore germination stage. Colonies were cultured in sporulation medium (1% potassium acetate, 0.1%

yeast extract, 0.05% glucose, and 2% agar). Cell division and sporulation occurred within 3 – 7 days and measured for their sporulation efficiency.

5.2.11 Lactic acid tolerance screening experiments

Precultures of *S. cerevisiae* strains were grown at 30 °C in YP medium containing 20 g/L of glucose. Cells were harvested at mid-exponential phase and adjusted to an initial cell density of 0.1 (OD₆₀₀) for further use. For fermentation performance assessment, strains were inoculated into 200 µL of sterile YPD40 medium containing 0, 10, 40, or 60 g/L of lactic acid in a honeycomb well plate. The well plate and cultures were incubated at 30 °C with constant shaking in a Bioscreen C automated growth curve analysis system (Growth Curves USA, NJ). Results are shown as cell optical density after 48 hours of growth.

5.2.12 Construction of a lactic acid-producing, industrial *S. cerevisiae* strain (J17XL)

Construction of an integration cassette and subsequent transformations followed the unmodified protocol listed in **Section 2.2.1**.

5.3 Results

5.3.1 Industrial strain screening overview

An exhaustive review and analysis of 24 industrial *Saccharomyces spp.* yeast strains was conducted (**Table 5.3**). Compared to the control strain JIN 03 (ATCC 4124), several strains appeared to excel in two conditions amenable to lactic acid production from lignocellulosic feedstocks: a rapid xylose fermentation rate and a strong tolerance in low pH conditions.

Several interesting trends appeared at the end of the screening process. We observed that only five strains were able to match the fermentation rate of the ATCC 4124 control strain in high concentrations of glucose (160 g/L) and no strain significantly exceeded the control. This suggests that the ATCC 4124 strain is considerably tolerant to high osmotic stress, whereas many industrial yeast do not seem to possess this tolerance. We also observed that few strains seemed to be impacted by high temperature (37 °C) fermentation beyond that of the control. When expressing a heterologous xylose fermentation pathway consisting of *XYL1*, *XYL2*, and *XYL3*, strains named as J1X (ATCC 2360), J2X (ATCC 4098), and so forth, the majority of transformed industrial strains performed comparably to the control ATCC 4124 strain. However, J5X (ATCC 4127), J18X (ATCC 56069), and J24X (ATCC 66348) were found to more rapidly ferment xylose and produce ethanol than the ATCC 4124 control.

Regarding the fermentation inhibitors acetic acid, furfural, and hydroxymethylfurfural (HMF), which are common in lignocellulosic hydrolysates, most strains were as tolerant as the ATCC 4124 control. However, JIN 09 (ATCC 9763), JIN 13 (ATCC 24858), and JIN 17 (ATCC 46523) were able to grow more rapidly than the control in the presence of these inhibitors, suggesting that they may be preferential strains for industrial lignocellulosic hydrolysate fermentations. In addition, several strains were comparable to or outperformed the ATCC 4124 control strain in fermentation media containing actual hydrolysate: JIN 16 (ATCC 38554), JIN 21 (ATCC 60493), and JIN 23 (ATCC 62914) were found to outperform the ATCC 4124 control while JIN 05 (ATCC 4127), JIN 06 (ATCC 4921), JIN 18 (ATCC 56069), JIN 24 (ATCC 66348), and JIN 26 (ATCC 96581) performed comparably.

Taking into account the relative performances of the strains in terms of low pH tolerance, fermentation inhibitor resistance, and xylose fermentation rates, we identified JIN 05 (ATCC

4127), JIN 17 (ATCC 46523), JIN 18 (ATCC 56069), and JIN 24 (ATCC 66348) as four strains to conduct further tests regarding lactic acid resistance and production.

After the initial large-scale screening, the four selected strains, hereby referred to as J5 (ATCC 4127), J17 (ATCC 46523), J18 (ATCC 56069), and J24 (ATCC 66348), were subjected to several additional screening tests to assess their tolerance specifically to lactic acid. These tests were conducted based on the hypothesis that strains with an initially higher tolerance to lactic acid would potentially produce higher lactic acid yields when expressing a heterologous *ldhA*, such as the *ldhA* gene and cassette used in **Chapters II, III, and IV**.

5.3.2 Lactic acid tolerance of ATCC 4127, ATCC 46523, ATCC 56069, and ATCC 66348

To produce high concentrations of lactic acid using an engineered *S. cerevisiae* strain, it would be amenable to have a strain which is capable of growing even in the presence of high extracellular lactic acid concentrations even without the use of buffers or neutralizing agents. By obtaining a lactic acid-tolerant parental strain, the associated high costs of using excess buffers and neutralizing agents could potentially be eliminated. This cost reduction is especially important for high-volume/low-value industrial chemicals such as lactic acid. Therefore, the four selected ATCC strains were grown in YPD medium containing 0, 10, 40, or 60 g/L of lactic acid (**Fig. 5.4**). In addition, the engineered EJ4L yeast strain, expressing a heterologous *ldhA* from *Rhizopus oryzae*, was used as a control. Because the EJ4L strain produces lactic acid, it was expected that it would grow significantly worse than the other four strains, which was readily apparent. All four industrial strains grew well in 0 g/L of lactic acid. However, J5 grew significantly worse when grown in 10 g/L of lactic acid compared to the other industrial yeast strains and none of the strains could grow in the presence of 60 g/L of lactic acid. Overall, it

seemed that J17 (ATCC 56069) appeared to have the most rapid growth in liquid YPD40 medium with 10 g/L or 40 g/L of lactic acid.

As a further test of the lactic acid tolerance of the strains, the four industrial strains and the non-*ldhA* expressing EJ4 engineered yeast strain were screened on agar plates containing YPD40 medium with 0 g/L or 40 g/L of lactic acid (**Fig. 5.5**). Surprisingly, the relative growth of the industrial strains on solid agar plates containing 40 g/L of lactic acid was not precisely the same as when grown in liquid medium containing 40 g/L of lactic acid. The largest difference was the performance of the J17 industrial yeast strain. Although the strain seemed to relatively perform the best in liquid medium, it displayed the smallest amount of cell growth on 40 g/L lactic acid in agar. However, compared to the EJ4 strain, all four industrial strains, and especially J18 and J24, grew significantly better. The EJ4 strain was in fact unable to grow at all in the presence of 40 g/L of lactic acid. This indicates that the screened industrial yeast strains may be better lactic acid producers when expressing the pITy3-*ldhA* cassette.

5.3.3 Construction of a lactic acid-producing, industrial *S. cerevisiae* strain (J17XL)

We constructed an industrial *S. cerevisiae* strain capable of producing lactic acid from xylose by introducing a fungal *ldhA* from *R. oryzae* into a xylose-fermenting *S. cerevisiae* using the materials and methods listed in **Section 2.2.1**. The integration of the pITy3 plasmid relies on the availability of a δ sequence of a Ty element of the yeast chromosome. However, the presence of δ sequences in the industrial yeast strains (specifically J5, J17, J18, and J24) has not yet been elucidated via sequencing. After repeated transformation attempts, we were only able to confirm successful integration of the pITy3-*ldhA* cassette into the genome of the J17 strain. Therefore, we suspect, that J5, J18, and J24 may lack the necessary δ sites required for successful integration of

the pITy3-*ldhA* plasmid. For the successful integration of *ldhA* into the J17 strain, we chose specifically used J17X, a J17 strain expressing a heterologous xylose pathway (following the materials and methods listed in **Section 5.2.3.**) for introduction of the pITy3-*ldhA* cassette in order to produce lactic acid from xylose by an engineered industrial yeast strain. The resulting strain, named J17XL, was used in further fermentation experiments. The lactate dehydrogenase enzymatic activity of J17XL grown in glucose or xylose was measured following protocol in **Section 2.2.4** and found to be not significantly different from the EJ4L strain (data not shown).

5.3.4 Lactic acid production from xylose by an engineered industrial yeast strain (J17XL)

After construction of the xylose-fermenting, lactic acid-producing industrial yeast strain J17XL (ATCC 46523 background), we assessed the ability of the strain to produce lactic acid from glucose and xylose. In YPD40 medium, there was no significant difference in lactic acid production or ethanol production compared to the EJ4L control strain (data not shown). However, when grown on YPX40 medium without the use of buffers or neutralizing agents, the J17XL strain produced significantly less ethanol than the EJ4L control strain (**Fig. 5.6**). The EJ4L strain reached peak lactic acid and ethanol titers of 12.4 g/L and 4.1 g/L, respectively, with yields of 0.41 g lactic acid/g xylose and 0.12 g ethanol/g xylose at 40 h. Comparatively, the J17XL strain reached its peak lactic acid titer of 13 g/L with a yield of 0.37 g lactic acid/g xylose at 70 h.

5.4 Discussion

In broad terms, *Saccharomyces spp.* can be divided up into two major categories: 1) industrial or 2) laboratory. As the name implies, industrial yeast strains are considered as such

due to their ability to resist harsh industrial fermentation conditions, which includes fermentation inhibitor-laden lignocellulosic hydrolysates. In many cases, industrial yeast are polyploid strains, whereas laboratory yeast are most commonly haploid strains [173, 190]. While the increased ploidy can aid the resistance of the yeast strain to fermentation conditions, polyploid can also increase the difficulty of introducing targeted genetic perturbations. However, with the CRISPR/Cas9 gene editing system, engineering polyploid yeast strains has become increasingly easy [191]. With the CRISPR/Cas9 system in mind, new studies to identify the phenotypic characteristics of industrial yeast strains would be beneficial.

Of the 22 industrial yeast strains in this study, six (ATCC numbers 4127, 4921, 56069, 6022, 60223, and 62914) have no peer-reviewed literature citing the ATCC nomenclature, and to our knowledge, have not been used in any major laboratory- or industrial-scale studies. Despite this, these six strains also did not have any significantly improved phenotypes compared to the highly-studied ATCC 4124 control strain (**Table 5.3**). With this in mind, we selected industrial yeast strains ATCC 4127, ATCC 46523, ATCC 56069, and ATCC 66348 for integration of the pITy3-*ldhA*-G418 cassette. However, after several attempted transformations, we were only able to confirm successful integration of *ldhA* in the ATCC 46523 strain (J17XL). Two connected possibilities exist for the failed integration into the three other industrial strains.

First, the absence or low occurrence rate of retrotransposon Ty elements could be a limiting factor. Because the pITy3 cassette integrates into δ sequences of the Ty element on a chromosome, it is a necessity that one or more of the loci exist in the target transformant strain. The yeast retrotransposon Ty has been measured as occurring roughly 35 times in the yeast genome [192]. It is well established that each Ty element contains roughly two long terminal repeats (LTR), known as δ sequences [193]. As such, it was first estimated that at least 80 δ

sequences exist within the yeast genome [192]. More recently, the occurrence of δ sequences has been estimated to be as high as 150-200 copies [194, 195]. However, until complete genome sequencing or other methods of quantifying the Ty δ sequences is conducted on ATCC 4127, ATCC 46523, and ATCC 66348 the possibility exists that these strains lack the necessary integration sites, which would explain why integration using the pITy3-*ldhA* cassette was unsuccessful.

Second, the three strains we could not successfully transform may have an increased sensitivity to the antibiotic gentamicin G (G418). Peptide synthesis is inhibited in eukaryotic cells by G418 through disruption of cellular elongation [196]. Integration of the pITy3 vector confers a resistance to G418 by integration of the NEO kanamycin resistance gene of transposon Tn903 [150, 197]. It is also known that G418 resistance increases proportionately to δ vector copy number, which relies on the presence of Ty δ sequences [150]. Together, this suggests that the industrial strains could have a low Ty δ sequence count, limiting integration of the G418 resistance supplied by the pITy3 cassette, resulting in no cell viability in the presence of G418 and ultimately a failed transformation.

As ATCC 46523 was the only successful pITy3-*ldhA*-G418 transformant (J17XL), it was the only strain grown in glucose and xylose media for comparison to the previously-constructed xylose-fermenting, lactic acid-producing EJ4L strain. We initially expected that J17XL would produce more lactic acid and less ethanol than the EJ4L strain for several reasons. First, the J17 parental strain was found to have a strong resistance to acetate and low pH conditions based on our agar plating assay (**Table 5.3**). Secondly, the xylose consumption rate and cell growth rate of J17X were found to be at least comparable to the ATCC 4124 laboratory strain (**Table 5.3**). Most

importantly, J17 seemed to have an increased lactic acid tolerance compared to EJ4 or EJ4L when grown or plated on media containing lactic acid (**Figs. 5.4 and 5.5**).

However, despite the apparent organic acid and low pH resistance of the J17 strain, when engineered to ferment xylose (J17X) and produce lactic acid (J17XL), it was unable to produce significantly more lactic acid than the EJ4L strain (**Fig. 5.6**). The J17XL strain did have the important benefit of producing no measureable ethanol, resulting in a homolactic fermentation, which is valuable for reducing industrial purification costs by removing the need to distill ethanol. As a final result, the J17XL strain produced a 0.5 g/L greater titer than the EJ4L strain, but as no ethanol was produced, the net theoretical yield of all products (lactic acid and ethanol) from consumed xylose was 53 % from the EJ4L control strain, but only 37 % from the J17XL industrial strain. Overall, these results provide some insight that introduction of the same pITy3-*ldhA*-G418 cassette into two different *S. cerevisiae* strains can provide two different phenotypes: one strain producing no ethanol (J17XL) and one strain producing substantial ethanol (EJ4L).

5.5 Conclusions

Collectively, this study has provided a useful dataset (**Table 5.3**) for other researchers to refer to when choosing an industrial *Saccharomyces spp.* for their unique purpose. This study builds on previous studies which have also aimed to evaluate a variety of industrial yeast strains with the intent to improve the available dataset of industrial yeast phenotypes [198, 199]. Furthermore, we demonstrate that, although lactic acid titer did not increase in our engineered industrial strain, phenotypes can vary significantly (no ethanol produced compared to significant ethanol production) simply by changing the choice of parental strain (**Fig. 5.6**). Further studies are underway to elucidate why the J17XL strain produces no ethanol and to determine why the

other attempted ATCC transformants (ATCC 4127, ATCC 56069, and ATCC 66348) were unable to integrate the pITy3-*ldhA*-G418 cassette.

5.6 Figures

Organic acid	Major industrial uses	Reference(s)
Lactic acid	Poly lactide precursor, food preservative	[49, 50, 80, 81, 112, 138, 159]
Succinic acid	Polyester precursor, 1,4-butanediol precursor	[51-53, 201]
Glycolic acid	Dyes, flavoring agent, skin care	[55]
Malic acid	Flavoring agent, pH modifier in cosmetics	[202-204]

Table 5.1 A list of organic acids which can be overproduced by engineered *Saccharomyces spp.*

The acid name, major industrial uses, and relevant references are shown.

Code name	ATCC Number	Isolation Origin	Reference(s)
JIN 01 or J1	ATCC 2360	Kefir	[205]
JIN 02 or J2	ATCC 4098	German white wine	[206]
JIN 03 or J3	ATCC 4124	Molasses distillery	[207]
JIN 04 or J4	ATCC 4126	Amylo process	[208]
JIN 05 or J5	ATCC 4127	American Concord grapes	N/A
JIN 06 or J6	ATCC 4921	French wine	N/A
JIN 08 or J8	ATCC 7754	Fleischmann bakers' yeast	[209]
JIN 09 or J9	ATCC 9763	Distillery	[210]
JIN 10 or J10	ATCC 20597	Mutant derived from ATCC 20598	[211]
JIN 11 or J11	ATCC 20598	N/A	[211]
JIN 12 or J12	ATCC 24855	Egyptian distillery	[212]
JIN 13 or J13	ATCC 24858	N/A	[212]
JIN 14 or J14	ATCC 24860	N/A	[213]
JIN 15 or J15	ATCC 26422	Sake	[214]
JIN 16 or J16	ATCC 38554	Canned cherries	[215]
JIN 17 or J17	ATCC 46523	Baker's yeast	[216]
JIN 18 or J18	ATCC 56069	Fermented banana	N/A
JIN 19 or J19	ATCC 60222	Egyptian baker's yeast	N/A
JIN 20 or J20	ATCC 60223	Alsa Briochin bakers' yeast	N/A
JIN 21 or J21	ATCC 60493	Canned strawberries	[215]
JIN 23 or J23	ATCC 62914	Frozen unbaked bread dough	N/A
JIN 24 or J24	ATCC 66348	Japanese soil	[217]
JIN 25 or J25	ATCC 66349	Candied apple	[217]
JIN 26 or J26	ATCC 96581	Spent sulfite liquor fermentation	[218]

Table 5.2 Listing of all 24 industrial *S. cerevisiae* strains which were screened in this study, along with their associated ATCC number, their origin of isolation, and a relevant reference if available.

	1	2	3	4	5	6	8	9	10	11	12	13
	2360	4098	4124	4126	4127	4921	7754	9763	20597	20598	24855	24858
YPD 160 g/L												
30 °C												
37 °C												
1 g/L acetate												
2 g/L acetate												
3 g/L acetate												
1 g/l furfural												
2 g/l HMF												
20% H												
FC												
SE												
XCR												
EPRx												
ODx												
CCR												
EPc												
ODc												
YPDga												
Hga												
VDga												
4.5pr												
5.0pr												
5.5pr												
6.0pr												
6.5pr												
SMga												
SCDga												
ODman												
EPRman												
ODmal												
EPRmal												
ODsuc												
EPRsuc												

Table 5.3(cont.)

	14	15	16	17	18	19	20	21	23	24	25	26
	24860	26422	38554	46523	56069	6022	60223	60493	62914	66348	66349	96581
YPD 160 g/L												
30 °C												
37 °C												
1 g/L acetate												
2 g/L acetate												
3 g/L acetate												
1 g/l furfural												
2 g/l HMF												
20% H												
FC												
SE												
XCR												
EPRx												
ODx												
CCR												
EPc												
ODc												
YPDga												
Hga												
VDga												
4.5pH pr												
5.0pH pr												
5.5pH pr												
6.0pH pr												
6.5pH pr												
SMga												
SCDga												
ODman												
EPRman												
ODmal												
EPRmal												
ODsuc												
EPRsuc												

Table 5.3 (cont.)

Table 5.3 A heat map indicating the relative performance of our industrial strain screening experiments. Our internal code name for each strain (JIN01, JIN02, etc.) is listed along the top row. The ATCC strain name is listed along the second row. The first column indicates the specific test with detailed experimental conditions shown in **Sections 5.2.1** through **5.2.10**. The control strain, JIN03 (ATCC 4124) is shown in the third results column from which all other strains were compared. Performance for a specific test that was greater than 10% lower than the ATCC 4124 control strain is indicated by a dark orange color. Performance 5-10% lower is indicated in light orange, performance within 5% lower or higher is indicated by no color, performance 5% higher is indicated by light purple, and performance greater than 10% higher is indicated by dark purple. Black boxes indicate the specific strain was not studied in the given test. **Abbreviations:** HMF, hydroxymethylfurfural; FC, flow cytometry; SE, sporulation efficiency; XCR, xylose consumption rate; EPR_x , ethanol production rate from xylose; OD_x , optical density production rate from xylose; CCR, cellobiose consumption rate; EP_c , ethanol production rate from cellobiose; YPD_{ga} , yeast extract peptone dextrose medium monitored by gas analysis; H_{ga} , 25% hydrolysate mixture monitored by gas analysis; VD_{ga} , Verduyn's minimal medium with complete supplement mixture monitored by gas analysis; pr, plate reader fermentation; SM_{ga} , synthetic complete medium with complete supplement mixture monitored by gas analysis; SCD_{ga} synthetic complete medium without complete supplement mixture monitored by gas analysis; OD_{man} , optical density production rate from mannose; EPR_{man} , ethanol production rate from mannose; OD_{mal} , optical density production rate from maltose; EPR_{mal} , ethanol production rate from maltose; OD_{suc} , optical density production rate from sucrose; EPR_{suc} , ethanol production rate from sucrose.

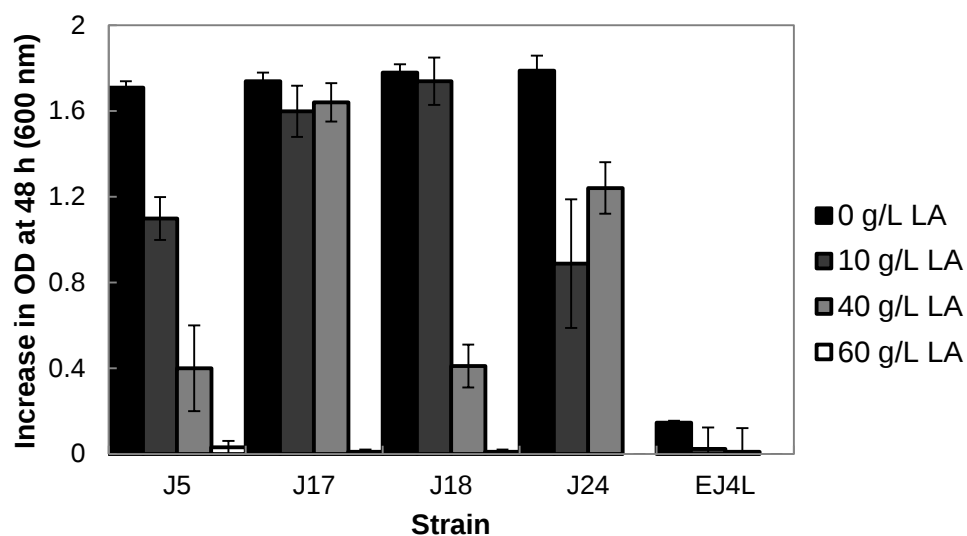


Fig. 5.4 Increase in optical density (OD₆₀₀ nm) at 48 h relative to 0 h optical density of four industrial *S. cerevisiae* strains and the engineered EJ4L strain in YPD medium containing 0, 10, 40, or 60 g/L of lactic acid. Black bars indicate 0 g/L lactic acid, dark grey bars indicate 10 g/L of lactic acid, light grey bars indicate 40 g/L of lactic acid, and white bars indicate 60 g/L of lactic acid. Error bars indicate standard deviation (n=2). Abbreviations: OD, optical density; LA, lactic acid.

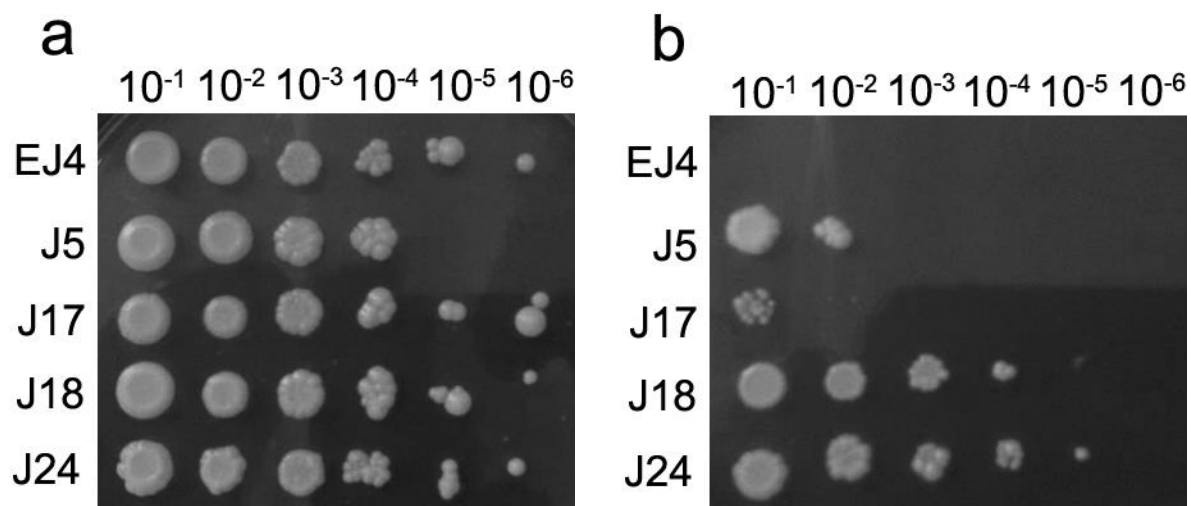


Fig. 5.5 Serial diluted single colonies of the engineered EJ4 strain and four industrial yeast strains were plated onto YPD40 agar plates containing **a** 0 g/L or **b** 40 g/L of lactic acid.

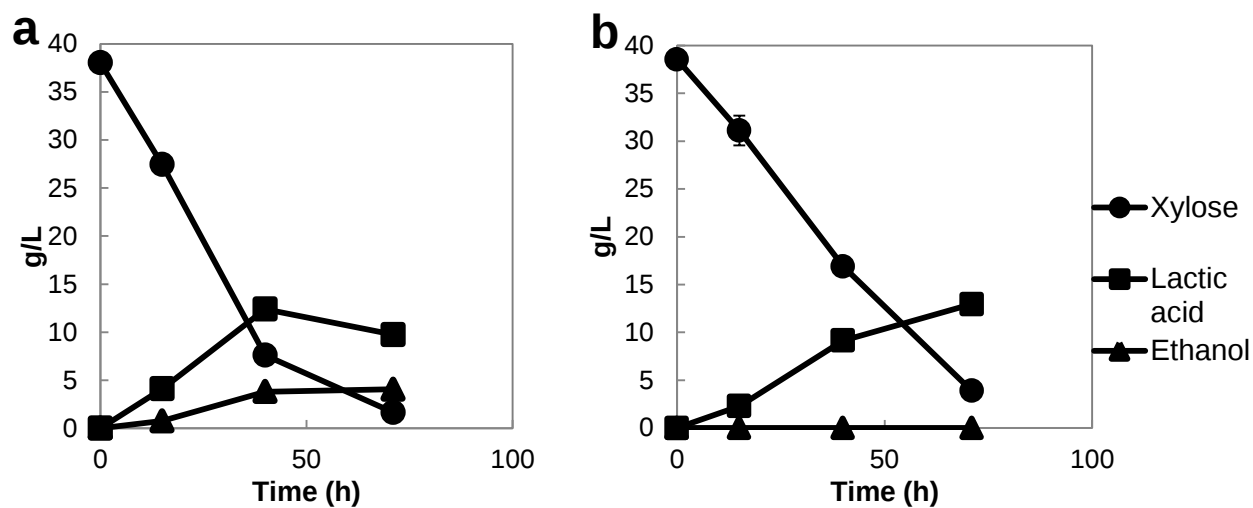


Fig. 5.6 Fermentation profile, over time, of two *ldhA*-expressing engineered *S. cerevisiae* strains. **a** Recombinant laboratory strain EJ4L grown in YPX medium. At 40 h, EJ4L fermented ~30 g/L of xylose (*closed circle*) producing 12.4 g/L of lactic acid (*closed square*). **b** Recombinant industrial strain JIN17XL grown in YPX medium. At 71 h, JIN17XL converted ~35 g/L of xylose (*closed circle*) into ~13 g/L of lactic acid (*closed square*). Ethanol (*closed triangle*) is also shown. The values are the mean of two independent experiments and the *error bars* indicate the standard errors.

CHAPTER VI PHENOTYPIC AND GENOTYPIC ANALYSIS OF ENGINEERED LACTIC ACID-PRODUCING YEAST TO ELUCIDATE UNDERLYING MECHANISMS

6.1 Introduction

The production of lactic acid at the industrial scale has increased dramatically in recent years, with global production levels estimated at between 260,000 metric tons [93] to as high as 367,000 metric tons [219] or even 726,000 metric tons [220]. Most lactic acid is currently produced through microbial fermentation of edible feedstocks containing glucose. To alleviate the concerns of disrupting the food supply by producing fuels and chemicals, such as lactic acid, from edible sugars, many studies have been conducted to convert inedible lignocellulosic sugars into these value-added products [221, 222]. Recently, our lab has developed several *Saccharomyces cerevisiae* yeast strains capable of efficiently fermenting xylose and cellobiose, two major lignocellulosic sugars, into lactic acid [49, 50]. A heterologous xylose assimilation pathway consisting of *XYL1*, *XYL2*, and *XYL3* allowed for fermentation of xylose [85], while a heterologous pathway of *cdt-1* and *gh1-1* allowed for assimilation and cleavage of the disaccharide cellobiose [148, 166]. A gene (*ldhA*) encoding for lactate dehydrogenase (LDH) from the fungus *Rhizopus oryzae* was then integrated into the genome of the xylose- and cellobiose-fermenting engineered yeast. In each case, the engineered strain had no genotypic disruption to the native ethanol pathway, consisting of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), allowing the strains to freely produce ethanol.

The content of this chapter is in preparation for submission with authorship by Timothy L. Turner, Guo-Chang Zhang, Lahiru Niroshan Jayakody, Stephan Lane, Heejin Kim, Whi Yeon Cho, and Dr. Yong-Su Jin, who was the director of the research.

Expectedly, we found that when fermenting glucose, both strains produced primarily ethanol [49, 50]. Conversely, we observed that when fermenting xylose, cellobiose, or a mixture of xylose and cellobiose, lactic acid was not only the major product, but the ethanol yield was generally negligible. The underlying mechanisms and biological basis for this phenotype was not understood at the time, but here, we disclose additional experiments and discussion which provides initial evidence to explain these results. Primarily, these experiments focus on overexpression and deletion of *JEN1* and *ADY2*, carboxylic acid transporters which naturally occur in *S. cerevisiae* yeast. Using the modern clustered regularly-interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) engineering methodology, here, we provide evidence that the *JEN1* and *ADY2* genes are at least partially essential for efficient lactic acid production from lignocellulosic sugars by engineered *S. cerevisiae*. In addition, we will investigate the impacts of *JEN1* and *ADY2* deletions on intracellular metabolite concentrations, based on the hypothesis that deletion of these carboxylate transporters will cause an increase of intracellular lactic acid, resulting in feedback inhibition of LDH, and ultimately slowing the overall fermentation and reducing the final lactic acid titer.

6.2 Materials and Methods

6.2.1 Flask fermentations and analysis

Yeast cells were cultured in YP medium (10 g/L yeast extract and 20 g/L peptone) containing glucose (YPD), xylose (YPX), cellobiose (YPC), maltose, mannose, or sucrose. Lactic acid (30 % in H₂O stock) used for YP fermentations only containing 10 g/L of lactic acid as a carbon source was obtained from Sigma-Aldrich (product number L1875). Concentrations of

the sugars were displayed as numbers following their initials (e.g., YPD160, YP medium containing 160 g/L of glucose). Stock cultures were maintained on YPD agar (20 g/L agar) plates in 4 °C. Yeast precultures were grown in YP medium containing 40 g/L of glucose in 5 mL total volume and harvested at mid-exponential phase. Fermentations were conducted with an initial volume of 50 mL in 250 mL Erlenmeyer Pyrex[®] flasks (Corning, MA). Flasks were shaken at 100 RPM on an Innova 2300 shaker (New Brunswick Scientific, CT) in a 30 °C incubation room. Cell optical density (OD) was measured via NanoDrop 200C (Thermo Fisher Scientific, MA) or BioMate 3 UV-visible spectrophotometer (Thermo Fisher Scientific, MA) and the initial OD was adjusted to ~1. Glucose, xylose, cellobiose, glycerol, acetate, ethanol, and lactic acid concentrations were determined by use of a 1200 Infinity series HPLC system (Agilent Technologies, CA) equipped with a refractive index detector using a Rezex ROA-Organic Acid H⁺ (8%) column (Phenomenex Inc., CA). The column was eluted with 0.005 N H₂SO₄ at a flow rate of 0.6 mL/min at 50 °C.

Intracellular metabolites were prepared as discussed in **Section 6.2.7**. Prepared samples were analyzed by a GC/MS system (Agilent Technologies Inc., CA) consisting of a 7,890 gas chromatograph; a 5,975 MSD was used to analyze the presence of 21 intracellular metabolites by comparing sample chromatographs to an internal library. GC was performed on a 30-m HP-5MS column with 0.25 mm inner diameter and 0.25 µm film thickness (Agilent Technologies Inc. CA) with an injection temperature of 250 °C, the interface set to 250 °C, and the ion source adjusted to 230 °C. The nitrogen carrier gas was set at a constant flow rate of 0.8 ml min⁻¹. The temperature program was initially 5 min at 40 °C followed by an oven temperature increase of 40 °C min⁻¹ to 280 °C for the final 1 min. The mass spectrometer was operated in positive electron impact mode at 69.9 eV ionization energy in m/z 30–800 scan range. The spectra of all

chromatogram peaks were evaluated using the HP Chemstation (Agilent Technologies Inc., CA).

6.2.2 Construction of a multicopy, δ -integration vector containing *ldhA* with a Hygromycin B selection and expression in a xylose-fermenting *S. cerevisiae*

An integration cassette (pITy3-*ldhA*-HygB) was designed with a Hygromycin B antibiotic selection marker based on the previously reported pITy3-*ldhA*-G418 cassette [49, 50]. The G418 encoding region was cut and a Hygromycin B encoding region was ligated into the plasmid in place of the G418 region. The Hygromycin B template was obtained through PCR cloning of the Hygromycin B encoding region of the well-known pAG32 episomal plasmids. *Escherichia coli* TOP10 were used for gene cloning and manipulation and were grown in Luria-Bertani medium; 100 μ g/mL of Hygromycin B was added to the medium when required. The pITy3-*ldhA*-HygB plasmid was transformed into the SR8 strain using a high-efficiency lithium acetate transformation method [103]. Yeast transformants were selected on YPD20 plates containing 250 μ g/mL Hygromycin B. The resulting strain, SR8HL (SR8 with Hygromycin B resistance and lactate dehydrogenase encoded by *ldhA*), was used for *JEN1* and *ADY2* overexpression experiments. Lactate dehydrogenase activity of several SR8HL transformants was measured as previously described [49, 50] as a secondary methods to confirm successful *ldhA* integration and expression.

6.2.3 Overexpression of *JEN1* and *ADY2* in lactic acid-producing *S. cerevisiae*

Yeast cells were cultured in yeast extract peptone medium (10 g/L yeast extract and 20 g/L peptone, YP) containing glucose (YPD), In order to construct an *S. cerevisiae* strain capable of converting glucose or xylose to lactic acid with additional copies of *JEN1* and *ADY2*, an

engineered yeast strain (SR8HL) capable of fermenting xylose was transformed with an episomal expression pRS42-series plasmid harboring the appropriate *JEN1* or *ADY2* sequence cloned from a D452-2 yeast template sequence of *JEN1* and *ADY2*. The final pRS42K (Kanamycin/G418) plasmid contained the *ADY2* (pRS42k-*ADY2*) or *JEN1* (pRS42K-*JEN1*) sequences with a GPD promotor and CYC terminator. *Escherichia coli* TOP10 were used for gene cloning and manipulation and were grown in Luria-Bertani medium; 50 µg/mL of kanamycin was added to the medium when required. The pRS42K, pRS42K-*JEN1*, and pRS42K-*ADY2* plasmids were transformed into the SR8HL strain using a high-efficiency lithium acetate transformation method [103]. Yeast transformants were selected on YPD20 plates containing 300 µg/mL G418. To maintain pRS42K-series plasmid stability in the yeast cell, 250 µg/mL of G418 was added to all precultures and fermentations. Successful integration was confirmed by phenotypic resistance to G418 and colony PCR of the transformed yeast colonies.

6.2.4 Incubation of lactic acid-producing *S. cerevisiae* with α -Cyano-4-hydroxycinnamic acid

α -Cyano-4-hydroxycinnamic acid (CHC, Sigma-Aldrich, MO) was stored as a powder in a 4 °C refrigerator. Due to low solubility in water, the CHC was dissolved into 100 % methanol at a concentration of 5 mM and added to fermentation media to a final concentration of 100 µM. For experiments using CHC, two controls were used: 1) containing autoclaved nanopure water at a volume equal to the CHC solution added to the experimental fermentations and 2) containing 100 % methanol without CHC at a volume equal to the CHC solution added to the experimental fermentations. Fermentation protocol followed methods listed in **Section 6.2.1** unless otherwise noted.

6.2.5 Construction of $\Delta JEN1$, $\Delta ADY2$, and $\Delta ADY2\Delta JEN1$ lactic acid-producing *S. cerevisiae* strains using CRISPR/Cas9

The pRS42N-Cas9 plasmid providing nourseothricin (NAT) resistance was introduced into the desired strains (EJ4 and EJ4L) for *JEN1* and *ADY2* deletion following a standard lithium acetate transformation protocol as listed in **Section 6.2.2**, except 120 $\mu\text{g/mL}$ NAT was used for selection in place of Hygromycin B in YP medium. A FastCloning method was employed to generate gRNA expression plasmids for *JEN1* and *ADY2* deletion: *JEN1* and *ADY2* sequences +/- 1 kb were obtained from the Saccharomyces Genome Database [223]. The Broad Institute sgRNA Designer [224] was used to obtain two optimal 20-bp sgRNA recognition sequences targeting *JEN1* and *ADY2* open reading frames, respectively. Finally, the 20-bp *URA3* recognition sequence from a previously reported gRNA expression plasmid (gRNA-ura-HYB, [90]) was replaced with *JEN1* and *ADY2* recognition sequences using the PCR-based FastCloning [225]. The resulting plasmids were designated as pRS42H- $\Delta JEN1$ gRNA and pRS42H- $\Delta ADY2$ gRNA. Two double-stranded 90 mer oligonucleotide donor DNA for disrupting *JEN1* and *ADY2* were PCR amplified using primer pairs T102-JdDNA-U/T103-JdDNA-D and T104-AdDNA-U/T105-AdDNA-D, respectively. The sgRNA plasmids along with donor DNA primers and confirmation PCR primers are listed in **Table 1.4**. The pRS42H- $\Delta ADY2$ gRNA plasmid was then transformed together with the donor DNA into the Cas9 expressing EJ4 and EJ4L strains. Cells were plated on a YPD-HygB-NAT plate and allowed to grow for 2-3 days until transformants were ready to pick. Transformants were then examined by colony PCR using primer pair T108/T109 to screen for *ADY2* mutants. *JEN1* was deleted in EJ4 and EJ4L strains following the same procedures using primer pair T106/T107 for colony PCR.

For deletion of both $\Delta ADY2\Delta JEN1$, the Hygromycin B resistance was dropped out in *ADY2*-deleted EJ4 and EJ4L strains by culturing them in NAT liquid media for 24-36 hours. An appropriate amount of the cell cultures were taken and plated onto a YPD-NAT plate to isolate single colonies, which were then replica plated onto a YPD-HygB plate to confirm the drop-out of the Hygromycin B resistance. The resulting colony was picked for subsequent deletion of $\Delta JEN1$ following the same procedure.

6.2.6 Construction of an *ADY2* complement strain from EJ4L $\Delta ADY2\Delta JEN1$

First, yeast genomic DNA from the parental EJ4 strain was prepared from YPD40 grown cells harvested at mid-log phase using the YeaStar Genomic DNA Kit (Zymo Research, CA) without modification to the manufacturer's protocol. Approximately 60 ng of genomic DNA was amplified via three cycles of PCR with Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB, MA) using primers *Ady2OE-F*: gtacgtacggatccACCTTGGGATATCGTTGGA and *Ady2OE-R*: gtacgtacccgcggGGCAAACGATAGACCTTTC providing *Bam*HI and *Sac*II cutsites, respectively, for an insert of the native *ADY2* gene with approximately 1 kb upstream and 650 bp downstream to include the native promoter and terminator. Fragment size of the amplified region was confirmed by gel electrophoresis. The pRS42N plasmid, conferring NAT resistance, was used as the host cassette and was digested at single cut sites with restriction enzymes *Bam*HI and *Sac*II, the plasmid was treated with calf intestinal alkaline phosphatase (CIP) to reduce self-annealing, and annealed with the *ADY2* cloned fragment. The resulting pRS42N-*Ady2* plasmid was transformed into TOP10 *E. coli* grown in Luria-Bertani medium containing appropriate selection antibiotics and used for gene cloning and manipulation. The empty pRS42N plasmid and the pRS42N-*Ady2* plasmid were individually transformed into the

EJ4L and EJ4L Δ ADY2 Δ JEN1 strains using a high-efficiency lithium acetate transformation method [103]. Yeast transformants were selected on YPD20 plates containing 300 μ g/mL NAT. Successful integration was confirmed by phenotypic resistance to NAT and colony PCR of the transformed yeast colonies.

6.2.7 Analysis of intracellular lactic acid and other metabolites and cell preparation

Yeast cells were grown in YP medium containing appropriate concentrations of carbon sources as indicated and harvested at mid-log phase. Cells were rapidly quenched using a fast filtration method as follows: 1) Cell cultures were standardized to OD 1 and centrifuged with the removal of supernatant, then washed twice with autoclaved nanopure water, centrifuging and removing the water between each wash; 2) Cells were resuspended in 1 mL of water and filtered onto a nylon membrane using a vacuum manifold; 3) Cells were washed while on the nylon membrane/vacuum manifold with 1 mL of water; 4) Filtered cells and the nylon membrane were placed into a 1.5 mL microcentrifuge tube containing 1 mL of 75% ethanol and vortexed briefly; 5) The 1.5 mL microcentrifuge tube was incubated in an 80 °C water bath for 3 minutes; 6) The 1.5 mL microcentrifuge tube was centrifuged for 1 minute at 15,000 RPM; 7) 500 μ L of supernatant was transferred into a new 1.5 mL microcentrifuge tube without disturbing the debris pellet; 8) The 500 μ L solution was vacuum dried on low heat for 3 hours; 9) One triplicate of samples were set aside for immediate measurement of intracellular lactic acid using the Lactate Assay Kit MAK064 (Sigma-Aldrich, MO) or pyruvic acid using the Pyruvate Assay Kit MAK071 (Sigma-Aldrich, MO) without modification to the manufacturer's protocols and another triplicate of samples were immediately derivatized to prepare for gas chromatography-mass spectrometry analysis.

To increase the volatility and detectability of the intracellular metabolites by GC-MS, dried samples from step 9 above were derivatized as follows: 1) 40 mg of methoxyaminehydrochloride was dissolved in 1 mL pyridine and vortexed to fully dissolve; 2) 5 μ L of the methoxyaminehydrochloride in pyridine solution was added to each dried sample and briefly centrifuged; 3) Samples were incubated at 30 °C for 90 minutes at 400 RPM in a Thermomixer R (Eppendorf, Germany) and then briefly centrifuged; 4) 45 μ L of Trifluor-N-methyl-N-(trimethylsilyl)acetamide was added to each microcentrifuge tube and briefly centrifuged; 5) Samples were incubated at 37 °C for 30 minutes at 400 RPM in a Thermomixer R (Eppendorf, Germany) and then briefly centrifuged; 8) Samples were transferred to an HPLC vial with a 250 μ L spring-bottom insert and tightly capped before being injected into the GC-MS.

6.2.8 Bioreactor fermentations

Yeast precultures were grown in YPD40X40 medium and harvested at mid-log phase, then washed twice with sterilized water to prepare inoculums for fermentations. The bioreactor fermentations were conducted in YP medium containing 10 g/L of glucose, 40 g/L of xylose, and 80 g/L of cellobiose using a BioFlo/CelliGen 115 bioreactor (New Brunswick Scientific Co., USA). An initial yeast cell concentration of ~ 1 (0.47 mg DCW/mL , OD_{600}) was used. Working volume was set at 1 L inside of a 2 L glass vessel. Aeration was maintained at a flow rate of 1.5 L/min of microfiltered ($0.22 \text{ }\mu\text{m}$) ambient air and an impeller rotation of 200 RPM. Temperature was maintained at 30 °C. NaOH (10 N) was added as needed to maintain a pH value of 4, 5, or 6 as indicated.

6.3 Results

6.3.1 Low extracellular pH slows lactic acid production by engineered yeast expressing *ldhA* in a simulated hydrolysate

Three individual 1L bioreactor fermentations inoculated with the *ldhA*-expressing EJ4L yeast strain were established with YP medium containing 10 g/L glucose, 40 g/L xylose, and 80 g/L cellobiose to simulate the sugar composition of a common lignocellulosic hydrolysate. The pH value for each bioreactor was constantly maintained at 4, 5, or 6 using automated addition of 10 N NaOH as needed. At pH 6, the EJ4L strain was able to efficiently consume all available sugars and produce 81.6 g/L of lactic acid with no ethanol accumulation (data not shown). However, the rate of fermentation and especially the lactic acid productivity was significantly slower in the pH 5 condition and the slowest in the pH 4 condition (**Fig. 6.1**). The specific lactic acid productivity for each condition was 0.45 g/L-h in pH 4, 0.52 g/L-h in pH 5, and 0.67 g/L-h in pH 6, representing a 49 % increased lactic acid productivity in the pH 6 condition as compared to the pH 4 condition. In addition, the cellobiose consumption rate was significantly slower as the pH decreased (**Fig. 6.2**), which could explain the decreased lactic acid productivity. In the pH 6 condition, cellobiose was consumed at a rate of 0.64 g/L-h compared to a rate of 0.13 g/L-h in the pH 4 condition, representing a nearly 4-fold increase from pH 4 to pH 6.

6.3.2 *JEN1* and *ADY2* overexpression provides no significant improvement to lactic acid production by the SR8HL strain

The *JEN1* and *ADY2* genes, which natively encode for carboxylate transporters in *S. cerevisiae*, were overexpressed in the *ldhA*-expressing engineered *S. cerevisiae* strain SR8HL.

The pRS42K, pRS42K-JEN1 and pRS42K-ADY2 plasmids were transformed into SR8HL, yielding three strains: SR8HL-Emp, SR8HL-J1, and SR8HL-A2 and used for YPD fermentations without the addition of buffers or neutralizing agents such as CaCO_3 . When fermenting glucose, no significant difference was observed in the entire fermentation profile, with lactic acid and ethanol yields shown in **Fig. 6.3**. All three strains displayed ethanol yields between 0.27 to 0.29 g ethanol/g glucose and lactic acid yields between 0.08 and 0.10 g lactic acid/g glucose, indicating no significant differences.

The previous experiment was repeated with xylose used in-place of glucose. However, none of the three strains could finish the fermentation likely due to the decrease in pH and increase in lactic acid because no CaCO_3 was added (data not shown), which is expected based on previous reports of a similar *ldhA*-expressing, xylose-fermenting strain (**Fig. 2.2b**) [49]. Therefore, the YPX40 experiment was repeated with the addition of 35 g/L CaCO_3 to ensure saturation of a neutralizing agent and assure complete xylose utilization. The overexpression of *JEN1* and *ADY2* on the SR8HL lactic acid-producing strain resulted in no significant change in the fermentation profile from xylose with CaCO_3 (**Fig. 6.4**). Consistently, no measureable ethanol was produced from xylose and a lactic acid yield of ~0.64 g lactic acid/g xylose was produced for both overexpression strains and the empty plasmid control, indicating that the overexpression of the *JEN1* or *ADY2* carboxylate transporters had no significant impact on lactic acid production.

6.3.3 Using α -cyano-4-hydroxycinnamic acid as a chemical inhibitor of lactic acid transporters in *ldhA*-expressing *S. cerevisiae*

Although overexpression of *JEN1* and *ADY2* had no significant impact on the production of lactic acid in the *ldhA*-expressing strain in either glucose or xylose, we hypothesized that

inactivation of one or both of these genes could impair lactic acid transport out of the cell, resulting in a decreased overall lactic acid production profile. As a preliminary test of this hypothesis, the EJ4L strain, expressing an *ldhA* from *Rhizopus oryzae*, was grown in YPX40 medium containing α -cyano-4-hydroxycinnamic acid (CHC, dissolved in methanol), a known inhibitor of several yeast transporters including *JEN1* and *ADY2* [226, 227].

The water and methanol control fermentations generated ethanol yields of 0.05 g ethanol/g xylose and 0.08 g ethanol/g xylose, respectively (**Fig. 6.5**). In comparison, the CHC fermentation ethanol production was significantly higher, with a yield of 0.15 g ethanol/g xylose, representing a yield 312 % and 185 % higher than the water and methanol control fermentations, respectively. Regarding lactic acid production, the water and methanol control fermentations generated lactic acid yields of 0.41 g lactic acid/g xylose and 0.43 g lactic acid/g xylose, respectively. Comparatively, the CHC fermentation produced significantly less lactic acid, with a yield of 0.26 g lactic acid/g xylose, a reduction in lactic acid production of 38 % and 41 % compared to the water and methanol control fermentations, respectively.

6.3.4 Lactic acid production from *ldhA*-expressing *S. cerevisiae* with $\Delta JEN1$, $\Delta ADY2$, and $\Delta ADY2\Delta JEN1$

The CHC fermentation indicated that loss of function of carboxylate transporters, such as Jen1 and/or Ady2, by chemical disruption, reduced lactic acid production in the EJ4L strain (**Fig. 6.5**). However, because CHC is a non-specific inhibitor, it is difficult to ensure that only the Jen1 and/or Ady2 transporters were inhibited rather than a wide-range of proteins. To precisely assess the impact of inactive Jen1 and/or Ady2 proteins, the six new strains were engineered, three based on the EJ4 parental strain and three based on the *ldhA*-expressing EJ4L strain: **1)**

EJ4 Δ JEN1, 2) EJ4 Δ ADY2, 3) EJ4 Δ ADY2 Δ JEN1, 4) EJ4L Δ JEN1, 5) EJ4L Δ ADY2, and 6) EJ4L Δ ADY2 Δ JEN1. The newly constructed strains were then tested in YPX medium without CaCO₃ and no significant differences were observed between the EJ4 control strain and the EJ4 Δ JEN1, EJ4 Δ ADY2, or EJ4 Δ ADY2 Δ JEN1 strains (data not shown).

When the EJ4L transporter deleted strains were grown in YPX medium, a significant difference in lactic acid production was observed in the EJ4L Δ ADY2 Δ JEN1 strain compared to the parental EJ4L control (**Table 6.6**). The lactic acid yields of the EJ4L, EJ4L Δ JEN1, and EJ4L Δ ADY2 strains were similar with yields of 0.45, 0.42, and 0.41 g lactic acid/g xylose, respectively. However, the EJ4L Δ ADY2 Δ JEN1 strain displayed a significantly lower lactic acid yield, generating only 0.29 g lactic acid/g xylose, a ~36 % reduction compared to the EJ4L parental strain. The ethanol yield among all strains with Δ JEN1 was also slightly increased, to yields between 0.13 to 0.15 g ethanol/g xylose compared to a yield of 0.08 g ethanol/g xylose from the parental EJ4L strain or an ethanol yield of 0.04 g ethanol/g xylose from the EJ4L Δ ADY2 strain.

6.3.5 Lactic acid uptake rate of Δ JEN1 and Δ ADY2 *S. cerevisiae* strains

The parental EJ4, EJ4 Δ JEN1, EJ4 Δ ADY2, and EJ4 Δ ADY2 Δ JEN1 strains, were grown in YP medium containing ~10 g/L of lactic acid and no other carbon source. The parental, control EJ4 strain consumed approximately 9.8 g/L of lactic acid over 50 hours which was similar to the EJ4 Δ ADY2 strain, which consumed 10.2 g/L of lactic acid in the same time (**Fig. 6.7**). However, a clear split is apparent in the two strains lacking the JEN1 gene. EJ4 Δ JEN1 and EJ4 Δ ADY2 Δ JEN1 consumed only 6.4 g/L and 5.9 g/L of lactic acid in 50 hours. Although the lactic acid uptake rate was the lowest in the EJ4 Δ ADY2 Δ JEN1 strain, this experiment suggests

that Jen1 plays a more significant role in import of lactic acid than Ady2, as evidenced by the significantly lower lactic acid uptake rate in the EJ4 Δ JEN1 strain as compared to the EJ4 Δ ADY2 strain.

6.3.6 Analysis of intracellular lactic acid and other metabolites of Δ JEN1 and Δ ADY2, *ldhA*-expressing *S. cerevisiae* strains

Although the JEN1 and ADY2 deletion experiment results shown in **Table 6.6** indicate the importance of these two monocarboxylate transporters in lactic acid production, the specific mechanism of the impact of their deletions was unclear. To assess the impact, the intracellular lactic acid concentrations of EJ4L and EJ4L Δ ADY2 Δ JEN1 were measured from the quenched crude cell extracts of YPX-grown yeast cells harvested during phase. Samples were collected and measured at 39 h and 48 h and results are presented as relative abundance of intracellular lactic acid as compared to extracellular lactic acid, with the EJ4L control strain set as a value of 1 **Fig. 6.8**. The ratio of intracellular lactic acid to extracellular lactic acid in the EJ4L Δ ADY2 Δ JEN1 was approximately 250% of the EJ4L parental strain, indicating that the deletion of the ADY2 and JEN1 monocarboxylate transporters measurably inhibits the rate at which lactic acid can be expelled from the intracellular mixture.

As a control for **Fig. 6.8**, EJ4 and EJ4 Δ ADY2 Δ JEN1 samples from **Fig. 6.7** were also assessed for their intracellular lactic acid concentrations. This test was designed to verify that the intracellular lactic acid measurements of **Fig. 6.8** were not merely the upper limit of intracellular lactic acid accumulation by the engineered *S. cerevisiae* strains. Predictably, the EJ4 Δ ADY2 Δ JEN1 strain, which consumed 39 % less lactic acid at 50 h than the parental EJ4 strain (**Fig. 6.7**), displayed a similarly lower intracellular lactic acid concentration of ~70 % of

the parental EJ4 strain (data not shown). As a final control experiment, the non-*ldhA*-expressing EJ4 strain was grown in YPX medium and the measurement of intracellular lactic acid resulted in error-range measurements, indicating that the intracellular lactic acid test was not providing a false-positive lactic acid measurement (data not shown).

Similarly, intracellular pyruvate concentrations of the EJ4L strain and the EJ4L Δ ADY2 Δ JEN1 grown in YPX medium and harvested at mid-log phase were assessed (**Fig. 6.9**). However, no significant difference in the concentration of intracellular pyruvate was observed between the parental EJ4 strain and EJ4 Δ ADY2 Δ JEN1 strain or the lactic acid-producing EJ4L and EJ4L Δ ADY2 Δ JEN1 strains. This suggests that the lactic acid-producing strains expressing *ldhA* or the Δ ADY2 Δ JEN1 strains maintain consistent intracellular pyruvate concentrations in-line with their respective parental strains.

A wider range of intracellular metabolites was assessed through GC-MS analysis (**Fig. 6.10**) to determine if the deletion of the carboxylate transporters could impact the relative abundance of intracellular metabolites. However, no significant differences were observed, suggesting that the double-deletion of *JEN1* and *ADY2* did not greatly impact the transport or synthesis of these metabolites.

6.4 Discussion

We have presented several experiments to improve our understanding of lactic acid production from lignocellulosic sugars. Our previous reports [49, 50] displayed that the production of lactic acid from *S. cerevisiae* strains expressing a *R. oryzae* *ldhA* without disruption to the native ethanol pathway would produce lactic acid as the major product from xylose and cellobiose, but ethanol would be the major product from glucose. Because the

fermentation rate was significantly slower in xylose and cellobiose than in glucose, we first assumed that the rate of the fermentation caused the carbon flux to shift away from ethanol towards lactic acid. To support this idea, previous reports indicate that the K_M of *R. oryzae* LDH on pyruvate is ~0.55 mM [113], which is lower than the K_M of the *S. cerevisiae* PDC, which has been measured between 2.29 and 3 mM [114]. This suggested that slower uptake of xylose and cellobiose compared to that of glucose resulted in lower intracellular pyruvate concentrations, providing LDH an opportunity to convert pyruvate into lactic acid before the pyruvate concentration is high enough for the lower affinity PDC to take action. To assess this, we conducted glucose fermentations with the SR8L strain at 30 °C, 23 °C, and 16 °C. However, the lactic acid yields from all three fermentations were similar despite reduced rates of glucose consumption and product formation [49]. Although this was a relatively simplistic design to assess the effect of the fermentation rate on lactic acid yields, it suggested that other aspects of yeast genetics or physiology must have a greater impact on lactic acid production from lignocellulosic sugars.

Based on internal RNA-sequencing data of glucose and xylose-grown *S. cerevisiae*, we noted that the relative RNA levels of *JEN1* and *ADY2* were ~55 times and ~10 times higher in xylose-grown cells than in glucose-grown cells (data not shown). Similarly, Lin et al. reported that the transcription levels of *JEN1* and *ADY2* were ~5 times and ~2 times higher in cellobiose-grown yeast than in glucose-grown yeast [228]. Jen1 and Ady2 are of particular interest because they have previously been reported as monocarboxylate transporters which transport several molecules, including lactic acid [118, 229, 230]. However, all prior lactic acid-related studies of the *JEN1* and *ADY2* genes have been limited to their expression in glucose-grown yeast and have therefore been eluded by the seemingly native upregulation of both genes in non-repressing

sugars [231], such as the lignocellulosic sugars xylose and cellobiose. With this upregulation in mind, overexpression of *JEN1* and *ADY2* looked promising to improve lactic acid production in glucose medium.

However, overexpression of *JEN1* and *ADY2* did not improve lactic acid yield when the SR8HL strain fermented glucose (**Fig. 6.3**) or xylose (**Fig. 6.4**). At least two possibilities exist to explain this result. In the case of xylose fermentations, because *JEN1* and *ADY2* are highly upregulated, based on RNA-sequencing results, Jen1 and Ady2 may already be saturated, resulting in no benefit from further expression of the encoding genes. However, when fermenting glucose, *JEN1* and *ADY2* are significantly downregulated, so overexpression of these genes should improve lactic acid transport out of the cell. Beyond the catabolic repression of *JEN1* and *ADY2* that occurs at the transcriptional level, even post-translational levels are rapidly degraded in the presence of glucose [232, 233]. Together, these results indicate that overexpression of *JEN1* and *ADY2* may be futile in terms of significantly improving the Jen1 and Ady2 activity in most conditions.

Although upregulation of *JEN1* and *ADY2* may have limited benefits, the present study provides evidence that inhibition or deletion of these genes will disrupt lactic acid production by engineered *S. cerevisiae* from xylose. First, CHC was used as a chemical inhibitor, reducing the yield of lactic acid from xylose by approximately 41 %. Although CHC has been reported to inhibit yeast monocarboxylate transporters [226, 227], it is non-specific, and therefore it is difficult to assess the molecular basis for the reduced lactic acid production from CHC-incubated yeast.

Because CHC is non-specific, a targeted deletion of *JEN1* and *ADY2* was then utilized. The CRISPR/Cas9 method [32] for gene deletion was used to create single deletion strains and

an *ADY2* and *JEN1* double-deletion strain. With the *EJ4LΔADY2ΔJEN1* strain, lactic acid yields from xylose were reduced by ~36 % (**Table 6.6**). However, no significant difference was observed with only single deletions of *JEN1* or *ADY2*. The failure of the single deletions to drastically impact lactic acid production may simply be a compensatory effect: when *JEN1* or *ADY2* are individually deleted, the other remaining gene may be upregulated in part because of their similar overall functions. Importantly, Jen1 and Ady2 have a closely-linked protein-protein interaction (**Fig. 6.11**). In part, the reduced lactic acid yield from the double-deletion strain is likely due to a reduced rate at which lactic acid can be exported from the intracellular cytoplasm. In turn, this reduced export results in feedback inhibition of LDH by the accumulated lactic acid. Evidence for this is shown by the relatively increased intracellular lactic acid concentration of the double-deleted strain compared to the parental strain (**Fig. 6.8**). Essentially, rapid and efficient export of lactic acid is necessary to maintain a stable intracellular pH and to reduce feedback inhibition of lactic acid on LDH. However, the double-deletion of *JEN1* and *ADY2* only reduced the lactic acid yield by 36 % rather than reducing it by 100 %. This could be due to numerous reasons, but most likely, other lactic acid transporters exist, as others have speculated [230].

Finally, because all of the lactic acid-producing strains displayed decreased xylose uptake rates (**Fig. 2.3**) compared to the non-lactic acid-producing strains, the increased lactic acid production and decreased ethanol production from xylose could be attributed to a slowed production and accumulation of pyruvate. Pyruvate serves as a major substrate in the lactic acid and ethanol pathways. In addition, the reported K_M value (binding affinity of the enzyme to the substrate) for *R. oryzae* lactate dehydrogenase (LDH) and *S. cerevisiae* pyruvate decarboxylase (PDC) differ. Whereas LDH has a K_M of 0.55 mM [113], PDC has a K_M value of 2.29 to 3 mM

[114]. This indicates that a slower xylose uptake by LDH-expressing strains compared to the non-LDH-expressing strains may result in reduced intracellular pyruvate concentrations, allowing the higher binding affinity LDH to overtake PDC, thus producing more lactic acid than ethanol on the xylose condition. This hypothesis could also explain why the EJ4L Δ JEN1 Δ ADY2 strain produces less lactic acid than the parental strain, as the EJ4L Δ JEN1 Δ ADY2 strain has a slightly increased xylose uptake rate as compared to the parental EJ4L strain.

To assess this hypothesis, we measured the intracellular pyruvate concentrations of xylose grown cells of EJ4, EJ4 Δ ADY2 Δ JEN1, EJ4L, and EJ4L Δ ADY2 Δ JEN1 (**Fig. 6.9**). Although minor variations in intracellular pyruvate were observed, a *t*-test of the results indicated that there was no statistically significant difference between the four strains (**Fig. 6.9**) in terms of intracellular pyruvate concentrations.

This result indicates that the intracellular pyruvate concentration among the parental and LDH-expressing strains, with or without *JEN1* and *ADY2* deletions, is relatively similar. Importantly, this provides evidence that decreased production of lactic acid by the double-deletion of *JEN1* and *ADY2* is likely not due to differences in intracellular pyruvate concentrations. Instead, the difference may be due to the relative increase of intracellular lactic acid in the EJ4L Δ JEN1 Δ ADY2 strain (**Fig. 6.8**). Specifically, this increased intracellular lactic acid likely acts as a feedback inhibitor of LDH, diminishing lactic acid production and allowing for PDC to provide the primary outlet for NAD⁺ regeneration.

6.5 Conclusions

Together, these experiments have elucidated the following results: 1) low extracellular pH is specifically inhibitory to lactic acid production from cellobiose using the *cdt-1/gh1-1*

cellobiose fermentation pathway, 2) overexpression of *JEN1* and *ADY2* is unlikely to improve lactic acid production from glucose due to the rapid transcriptional and post-translational degradation, but also is unlikely to improve lactic acid production from xylose or cellobiose due to the natively highly upregulated status of both genes in the presence of these sugars, 3) *JEN1* and *ADY2* deletion significantly inhibits lactic acid production from lignocellulosic sugars likely due to an increased intracellular lactic acid concentration relative to the extracellular concentration. Moving forward, future studies will focus on identifying other native lactic acid transporters and generating mutant Jen1 and Ady2 proteins which are less susceptible to the glucose-signaled endocytosis, with the intent of improving lactic acid production from lignocellulosic hydrolysate mixtures which contain small, but significant, concentrations of glucose.

6.6 Figures

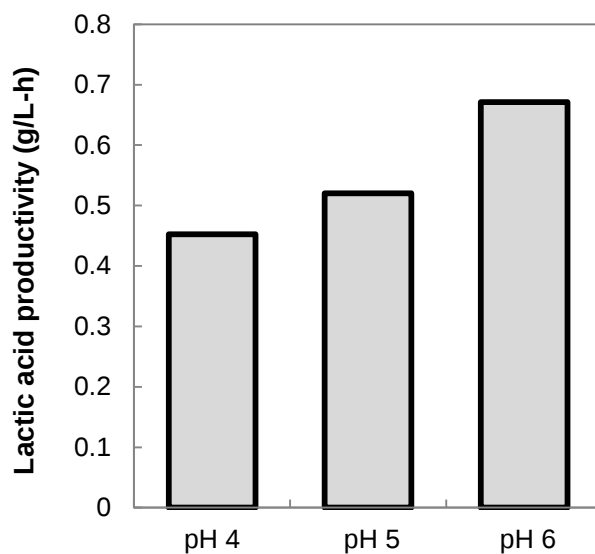


Fig. 6.1 Individual bioreactor fermentations containing YP medium with 10 g/L glucose, 40 g/L xylose, and 80 g/L cellobiose with pH maintained at 4, 5, or 6 via 10 N NaOH addition displaying the lactic acid productivity (g/L-h) at ~74 h.

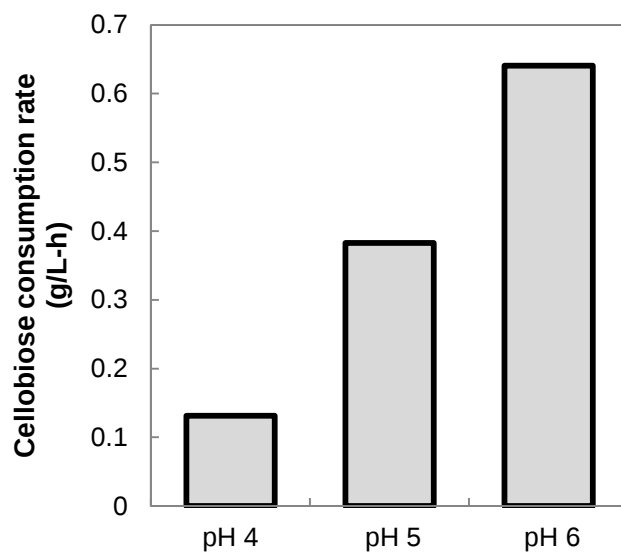


Fig. 6.2 Individual bioreactor fermentations containing YP medium with 10 g/L glucose, 40 g/L xylose, and 80 g/L cellobiose with pH maintained at 4, 5, or 6 via 10 N NaOH addition displaying the rate of cellobiose consumption (g/L-h) at ~74 h.

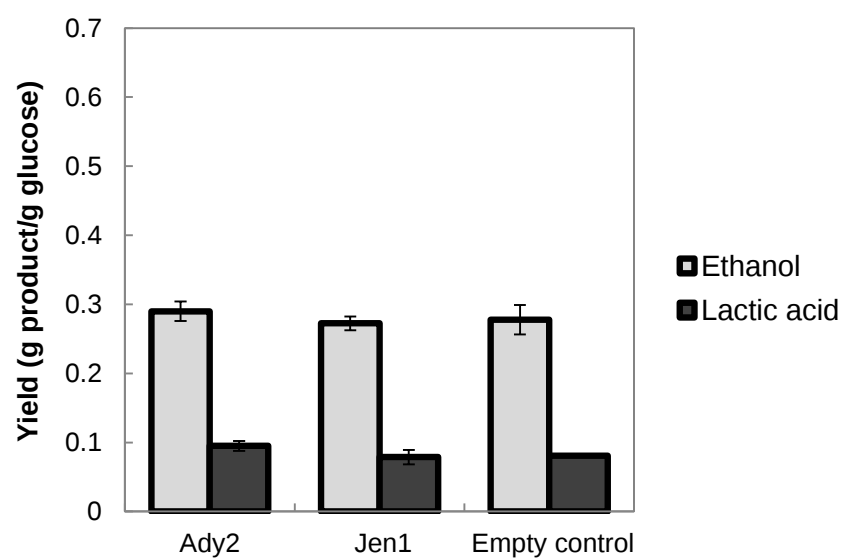


Fig. 6.3 Flask fermentations of the SR8HL strain overexpressing *ADY2*, *JEN1*, or an empty pRS42K plasmid grown in YP glucose medium. The yields of lactic acid and ethanol (g product/g glucose) are shown at ~6 h. The values are the means of two independent experiments and the errors indicate the standard deviation.

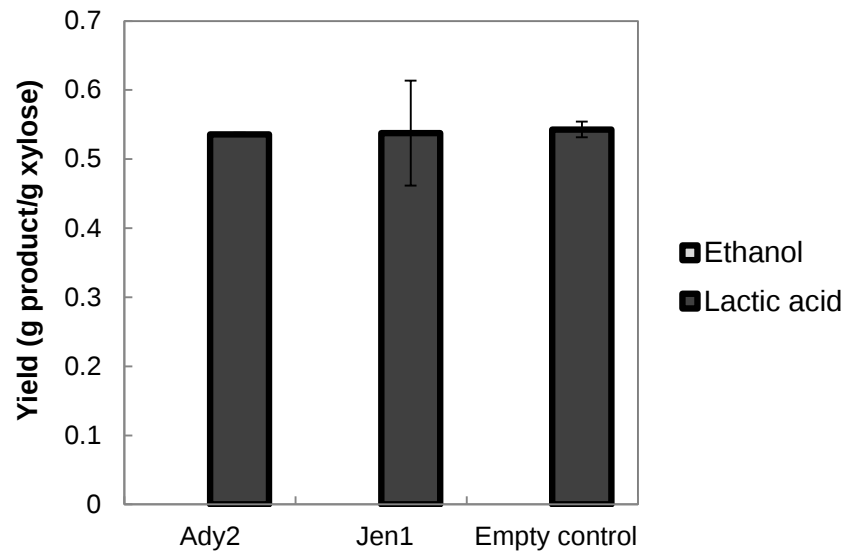


Fig. 6.4 Flask fermentations of the SR8HL strain overexpressing *ADY2*, *JEN1*, or an empty pRS42K plasmid grown in YP xylose medium. The yields of lactic acid and ethanol (g product/g glucose) are shown at ~70 h; no ethanol production was observed. The values are the means of two independent experiments and the errors indicate the standard deviation.

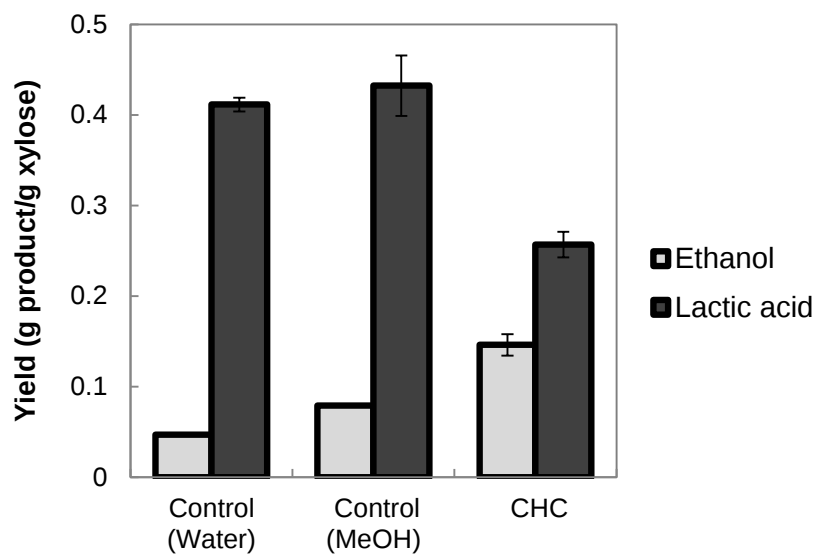


Fig. 6.5 Flask fermentations of the EJ4L strain grown in YP xylose medium containing water (control), methanol (control), or α -cyano-4-hydroxycinnamic acid (CHC) dissolved in methanol. The yields of lactic acid and ethanol (g product/g xylose) are shown at ~45 h. The values are the means of two independent experiments and the errors indicate the standard deviation.

Abbreviations: MeOH, methanol; CHC, α -cyano-4-hydroxycinnamic acid.

	Ethanol	Lactic acid
EJ4L	0.08 $\pm$ 0.02	0.45 $\pm$ 0.02
EJ4L Δ <i>JEN1</i>	0.13 $\pm$ 0.02	0.42 $\pm$ 0.01
EJ4L Δ <i>ADY2</i>	0.04 $\pm$ 0.01	0.41 $\pm$ 0.01
EJ4L Δ <i>ADY2</i> Δ <i>JEN1</i>	0.15 $\pm$ 0.01	0.29 $\pm$ 0.02
EJ4L Δ <i>ADY2</i> Δ <i>JEN1</i> -empty	0.15 $\pm$ 0.04	0.30 $\pm$ 0.01
EJ4L Δ <i>ADY2</i> Δ <i>JEN1</i> / <i>ADY2</i>	0.13 $\pm$ 0.03	0.39 $\pm$ 0.02

Table 6.6 Ethanol and lactic acid yields from Δ *JEN1* and Δ *ADY2* strains, control strains, and a Δ *ADY2* strain with a recovered *ADY2* expression. Values are the average of duplicate experiments with error ranges shown as +/- values; $p \leq 0.05$.

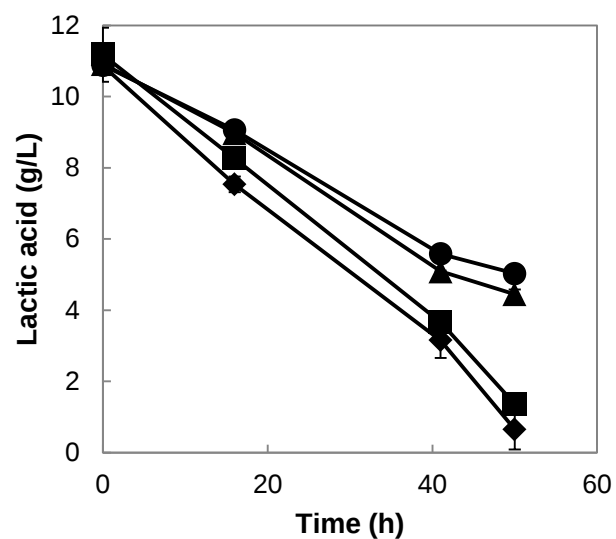


Fig. 6.7 Fermentation profile showing the change in lactic acid concentration (g/L) overtime in YP medium containing ~10 g/L of lactic acid and no other carbon source. Four strains are shown: EJ4 (*closed square*), EJ4ΔJEN1 (*closed triangle*), EJ4ΔADY2 (*closed diamond*), and EJ4ΔADY2ΔJEN1 (*closed circle*). The values are the mean of two independent experiments and the *error bars* indicate the standard errors.

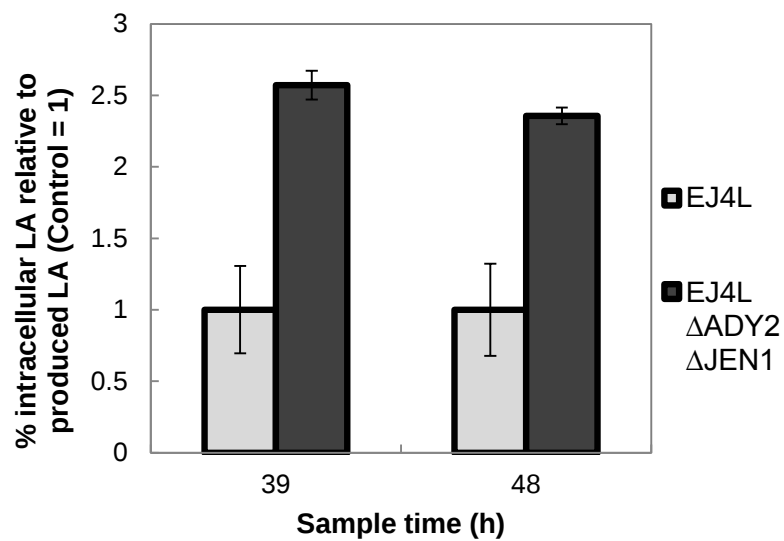


Fig. 6.8 Intracellular lactic acid measurement of YPX-grown EJ4L and EJ4LΔADY2ΔJEN1 strains harvested at 39 h and 48 h. Results are shown as fold-increase of intracellular lactic acid concentration relative to extracellular lactic acid compared to the EJ4L control (standardized to 1). The values are the mean of three independent experiments and the *error bars* indicate the standard errors.

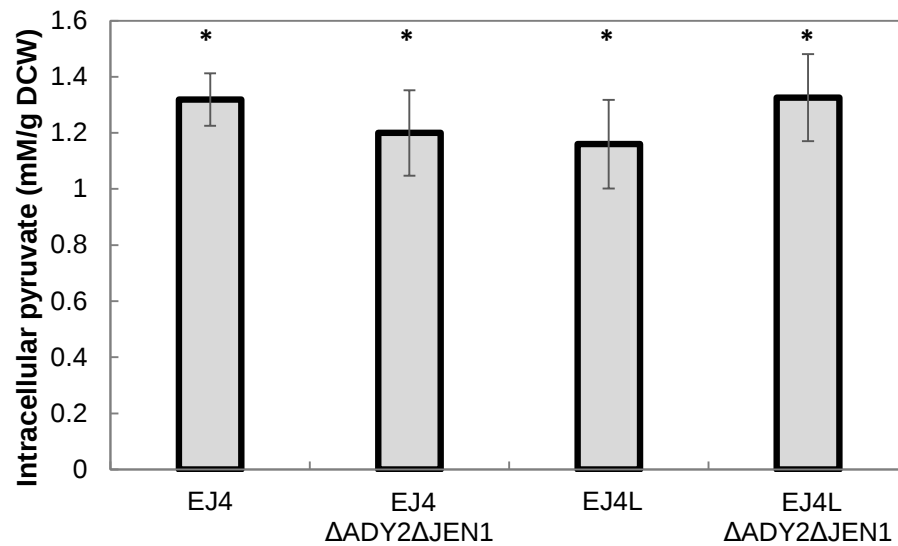


Fig. 6.9 Intracellular pyruvate concentrations (mM/g DCW) of YPX-grown yeast harvested at mid-log phase. EJ4 serves as the control for the EJ4 Δ ADY2 Δ JEN1 strain and EJ4L serves as the control for the EJ4L Δ ADY2 Δ JEN1 strain. A *t*-test was applied for each pair of control/experimental strains, indicating no statistically significant difference (*) in intracellular pyruvate concentrations was detected in Δ ADY2 Δ JEN1 strains. The values are the mean of three independent experiments and the *error bars* indicate the standard errors.

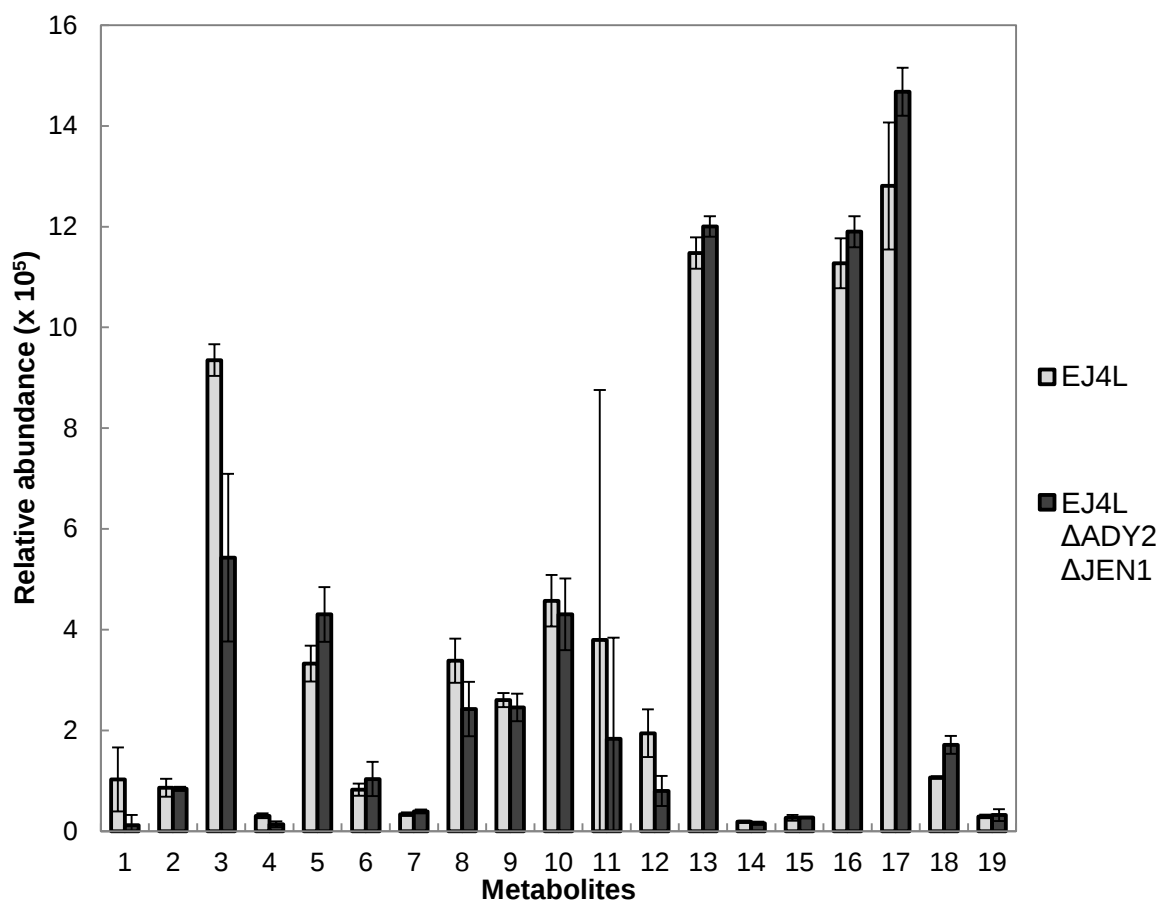


Fig. 6.10 Intracellular metabolite analyses by GC-MS of YPX-grown EJ4L and EJ4LΔADY2ΔJEN1 strains harvested at 48 h. The relative abundance of each metabolite is shown. The values are the mean of three independent experiments and the *error bars* indicate the standard errors. The metabolites are associated to the X-axis numbers as follow: **1)** phosphoric acid, **2)** diterbutylphenol, **3)** glutamic acid, **4)** phenylalanine, **5)** lyxose, **6)** arabitol, **7)** glucopyranose, **8)** ornithine, **9)** tetradecanoic acid, **10)** lysine, **11)** mannitol, **12)** tyrosine, **13)** hexadecenoic acid, **14)** inositol, **15)** *n*-ocadecan-1-ol, **16)** octadecanoic acid, **17)** trehalose, **18)** ergocalciferol, **19)** mannitol.

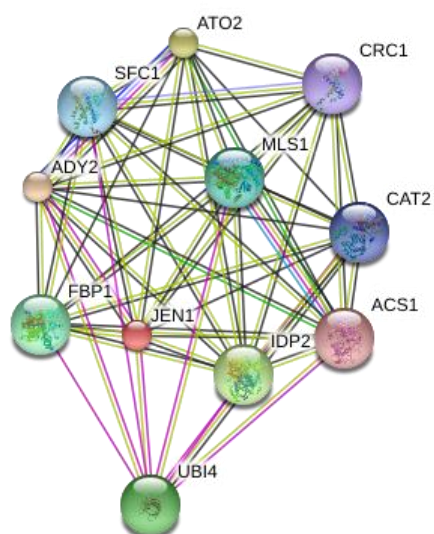


Fig. 6.11 Predicted protein-protein interaction of Jen1 and Ady2. Currently, the 3D protein structures of both proteins are unknown. The brown, light green, and pink interaction lines indicate co-expression, data obtained from text-mining abstracts, and experimentally determined data, respectively. This figure was obtained from www.string-db.org [234].

CHAPTER VII SUMMARY AND FUTURE STUDIES

7.1 Summary

Efficient and cost-effective production of lactic acid from inedible lignocellulosic feedstocks would help to push our global economy towards a renewable future with less reliance on finite fossil fuels. In particular, this is due to the ability for lactic acid to be formed into a biodegradable polyester known as polylactide (PLA), which can serve as a replacement for some petroleum-derived plastics. The focus of this dissertation was to elucidate mechanisms necessary for improving lactic acid production from lignocellulosic sugars by an engineered *Saccharomyces cerevisiae* yeast.

In **Chapter II**, the first known *S. cerevisiae* strain capable of fermenting xylose into lactic acid was constructed. This was achieved by expressing a heterologous xylose pathway (*XYL1*, *XYL2*, and *XYL3*) from *Pichia stipitis* into a D452-2 background *S. cerevisiae* strain. Deletions of *PHO13* and *ALD6* further improved the xylose fermentation profile. Then, a heterologous lactate dehydrogenase (*ldhA*) from *Rhizopus oryzae* was integrated into the yeast genome, allowing for the conversion of pyruvate to lactic acid. Surprisingly, lactic acid was the major product when xylose was fermented, but glucose was the major product when ethanol was fermented. A yield of ~0.69 g lactic acid/g xylose was achieved as a final result.

In **Chapter III**, a mixture of glucose, xylose, and cellobiose was co-consumed by an engineered *S. cerevisiae* to produce primarily lactic acid. This was achieved by using the same cassettes for xylose metabolism and lactic acid production, but in addition, multiple copies of a cellodextrin transporter (*cdt-1*) and a β -glucosidase (*gh1-1*) were expressed in the strain. As in **Chapter II**, it was observed that almost no ethanol was produced when xylose was fermented.

Similarly, when cellobiose was fermented, almost no ethanol was produced. When fermentation a mixture of glucose (10 g/L), xylose (40 g/L), and cellobiose (80 g/L), a lactic acid yield of 0.65 g lactic acid/g sugar was achieved. As a final result, we were able to achieve a titer of over 120 g/L lactic acid by maintaining pH 6 and feeding additional lignocellulosic sugars.

In **Chapter IV**, we applied the cellobiose-fermenting, lactic acid-producing strain from **Chapter III** to dairy products, such as lactose, shelf-stable milk, and cheese whey in order to produce lactic acid. This study served as a proof of concept for the application of engineered yeast to produce value-added products from dairy industry byproducts, such as contaminated milk or, through further improvements, highly acid whey from Greek yogurt production.

In **Chapter V**, we investigated numerous industrial *Saccharomyces spp.* strains to assess their tolerance in a variety of industrially-relevant conditions, especially conditions related to lignocellulosic hydrolysate fermentations. Four industrial strains (ATCC 4127, ATCC 46523, ATCC 56069, and ATCC 66348) were selected as the overall top-performing strains in terms of resistance to low pH, xylose-fermentation capability, and resistance to commonly-occurring lignocellulosic hydrolysate fermentation inhibitors. An *ldhA* cassette and the xylose fermentation cassettes from **Chapter II** were integrated into the ATCC 46523 genome. As a result, the engineered strain did not produce significantly more lactic acid than the control strain despite the ATCC 46523 strain displaying increased tolerance in low pH and high extracellular lactic acid concentration conditions. However, ethanol production by the ATCC 46523 transformant was lower than the control. This suggests that selection of an ideal starting strain when seeking to maximize lactic acid production from xylose or cellobiose could be beneficial, but further screening for strain selection is necessary.

In **Chapter VI**, several genetic perturbations related to *JEN1* and *ADY2* were undertaken to elucidate underlying mechanisms of lactic acid production from lignocellulosic sugars. Most importantly, we provided evidence that *JEN1* and *ADY2* are upregulated in the presence of xylose, or more accurately, are not downregulated as is the case when in the presence of glucose. This aligns with other reports of upregulation when fermenting cellobiose and also of rapid proteolytic degradation of Jen1 and Ady2 when in the presence of glucose. Through double deletion of *JEN1* and *ADY2*, lactic acid production from xylose was reduced by ~36 % compared to the control strain, indicating the importance of *JEN1* and *ADY2* for lignocellulosic sugar conversion to lactic acid.

7.2 Future studies

Although these studies have made a considerable effort to improve the production of lactic acid from lignocellulosic feedstocks, still, several major barriers must be overcome before industrial-scale production can ensue. Importantly, development of lactic acid-producing *S. cerevisiae* which can grow without severe hindrance in lignocellulosic hydrolysate is a necessity for cost-effective industrial fermentations. As a rule of thumb, industrial-scale fermentations should also reach productivity of at least 2 g/L-h, of which the strains developed in the present studies are unable to do so from xylose and cellobiose, although higher initial cell inoculums may alleviate this issue. Finally, continued investigation of the underlying mechanisms for lactic acid production from engineered yeast would be beneficial, especially for identifying new lactic acid transporters in addition to creating mutant Jen1 and Ady2 transporters which are not as susceptible to degradation in the presence of glucose. With these and other goals reached,

efficient industrial-scale lactic acid production from lignocellulosic feedstocks could one day be achieved.

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