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Strategies for Increased Lactic Acid Production from Algal Cake Fermentations at Low pH by *Lactobacillus casei*

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STRATEGIES FOR INCREASED LACTIC ACID PRODUCTION FROM ALGAL CAKE FERMENTATIONS AT LOW PH BY *LACTOBACILLUS CASEI*

by

Tom Overbeck

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ABSTRACT

Strategies for Increased Lactic Acid Production from Algal Cake Fermentations at Low pH by *Lactobacillus casei*

by

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We explored using de-oiled algal biomass (algal cake) as a low-value substrate for production of lactic acid in fermentations with *Lactobacillus casei*, and strategies for increasing lactic acid production at low pH. *L. casei* 12A algal cake (AC) fermentations showed carbohydrate and amino acid availability limit growth and lactic acid production. These nutritional requirements were effectively addressed with enzymatic hydrolysis of the AC using α-amylase, cellulase, and pepsin. Producing 0.075 g lactic acid per g AC from AC digested with all three enzymes. We explored heterologous expression of the cellulase gene (*celE*) from *Clostridium thermocellum* and the α-amylase gene (*amyA*) from *Streptococcus bovis* in *L. casei* 12A. Functional activity of CelE was not detected, but low-level activity of AmyA was achieved, and increased > 1.5-fold using a previously designed synthetic promoter. Nonetheless, the improvement was insufficient to significantly increase lactic acid production. Thus, substantial

optimization of *amyA* and *celE* expression in *L.* casei 12A would be needed to achieve activities needed to increase lactic acid production from AC.

We explored transient inactivation of MutS as a method for inducing hypermutability and increasing adaptability of *L. casei* 12A and ATCC 334 to lactic acid at low pH. The wild type cells and their Δ*mutS* derivatives were subject to a 100-day adaptive evolution experiment, followed by repair of the Δ*mutS* lesion in representative isolates. Growth studies at pH 4.0 revealed that all four adapted strains grew more rapidly, to higher cell densities, and produced significantly more lactic acid than untreated wild-type cells. The greatest increases were observed from the adapted Δ*mutS* derivatives. Further examination of the 12A adapted Δ*mutS* derivative identified morphological changes, and increased survival at pH 2.5. Genome sequence analysis confirmed transient MutS inactivation decreased DNA replication fidelity, and identified potential genotypic changes in 12A that might contribute to increased acid lactic acid resistance. Targeted inactivation of three genes identified in the adapted 12A Δ*mutS* derivative revealed that a NADH dehydrogenase (*ndh*), phosphate transport ATP-binding protein PstB (*pstB*), and two-component signal transduction system (TCS) quorum-sensing histidine kinase (*hpk*) contribute to increased acid resistance in 12A.

(151 pages)

PUBLIC ABSTRACT

Strategies for Increased Lactic Acid Production from Algal Cake Fermentations at Low pH by *Lactobacillus casei*

Tom J. Overbeck

The algal biodiesel production process generates de-oiled algal biomass (algal cake) as a waste by-product. Algal cake (AC) is high in protein and carbohydrate, which makes it a promising low-value substrate for production of other valuable bioproducts. We explored use of AC as a substrate for production of lactic acid via fermentation with *Lactobacillus casei*, as a way of reducing the production costs of algal biodiesel and lactic acid. Initial fermentations of AC with *L. casei* 12A indicated that one or more nutrients were limiting growth and lactic acid production. By conducting fermentations with different nutrient supplementations, we determined the limiting nutrients were carbohydrate and amino acids. Enzyme treatments of AC with α-amylase, cellulase (endo-1,4-β-D-glucanase), and pepsin (non-specific protease), revealed these enzymes effectively hydrolyzed the starch, cellulose, and polypeptides, respectively, in AC and enabled increased fermentation of this substrate by *L. casei*. With an optimized level of 0.075 g lactic acid per g AC produced when the AC was digested by all three enzymes. Those findings confirm AC is a promising, low-value substrate for production of valuable bioproducts such as lactic acid.

With carbohydrate availability determined to be the primary nutrient limiting growth and lactic acid production, we then explored heterologous expression of the cellulase gene (*celE*) from *Clostridium thermocellum* and the α-amylase gene (*amyA*)

from *Streptococcus bovis* 148 in *L. casei* 12A, as a potential means of reducing enzyme costs for AC fermentation. Functional activity of CelE in *L. casei* 12A was not detected, but low-level activity of AmyA was achieved. AmyA activity was increased greater than 1.5-fold by using a synthetic promoter to drive expression of *amyA*, but the improvement was not sufficient to significantly increase lactic acid production by *L. casei* in AC fermentations. Thus, further optimization of *amyA* and *celE* in *L.* casei 12A would be needed to obtain the enzyme activities required for efficient AC fermentation with *L. casei*.

The production cost of lactic acid is reduced when fermentations are conducted at low pH, because the undissociated form of the acid can more efficiently recovered from the fermentation medium. Thus, improving the ability of *L. casei* to grow and ferment carbohydrates to lactic acid at or below the pKa of lactic acid would make this strain even more attractive to industry. Adaptive laboratory evolution is a useful tool for increasing the fitness of an organism to defined conditions. We explored transient inactivation of the gene encoding the DNA mismatch repair enzyme MutS as a method for inducing hypermutability and increasing adaptability of *L. casei* to lactic acid at low pH. To accomplish this, the MutS gene was inactivated in *L. casei* 12A and ATCC 334 by two-step gene replacement, then wild type cells and their Δ*mutS* derivatives were subject to a 100-day adaptive evolution process. At the end of the process, the Δ*mutS* lesion was repaired in selected isolates. Growth studies at pH 4.0 revealed that all four adapted strains grew more rapidly, to higher cell densities, and produced significantly more lactic acid than unadapted wild-type cells. The greatest increases in growth and lactic acid production were observed from the adapted Δ*mutS* derivatives. Examination of the 12A

adapted Δ*mutS* derivative by scanning electron microscopy revealed changes to the cell surface, and a significant decrease in cell volume. Additionally, survival of the 12A adapted Δ*mutS* derivative at pH 2.5 was also significantly improved. Genome sequence analysis confirmed MutS inactivation decreased DNA replication fidelity. It also showed the adapted ATCC 334 Δ*mutS* derivative had suffered lesions in additional genes associated with replication fidelity, and identified potential genotypic changes in 12A that might contribute to increased lactic acid resistance. Targeted inactivation of three genes that had suffered three non-sense mutations identified in the adapted 12A Δ*mutS* derivative revealed that co-inactivation of a NADH dehydrogenase (*ndh*) and phosphate transport ATP-binding protein PstB (*pstB*) increased growth and lactic acid production by *L. casei* 12A at pH 4.0, and co-inactivation of a two-component signal transduction system (TCS) quorum-sensing histidine kinase (*hpk*) and *pstB* increased survival at pH 2.5.

DEDICATION

I would like to dedicate my dissertation to my fallen brothers Harry “Buck” Winkler, Michael Cerrone, Gregory “Shorty” Millard, Clayton Dunn, Michael Jaurigue, Joshua Boyd, and Rodolfo “Rudy” Rodriguez for their inspiration, companionship, and sacrifices; and to my wife Kylie Owens for her unwavering and invaluable support.

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

Introduction

Renewable Energy

Fossil carbon-based sources (i.e., petroleum, coal, and natural gas) have been the most significant contributors to past and present energy requirements, and will likely continue to be for the foreseeable future [38]. Based on the current and expected human population and economic growth, global energy consumption is projected to increase at least two-fold by midcentury [24]. Concurrent growth of the human population and its dependency on fossil based fuels will eventually exhaust the world's supplies of easily exploitable fossil-derived energy resources [38]. As a result, there is growing pressure to develop alternative sustainable and renewable resources that can contribute to both current and future energy demands.

Energy consumption within the United States is expected to grow by 7 percent from 98 quadrillion Btu, in 2011 to 104 quadrillion Btu in 2035 [40]. However, U.S. consumption of liquid fuels is expected to decrease from 37.0 quadrillion Btu in 2011 to 36.1 quadrillion Btu in 2040, due in part to a projected increase in the consumption of biofuels over this period. Liquid biofuels currently contribute 1.3 quadrillion Btu to U.S. energy needs, and this figure is projected to rise to 2.1 quadrillion Btu by 2040 [40]. While this trend is encouraging, additional research and development is needed if liquid biofuels are to provide a meaningful alternative for global liquid fuel demand.

Further pressure to develop renewable alternatives comes from the growing need to decrease the negative environmental impacts of using fossil-based fuels. Greenhouse gas (GHG) emissions, especially CO_2 emissions associated with the use of these fuels, have been linked to global climate change and other adverse environmental effects, such as ocean acidification [38]. For the past 650,000 years, atmospheric CO_2 concentrations were between 210 and 300 ppm and has been highly correlated with temperature swings. Due to emissions from fossil fuel consumption over the past 50 years, CO_2 concentrations have been rising and now exceed 400 ppm [24, 41]. Growing concerns have been raised about the environmental impacts imparted from the use of fossil carbon-based fuels as the most substantial contributor to the worlds energy supply. While some measures have been implemented to regulate emission levels, and technological advances have contributed to a decline in emission rates, additional measures are urgently needed to control the increases in the GHG levels currently being observed. What can be said with certainty is that if atmospheric CO_2 concentrations continue to increase without severe intervention, concentrations will reach levels by mid-century that have not been present on the planet in at least the past 650,000 years, and catastrophic environmental changes are a likely consequence [24].

Fortunately, various sustainable energy systems are being developed for the production of cost-competitive, cleaner fuels and chemicals from renewable resources [38]. In the future, biofuels are poised to provide environmental, economic, and social benefits to communities as well as energy efficiency for industrial production [13]. Liquid biofuel production and consumption as an alternative to using fossil fuel resources is an area that is positioned to undergo rapid growth.

Growth of the global human population will not only affect the growing energy demand, but will also have repercussions on the world's food supply. World population is expected to grow from 6.2 to 9.5 billion by 2050 with a projected increase in food demand of nearly double what it currently is [31]. Rising food prices and diminishing agricultural land area (due to expanding development and climate change) place global food security in jeopardy [31]. Plant biomass is the only accessible non-fossil source of carbon, with energy stored in the form of chemical bonds [38]. This makes it a promising feedstock to produce biofuels. However, the use of land for production of biofuels has stemmed a "food versus fuel" debate, which argues that growing crops for biofuel production exacerbates the problem of malnourishment worldwide [26]. There are many factors to consider for the feasibility and overall benefit to using biofuels. The major factors that will determine the impacts of biofuels include their contribution to land use change, the feedstock used, and issues with technology and scale [29]. This creates greater need for biofuel sources which do not compete with terrestrial agricultural land for food crops [31]. Hence, the quality of life for future generations is dependent on realization of four important objectives: energy security, reduced GHG emissions, biodiversity, and the sustainability of the food supply [33].

Biodiesel

Biodiesel is a liquid biofuel that has many important technical advantages over petrodiesel: inherent lubricity, low toxicity, derivation from a renewable and domestic feedstock, superior flash point and biodegradability, negligible sulfur content, and lower exhaust emissions [25]. Production of biodiesel has been growing and is projected for

continued growth. The U.S. National Biodiesel Board estimates that the biodiesel production capacity for 2008 in the U.S. was 2.24 billion gallons per year, compared to 23 million gallons per year in 2003, with more growth predicted [25]. There are four major feedstock categories for production of biodiesel: algae, oilseeds, animal fats, and various low-value materials such as used cooking oils, greases, and soapstocks [25].

Algae have been researched as a biomass source for fuel production since the 1950's [1]. Algae are photosynthetic organisms that use sunlight (the most abundant source of energy), CO_2, and other available raw materials to synthesize and accumulate large quantities of neutral lipids and carbohydrates as well as other valuable co-products [15, 29]. Much of the research focus for utilizing algae as a fuel source has been for biodiesel production due to the high oil content of many algal strains which is only a processing step or two away from biodiesel [1]. The lipids present in algae are harvested using an acid pretreatment (e.g. sulfuric acid with methanol), and recovered in chloroform. Recovery methods and conversion of the lipids present in algae have received much attention for improving the process for higher yields and efficiency in the production of biodiesel.

Multiple factors make the production of biofuel from algal biomass an appealing alternative to current fuel sources. Algae can tolerate and utilize substantially higher levels of CO_2 than terrestrial plants, and can thus utilize CO_2 for carbon capture and greenhouse gas sequestration while cultivating biomass [29]. When compared to other oil feedstock's, algae give a much higher oil yields (the oil mass of some algae is greater than 80 percent of the dry weight of the algal biomass) and so are recognized as the most efficient biomass for producing fuel [13]. Algae are reportedly capable of producing 30

times more oil per unit of land than terrestrial oilseed crops [29]. Algal biofuel production has the advantage over other biofuel feedstocks in that it does not require arable land, and can be grown in saline/costal seawater and on undesirable agricultural lands [29, 35]. Consequently, there is little competition with agriculture for land resources. Due to the high protein concentrations within many species of algae, many researchers believe that algae could play a large role in providing a protein rich food source to livestock as a method for feeding the global population [1]. The minimized land, water, and energy needed to produce higher quantities of algal oils when compared to other terrestrial biofuel production platforms, makes this resource a promising substrate for biofuel production [13, 31].

The main nutrients needed for the production of algae for biodiesel production are nitrogen and phosphorus [35]. To meet the potential biofuel market in Europe alone, approximately 25 million tons of nitrogen and 4 million tons of phosphorus are needed, which is about twice the amount that is presently produced as fertilizer in Europe [35]. One major drawback to the use of fertilizers is the required energy input for their production, which affects both the cost and the environmental impacts. This makes them major contributors to net energy consumption and yields a net negative energy balance in the overall biodiesel production process [1]. Because nitrogen and phosphorus are nonrenewable resources, the ability to recycle these nutrients would improve net energy yield in biofuel production [29]. Even with the potential benefits of using algae as biodiesel source, it is still not economically cost competitive with petroleum diesel, at $0.15/gal and $0.05/gal respectively [20]. The technologies to use algae as a viable alternative to fossil fuels are still lacking; the energy balance of producing the fuel is still

too high, and the economics don't compare [1]. With current technology, growing algae without a cost-effective nitrogen supply and purely for biodiesel production makes obtaining a positive energy balance unachievable [1]. For the replacement of current fossil fuel resources, production of algal based biofuels needs to take place on a much larger scale at far lower cost [35].

Using wastewater as a fertilizer for algal biofuel production can provide sustainable bioremediation of wastewaters for environmental and economic benefits, and limit competition with arable crops for agricultural resources [29]. This practice would also increase the net energy gain by recycling nitrogen and phosphorus, thus making the overall process more profitable. By also using a processing system that incorporates sequential production of various value-added products from the algal biomass (i.e. residual proteins or carbohydrates), the cost of production could be substantially reduced, increasing the profit margin as well as the net energy gain [31].

Biorefinery

A biorefinery involves conversion of multiple components of the biomass material to produce useable products such as fuel, power, and other value-added chemicals [22, 30]. Because algal biofuel production is currently not cost feasible as a fossil fuel replacement, the biorefinery approach has been proposed to allow for algal biomass-based products to be produced in a commercially viable manner [27]. For example, a biorefinery approach for the utilization of the high protein content within algae as a carbon source for growth by *Escherichia coli*, through metabolic engineering,

has been demonstrated for the production of additional biofuels and the recycling of nitrogen within the system [16, 17].

Algal biomass has significant concentrations of proteins, carbohydrates, and other nutrients that can be used for the production of co-products, making it a strong candidate for the biorefinery concept [22, 30]. Wijffels, et al. [36] further demonstrated that the production of algae for coproduction of biodiesel and bulk chemicals could become economically feasible, and with the additional technological advances in this area could greatly reduce that the cost price for the production of algal biodiesel.

Lactic Acid

Lactic acid (2-hydroxypropionic acid), $CH_3CHOHCOOH$, is the most widely occurring hydroxycarboxylic acid [9, 10, 19]. It is also the simplest hydroxy acid with an asymmetric carbon; existing in two optically active configurations, with the L(+) isomer produced by mammals and moulds, and both D(-) and L(+) enantiomers produced by bacterial systems [12, 37]. Lactic acid can be produced by chemical synthesis, which yields a racemic mixture of D(-) and L (+) isomers. However, it is most commonly produced through microbial fermentation processes [9, 12, 19, 28]. Allowing for production of desired stereoisomers that can then be combined in different percentages to alter properties, such as crystallization in the production of Poly(lactic acid) (PLA) [12].

Lactic acid has many versatile industrial applications in food, pharmaceuticals, textile, leather, chemical, and eco-friendly thermoplastics [12, 19]. Currently, food-related applications in the U.S. account for approximately 85% of the lactic acid demand where it is used as food acidulant, emulsifying agent, flavoring, preservative, and pH

buffering agent [10]. Lactic acid shows potential growth opportunities in marketable uses such as a substrate for industrial production of biodegradable polymers, oxygenated chemicals, "green" chemicals/solvents, and plant-growth regulators [10]. For example, PLA is a thermoplastic, high-strength polymer that can be made from renewable resources and is currently being used in the industrial packaging field and biocompatible/bioabsorbable medical device market [12]. These growing uses for lactic acid make it an attractive target as a co-product in a biofuels-based biorefinery.

Lactobacillus casei

Biotechnological production of lactic acid is chiefly carried out through fermentation of carbohydrates by certain lactic acid bacteria (LAB) or filamentous fungi [19]. LAB are characterized by being gram-positive, non-sporing, aerotolerant, tolerance to low pH (< 5.0), tolerance to temperatures above 40°C, and being nutritionally fastidious [4, 37]. LAB are classified into two groups based on end-products from glucose: homofermentative and heterofermentative. Homofermentative LAB produce virtually one product—lactic acid, through carbohydrate metabolism, while heterofermentative LAB produce lactic acid and other metabolic products, principally acetic acid and ethanol [19, 37]. Species of LAB that are most commonly used for the production of lactic acid from different carbon sources are *Lactococcus lactis*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, and *Lactobacillus casei* [19].

Lactobacillus casei is an aciduric, rod-shaped, facultatively heterofermentative LAB that can be isolated from many diverse ecological niches such as raw and fermented milk and meat or plant products as well as the oral, intestinal, and reproductive tracts of

humans and animals [5, 6, 8]. This broad ecological distribution reflects the metabolic flexibility of *L. casei*, which can utilize and ferment a diverse array of simple and complex carbohydrates as well as sugar alcohols [6].

Investigations on development of biotechnological processes for lactic acid production has been focused on making the process more effective and economical [19]. The utilization of various types of low value carbohydrate sources for lactic acid fermentation, such as sugars obtained from agricultural byproducts, can add considerable value to the process [12]. Therefore, by using a biorefinery type approach for the coproduction of biodiesel from algae and lactic acid from de-oiled algae biomass (algal cake which is currently generated as a waste by-product) would improve the economics and sustainability of biodiesel production.

Fermentation at Low pH

Increased cost feasibility can also be incurred by more efficient product recovery. Lactic acid produced through fermentation requires extensive purification processes that can represent 10-50% of the total production cost [14, 34]. Lactic acid, with a pKa of 3.86, is more easily and efficiently extracted from the fermentation broth when in the undissociated protonated form [11, 14]. Consequently the profability of lactic acid production can be increased with fermentations occuring at a pH close to or below the pKa, where a higher ratio of the acid produced is undissociated.

Lactic acid produced through fermentation by *L. casei* is exported out of the cell in the dissociated ionic form via the proton-lactate symporter, consequently decreasing the extracellular pH (pH_o) and increasing the undissociated lactic acid concentration [5].

The undissociated lactic acid is membrane soluble and passively diffuses into the cytoplasm, where it then dissociates into the ionic form, lowing the internal pH (pH_i) of the cell [23]. Once pH_i reaches a critical threshold, cellular functions will become impeeded and cell viability will consequently be decreased [3, 5, 18, 21].

The acid tolerance response (ATR) is an adaptive response utilized by LAB and other bacteria for survival under low pH conditions [2]. Characterization of the ATR in *L. casei* and other bacteria has provided valuable insight into potential strategies for optimizing fermentations at low pH, and potentially allowing for more efficient recovery of lactic acid in industrial applications [5, 34, 39]. Incorporating these strategies into *L. casei* fermentations of algal cake (AC) to lactic acid should further reduce processing costs, and increase the cost feasability of algal biodiesel production via a biorefinery approach.

Hypothesis

The Utah State University Biofuels Center (USU-BC) has used algae to produce renewable biodiesel. The algae are harvested and processed for lipid extraction and subsequent chemical conversion to biodiesel. The process generates AC as low-value byproduct after extraction of the lipids. One of the algal species used is a Great Salt Lake isolate, designated USU080. Qualitative and quantitative analyses of the monosaccharide and amino acid composition from the generated USU080 AC has indicated it has a composition of 6.73% carbohydrate, with glucose comprising 56.3% of the total monosaccharide composition, and 24.37% amino acids. Thus, USU080 AC is a

promising low-value substrate for lactic acid production through fermentations using *L. casei*.

It is the hypothesis of this study that *L. casei* can be engineered to grow on the de-oiled algal biomass (AC) of USU080, and effectively ferment this substrate into lactic acid under low pH conditions.

Objectives

To explore this hypothesis, AC from USU080 generated as a waste material from biodiesel production was utilized as a substrate for production of lactic acid through fermentations by *L. casei* 12A, a plant isolate. Experiments were performed to construct genetically engineered strains for a more efficient conversion of substrate to product at low pH. Research described in this dissertation addressed the following objectives:

1. Characterization of baseline conditions for growth and metabolites produced from growth on USU080 by *L. casei* 12A.
2. Genetic manipulation of *L. casei* for improved lactic acid production from USU080 AC.
3. Implementing strategies for optimizing lactic acid fermentations by *L. casei* at low pH.

With no previous research on fermentations of AC with *L. casei*, it was important to establish baseline conditions for growth and lactic acid production. To accomplish Objective 1, growth and lactic acid production of *L. casei* 12A on USU080 AC solutions

supplemented with a multi-vitamin mixture, casamino acids and/or glucose was measured, as was AC enzymatically pretreated with cellulase, α-amylase and/or pepsin.

Work from Objective 1 revealed carbohydrate availability to be the primary limiting factor for growth and lactic acid production by *L. casei* 12A from AC, and that enzymatic hydrolysis of AC with cellulase and α-amylase was effective in addressing this limitation. To accomplish Objective 2, codon optimized cassettes containing a *Clostridium thermocellum* cellulase gene, endo-1,4-β-glucanase E (CelE) or *Streptococcus bovis* 148 α-amylase (AmyA) were cloned separately into *L. casei* 12A. Enzyme activity from these clones was measured, and low-level AmyA activity was confirmed, but no CelE activity was detected.

Previous research using adaptive evolution of *L. casei* under low pH conditions was demonstrated to increase lactic acid tolerance [39]. To accomplish Objective 3, we modified this approach and examined the effect of transient inactivation of the mismatch repair (MMR) process during adaptive evolution. The *mutS* gene was deleted in its entirety from the genomes of *L. casei* strains 12A and ATCC334 genomes. The wild-type parental strains and their Δ*mutS* derivatives were sequentially passaged through decreasing pH and increasing lactic acid concentrations over a 100-day period. Restoration of *mutS* at the end of the adaptive evolution process was used to stop hypermutability and the adapted strains were analyzed for genotypic and phenotypic changes.

L. casei strains with increased lactic acid tolerance were obtained from the adaptive evolution process with the largest increases observed in the Δ*mutS* derivatives. Genotypic changes responsible for the observed phenotype were identified in *L. casei*

12A. Targeted inactivation of three genes that had suffered three non-sense mutations identified in the adapted 12A Δ*mutS* derivative revealed that co-inactivation of a NADH dehydrogenase (*ndh*) and phosphate transport ATP-binding protein PstB (*pstB*) increased growth and lactic acid production by *L. casei* 12A at pH 4.0, and co-inactivation of a two-component signal transduction system (TCS) quorum-sensing histidine kinase (*hpk*) and *pstB* increased survival at pH 2.5.

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CHAPTER 2

LITERATURE REVIEW

Fermentation of Algal Cake

Microalgae are a rich source of carbohydrates, proteins, and lipids [5]. Not surprisingly, microalgal biomass has been utilized by people for thousands of years [65]. Biotechnological applications of microalgal biomass began in the 1950's, and it has since been used in the health, food, cosmetic, and energy industries [63, 65, 75]. Using lipids from microalgae as a biodiesel feedstock has gained considerable attention recently, because of its advantages over petroleum diesel and other oil feedstocks [2, 38, 63, 76]. However, biodiesel production is not cost competitive with petroleum diesel [23]. Increasing the cost feasibility of the production process can be achieved through using a biorefinery approach, where the remaining proteins and carbohydrates in the de-oiled algal biomass (algal cake) are converted to value added co-products [25, 62, 66, 67, 77].

In 1983, Nakas et al. [39] explored the potential of fermenting microalgal biomass using *Clostridium pasteurianum* to produce the organic chemicals butanol, 1,3-propanediol, ethanol, and acetate. Those investigators concluded that algal biomass is a viable eco-friendly substrate for bacterial fermentations [39]. Since then, researchers have been investigating the potential of using microalgal biomass as fermentable substrate to produce valuable chemicals. For example, lactic acid bacteria (LAB); *Lactobacillus amylovorus* [21, 24], *Lactobacillus coryniformis* subsp. *torquenns* [44], *Lactobacillus lactis* [17], *Lactobacillus paracasei* LA104 [43], and *Lactobacillus pentosus* [70] and other microorganisms like *Rhodobium marinum* A-501 [21, 24],

Saccharomyces cerevisiae [17], and *Thermoanaerobacterium* sp. [57] have been used to produce; lactic acid [17, 43, 44, 70], ethanol [17], and hydrogen [21, 24, 57, 73].

While these studies have provided valuable insight into using algal biomass as a fermentative substrate, additional research is needed to incorporate algal cake (AC) fermentations in a biorefinery approach towards biodiesel production. Most of the fermentations in these studies were performed on algal biomass that contained their native lipids [21, 24, 39, 43, 44, 57], which is very different from the AC produced from the biodiesel process. Thus, it cannot be assumed that the same growth and organic chemical production would occur using AC as a substrate. Additionally, some of these studies addressed perceived nutritional requirements by means of chemical pretreatments (e.g. acid hydrolysis) to release fermentable carbohydrates [17, 21, 57, 70]. None of the algal biomass fermentations provide a detailed characterization of the nutritional requirements needed for growth and chemical production. Such information is essential because it would reveal new insights for increasing growth and chemical production on this substrate. For example, acid hydrolysis has been demonstrated to release some fermentable carbohydrates, but enzymatic hydrolysis is preferable because of substrate specificity and the absence of toxic and corrosive chemicals [43, 79]. For these reasons, research detailing the nutritional requirements needed for growth and organic chemical production from AC, as well as exploring enzymatic methods for addressing any identified nutritional requirements would be of value to the algal biodiesel industry.

Amylase and Cellulase

Starch is a homopolymeric carbohydrate made of two α-glucan components, amylose and amylopectin. Amylose is a long, linear polymer comprised mostly of α-1,4 linked glucose moieties, and typically makes up 20-35% of starch [72]. Amylopectin is a shorter but much larger molecule due to a heavily branched structure, resulting from glucose moieties joined through ≈ 95% α-1,4 and 5% α-1,6 linkages. Starch is synthesized through a complex biological process, beginning with glucose produced by photosynthesis, and used as a carbohydrate storage component in green algae and many green plants [58, 72].

Starch is hydrolyzed by enzymes called amylases. Amylase is a family of at least three groups of enzymes; α-amylase, β-amylase, and γ-amylase. α-amylase is an endo-hydrolase that catalyzes the hydrolysis the α-1,4 linkages, releasing smaller carbohydrate units such as glucose and maltose [68]. β-amylase is an exo-hydrolase that catalyzes the hydrolysis of α-1,4 linkages at the non-reducing end of the homopolymer, successively releasing maltose units [68]. γ-amylase cleaves the α-1,6 linkages and the terminal α-1,4 linkages at the non-reducing end, releasing glucose [68]. The enzyme of major interest in industrial applications is α-amylase because of its endo-hydrolase activity, which results in rapid starch hydrolysis. α-amylase is produced by a broad range of organisms, including plants, animals, and microorganisms [68]. However, relatively few lactic acid bacteria (LAB) produce native amylases, and those that do have either low lactic acid yields or low enantioselectivity [35, 46].

Cellulose is also a homopolymeric carbohydrate, comprised solely of linear β-1,4 inked glucose units. Cellulose is the main carbohydrate component of algal and plant cell walls [58]. Cellulose is hydrolyzed by enzymes called cellulases, which like amylase, is a family with at least three groups of enzymes; endo-β-1,4-D glucanase, exo-β-1,4-D glucanase, and β-glucosidases [29]. Endo-1,4-β-D glucanase is an endo-hydrolase that catalyzes the hydrolysis of random β-1,4 linkages, releasing smaller glucan chains of varying length [29]. Exo-β-1,4-D glucanase is an exo-hydrolase that catalyzes the hydrolysis of β-1,4 linkages at the ends of cellulose chains, releasing β-cellobiose units [29]. β-glucosidases catalyze the hydrolysis of β-cellobiose units produced by the other enzymes, releasing glucose [29]. The endo-β-1,4-D glucanase is of greatest industrial interest because of its endo-hydrolase activity, which results in more rapid hydrolysis of cellulose. While β-glucosidases have been characterized in many LAB, no native LAB endo-β-1,4-D glucanases or exo-β-1,4-D glucanases have been identified in these bacteria [1, 35, 74].

For over the past 25 years, some attention has been paid to engineering amylolytic and cellulolytic LAB strains [3, 4, 41, 48, 50, 56, 59, 60], with greater success in achieving heterologous expression of α-amylases than endo-1,4-β-D glucanases in LAB [35]. The most successful heterologous α-amylase expression levels have been achieved using the α-amylase from *Streptococcus bovis* 148 [35, 41, 45, 47, 59], with the highest level of expression (0.9 U/ml) reported by Narita, et al. [40]. Successful heterologous expression of endo-1,4-β-D glucanases has also been reported, with CelA and CelE from *Clostridium thermocellum* giving the highest activity [3, 8, 48, 56, 60]. The highest level

of cellulase expression (1.9 U/ml) was reported by Bates et al. with CelE [4]. To date, there has been one previous report of heterologous expression of α-amylase in *L. casei* [41], but no reports on expression of an endo-1,4-β-D glucanase [35].

Since the carbohydrate composition of green algal biomass is largely present as starch and cellulose [58], research investigating heterologous expression of α-amylase and an endo-1,4-β-D glucanase is an appealing economic strategy to increase the amount of fermentable carbohydrates in for lactic acid production by LAB species such as *Lactobacillus casei*.

Adaptive Evolution

Mutations can have varying effects upon the fitness of an organism, and can be classified as neutral, deleterious, or beneficial. Beneficial mutations can be described as mutations that confer a growth advantage in a specific environment [15]. While beneficial mutations increase an organism's fitness, deleterious mutations that decrease the fitness occur at a substantially higher rate [7, 10, 22, 26, 51]. To avoid deleterious mutations, mutation rates of bacterial populations in stable environments are generally kept low by mechanisms that ensure DNA replication and repair fidelity [12, 14, 19, 27, 28, 42]. However, increased mutational rates can be beneficial to bacterial populations under stressed conditions, where an increased probability of creating beneficial mutations can help cells overcome a selective pressure [10, 15, 19, 51, 55, 69].

Cells with increased mutation rates (mutators) have been observed as a small percentage of bacterial populations, and shown to have increased adaptability under stressed conditions [6, 16, 31, 52, 64, 71]. Mutator phenotypes have been attributed to

mutations in DNA replication and repair enzymes (mutator alleles) [9, 13, 18, 32, 33], with strong mutators observed in cells with a deficient mismatch repair (MMR) system. [36, 49, 53, 54, 82]. The MMR process is highly conserved in prokaryotes and eukaryotes and ensures high DNA replication fidelity by correcting base-pair mismatches and small insertion or deletion loops [10, 20, 30, 37, 61]. In *E. coli* and other organisms, MMR is initiated by the MutS enzyme during DNA replication, which recognizes and binds base-pair mismatches in association with the β-clamp of the DNA polymerase, resulting in a ATP-dependent conformational change [20, 30, 37]. This conformational change induces binding of the MutL protein and subsequent activation of the endonuclease MutH, allowing for excision of the bp mismatch in the unmethylated daughter strand [20, 30, 37].

Adaptive laboratory evolution has been used to gain insight into the role that DNA replication and repair systems such as MMR have upon a microorganism's ability to evolve under specified growth conditions, and to create "improved" strains with beneficial characteristics for biotechnological/industrial processes [11, 34, 78, 80, 82]. The adaptive evolution process allows for the creation of these improved strains, with increased tolerance to various defined stresses, by coupling the desired phenotype to growth [78, 80]. To date, most adaptive evolution experiments have been conducted on model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* [11].

In LAB, adaptive evolution has been used to increase lactic acid tolerance in *L. casei* Zhang, by serial passage into fermentation medium with decreasing pH and increasing lactic acid concentrations [81]. The results of that study produced an adapted strain with increased lactic acid tolerance, and revealed potential mechanisms for acid

tolerance in *L. casei*. This study explored a novel adaptive evolution approach using transient *mutS* deletion to select *L. casei* strains that could tolerate lactic acid at very high concentrations. Genetic characterization of these mutants provided invaluable insight into the acid resistance mechanisms in *L. casei*.

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CHAPTER 3[1]

FERMENTATION OF DE-OILED ALGAL BIOMASS BY *LACTOBACILLUS CASEI* FOR PRODUCTION OF LACTIC ACID

Abstract

De-oiled algal biomass (algal cake) generated as waste byproduct during algal biodiesel production is a promising fermentable substrate for co-production of value-added chemicals in biorefinery systems. We explored the ability of *Lactobacillus casei* 12A to ferment algal cake for co-production of lactic acid. Carbohydrate and amino acid availability were determined to be limiting nutritional requirements for growth and lactic acid production by *L. casei*. These nutritional requirements were effectively addressed through enzymatic hydrolysis of the algal cake material using α-amylase, cellulase (endo-1,4-β-D-glucanase), and pepsin. Results confirm fermentation of algal cake for production of value-added chemicals is a promising avenue for increasing the overall cost competiveness of the algal biodiesel production process.

Introduction

Increasing global demand for sustainable energy and chemicals has generated considerable interest in "green" bio-based products and technologies [1, 51]. Algal

[1] Reprinted from Overbeck T, Steele JL, Broadbent JR (2016) Fermentation of de-oiled algal biomass by *Lactobacillus casei* for production of lactic acid. Bioprocess Biosyst Eng. 39:1817-1823 with permission (Appendix B).

biodiesel production, for example, has emerged as a promising liquid biofuel with many advantages over petrodiesel and other biodiesel feedstocks [24, 27, 41, 48]. Algal biodiesel production involves harvesting algal biomass and extracting the lipids present in the cell envelope, using acid hydrolysis and chloroform extraction, which are then converted to biodiesel via transesterification. Glycerol and de-oiled algal biomass (algal cake; AC) are separately generated as waste products [24].

While much research has focused upon increasing oil yields and improving the production process, algal biodiesel production is not presently cost competitive with petrodiesel [48]. Utilizing a biorefinery approach in the production of algal biodiesel, wherein fuel production is coupled with the conversion of unused biomass into value-added co-products, such as bioethanol, biohydrogen, or lactic acid, have been demonstrated to increase the cost competitiveness of biodiesel [13, 22, 34, 40, 43–46, 49]. Thus, fermentation of chemically pretreated AC is an attractive option for co-generation of new bio-based products in biorefinery approaches for production of algal-based biodiesel [13, 34, 46].

Lactic acid has applications in the food, cosmetic and chemical industries, and is primarily produced through fermentation processes using lactic acid bacteria (LAB) rather than chemical synthesis because of the ability to produce one desired stereoisomer of lactic acid, rather than a racemic mixture of D and L-lactic acid [11, 14, 20]. L-Lactic acid, for example, is the preferred stereoisomer for the production of the versatile biodegradable polymer (poly) lactic acid (PLA) [3, 11, 14, 15, 19]. Production costs for lactic acid are primarily dependent upon the costs of the raw materials used as fermentable substrates, creating substantial interest in the utilization

of low value fermentable substrates [3, 15, 19]. Consequently, co-production of lactic acid from AC has the potential to increase the cost feasibility of both production processes.

Lactobacillus casei is an aciduric, rod-shaped, facultatively heterofermentative LAB that produces L-lactic acid as its primary metabolic end-product. *L. casei* shows high metabolic flexibility, and can be isolated from a diverse array of ecological niches [6–8]. *L. casei* 12A was isolated from corn silage, and like many other *L. casei* plant isolates has the ability to ferment a wide-array of carbohydrates associated with this nutritionally variable niche, including cellobiose, panose, isomaltose, maltose and maltotriose [7, 8, 47].

In this study, we examined the ability of *L. casei* 12A to ferment AC for the production of lactic acid, and identified the key factors that limit growth and lactic acid production on this substrate. Results showed enzymatic pretreatment of the AC to liberate carbohydrate and amino nitrogen enabled rapid growth and lactic acid production by *L. casei* 12A, and revealed strategies to optimize co-production of lactic acid without the need for chemical or enzymatic pretreatments.

Materials and Methods

Growth Conditions

Lactobacillus casei 12A was maintained at −80 °C in MRS broth (Difco Laboratories, Detroit, MI) containing 10 % glycerol. Working cultures were prepared by two successive transfers in APT broth (HiMedia, Mumbai, India) with incubation at

37 °C for 17 h. Inoculations were conducted at 1 % (v/v) after standardizing to an optical density at 600 nm (OD600) of 1.0 in APT.

The AC was obtained from the Utah State University Biofuels Center (USU-BC) after acid hydrolysis and chloroform extraction of lipids from a Great Salt Lake algal isolate designated USU080. The AC was air dried at RT by the USU-BC and stored at −20 °C. A qualitative and quantitative analysis of amino acid and monosaccharide composition of the USU080 AC was performed by SGS M Scan Inc. (West Chester, PA) using acid hydrolysis with subsequent reverse phase high pressure liquid chromatography (RP-HPLC) and high-performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD), respectively.

Growth experiments were performed using USU080 AC material suspended in 1.0 % meta-phosphoric acid buffer (Sigma Aldrich, St. Louis, MO) titrated to pH 6.5. Small batch (10 mL) fermentations were conducted at 37 °C and 200 rpm in a Lab-Line 4628 shaking incubator (Dubuque, IA) for 24 h. Cells were plated on MRS agar every 2 h between 0 and 10 h, and at 20 and 24 h and colonies enumerated after 48 h of incubation in BBL GasPak jars with envelopes (Becton, Dickinson and Company, Franklin Lakes, NJ). APT broth medium, which contains 1 % glucose, was used as the positive control for growth and lactic acid comparisons. All experiments were performed using a minimum of two biological-experimental replicates. Standard error of the mean was determined for each experimental sample time point.

Determination of Nutritional Requirements

2 % (w/v) AC solutions were supplemented with a multi-vitamin mixture (0.0008 mM D-biotin, 0.0022 mM folic acid, 0.0081 mM niacinamide, 0.0646 mM pyridoxal and pyridoxine HCl, 0.0005 mM riboflavin, and 0.0021 mM Ca-pantothenate), 0.25 % casamino acids, or 0.87 % d-glucose (calculated to deliver the 1 % monosaccharide concentration present in APT, based upon HPLC analysis of the AC). AC supplemented with 0.87 % D-glucose was additionally supplemented with the vitamin mixture and/or 0.25 % casamino acids. Growth of *L. casei* 12A in these suspensions was performed as described above.

Enzymatic Hydrolysis of Algal Cake

AC solutions were treated with 0.4 % (w/v) pepsin from porcine gastric mucosa (P07012_Sigma; Sigma Aldrich), and/or 1200 U porcine pancrease α-amylase (A3176_Sigma; Sigma Aldrich), and/or 5 U cellulase (endo-1,4-β-d-glucanase) from *Aspergillus niger* (C1184_Sigma; Sigma Aldrich) [25, 36]. Enzyme treatments were prepared by suspension of the enzymes in 5 mL 1.0 % meta-phosphoric acid buffer at pH of 1.5, 6.9, or 4.8, respectively, then sterilized by passage through a 0.2 μm cellulose acetate filter (VWR, Randor, PA) into an AC solution titrated to the same pH and calculated to give a final concentration of 15 % (w/v) AC (calculated to deliver the 1 % monosaccharide concentration present in APT, based upon HPLC analysis of the AC) after all enzyme additions. AC/pepsin solutions were incubated at 37 °C and 200 rpm for 3 h, AC/α-amylase solutions were incubated at 37 °C and 200 rpm for 16 h, and AC/cellulase solutions were incubated at 50 °C and 250 rpm for

24 h [25, 36]. Enzymes were inactivated by boiling for a period of 5 min at the end of each treatment. Combinatorial enzyme digestions were performed sequentially in order of increasing pH conditions, with enzyme inactivation after each individual enzyme treatment.

Lactic Acid Determination

All remaining supernatant from each sample was collected after 24 h growth experiments by centrifugation at 5000×g/min at 0 °C for 10 min using a Sorvall RT6000B refrigerated centrifuge (Dupont, Wilmington, DE) and sterilized by passage through a 0.2 μm cellulose acetate filter. Supernatant samples were stored at −20 °C prior to analysis. Quantitative D/L-lactic acid analyses were performed using the R-Biopharm AG (Darmstadt, Germany) D/L-lactic acid UV-method test kits as directed by the supplier, except that the total volume of the assay was decreased from 1 to 0.5 mL while maintaining the proportions described in the manufacturer's instructions. A Bonferroni correction for multiple pairwise comparisons was used to identify statistically significant differences in lactic acid yields from each treatment [30].

Determination of Algal Cake Composition

Supernatant samples from 50 mL 15 % AC solutions treated with pepsin, α-amylase, and cellulase were collected by centrifugation at 5000×*g*/min at RT for 10 min, and stored at −20 °C until needed. The Somogyi–Nelson method was used for quantification of reducing sugars [29] with measurement at OD_{540}. Utah State

University Analytical Laboratories performed quantitative compositional analysis of the USU080 AC using the Dumas (combustion) method to measure proteins [50], and the loss on ignition method to calculate and measure moisture and ash, respectively [39, 42]. Determination of the fat and total carbohydrate content of the AC was performed using the chloroform–methanol method [12] and carbohydrate by difference method [26], respectively.

Results and Discussion

Determination of Nutritional Requirements

Qualitative and quantitative analysis of amino acid and monosaccharide composition of acid hydrolyzed USU080 AC indicated it contained 6.7 % carbohydrate (predominantly glucose, Table A-1) and 24.4 % protein (Table A-2), including all 16 amino acids that can be detected following acid hydrolysis. However, fermentations of 15 % AC solutions with *L. casei* 12A revealed growth and lactic acid production was significantly lower ($P < 0.05$) than that achieved in APT broth. Since carbohydrate is the primary energy source for growth of *L. casei*, and lactic acid is the major end-product of carbohydrate use, this finding suggested one or more nutrients was limiting in the AC. To identify the limiting nutrient(s), 2 % AC solutions were supplemented with glucose as the carbohydrate source, casamino acids for an amino acid source, or a mixture of essential and stimulatory vitamins previously determined to restore growth of *L. casei* on corn stover hydrolysate (unpublished data). Supplementation with glucose significantly increased growth to a level comparable to that achieved in APT

broth (Fig. 3-1a), and lactic acid production was also significantly increased from 1.67 ± 0.65 mM (0.15 ± 0.04 g/L) to 37.2 ± 7.75 mM (3.35 ± 0.46 g/L). However, lactic acid yields remained significantly lower than that attained with APT (82.4 ± 10.7 mM, 7.42 ± 0.88 g/L) (Fig. 3-1c). Supplementation with casamino acids or vitamins did not increase growth or lactic acid production (Fig. 3-1a, c), indicating that carbohydrate availability was the primary limiting factor for lactic acid production by *L. casei* 12A from AC.

To explore the basis for the lower yield of lactic acid from AC supplemented with glucose compared to APT, AC with glucose was further supplemented with the vitamin mixture and/or casamino acids. Supplementation with vitamins did not provide a significant increase in growth or lactic acid production (50.3 ± 7.89 mM, 4.53 ± 0.42 g/L), but supplementation with casamino acids significantly increased growth and lactic acid production (68.9 ± 20.85 mM, 6.21 ± 1.27 g/L) compared to AC with glucose alone (Fig. 3-1b, c). The growth and lactic acid production on AC with glucose and casamino acids was not significantly different from that achieved on APT. The level of lactic acid production (75.1 ± 2.83 mM, 6.77 ± 0.17 g/L) achieved by supplementation with all three nutrients was not significantly higher than that achieved by supplementation with glucose and casamino acids or that on APT. These findings indicated that carbohydrate and amino acid availability in AC must be enhanced for *L. casei* 12A to attain the same biomass and lactic acid yields seen with APT.

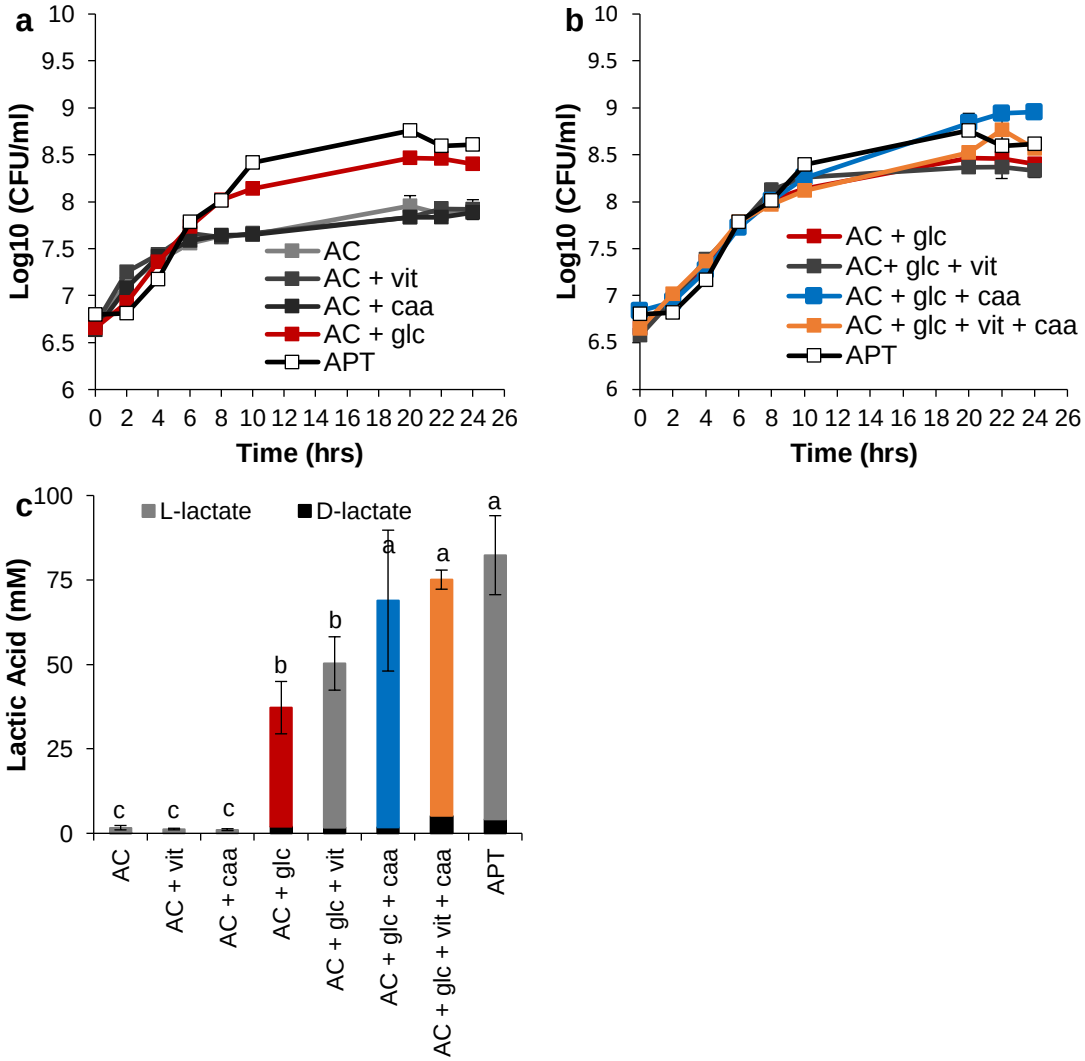

Fig. 3-1 Growth of *L. casei* 12A on 2 % algal cake (AC) and 2 % AC solutions supplemented solely with a defined vitamin mixture, 0.25 % casamino acids, and 0.87 % glucose (a), and 2 % AC with 0.87 % glucose solutions additionally supplemented with a defined vitamin mixture and/or 0.25 % casamino acids (b), using the laboratory medium APT as a control. c D-(Black) and L-(gray) lactic acid produced by *L. casei* 12A on each medium. Growth mediums with the same letter are not significantly different ($P < 0.05$).

Enzymatic Hydrolysis of Algal Cake

Enzymatic hydrolysis of the AC was used to better characterize the carbohydrate and amino acid limitations affecting growth and lactic acid production by *L. casei* 12A on this substrate. Cellulose is the primary component of algal cell walls and starch is used as a carbohydrate storage component in green algae, making them both promising sources of carbohydrates within AC [35]. Cellulase (endo-1,4-β-D-glucanase) and α-amylase were examined for their prospective ability to hydrolyze cellulose and starch, respectively, in the AC, and thus generate fermentable mono- and disaccharides that are needed by *L. casei* 12A to increase biomass and lactic acid yields.

While *L. casei* 12A is known to contain a gene encoding a cell envelope anchored proteolytic enzyme, and this enzyme is functional in other strains [5, 16, 23], our results suggest that it does not release the levels of amino nitrogen from proteins in AC needed to support growth and lactic acid production by 12A. Pepsin is a nonspecific protease that cleaves peptide bonds with an aromatic amino acid on either side, and we wanted to determine whether this broad activity could hydrolyze AC proteins into polypeptides that could better support growth of *L. casei* 12A.
As is shown in Fig. 3-2 pepsin, α-amylase, or cellulase treatment of 15 % AC each resulted in significant increases in growth and lactic acid production. Lactic acid yields from these treatments went from 5.21 ± 0.39 mM (0.47 ± 0.02 g/L) to 35.0 ± 4.07 mM (3.15 ± 0.33 g/L), 32.9 ± 5.36 mM (2.96 ± 0.35 g/L) and 47.5 ± 1.80 mM (4.28 ± 0.11 g/L), respectively, demonstrating the effectiveness of enzymatic treatments to enhance the content of fermentable sugar and polypeptides in AC. Further increases

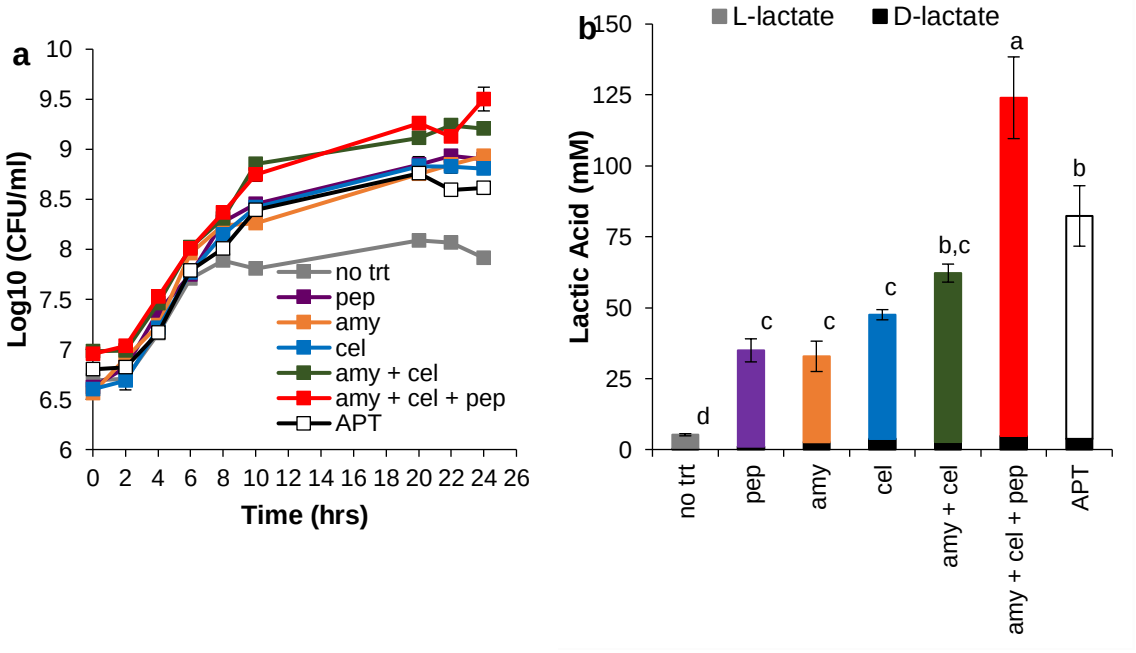

Fig. 3-2 (a) Growth of *L. casei* 12A on 15 % algal cake (AC) (no trt) solutions enzymatically treated with pepsin, α-amylase, cellulase, α-amylase and cellulase, or pepsin, α-amylase and cellulase using the laboratory medium APT as a control (1 % glucose). b D-(Black) and L-(gray) lactic acid produced by *L. casei* 12A on each medium. Growth mediums with the same letter are not significantly different ($P < 0.05$).

in growth were observed when the AC was digested with α-amylase and cellulase or with α-amylase, cellulase, and pepsin (Fig. 3-2a). However, the amount of lactic acid produced from the AC digested with α-amylase and cellulase (62.2 ± 3.18 mM, 5.60 ± 0.13 g/L) was not significantly different from APT (82.4 ± 10.7 mM, 7.42 ± 0.88 g/L), or from any of the single enzyme digest (Fig. 3-2b). In contrast, the lactic acid yield from the triple-enzyme digested AC (124.0 ± 14.4 mM, 11.17 ± 1.22 g/L) was significantly higher than that achieved on any other growth medium (Fig. 3-2b). This finding emphasized the importance of addressing both the

carbohydrate and amino acid availability for optimized biomass and lactic acid production by *L. casei* in AC.

The significantly higher level of lactic acid produced from *L. casei* 12A fermentation of enzyme treated AC versus the positive control APT is potentially explained by an underestimation of the carbohydrate concentration in the AC using the acid hydrolysis method. To explore this possibility, the Somogyi–Nelson method was used to determine the amount of reducing sugars present in undigested 15 % AC as well as the α-amylase, cellulase, and pepsin digested solution. The undigested 15 % AC solution was determined to have 0.336 ± 0.002 g/L reducing sugars, indicating the increase in lactic acid production (35.0 ± 4.07 mM, 3.15 ± 0.33 g/L) by *L. casei* on AC digested with pepsin alone was likely a result of amino nitrogen metabolism, addressing cellular energy requirements. The triple-digested AC was determined to have 11.78 ± 1.28 g/L reducing sugars, revealing that the AC material contains a minimum of 7.85 ± 0.85 % carbohydrate, and not 6.73 % as originally measured by acid hydrolysis. This finding confirms the significantly higher level of lactic acid observed from the triple-enzyme treated AC is due to a higher concentration of carbohydrate in AC versus APT.

Underestimation of the sugar content in the acid hydrolyzed AC may be due to the difficulties associated with hydrolyzing the β-1,4-glycosydic linkages within the crystalline cellulose structure [4, 18, 32]. Furthermore, because the Somogyi–Nelson method only measures reducing sugars, and not additional non-reducing sugar moieties released as polysaccharides from the enzyme digestions, it was likely that even 7.85 % carbohydrate was an underestimation of the carbohydrate composition of the AC

material. To better characterize the carbohydrate content of USU080 AC, a more complete compositional analysis was performed. Those analyses revealed the sample contained 7.8 ± 0.78 % moisture, 19.5 ± 1.95 % ash, 0.73 ± 0.074 % fat, 41.0 ± 4.1 % protein and 30.97 ± 6.9 % carbohydrate. The high protein and carbohydrate content further highlights the opportunity to increase *L. casei* biomass and lactic acid yields through enzymatic hydrolysis of these two major AC biomass components.

With limited amounts of available carbohydrates in the AC solution solely digested with pepsin, the observed significant increase in growth and lactic acid production can likely be attributed to the deamination and subsequent metabolism of available amino nitrogen to meet biomass and cellular energy needs, with lactic acid as an end-product. Under conditions of limited amino acid availability, such as in the AC solutions digested with α-amylase and/or cellulase, the increased levels of available carbohydrates are likely used to generate cellular energy, resulting in increased levels of lactic acid, and for *de novo* amino acid biosynthesis. When amino nitrogen and sugar are both provided, as was the case with the triple-enzyme treated AC, biomass and lactic acid production from the AC material were each favored.

Conclusions

In summary, we have demonstrated that AC is a viable fermentable substrate for possible incorporation into a biorefinery approach to biodiesel production with co-production of lactic acid by *L. casei*. To optimize the production of lactic acid or other value-added end products, however, carbohydrate and amino acid availability must be

enhanced. These limitations can be overcome by enzymatic hydrolysis with a nonspecific protease (e.g., pepsin), α-amylase, and cellulase. Producing an optimized level of 0.075 g lactic acid per g AC when the AC is digested by all three enzymes. Alternatively, heterologous expression of α-amylase and cellulase by Lactobacilli has been previously reported [2, 9, 10, 17, 21, 28, 31, 33, 37, 38], and could provide an attractive means for increasing lactic acid production from AC without the need of costly chemical or enzyme pretreatments.

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CHAPTER 4

HETEROLOGOUS EXPRESSION OF α-AMYLASE AND CELLULASE IN *LACTOBACILLUS CASEI* 12A

Abstract

Algal biodiesel waste byproduct (algal cake) can be fermented by *Lactobacillus casei* 12A for co-production of lactic acid, but significant increases in lactic acid production are achieved by increasing carbohydrate availability. This may be performed by enzymatic hydrolysis of the cellulose and starch in algal cake using cellulase and α-amylase, respectively. To increase the cost-feasibility of a biorefinery type approach, we explored heterologous expression of the cellulase gene (*celE*) from *Clostridium thermocellum* and the α-amylase gene (*amyA*) from *Streptococcus bovis* 148 in *L. casei* 12A. Functional expression of *celE* in *L. casei* 12A was not detected. However, secretion of AmyA was shown, and a 1.5-fold increased level of activity was attained using a synthetic promoter previously designed for *Lactobacillus plantarum*. Nonetheless, the levels of amylase activity attained were insufficient to significantly increase lactic acid production by *L. casei* 12A in AC fermentations. Thus, substantial optimization of *celE* and *amyA* expression in *L.* casei 12A would be needed to obtain the activities required for reducing enzyme costs in a biorefinery that incorporates AC fermentation by *L. casei*.

Introduction

Growing interest in sustainable energy and chemicals has increased demand for many bio-based products, including biodiesel [3, 35]. Algal biodiesel has emerged as a promising alternative to non-renewable liquid fossil fuels [14, 17, 32, 34]. However, algal biodiesel cannot currently be produced in a manner that is cost-competitive as compared to the traditional alternatives [1]. A biorefinery strategy, where the manufacture of bio-based products is coupled with the generation of value added co-products, has been demonstrated to be an effective means for increasing the overall cost-competitiveness [9, 29]. We previously examined the potential to ferment de-oiled algal biomass (algal cake), generated as a waste byproduct, using *Lactobacillus casei* 12A for co-production of the industrially valuable chemical lactic acid [24]. That work revealed carbohydrate availability to be the primary limiting factor for growth and lactic acid production and showed this limitation could be effectively addressed by enzymatic pre-treatments with endo-1,4-β-D glucanase (cellulase) and α-amylase, with 0.04 g lactic acid per g of algal cake produced when digested with both enzymes together [24]. Thus, heterologous expression of cellulase and α-amylase by *L. casei* 12A presents an attractive means for increasing lactic acid production in AC fermentations, without the need of pretreatments [24].

Heterologous expression of cellulase and α-amylase in lactic acid bacteria (LAB), such as *L. casei*, has previously been described [2, 19, 25, 27, 30, 31]. In LAB, the highest levels of expression were reported using the cellulase gene (*celE*) from *Clostridium thermocellum* [6], and the α-amylase gene (*amyA*) from *Streptococcus bovis*

148 [18]. In this study, codon optimized cassettes of *celE* from *C. thermocellum* and *amyA* from *S. bovis* 148 were synthesized and cloned into *L. casei* 12A, then enzyme activity was measured. No functional cellulase activity was detected, but low level amylase activity was found in the supernatant. Amylase activity was increased approximately 1.5-fold by replacing the constitutive phosphoglyceromutase promoter initially used in the amylase cassette with a highly active synthetic promoter designed for *Lactobacillus plantarum*. However, the amylase activity was still insufficient to release enough fermentable carbohydrates from AC to significantly increase growth and lactic acid production by *L. casei* 12A.

Materials and Methods

Strains and Plasmids

Bacterial strains and plasmids used in this work are listed in Table 4-1. *Lactobacillus casei* and *Escherichia coli* strains were maintained in a laboratory culture collection at -80°C in 15% glycerol stocks. *L. casei* strains were propagated in MRS (Difco Laboratories, Detroit, MI) or APT (HiMedia, Mumbai, India) broth, with 2.5 μg/ml erythromycin (Ery) when appropriate. *E. coli* strains were propagated with aeration at 200 rpm and 37°C in LB broth (Difco Laboratories), with 100 μg/ml Ery when appropriate.

Table 4-1 Bacterial strains and plasmids used for heterologous expression of *celE* and *amyA* in *Lactobacillus casei* 12A.

Strains and plasmids	Relevant Characteristics	Source
Escherichia coli DH5α		Lab
Lactobacillus casei 12A		Lab
pDW2	*L. casei* cloning vector	Unpublished work
pDW2:*celE*-I	native promoter, native RBS, codon optimized *celE* CDS, NICE terminator	This study
pDW2:*amyA*-I	Ppgm, RBS, codon optimized *amyA* CDS, NICE terminator	This study
pDW2:*amyA*-II	Pp11, RBS, codon optimized *amyA* CDS, NICE terminator	This study

Lab = Broadbent laboratory culture collection.

Plasmid Construction

The *Clostridium thermocellum* endo-β-1,4-D glucanase gene *celE* from and *Streptococcus bovis* 148 α-amylase gene *amyA* coding sequences (CDS) were codon-optimized for *L. casei* using the OPTIMIZER codon optimization online tool [26]. After codon optimization, the CDSs were analyzed for direct and indirect repeats greater than 7 bp in length using the Unipro UGENE software [23]. Repeats larger than 11 bp were eliminated by changing codons within repeats with similar usage weights (e.g. two codons for Tyr; UAU with a weight of 1.00 and UAC with a weight of 0.898) to the second most frequently used codon for *L. casei*. After elimination of the larger repeats, the overall number of repeats was further reduced by changing random codons with similar usage weights throughout the CDS. After reducing the number of repeats, the native promoter, native ribosomal binding site (RBS), and NICE terminator [16] were

added to the codon-optimized CDS for *celE*, and the phosphoglyceromutase promoter (Ppgm) and associated RBS from *L. casei* ATCC334, and NICE terminator were added to the codon optimized CDS for *amyA*, resulting in our full gene constructs. The assembled full gene constructs were subsequently analyzed using the Custom Gene Entry online tool (Integrated DNA Technologies (IDT) Coralville, IA) to identify problem regions containing a high amount of 8 bp repeats within a 150 bp region. Identified problem regions within the CDS were eliminated by again using alternate codons as previously described. No changes were made to the promoter, RBS and terminator sequences. The CDSs were further analyzed for catabolite response elements (cre) as previously described for *Lactobacillus acidophilus* [4, 5]. The final gene construct sequences (*celE*-I and *amyA*-I) 2,408 bp and 2,778 bp respectively (Fig. 4-1, Table A-3), were ordered as synthetic gene constructs from IDT and were delivered in the vector AmpBlunt.

The full *celE*-I and *amyA*-I constructs were PCR amplified from the synthetic constructs using Phusion high-fidelity DNA polymerase kit (New England Biolabs, Ipswich, MA), with primers *cel*1 x *nice* and *amy*1 x *nice*, respectively, which include *Xba*I and *Pst*I linkers (Table 4-2). To replace Ppgm in *amyA* with a more active promoter (Phrommao and Steele, unpublished data Fig. A-2) we designed a cassette comprising the synthetic promoter p11 (Pp11) sequence and it's RBS (113 bp) [28] plus the first 30 codons of the *amyA*-I construct, except that the last two codons were changed to introduce a *Pvu*I linker (Table A-3). This cassette was synthesized by IDT. The Ppgm region in the *amyA*-I construct was replaced by the synthetic Pp11 construct via Phusion

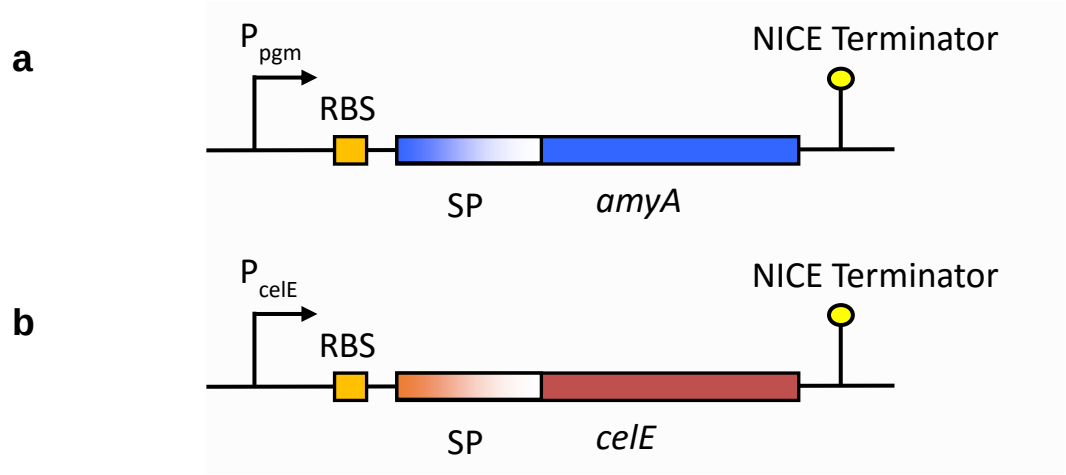

Fig. 4-1 Schematic of (a) *amyA*-I construct containing *Lactobacillus casei* constitutive phosphoglyceromutase promoter (P_{pgm}) and ribosomal binding site (RBS), *L. casei* codon optimized *Streptococcus bovis* 148 α-amylase gene *amyA* coding sequences (CDS) with native signal peptide (SP), and NICE terminator, and (b) *celE*-I construct containing *L. casei* codon optimized *Clostridium thermocellum* endo-β-1,4-D glucanase gene *celE*, native *celE* promoter (P_{celE}) and RBS, and NICE terminator.

Table 4-2 PCR primers used for heterologous expression of *celE* and *amyA* in *Lactobacillus casei* 12A. Underlined regions represent restriction enzyme linkers.

Primer	Sequence	Restriction Site
*cel*1	CGGTCTAGAGGGTTTCCAACAAA	*Xba*I
*cel*2	CCGGTAGCAGCGTTAGTA	
*cel*3	ACCTAGCGGTATCATTGC	
*cel*4	TGGAATGGATGGGTGGTAC	
*cel*5	GCA CAT TTT GGG CCG TTA	
*amy*1	CGGTCTAGATTTCCAATATACCAGAGA	*Xba*I
*amy*2	TTGCCAGCTGAGATATGACG	
*amy*3	ACCGATAAGGTTTCAGGCC	
*amy*4	ACCCAAAACGCTTTGTTGAC	
*amy*5	CGGTCAAGGTCAAGGTTGTT	
amyX	AATATCTAGACGATCGGCGCCGG	*Pvu*I
nice	CGGCTG CAGTTATAAATAAAAAAACCAC	*Pst*I
p11-f	CGGTCTAGAAGCGCTATAGTTG	*Xba*I
p11-r	CATCGATCGCGGTCAAGA	*Pvu*I

PCR of the Pp11 and *amyA*-I constructs with primers *p11-f* x *p11*-r and *amyX* x *nice*, respectively, which respectively include *Xba*I and *Pvu*I, and *Pvu*I and *Pst*I linkers. After digestion of the amplified products with *PvuI*, the digested products were ligated, and the ligation reaction was amplified by Phusion PCR with primers *p11-f* x *nice*, with *Xba*I and *Pst*I linkers. The *amyA* construct with Pp11 was designated *amyA*-II. The amplified *celE*-I, *amyA*-I, and *amyA*-II constructs were digested and ligated into the *L. casei* cloning vector pDW2 (Welker, Steele and Broadbent, unpublished data, Fig. A-1).

Transformation of recombinant plasmids into *E. coli*, followed by plating, plasmid purification, and screening was performed as previously described [8]. The recombinant plasmids were recovered from *E. coli* and the insert regions sequenced to verify construct fidelity. Next, the individual plasmids were transformed into *L. casei* 12A competent cells, prepared by growth in MRS with 1% glycine to an OD_{600} between 0.6 and 0.8, pretreated with sterile dH_2O for 30 min [33]. Electroporation was performed in 2 mm cuvettes using a Bio-Rad Gene Pulser (Hercules, CA) set at 2,500 V, 25 μF, and 400 Ω, following the protocol of Welker, et al. [33]. Electroporated cells were recovered in MRS with 0.5 M sucrose and plated onto MRS with 2.5 μg/ml Ery, following the outlined protocol [33]. Genomic DNA of the *L. casei* 12A Ery^R colonies was isolated by lysing the cells with lysozyme and proteinase K, cleaning with chloroform, and precipitation with ethanol following the protocol of Broadbent, et al. [8]. Genomic DNA was screened by PCR with Mastermix Taq DNA polymerase (5 Prime Inc. Gaithersburg, MD) using the primers *cel*1 x *nice*, *amy*1 x *nice*, and *p11-f* x *nice* for the *celE*-I, *amyA*-I, and *amyA*-II constructs, respectively. DNA sequencing of these PCR products, with the

primers *cel*1-5, *amy*1-5, *p11-f*, *p11-r*, and *nice* (Table 4-2), confirmed the fidelity of these constructs in *L. casei* 12A.

Enzymatic Assays

E. coli and *L. casei* 12A pDW2:*celE*-I transformants and corresponding cells containing only pDW2 (controls) were streaked on LB or MRS plates, respectively, containing 0.2% carboxymethylcellulose (CMC) and incubated at 37°C for 48 h. After incubation, the plates were stained with Gram's iodine solution for 3-5 min and examined for zones of clearing around colonies, indicating CMC hydrolysis from glucanase activity [12]. Working cultures of *L. casei* pDW2:*celE*-I and pDW2 (control) were prepared by two successive transfers in MRS with 0.5% CMC instead of glucose plus 2.5 μg/ml Ery (MRS-CMC), with incubation at 37°C for 17 h. Working cultures were used to inoculate (1 % v/v) 10 ml MRS-CMC and incubated at 37°C for 24 h. After incubation the cells were pelleted by centrifugation at 5000×g/min at 0°C for 10 min using a Sorvall RT6000B refrigerated centrifuge (Dupont, Wilmington, DE), then 50 μl of supernatant was transferred to 1 ml 1.5% CMC in 50 mM Tris(HCl) at pH 7.0, and incubated for 2 h at 37 or 60°C with shaking at 250 rpm [31]. The amount of reducing sugars in the CMC-supernatant mixture was then measured using the Somogyi-Nelson method [20] to quantify cellulase activity [2, 22, 25, 27, 31].

E. coli and *L. casei* 12A pDW2:*amyA*-I and pDW2:*amyA*-II transformants and their respective pDW2 controls were streaked on LB or MRS plates, respectively, that contained 1% soluble starch (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 37°C for 48 h. After incubation, the cells were stained with Gram's iodine

and examined for zones of clearing, which appear as a consequence of starch hydrolysis [30, 31]. To determine whether α-amylase was secreted, working cultures of *L. casei* 12A pDW2:*amyA*-I, pDW2:*amyA*-II, and the pDW2 control were prepared by two successive transfers in APT broth with 2.5 μg/ml Ery, with incubation at 37°C for 17 h. These cultures were used to inoculate (1% v/v) 25 ml APT broth with 2.5 μg/ml Ery, and incubated at 37°C for 6 h. Next, the cells were pelleted by centrifugation at 8000×g/min at 4 °C for 20 min then the supernatant was collected and stored on ice. The cell pellets were washed with 50 mM sodium phosphate buffer at pH 7, re-pelleted, and suspended in 2.5 ml of the same buffer [10]. A 1 ml sample of the cell suspension was enzymatically lysed by addition of 25 μl of 100 mg/ml lysozyme from chicken egg white (Sigma Aldrich, St. Louis, MO) in 10 mM Tris-HCl at pH 8 and 50 U of mutanolysin from *Streptomyces globisporus* ATCC 21553 (Sigma Aldrich), with incubation at 37°C for 30 min [7]. After the enzyme treatment, cells were mechanically disrupted by addition of 1.8 g of 0.1 mm glass beads (Research Products International, Mount Prospect, IL) and vortexing for 10 min [7, 10, 15]. Glass beads and cell debris were pelleted by centrifugation at 9000×g/min at RT for 15 min, and the aqueous cell lysate was collected. Amylase activity in the supernatant and crude cell lysate preparation was measured directly using the Phadebas kit (Magle Life Sciences, Lund, Sweden) following the manufacturers protocol, with the pDW2 control as a blank and an increase in the sample volume to 1 ml [15]. A two-tailed students t-test was performed to identify statistically significant ($P < 0.05$) differences in amylase activity between *amyA*-I and *amyA*-II at each time point.

To determine if amylase activity increased with fermentation time, and if the measured activity was sufficient to significantly increase lactic acid production from starch, amylase activity and lactic acid levels were measured in supernatant samples from 120 h fermentations of *L. casei* 12A pDW2:*amyA*-I and pDW2:*amyA*-II transformants, using pDW2 as a control. Working cultures were prepared by two successive transfers in MRS broth with 0.5% soluble starch instead of glucose and 2.5 μg/ml Ery (MRS-ST), with incubation at 37°C for 17 h. These cultures were used to inoculate (1% v/v) 40 ml MRS-ST broth, and incubated at 37°C. Cultures were plated after inoculation and every 24 h for 120 h. Samples (5 ml) were collected and centrifuged at 5000×g/min and 0 °C for 10 min. A 1 ml sample of the supernatant was collected for measuring amylase activity as previously described, and the remaining supernatant was sterilized by passage through a 0.2 μm cellulose acetate filter and stored at −20 °C for subsequent lactic acid analysis.

Quantitative D/L-lactic acid analyses were performed using the R-Biopharm AG (Darmstadt, Germany) D/L-lactic acid UV-method test kits as directed by the supplier, except that the total volume of the assay was decreased from 1 to 0.5 mL while maintaining the proportions described in the manufacturer's instructions [24]. A Bonferroni correction for multiple pairwise comparisons was used to identify statistically significant differences in lactic acid yields from each sample at each time point [21].

Amylase Hydrolysis of Algal Cake

AC derived from the green algae USU080 was obtained from the Utah State University Biofuels Center (USU-BC). A α-amylase (A3176_Sigma; Sigma Aldrich) enzyme suspension was prepared in 5 mL 1.0 % meta-phosphoric acid buffer at pH 6.9, and sterilized by passage through a 0.2 μm cellulose acetate filter (VWR, Randor, PA) into an AC solution titrated to the same pH and calculated to give a final concentration of 15 % (w/v) AC and 1 U/ml α-amylase [24]. The AC/α-amylase solutions were incubated for 72 h at 37 °C with shaking at 200 rpm. Then the enzyme was inactivated by boiling the solution for 5 min [24]. After the enzyme treatment, the AC solution was titrated to pH 6.5 with 37% HCl.

Growth experiments were performed on 15% USU080 AC with 1.0 % meta-phosphoric acid buffer at pH 6.5, 15% AC treated with 1 U/ml α-amylase for 72 h, or APT broth (control). Fermentations, plating, supernatant collection, and lactic acid measurements were carried out as previously described [24]. The measured growth and lactic acid yields from this experiment were compared to the results obtained previously by Overbeck et al. [24], from AC treated with 180 U/ml α-amylase for 16 h. A Bonferroni correction for multiple pairwise comparisons was used to identify statistically significant differences in lactic acid yields from each sample [21].

Results and Discussion

Cellulase Activity

DNA sequence analysis confirmed the fidelity of the pDW2:*celE*-I construct in *E. coli* and *L. casei* 12A. However, no cellulase activity on the CMC plates was detected for either host. Additionally, no increase in the amount of reducing sugars was detected from *L. casei* 12A pDW2:*celE*-I CMC-supernatant mixtures at 37 or 60°C, using the Somogyi-Nelson method to quantify cellulase activity. Negative results from both assays indicate a lack of functional cellulase expression by *L. casei* 12A pDW2:*celE*-I. Bates et al. [6] previously reported approximately 2 U/ml cellulase activity from heterologous expression of the *celE* gene in *Lactobacillus plantarum*, using the Somogyi-Nelson method. In that study (Bates et al. [6]) cellulase activity was measured, in liquid cultures grown in a medium containing yeast extract, tryptone, casamino acids, sodium chloride, and glucose [6]. Our experiments showed this approach results in carryover of reducing sugars from the culture supernatant, and presence of those sugars in the sample during the assay can give a false indication of cellulase activity (unpublished data). This finding was discovered by incorporating the empty vector *L. casei* 12A pDW2 control culture in the assay. Because Bates et al. [6] only mentions measuring activity for the *L. plantarum* strain harboring the *celE* gene, and did not include a control, it cannot be known for certain whether the reported activity is correct. It should be noted that those authors [6] did report observing zones of hydrolysis on CMC plates which would indicate cellulase activity.

The *celE* construct used in this study was modeled after the work of Bates et al. [6], which used native *celE* promoter, RBS and signal peptide. The primary differences in our construct involved codon optimization of the CDS and incorporation of a NICE terminator. However, these substitutions would not be expected to diminish or prevent expression of *celE*. Thus, we are unable to ascertain the reason *celE* was not expressed in *L. casei* 12A.

Amylase Activity

DNA sequence analysis also confirmed the fidelity of pDW2:*amyA*-I and pDW2:*amyA*-II constructs in *E. coli* and *L. casei* 12A. Additionally, zones of clearing were visible on iodine-stained starch plates with *E. coli*, *L. casei* 12A pDW2:*amyA*-I and pDW2:*amyA*-II transformants, indicating functional amylase activity in all three hosts. Assays for amylase activity in the supernatant and crude cell lysate prepared from 6 h cultures of *L. casei* 12A pDW2: *amyA*-I was determined to contain 2.62 and 0.05 U/L, respectively. Our finding that 98.13 % of the amylase activity was present in the supernatant confirmed that the α-amylase was produced and secreted by *L. casei* 12A pDW2:*amyA*-I. The levels of amylase activity in the supernatant and crude cell lysate samples of 6 h cultures of *L. casei* 12A pDW2: *amyA*-II were higher (9.93 and 0.26 U/L, respectively), which confirmed replacement of Ppgm with Pp11 [28] increased activity of AmyA in *L. casei*.

To explore whether longer fermentation time would increase total α-amylase activity, fermentations were conducted with *L. casei* 12A pDW2:*amyA*-I or pDW2:*amyA*-II for a period of 120 h in MRS-ST. A steady increase in amylase activity was observed

over the duration of the fermentations, with the maximum measured amylase activities for *L. casei* 12A pDW2:*amyA*-I and pDW2:*amyA*-II at 120 h being 31.8 and 53.7 U/L, respectively (Fig. 4-3a). However, concomitant increases in lactic acid production were not observed (Fig. 4-3b), indicating α-amylase expression by these cells was not high enough to release a significant amount of fermentable carbohydrates, and that further optimization of the *amyA* cassette or *L. casei* 12A would be needed to realize this goal.

Narita et al. [18] reported that incorporation of a 5'-untranslated leader sequence (UTLS) from *Lactobacillus acidophilus* increased heterologous expression of the *S. bovis* 148 *amyA* gene by LAB to 900 U/ml (0.9 U/L). Since this is the highest level of amylase expression reported for LAB, we were curious to see if addition of a comparable level of α-amylase activity significantly increased the growth and lactic acid yields by *L. casei* 12A in AC fermentations. As shown in Fig. 4-3a, growth of *L. casei* 12A on AC digested with 1,000 U/L (1.0 U/ml) for 72 h was significantly increased versus the untreated AC, and reached a level that was comparable to growth on AC digested with 180 U/ml. However, there was not a significant increase in lactic acid production for AC digested with 1 U/ml (14.04 ± 2.93, 1.05 ± 0.26 g/L) when compared to untreated AC (5.95 ± 2.83 mM, 0.54 ± 0.26 g/L) (Fig. 4-3b). Thus, while 1 U/ml of amylase activity did release enough carbohydrates to increase growth, it did not hydrolyze enough starch to improve lactic acid production within a 72 h period.

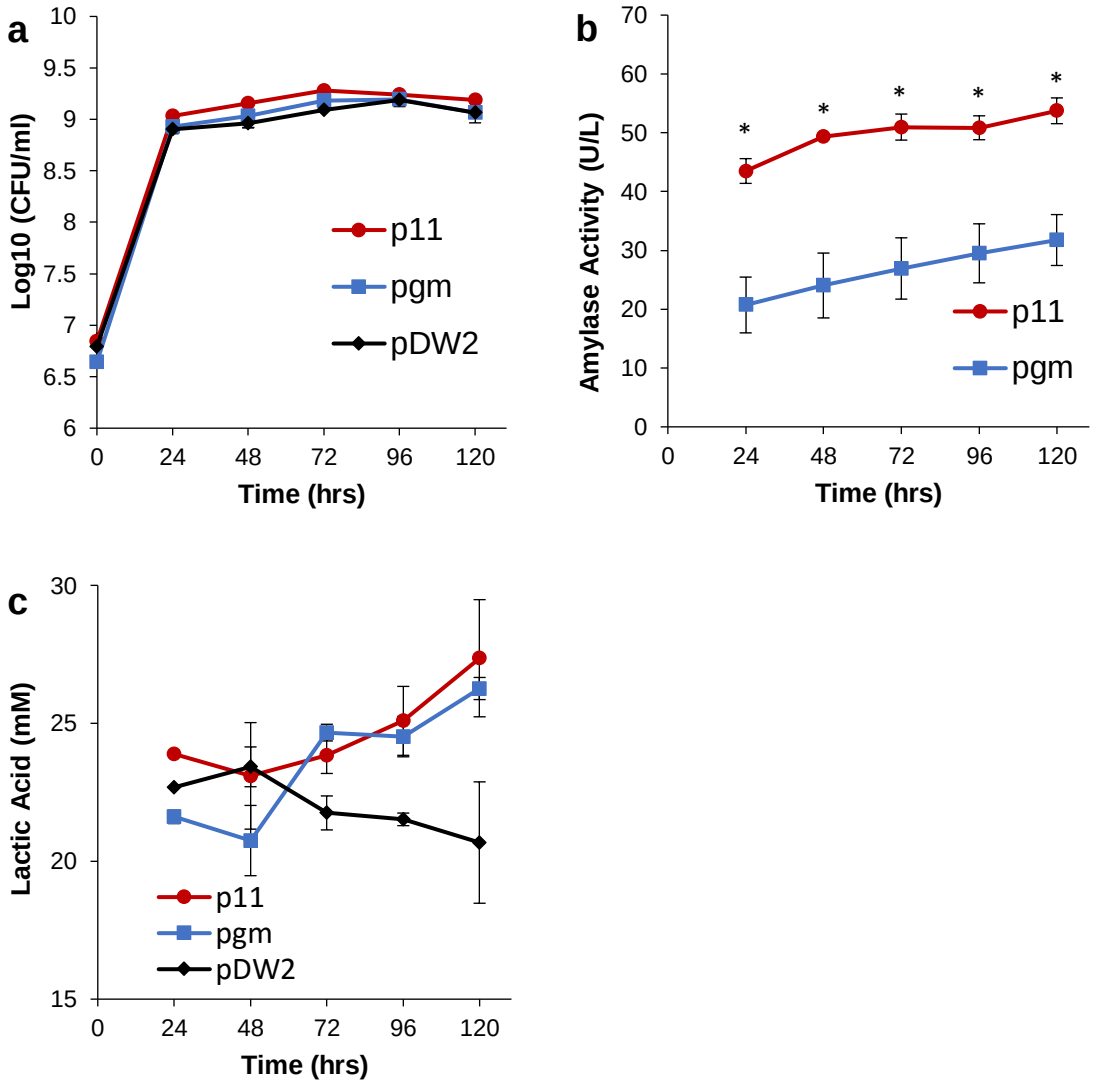

Fig. 4-2 Growth (a), amylase activity (b), and lactic acid yields (c) from *L. casei* 12A pDW2:*amyA*-I (circles), pDW2:*amyA*-II (diamonds), and the pDW2 (squares) on MRS-ST. *L. casei* 12A pDW2 was used as a blank for the amylase activity (b), with a value of 0 U/ml at each time point. Error bars represent SEM. Significant differences ($P < 0.05$) between cultures at each time point are indicated with a *.

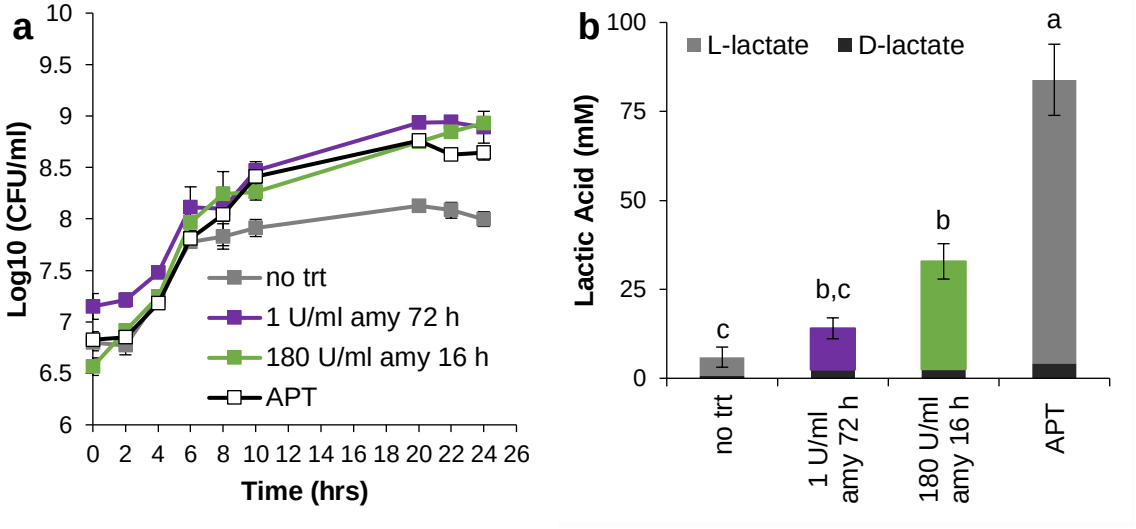

Fig. 4-3 (a) Growth of *L. casei* 12A on 15% algal cake (AC) solutions treated with 1 U/ml α-amylase for 72 h, 180 U/ml α-amylase for 16 h, or APT broth (control). (b) D-(Black) and L-(gray) lactic acid produced by *L. casei* 12A on each medium. Error bars represent SEM. Samples with the same letter are not significantly different ($P < 0.05$).

Conclusion

This study investigated heterologous expression of *celE* and *amyA* by *L. casei* 12A to improve lactic acid production from AC. No functional cellulase activity was observed from the codon-optimized *celE* construct, and strategies to obtain functional activity are unclear. In contrast, a low level of amylase activity was obtained for the Ppgm-driven *amyA* construct, and results showed expression could be increased using a highly active synthetic Pp11 promoter [28]. However, the increase in amylase activity achieved using Pp11 was not sufficient to increase lactic acid production by *L. casei* 12A in AC fermentations. Moreover, in vitro studies showed the highest reported level of

amyA expression by LAB significantly increased growth of *L. casei* 12A in 15% AC, but did not significantly increase in lactic acid production. Thus, methods previously shown to reduce production cost of enzymes in an industrial setting, such as an on-site [13] or integrated [11] approaches with efficient enzyme producing microorganisms may be more practical strategies for digesting AC material to increase lactic acid production.

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CHAPTER 5

DEVELOPMENT OF A TRANSIENT MUTS-BASED HYPERMUTATION SYSTEM FOR ADAPTIVE EVOLUTION OF *LACTOBACILLUS CASEI* TO LOW PH

Abstract

This study explored transient inactivation of the gene encoding the DNA mismatch repair enzyme MutS as a tool for adaptive evolution of *Lactobacillus casei*. MutS deletion derivatives of *L. casei* 12A and ATCC 334 were constructed and subject to a 100-day adaptive evolution process to increase lactic acid resistance at low pH. Wild-type parental strains were also subject to this treatment. At the end of the process, the Δ*mutS* lesion was repaired in representative 12A and ATCC 334 Δ*mutS* isolates. Growth studies in broth at pH 4.0 (titrated with lactic acid) showed all four adapted strains grew more rapidly, to higher cell densities, and produced significantly more lactic acid than untreated wild-type cells. However, the adapted Δ*mutS* derivatives showed the greatest increases in growth and lactic acid production. Further characterization of the 12A adapted Δ*mutS* derivative revealed it had a significantly smaller cell volume, a rougher cell surface, and significantly better survival at pH 2.5 relative to *L. casei* 12A. Genome sequence analysis confirmed transient *mutS* inactivation decreased DNA replication fidelity in both *L. casei* strains, and identified genetic changes that might contribute to the lactic acid resistant phenotypes of adapted cells. Targeted inactivation of three genes that had acquired nonsense mutations in the adapted 12A Δ*mutS* derivative showed that a NADH dehydrogenase (*ndh*), phosphate transport ATP-binding protein PstB (*pstB*), and

two-component signal transduction system (TCS) quorum-sensing histidine protein kinase (*hpk*) act in combination to increase lactic acid resistance in *L. casei* 12A.

Importance

Adaptive evolution has been applied to microorganisms to increase industrially desirable phenotypes, including acid resistance. We developed a method to increase the adaptability of *Lactobacillus casei* 12A and ATCC 334 through transient inactivation of the DNA mismatch repair enzyme MutS. Here, we show this method was effective in increasing resistance of *L. casei* to lactic acid at low pH. Additionally, we identified three genes that act in combination to increase acid resistance in *L. casei* 12A. These results provide valuable insight on methods to enhance an organism's fitness to complex phenotypes through adaptive evolution and targeted gene inactivation.

Introduction

Genetic mutations can have varying effects upon the fitness of an organism, and may be classified as neutral, deleterious, or beneficial. Beneficial mutations confer a growth advantage in a specific environment and therefore increase an organism's fitness [1]. In contrast, deleterious mutations decrease fitness and these mutations typically occur at a substantially higher rate than beneficial mutations [2]. To avoid deleterious mutations, the rate of genetic change in bacterial populations in stable environments is kept very low by mechanisms that ensure DNA replication and repair fidelity [3–5].

However, increased mutational rates can be beneficial to bacterial populations under adverse conditions, where mutations might help cells to overcome a selective pressure [2].

Cells with increased mutation rates (mutators) often occur as a small percentage of bacterial populations, and these cells display increased adaptability under stress conditions [6–8]. Mutator phenotypes are attributed to mutations in DNA replication and repair enzymes (mutator alleles) [9, 10], with strong mutators observed in cells with a deficient mismatch repair (MMR) system [11–13]. The MMR process is highly conserved in prokaryotes and eukaryotes, and ensures high DNA replication fidelity by correcting base-pair mismatches as well as small insertion or deletion loops [2]. In *E. coli* and other bacteria, MMR is initiated by the MutS enzyme during DNA replication, which recognizes and binds base-pair mismatches in association with the β-clamp of the DNA polymerase, resulting in a ATP-dependent conformational change [14]. This conformational change induces binding of the MutL protein and subsequent activation of the endonuclease MutH, allowing for excision of the mismatched base from the unmethylated daughter strand [14].

Adaptive laboratory evolution has been used to gain insight into the role that DNA replication and repair systems such as MMR have upon a microorganism's ability to evolve under specified growth conditions, and to select for "improved" strains with beneficial characteristics for biotechnological/industrial processes [13, 15, 16]. The adaptive evolution process allows for the emergence of these improved strains, with increased tolerance to various defined stresses, by coupling the desired phenotype to growth [16].

Lactobacillus casei is an aciduric heterofermentative lactic acid bacterium (LAB) that produces the industrially valuable chemical lactic acid, as its major metabolic end-product through carbohydrate fermentation. Costs for industrial production of lactic acid from microorganisms such as *L. casei* can be significantly reduced if fermentations are conducted at low pH, since lactic acid (pKa 3.86) is more efficiently extracted in the undissociated form [17, 18]. Like many other bacteria, survival of *L. casei* at low pH is enhanced by induction of an acid tolerance response (ATR) [19–21], and physiological characterization of this ATR has provided valuable insight for conducting *L. casei* fermentations at low pH [19, 20]. Moreover, adaptive evolution has also been successfully used to increase lactic acid resistance in *L. casei* [22].

It was our hypothesis that the adaptive laboratory evolution of *L. casei* to complex phenotypes such as increased lactic acid resistance at low pH could be enhanced through deliberate use of mutator cells. To test this idea, we developed a system for transient inactivation of *mutS* in *L. casei,* then performed adaptive evolution experiments to select for mutants that grew in broth adjusted to pH 4.0 with lactic acid. Mutants with the desired growth properties were isolated and, after restoration of *mutS*, the adapted strains were analyzed in more detail for genotypic and phenotypic changes. Genome resequencing confirmed transient MutS inactivation decreased DNA replication fidelity, and identified genetic changes that contribute to lactic acid resistance. Targeted mutagenesis confirmed a role for three genes in increased lactic acid resistance at low pH in *L. casei* 12A.

Results

Adaptive evolution

DNA sequence analysis confirmed deletion of the full *mutS* coding sequence (CDS) from the genomes of *L. casei* 12A and ATCC 334, which yielded mutator cells with impaired DNA mismatch repair for adaptive evolution experiments. Those strains, and wild type (wt) 12A and ATCC 334 were then subject to serial passage in MRS with decreasing pH (5.5 to 4.0), acidified with lactic acid, for a period of 100 days to select for derivatives with increased tolerance to lactic acid at low pH. At the end of the adaptive evolution process, seven lactic acid tolerant isolates were collected from each of the adapted strain suspensions, and the isolate which showed the most rapid growth rate in MRS broth at pH 4.0 was selected as the representative isolate. Next, the *mutS* lesion was successfully repaired in the representative *L. casei* 12A:Δ*mutS* and 334:Δ*mutS* adaptive evolution isolates (generating strains *L. casei* 12A-MAE and 334-MAE), to restore functional MutS activity and yield stable genetic derivatives for subsequent phenotypic and genotypic analyses.

Characterization of the acid tolerant phenotype in adapted cells

Growth studies in MRS at pH 4.0, adjusted with lactic acid, with wt *L. casei* 12A and ATCC 334, the wt adapted strains (12A-AE and 334-AE), and the Δ*mutS* adapted strains with the *mutS* lesion repaired (12A-MAE and 334-MAE), showed all four adapted strains grew significantly more rapidly and to a significantly higher final cell density than the respective unadapted parental wt strains (Fig. 5-1A, B). Moreover, the 12A-MAE and

334-MAE strains grew significantly more rapidly and to significantly higher cell densities than 12A-AE and 334-AE, respectively. (Fig. 5-1A, B). Additionally, significant decreases in pH and increases in total lactic acid production were observed by all four adapted strains when compared to their parental wt (Fig. 5-1C, D). While the lowest pH values were observed in both MAE strains, the terminal pH of 12A-MAE was not significantly different from 12A-AE. However, both 12A-MAE and 334-MAE did produce significantly more lactic acid during the pH 4.0 growth experiment than any of the other strains (Fig. 5-1D).

To determine whether the adaptive evolution process had also increased resistance to very low pH, early stationary phase cells were challenged at pH 3.0, 2.5, and 2.0 for 1 h. As shown in Fig. 5-2, a high percent survival by all three strains was observed at pH 3.0, and no survival at pH 2.0. However, results from pH 2.5 revealed a greater than 10-fold increase in the percent survival of strain 12A-MAE compared to the parental wt strain, while 12A-AE showed no detectable survival.

Morphological examination by scanning electron microscopy of 12A and 12A-MAE cells collected from steady state fermentations at pH 3.8 showed the cell surface of 12A-MAE had a rougher cell surface compared to wt 12A cells (Fig. 5-3). Additionally, a significant decrease in the length and width of the 12A-MAE cells was observed (1.25 ± 0.10 and of 0.436 ± 0.010 μm, respectively) when compared to 12A wt cells (1.60 ± 0.11 and 0.630 ± 0.014 μm, respectively).

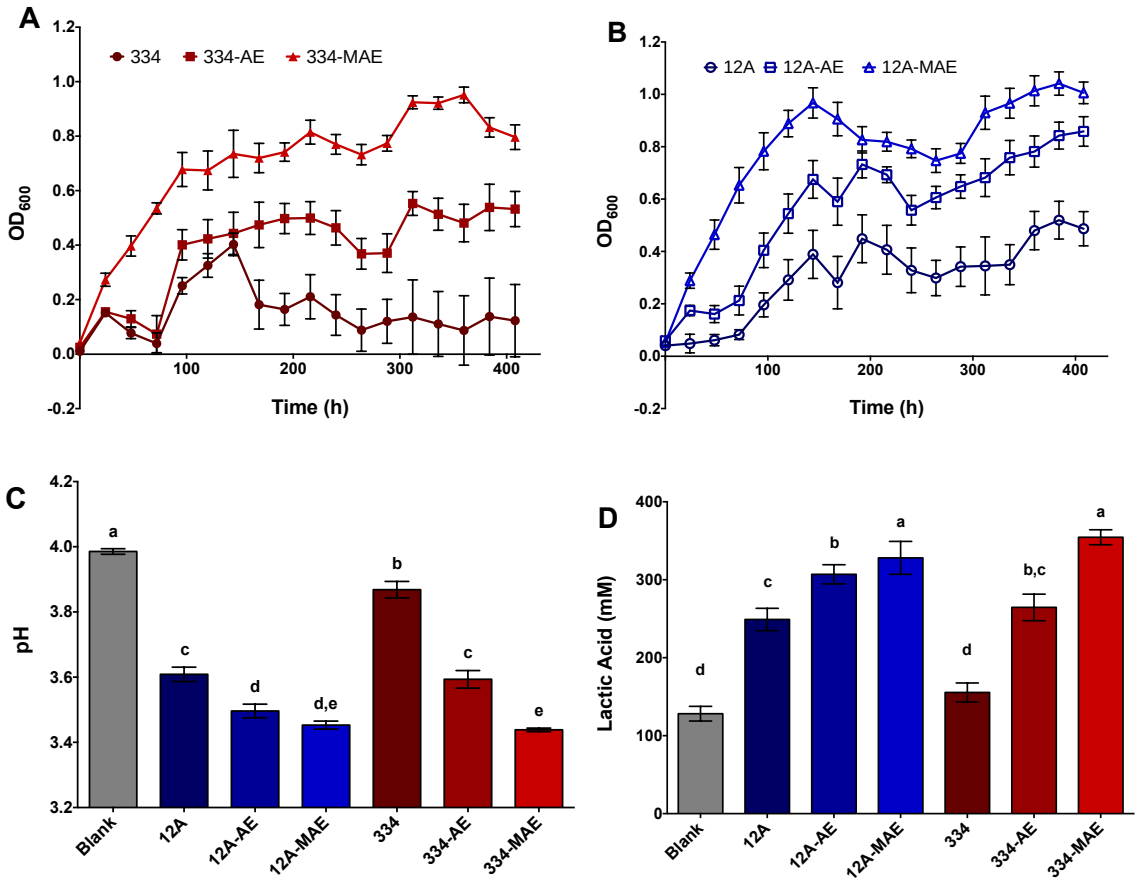

Fig. 5-1 Growth of the *L. casei* ATCC 334 (A) and 12A (B) parental wild-type strains (334 and 12A), the wild-type acid adapted strains (334-AE and 12A-AE), and the Δ*mutS* acid adapted derivatives (334-MAE and 12A-MAE) in MRS at pH 4.0, titrated with lactic acid, as well as the terminal pH (C) and lactic acid concentrations (D) after growth experiments. Error bars represent the SEM, and treatments with the same letter are not signficantly different.

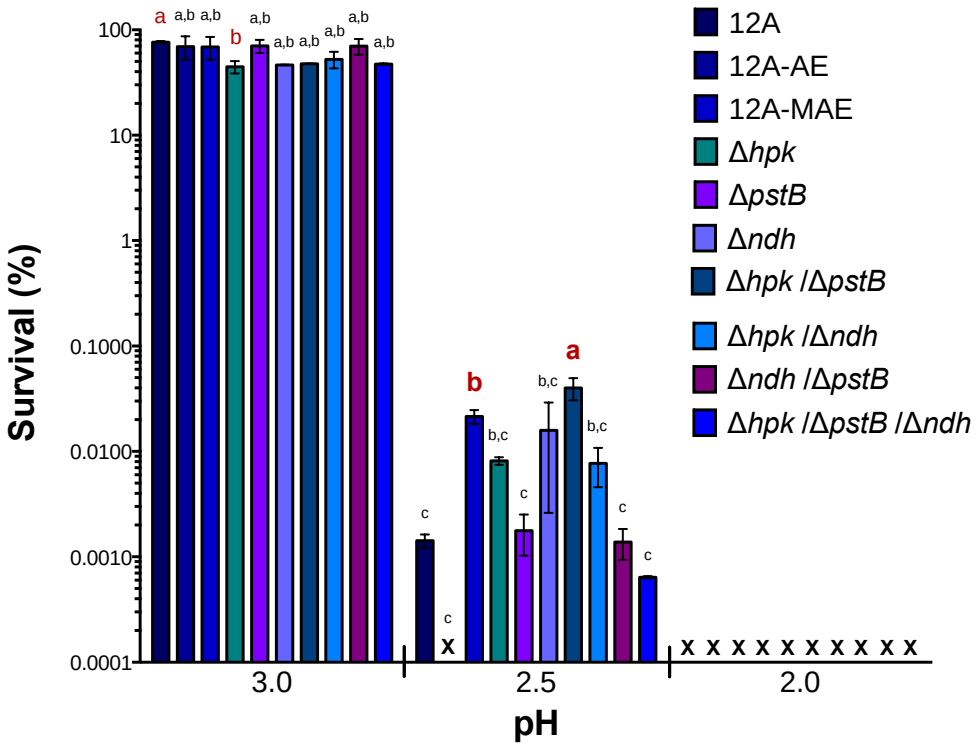

Fig. 5-2 Percent survival of the *L. casei* 12A parental wild-type strain (12A), the wild-type acid adapted strain (12A-AE), the Δ*mutS* acid adapted derivative (12A-MAE), and deletion derivatives 12A:Δ*hpk*, 12A:Δ*pstB*, 12A:Δ*ndh*, 12A:Δ*hpk*/Δ*pstB*, 12A:Δ*hpk*/Δ*ndh*, 12A:Δ*ndh*/Δ*pstB*, and 12A:Δ*hpk*/Δ*pstB*/Δ*ndh* after acid challenge at pH 3.0, 2.5, and 2.0 for 1 h[2]. Error bars represent the SEM, and treatments with the same letter within a pH group are not signficantly different.

[2] *L. casei* 12A:Δ*hpk*, 12A:Δ*pstB*, 12A:Δ*ndh*, 12A:Δ*hpk*/Δ*pstB*, 12A:Δ*hpk*/Δ*ndh*, 12A:Δ*ndh*/Δ*pstB*, and 12A:Δ*hpk*/Δ*pstB*/Δ*ndh* deletion derivatives are discussed later in the chapter.

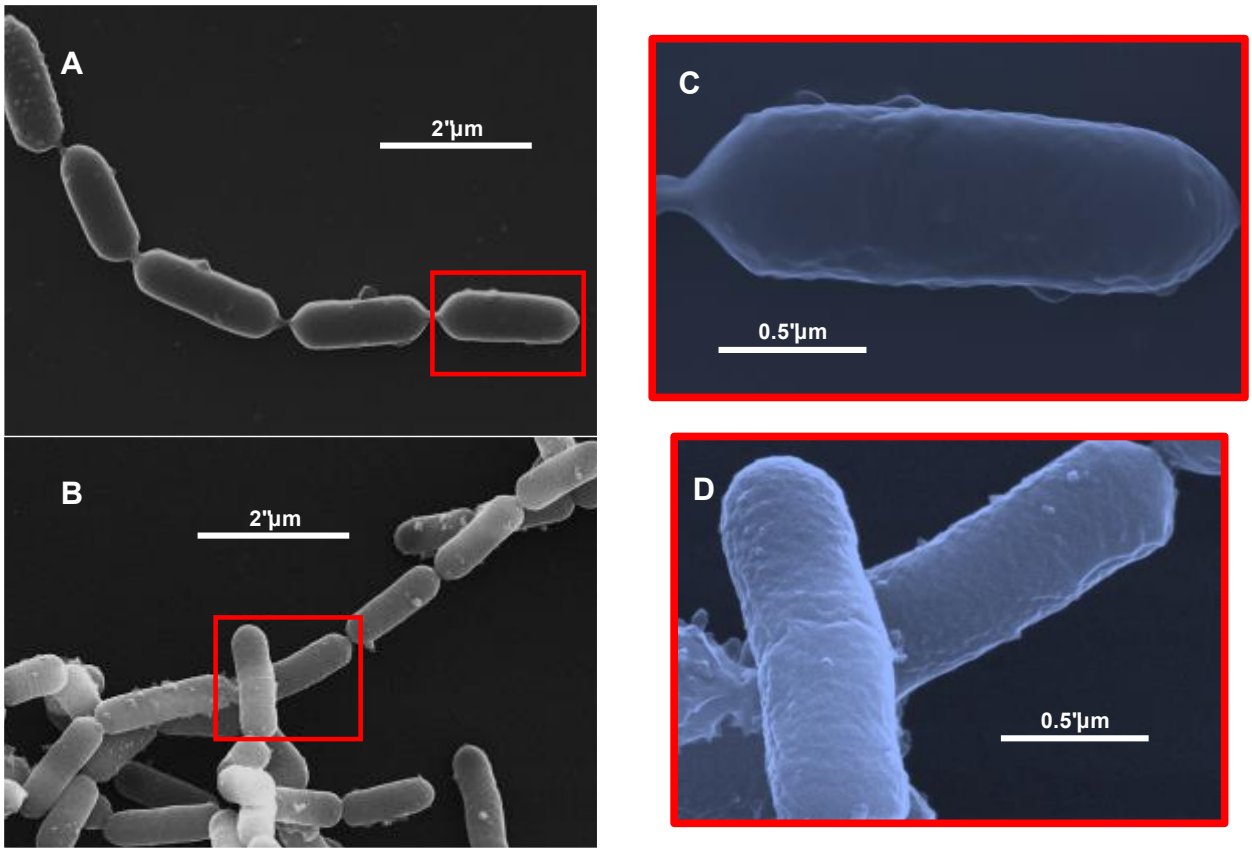

Fig. 5-3 Scanning electron microscope images of *L. casei* 12A parental wild-type cells (A, C), and the 12A Δ*mutS* acid adapted derivative (12A-MAE) (B, D). Images A and B depict multiple cells, and the red boxes within these images represent the areas where images C and D were captured, respectively. Images C and D provide close examination of the cell surface.

Genotypic characterization of acid tolerant mutants.

Whole genome resequencing of ATCC 334 acid adapted strains revealed that strain 334-MAE had accumulated over one thousand mutations in the genome. Further analysis revealed that several of these mutations had occurred within genes for other DNA repair enzymes, with nonsense mutations occurring in three DNA repair enzymes (Table A-4). In contrast, sequencing of 12A-AE identified 19 mutations, of which 13 were within predicted ORFs. Three mutations were predicted to be silent, five to result in an amino

acid substitution, one to result in the loss of a stop codon, and four to introduce nonsense mutations (Table A-5). Resequence data for 12A-MAE showed there was roughly a 3.3-fold increase in the number of mutations relative to strain 12A-AE, with 62 total mutations. Of these, 55 were predicted to occur within ORFs, with 18 predicted to be silent, 30 to introduce an amino acid substitution, one to result in the loss of a start codon, and six to create nonsense mutations (Table A-6). Surprisingly, no mutations were shared between 12A-AE and 12A-MAE. The nonsense mutations in 12A-AE and 12A-MAE all resulted from a single bp deletion. The six nonsense mutations identified in 12A-MAE affected genes encoding two 51 kDa hypothetical membrane proteins (*hyp*I, LCA12A_2604 and *hyp*II, LCA12A_0710), sortase A (LPXTG specific, *srtA*, LCA12A_2465), a NADH dehydrogenase (*ndh*, LCA12A_1744), phosphate transport ATP-binding protein PstB (*pstB*, LCA12A_2227), and a two-component signal transduction system (TCS) quorum-sensing histidine kinase (*hpk*, LCA12A_1895). The presence of all six nonsense mutations in 12A-MAE was verified by DNA sequence analysis of site-specific PCR products. Interestingly, when the day-100 acid-adapted populations were screened for the presence of these six nonsense mutations only the *hyp*I, *srtA*, and *ndh* were detected. Moreover, all three were evidenced as minor peaks within the DNA sequencing chromatograms, indicating the mutations were present within a subset of the day-100 culture population.

To explore the relationship between some of the nonsense mutations and the phenotypic characteristics of 12A-MAE, we introduced single, double, and triple gene deletions for *hpk*, *pstB*, and *ndh* in wt *L. casei* 12A. All seven deletion derivatives were successfully constructed and confirmed by DNA sequence analysis (Table 5-1). Growth

studies with these derivatives in MRS at pH 4.0, titrated with lactic acid, showed *L. casei* 12A:Δ*ndh*/Δ*pstB* had a significantly increased ability to grow at pH 4.0, when compared to wt 12A (Fig. 5-4). Furthermore, *L. casei* 12A:Δ*ndh*/Δ*pstB* also significantly decreased the final pH and increased lactic acid production relative to wt 12A. While the decrease in pH was not significantly different from that seen with 12A-MAE, *L. casei* 12A:Δ*ndh*/Δ*pstB* did produce significantly less lactic acid than 12A-MAE.

The seven *L. casei* 12A deletion derivatives were also tested for their ability to survive a one-hour acid challenge at pH 3.0, 2.5, and 2.0. None of the strains exhibited survival at pH 2.0 (Fig. 5-2). At pH 3.0 a significant decrease in the percent survival of 12A:Δ*hpk* was observed when compared to wt 12A, while the other strains were not significantly different from the 12A wt or 12A:Δ*hpk*. At pH 2.5, 12A:Δ*hpk*/Δ*pstB* displayed significantly higher survival than any of the other deletion derivatives, as well as wt 12A, 12A-AE, and even 12A-MAE. Survival of strains 12A: Δ*hpk*, 12A:Δ*ndh*, and 12A:Δ*hpk*/Δ*ndh* was not significantly different from 12A-MAE, 12A, or 12A-AE.

L. casei 12A:Δ*ndh*/Δ*pstB* and 12A:Δ*hpk*/Δ*pstB* deletion derivatives were examined for morphological changes by SEM, and no significant decrease in cell length and width (1.54 μm and 0.617 μm, and 1.58 μm and 0.651 μm, respectively) was observed for either deletion derivative. Revealing inactivation of *ndh*, *pstB*, and *hpk* do not contribute to the observed decreased cell volume of *L. casei* 12A-MAE.

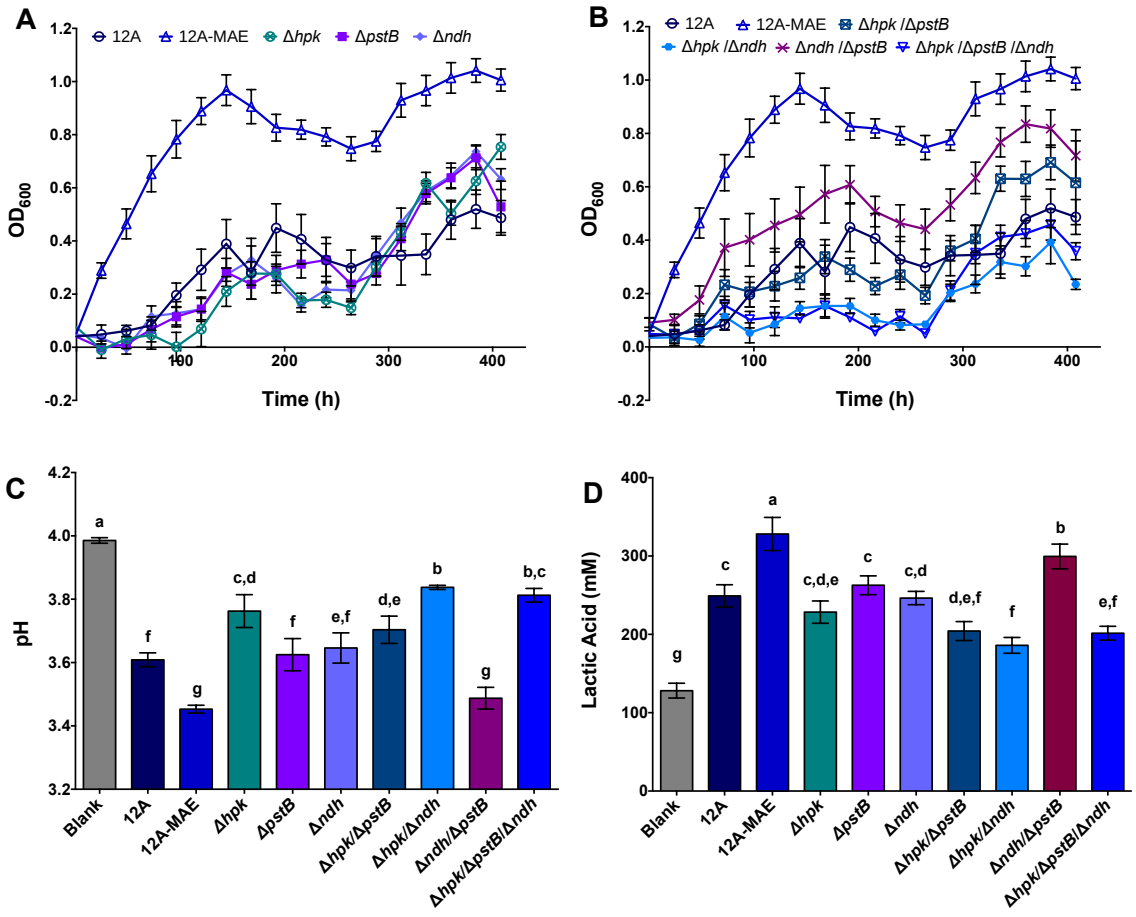

Fig. 5-4 (A, B) Growth of the *L. casei* 12A parental wild-type strain (12A), the 12A Δ*mutS* acid adapted derivative (12A-MAE), and deletion derivatives 12A:Δ*hpk*, 12A:Δ*pstB*, 12A:Δ*ndh*, 12A:Δ*hpk*/Δ*pstB*, 12A:Δ*hpk*/Δ*ndh*, 12A:Δ*ndh*/Δ*pstB*, and 12A:Δ*hpk*/Δ*pstB*/Δ*ndh* in MRS at pH 4.0, titrated with lactic acid, as well as the terminal pH (C) and lactic acid concentrations (D) after growth experiments. Error bars represent the SEM, and treatments with the same letter are not signficantly different.

Discussion

Under stressed conditions hypermutability can be leveraged to increase an organism's fitness and achieve a desired phenotype through adaptive laboratory evolution [1–3]. High mutation rates resulting from a deficient MMR process have been previously

shown to increase the fitness of an organism in defined environments [13, 23]. In this study, we explored the potential for coupling transient inactivation of the MMR process with adaptive evolution to obtain *L. casei* strains with increased lactic acid resistance at low pH. While both the wt and Δ*mutS* adapted strains (AE and MAE, respectively) each evolved to grow significantly better than unadapted wt cells at low pH, genome resequencing of the mutants confirmed *mutS* inactivation decreased DNA replication fidelity and yielded derivatives with a greater number of genetic changes (Table S2 and S3). Previous studies investigating the effect of inactive MMR on the mutation rate have shown increased mutation rates from 10 to 1,000-fold [12, 13, 23, 24]. Our observed 3.3-fold increase in the number of mutations in 12A-MAE is lower than the rates previously reported. However, we believe this difference is explained by the difference between methods used to identify mutations. In previous studies the mutation rate was calculated for hypermutable cells using reversion assays [12, 13, 23, 24], whereas we determined the number of mutations using genome resequencing after the adaptive evolution process. We expect that many mutations not selected for under our strong selective pressure were incidentally filtered out during the extended adaptive evolution process, reducing the final number of mutations observed in 12A-MAE.

Interestingly, genome sequence data for *L. casei* 334-MAE revealed a substantially higher number of mutations than were found in 12A-MAE. Examination of these mutations showed many had occurred in genes for other DNA repair enzymes, which suggested that the high number of mutations observed in 334-MAE is likely a result of even lower DNA replication fidelity during the adaptive evolution process. Growth studies in MRS at pH 4.0 show wt *L. casei* ATCC 334 is inherently less tolerant

to low pH when compared to 12A (Fig. 5-1A, B). Since hypermutability may increase cell adaptability under stressed conditions [7], it is possible that the additional decrease in replication fidelity observed in 334-MAE created a strong hypermutable background that enabled adaptation to low pH. However, the 334-MAE genome sequence also revealed that restoration of the *mutS* gene in this strain would not restore replication fidelity, and illustrates one possible risk of using MutS as a target for inducing transient hypermutation.

Given the lack of genomic stability in 334-MAE and the finding that growth and lactic acid production at low pH by *L. casei* 12A derivatives was superior, we selected the latter strains for more detailed characterization. Growth studies after adaptive evolution confirmed the laboratory-derived mutator cells (Δ*mutS*) yielded 12A mutants (12A-MAE) with significantly better growth and lactic acid production at pH 4.0 than wt adapted cells (12A-AE) (Fig. 5-1B, C, D). Genome resequencing showed these two strains had acquired different sets of mutations, and acid challenge studies revealed other phenotypic differences. The 12A-MAE strain had significantly better survival at pH 2.5 versus wt cells, while the 12A-AE strain had significantly reduced survival relative to the wt (Fig. 5-2). Thus, 12A-MAE appears to have accrued beneficial mutations during the adaptive evolution process with respect to survival at pH 2.5, while 12A-AE apparently acquired mutations that were deleterious for that environment. This finding also shows that while a desired phenotype such as growth at low pH can successfully be selected for using adaptive evolution, undesirable outcomes in other associated phenotypes such as survival at low pH can incidentally emerge from the process.

Previous studies on adaptive evolution [22, 25] and adaptation to lactic acid stress [26–28] have sometimes revealed changes to the cell surface and reduced cell volume. We observed similar changes to 12A-MAE compared to wt 12A (Fig. 5-3). Decreased cell volume is sometimes associated with a diminished growth rate resulting from nutrient limitations [29–32], but this likely does not explain the differences observed here since 12A and 12A-MAE cells were grown in the same medium and under the same conditions. Alternatively, it is possible that a decrease in cell volume by 12A-MAE may allow this strain to better combat acid stress by reducing the volume of intracellular space to buffer and/or increasing the concentration of intracellular enzymes involved in acid resistance. However, the genetic basis for the observed decrease in cell volume remains unclear.

To better explore the genetic basis for the lactic acid resistant phenotype of 12A-MAE, we screened the final 12A:Δ*mutS* adapted culture for each of the six nonsense mutations identified in 12A-MAE. Only the nonsense mutations in *hyp*I, *srtA*, and *ndh* were detected in sequencing chromatograms from the population of cells, but this finding does not preclude the possibility that mutations to *hyp*II, *pstB*, and *hpk* were present in a smaller subset of the final 12A:Δ*mutS* adapted culture (and so were not apparent in the sequencing chromatograms). Since all six nonsense mutations were present in 12A-MAE, we targeted three genes for further study: *pstB*, *hpk*, and *ndh*. The *pstB* gene is located within a phosphate (Pho) regulon, controlled by a TCS, and the *hpk* gene is part of a TCS located within a bacteriocin gene cluster. These genes were chosen because of their roles in signal transducing pathways [33, 34], while the *ndh* gene was chosen because it's nonsense mutation was detected in the final 12A:Δ*mutS* adapted culture.

Growth studies with seven single, double, and triple deletion mutants in MRS at pH 4.0, revealed that the combined deletion of *pstB* and *ndh* (12A:Δ*ndh*/Δ*pstB*) increased growth, and significantly increased the ability of 12A to produce lactic acid at pH 4.0 (Fig. 5-4B-D). No enhancement of growth or lactic acid production was observed with any of the other deletion mutants (Fig. 5-4). Similarly, acid challenge tests showed the 12A:Δ*hpk*/Δ*pstB* double mutant had significantly improved survival at pH 2.5, even compared to 12A-MAE (Fig. 5-2). Improved survival at pH 2.5 relative to wt 12A was not observed for any of the other deletion constructs. Our discovery that one *pstB* double deletion mutant improved growth and lactic acid production at low pH, while another improved survival at pH 2.5 indicates intracellular phosphate pools influence both phenotypes. Our findings also demonstrate that the physiological basis for these two phenotypes does not fully overlap.

The Pho regulon is responsible for inorganic phosphate (Pi) uptake by Pst proteins, and is controlled by a TCS in all bacteria containing a Pho regulon characterized to date [33, 35]. Studies in *Escherichia coli* have revealed this regulon is not only important for regulation of phosphate uptake, but also affects genes involved in virulence and pathogenesis, secondary metabolite production, nutritional regulation, and stress responses, including the ATR [35–38]. The Pho regulon in *E. coli* is tied to the expression of acid shock proteins [39, 40], sigma factors [35, 37, 38], chaperones [38], and acid resistance systems, including glutamate dependent acid resistance (GDAR) [38]. Inactivation of *pst* in *E. coli* up-regulates the Pho regulon, and increases the expression of genes involved in the ATR [38]. Inactivation of the TCS (TC04) from the *L. casei* Pho regulon in strain BL23 resulted in a decreased ability to grow at pH 3.75 and increased

tolerance to antibiotics that target the cell envelope (nisin and bacitracin) [42]. Those authors concluded the *L. casei* BL23 Pho regulon is involved in cell envelope stress tolerance [42]. Additionally, cross-talk with other TCS involved in secondary metabolite production, such as bacteriocins, is a common feature of the Pho regulon [35]. Inactivation of *pstB* alone did not have any detectable effect on growth and lactic acid production by *L. casei* 12A at pH 4.0, or survival at pH 2.5 (Fig. 5-4A). However, characterization of 12A:Δ*hpk*/Δ*pstB* and 12A:Δ*ndh*/Δ*pstB* double mutants show the Pho regulon is tied to these phenotypes (see above). Moreover, Our finding that the double deletion 12A:Δ*hpk*/Δ*pstB* had increased survival at pH 2.5 suggest potential cross-talk between the 12A Pho regulon and at least one TCS quorum-sensing HPK (*hpk*).

The ability to sense and respond to extracellular stresses is essential for bacterial survival under adverse conditions such as low pH. In lactobacilli and other gram-positive organisms, TCSs comprised of a HPK and response regulator (RR) often serve as stress sensors, and induce expression of genes that enhance cells' ability to survive [41–45]. The HPKs in gram-positive bacteria are usually part of the HPK_{10} subfamily [46, 47], some of which participate in quorum sensing through recognition of an inducing peptide pheromone (IP), and subsequently induce regulation of bacteriocin operons [46, 47]. The *hpk* gene of *L. casei* 12A is 1,299 bp in length and belongs to the HPK_{10} subfamily, and has a 95% DNA and 94% amino acid sequence similarity (with 100% sequence similarity in the conserved H, N and G regions) to the HPK Prck in *L. paracasei* E93490 [46]. The 3' end of the *L. casei* 12A *hpk* gene (432 amino acids), which contains the H, N and G regions, is also highly similar (95% DNA and 98.6% amino acid sequence similarity) to a smaller HPK_{10} (280 amino acids) in the TC13 system in *L. casei* BL23 [42], which is also

located within the same quorum sensing bacteriocin gene cluster. Inactivation of TC13 in *L. casei* BL23 decreased ability to grow at pH 3.75 [42]. Growth studies with *L. casei* 12A:Δ*hpk* at pH 4.0 show this strain also grew more poorly than the wt 12A over most of the assay, but ultimately did reach a significantly higher final cell density (Fig. 5-4A). However the terminal pH for the 12A:Δ*hpk* mutant was significantly higher than that of the wt 12A. Our findings also show that inactivation of *hpk* and *pstB* together can significantly increase survival at pH 2.5, so the role of this TCS in acid adaptation remains unclear.

NAD is a cofactor in essential biological processes. The ratio of NAD^+/NADH plays an important role in regulating the intracellular redox state and the metabolism of cells [48, 49]. Metabolism of carbon sources (e.g., glucose) during growth results in the reduction of NAD^+ to NADH. To maintain growth, NAD^+ must be regenerated through oxidation of NADH. This can be accomplished by coupling oxidation of NADH to the reduction of metabolic intermediates (i.e., fermentation) [50], or through reduction of terminal electron acceptors by NADH dehydrogenases [51, 52]. Consequently, strains deficient in NADH dehydrogenase activity are expected to have a decreased NAD^+/NADH ratio. Surprisingly, *Propionibacterium acidipropionici* mutants adapted to increased propionic acid levels with decreased NADH dehydrogenase expression [53] showed an increased NAD^+/NADH ratio at low pH [54]. *Lactobacillus casei* 12A contains two potential NADH dehydrogenases, the one targeted here and another located in cluster with a putative pheromone precursor. Inactivation of one these NADH dehydrogenases may increase fermentative metabolic flux in *L. casei* 12A cells, and alter the NAD^+/NADH ratio. Additionally, NAD^+ is an essential cofactor for DNA ligases in

all bacteria, involved in DNA replication, recombination, and repair reactions [55–57]. Therefore, an increased concentration of NAD^+ could also be beneficial in combating DNA damage caused by low pH. While inactivation of *ndh* alone had no detected influence on growth or lactic acid production at pH 4.0 by *L. casei* 12A, this phenotype was observed in the 12A:Δ*ndh*/Δ*pstB* double mutant. That finding indicates an altered NAD^+/NADH ratio may contribute to increased growth and lactic acid production at low pH.

In summary, we have demonstrated that transient MutS inactivation can be effectively combined with adaptive evolution to select for *L. casei* mutants with complex phenotypes such as lactic acid resistance. Genome resequencing confirmed *mutS* inactivation decreased DNA replication fidelity during the adaptive evolution process, and yielded mutants with significantly better growth and lactic acid production at low pH compared to adapted wt cells. However, hypermutation in 334:Δ*mutS* showed the potential of this approach to affect other genes associated with replication fidelity and prevent restoration of a stable genotype. Genome resequencing and directed mutagenesis also revealed that inactivation of a NADH dehydrogenase (*ndh*) and phosphate transport ATP-binding protein PstB (*pstB*) can increase the ability of *L. casei* 12A to grow and produce lactic acid at pH 4.0. Additionally, the survival of *L. casei* 12A at pH 2.5 can be increased by inactivation of a two-component signal transduction system (TCS) quorum-sensing HPK (*hpk*) and *pstB*.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this work are listed in Table 5-1. *L. casei* and *E. coli* strains were maintained in a laboratory culture collection at -80°C in 15% glycerol stocks. *L. casei* strains were propagated without aeration at 37 °C in MRS broth (Difco Laboratories, Detroit, MI), with 2.5μg/ml erythromycin (Ery) when appropriate. *E. coli* strains were propagated with aeration at 200 rpm and 37 °C in LB broth (Difco Laboratories), with 50 μg/ml ampicillin (Amp) or 100 μg/ml Ery when appropriate.

Gene deletions

The *mutS* gene (2.58 Kb) was deleted in its entirety from *L. casei* 12A and ATCC 334 genomes by gene replacement as described previously [58]. A list of the PCR primers used in this study can be found in Table 5-2. Genomic *L. casei* DNA was isolated by lysing the cells with lysozyme and proteinase K, cleaning with chloroform, and precipitation with ethanol, as previously described [88]. Briefly, 5' and bp 3' fragments upstream and downstream, respectively, of the *mutS* gene were first obtained by PCR amplification of *L. casei* 12A genomic DNA using the Phusion high-fidelity DNA polymerase kit (New England Biolabs, Ipswich, MA). A 960 bp 5' fragment was obtained with primers *mut*5'-f x *mut*5'-r, which respectively contain *Xba*I and *Pst*I restriction linkers, while a 1,210 bp 3' fragment was obtained with primers *mut*3'-f x *mut*3'-r, which respectively contain *Pst*I and *Xma*I linkers. Ends of the 5' and 3' PCR

Table 5-1 Bacterial strains and plasmids used in *Lactobacillus casei* adaptive evolution study.

Strains and plasmids	**Relevant Characteristics**	**Source**
E. coli DH5α	Cloning host	Lab
E. coli EC1000	Cloning host, *repA*$^{+}$	Lab
L. casei ATCC334		
334:Δ*mutS*	*mutS* gene deletion	This study
334-AE	334 final adaptive evolution representative isolate	This study
334-MAE	334:Δ*mutS* adaptive evolution isolate with *mutS* restored	This study
L. casei 12A		
12A:Δ*mutS*	*mutS* gene deletion	This study
12A-AE	12A final adaptive evolution isolate	This study
12A-MAE	12A:Δ*mutS* adaptive evolution isolate with *mutS* restored	This study
12A:Δ*hpk*	*hpk* gene deletion	This study
12A:Δ*pstB*	*pstB* gene deletion	This study
12A:Δ*ndh*	*ndh* gene deletion	This study
12A:Δ*hpk*/Δ*pstB*	*hpk* and *pstB* gene deletions	This study
12A:Δ*hpk*/Δ*ndh*	*hpk* and *ndh* gene deletions	This study
12A:Δ*ndh*/Δ*pstB*	*ndh* and *pstB* gene deletions	This study
12A:Δ*hpk*/Δ*pstB*/Δ*ndh*	*hpk*, *pstB*, and *ndh* gene deletions	This study
pBS1	pCJK47 *lacZ*$^{-}$ derivative, *repA*$^{-}$; P-*pheS*$^{+}$; Ery^{R}	[62]
pBSΔ*mutS*	pBS1; *mut*5'-f x *mut*5'-r; *mut*3'-f x *mut*3'-r	This study
pBS*mutS*	pBS1; *mut*5'-f x *mut*3'-r	This study
pBSΔ*hpk*	pBS1; *hpk*2 x *hpk*3; *hpk*4 x *hpk*5	This study
pBSΔ*pstB*	pBS1; *pst*5'-f x *pst*5'-r; *pst*3'-f x *pst*3'-r	This study
pBSΔ*ndh*	pBS1; *ndh*2 x *ndh*3; *ndh*4 x *ndh*5	This study

Lab = laboratory culture collection.

Table 5-2 PCR primers used in *Lactobacillus casei* adaptive evolution study. Restriction sites within the primer DNA sequence are underlined.

Primer	Sequence	Restriction Site
*mut*5'-f	GTTTTCTAGATTTCCAAGGATCAACTGAACGGCTG	*Xba*I
*mut*5'-r	GTTTTCTGCAGTTCGTCCTTTGACTTTCTTTGACGTTCTATATG	*Pst*I
*mut*3'-f	GTTTTCTGCAGTTTTATGCCAAAGATTCACCAACTATCGG	*Pst*I
*mut*3'-r	GTTTTCCCGGGTTGCGGTTCGTTAAAAATCGGCTG	*Xma*I
*mut*1	TATTGGTACGGTATTGTG	
*mut*2	TACATAAGAACCTTGAAG	
*mut*3	GTCAGAATTTTACTGGTG	
mut4	GTACCAAAAGCAACTCGG	
mut5	GAACGCAAACAGACACTG	
mut6	TCAAAGTTGACGCACCTG	
mut7	GTTTGAACATTTTCTCAAG	
*hpk*2	AAAAAAGGATCCGAAAAGATATGATATACTGTCACC	*Bam*HI
*hpk*3	AAAAAAAAGCTTGACCTCTGAATGCTGAGTGGGA	*Hind*III
*hpk*4	AAAAAAAAGCTTATGACCAACCACCAAACGATGCTG	*Hind*III
*hpk*5	AAAAAACCCGGGAGCGATACCTCCTTCAGGTTTCCC	*Xma*I
*hpk*1	AGTCAAATTCTGCGAAGGCTTGTG	
*hpk*6	TAAACAAAACGGCCATGCGTGTCC	
*pst*1	CGTTCTAGATTAGCCGTGCATATTT	*Xba*I
*pst*2	AATGAATTCCCTCCTAGCCGAA	*Eco*RI
*pst*3	GTGGAATTCATGCGACGACTTT	*Eco*RI
*pst*4	CGTGCATGCTTGATTACATGATTAG	*Sph*I
*pst*5	GCGGCCCTGATTTATACA	
*pst*6	GCGTTCTTCTACAGCGGAA	
*pst*7	AGCGCACATGAAGATCGT T	
*pst*8	CCTGCTGGGAACCTTGAC	
*ndh*2	AAAGGATCCTGATTCCGGCACCGTTGAAGTTGG	*Bam*HI
*ndh*3	AAAAAGCTTCACGTCTGCGAAGTCATTATTAGT	*Hind*III
*ndh*4	AAAAAGCTTACTGATCGCTATGATGTGAAGCAG	*Hind*III
*ndh*5	AAAGAATTCCAGCGCCAACTCCCTGCTGCCCAG	*Eco*RI
*ndh*1	GTTGTGAGTCAAGAAGACGTGAAG	
*ndh*8	AACTTTGCCGAATGGATCGGTCTC	
*hyp*I-f	CAACGCCAACGCAGTCAT	
*hyp*I-r	GGTGCTTTGTCCGCAGTT	
srtA-f	GCGAGGTAATCAGAGTGACAA	
srtA-r	GCAACTGTCTGACGAGTCAA	
ndh-f	GGAACAAGTCGATGCCGATA	
ndh-r	GGCGGTGGTAGCTTCTTT	
pstB-f	CAGTGATTACACGGCGTTCT	
pstB-r	GACACCCGGAATGAGATCAT	
*hyp*II-f	CAAGCACCTTCCCTAGATGAA	
*hyp*II-r	GTGGTTGGTTGCGTACTGAT	
hpk-f	CAACACTATCGCTACTTGGTGAA	
hpk-r	GCGGCTAGTATGAGTAGAGTAA	

products were filled with Mastermix Taq DNA polymerase (5 Prime Inc., Gaithersburg, MD), then separately blunt-end ligated into the cloning vector pGEMT (Promega Inc., Madison, WI), and transformed by electroporation into *E. coli* DH5α. Transformants were selected by blue-white colony screening on LB with 50 μg/ml ampicillin, X-gal, and IPTG, then plasmid DNA (pDNA) was collected from Amp^R and screened colonies by restriction enzyme digestions (*Xba*I and *Pst*I or *Pst*I and *Xma*I, respectively) to confirm the presence of appropriately sized insert DNA. After recovery of digested inserts of positive clones from preparatory agarose gels, a triple ligation mix, including digested 5' and 3' inserts, was used to assemble the fragments into the multiple-cloning-site (MCS) of the *Xba*I and *Xma*I digested vector pBS1 [58].

The ligated DNA was transformed into *E. coli* EC1000, and transformants were selected on LB with 100 μg/ml Ery. Recombinant plasmid DNA (pDNA) was recovered from Ery^R colonies, screened by PCR and restriction enzyme digest, and confirmed by DNA sequencing. Once confirmed, the recombinant plasmid, designated pBSΔ*mutS*, was transformed as described previously [89] into *L. casei* 12A and ATCC 334 competent cells, prepared by growth in MRS with 1% glycine and 0.9 M NaCl, respectively, to an optical density at 600 nm (OD_{600}) between 0.6 and 0.8, and pretreated with sterile dH_2O for 30 min. Electroporation was performed in 2 mm cuvettes using a Bio-Rad Gene Pulser (Hercules, CA) set at 2,500 V, 25 μF, and 400 Ω. Electroporated cells were recovered in MRS with 0.5 M sucrose and plated onto MRS with 2.5 μg/m Ery [59].

Genomic DNA of *L. casei* Ery^R colonies was isolated as described, then screened by PCR using the primers *mut*1 x *mut*3 to identify merodiploids with pBSΔ*mutS* integrated into the wt *mutS* locus by a single-crossover event (which produced products

for both the wt *mutS* (4.0 kb amplicon) and the Δ*mutS* (1.5 kb amplicon) gene). Merodiploids were passaged by 1% (v/v) transfers in MRS without antibiotic selection and plated onto Brain Heart Infusion (BHI; Becton-Dickson) with 7 mM 4-cloro-dl-phenylalanine (ClPhe; Sigma, St. Louis, MO), as previously described [58]. To screen for colonies that had lost *mutS*, PCR was performed with primers *mut*1 x *mut*3. DNA sequencing from the 5'-flanking DNA upstream of the deletion site to a point downstream using primers *mut*1 x *mut*3, and from the 3'-flanking DNA downstream of the deletion site to a point upstream using primers *mut*2 x *mut7*, was used to confirm the deletion of *mutS* in its entirety from the genome of *L. casei* 12A and ATCC 334 [58].

The *L. casei* 12A genes encoding a two-component signal transduction system (TCS) quorum-sensing histidine kinase (*hpk*, LCA12A_1895), phosphate transport ATP-binding protein PstB (*pstB*, LCA12A_2227), and NADH dehydrogenase (*ndh*, LCA12A_1744) were also inactivated by gene replacement using a similar approach. To generate a 1,307 bp deletion that removed the entire *hpk* ORF, a 550 bp 5' fragment was obtained with primers *hpk*2 x *hpk*3, which respectively contain *Bam*HI and *Hind*III restriction linkers, and a 599 bp 3'fragment was obtained with primers *hpk*4 x *hpk*5, which respectively contains *Hind*III and *Xma*I linkers. The 5' and 3' fragments were cloned and verified in *E. coli* DH5α using pGEMT, in *E. coli* EC1000 using pBS1, and in *L. casei* 12A with the recombinant pBSΔ*hpk* as described for *mutS*. Screening of DNA from *L. casei* Ery^R colonies was performed by Taq PCR with the primers *hpk*1 x *hpk*6 to identify merodiploids with pBSΔ*hpk* integrated into the *hpk* locus (which gave products for both the wt *hpk* (2.6 Kb amplicon) and the Δ*hpk* (1.3 Kb amplicon) gene). Non-selective passage and subsequent screening for the wt *hpk* or Δ*hpk* locus in isolates was

performed as described for *mutS*, with the primers *hpk*1 x *hpk*6. DNA sequencing from the 5'-flanking DNA upstream of the deletion to a point downstream from the 3'-flanking DNA was used to confirm full deletion of the *hpk* ORF in *L. casei* 12A:Δ*hpk*.

To remove the entire *pstB* ORF (774 bp), a 1,064 bp 5' fragment was generated with primers *pst*1 x *pst*2, which respectively contain *Xba*I and *Eco*RI restriction linkers. A 1,084 bp 3'fragment was obtained with primers *pst*3 x *pst*4, which respectively contain *Eco*RI and *Sph*I linkers, and the 5' and 3' fragments were cloned and verified in *E. coli* DH5α using pGEMT, in *E. coli* EC1000 using pBS1, and in *L. casei* 12A with the recombinant pBSΔ*pstB* as previously described. Screening of DNA from *L. casei* Ery^R colonies was performed by Taq PCR with the primers *pst*5 x *pst*8 to identify merodiploids with pBSΔ*pstB* integrated into the *pstB* locus (which gave products for both the wt *pstB* (1 Kb amplicon) and the Δ*pstB* (200 bp amplicon) gene). Non-selective passage and subsequent screening for the wt *pstB* or Δ*pstB* locus was performed as described with the primers *pst*6 x *pst*7. DNA sequencing from the 5'-flanking DNA upstream of the deletion to a point downstream from the 3'-flanking DNA using primers *pst*1 x *pst6* was used to confirm full deletion of the *pstB* coding sequence (CDS) in *L. casei* 12A:Δ*pstB*.

To generate a 561 bp (amino acids 19-205) in-frame deletion of the *ndh* ORF in *L. casei* 12A. A 824 bp 5' fragment was first obtained with primers *ndh*2 x *ndh*3, which respectively contains *Bam*HI and *Hind*III restriction linkers. A 742 bp 3'fragment was then obtained with primers *ndh*4 x *ndh*5, which respectively contains *Hind*III and *Eco*RI linkers. The 5' and 3' fragments were cloned into *E. coli* DH5α using pGEMT, in *E. coli* EC1000 using pBS1, and in *L. casei* 12A, using the recombinant pBSΔ*ndh* as described.

Screening of DNA from *L. casei* Ery^R colonies was performed by PCR with the primers *ndh*1 x *ndh*8 to identify merodiploids with pBSΔ*ndh* integrated into the *ndh* locus (which gave products for both the wt *ndh* (1.6 Kb amplicon) and the Δ*ndh* (1.1 Kb amplicon) gene). Non-selective passage and subsequent screening for the wt *ndh* or Δ*ndh* locus was performed as described, with the primers *ndh*1 x *ndh*8, and DNA sequencing from the 5'-flanking DNA upstream of the deletion to a point downstream from the 3'-flanking DNA confirmed the desired in-frame deletion was used to verify the desired deletion in the *ndh* coding sequence (CDS) in *L. casei* 12A:Δ*ndh*.

Finally, the *hpk*, *pstB,* and *ndh* genes were deleted in combination to generate a total of seven *L. casei* 12 double- and triple-gene deletion mutants (Table 5-1). The *pstB* gene was inactivated in *L. casei* 12A:Δ*hpk* and *L. casei* 12A:Δ*ndh* as described, resulting in *L. casei* 12A:Δ*hpk*/Δ*pstB* and *L. casei* 12A:Δ*ndh*/Δ*pstB*, respectively. The *ndh* gene was inactivated in *L. casei* 12A:Δ*hpk* and *L. casei* 12A:Δ*hpk*/Δ*pstB,* resulting in *L. casei* , 12A:Δ*hpk*/Δ*ndh* and *L. casei* 12A:Δ*hpk*/Δ*pstB*/Δ*ndh*, respectively.

Adaptive evolution

The approach in this study used for adaptive evolution of *L. casei* to low pH was based upon the work of Zhang, et al. [22]. The adjustment of pH in MRS broth below the standard pH 6.5 was attained by addition of 85% lactic acid (MP Biomedicals LLC, Solon, OH). Working cultures of *L. casei* 12A, 12A Δ*mutS,* ATCC 334, and 334:Δ*mutS*, were prepared by direct inoculation of 10 ml MRS broth from glycerol stocks with incubation at 37°C for 17 h, followed by transfer (1%, v/v) into 10 ml MRS, pH 5.5 (Day 0 cultures). Cultures were incubated at 37ºC and transferred at mid-exponential phase

(OD_{600} 2.0 – 3.0) to fresh MRS, pH 5.5 for 10 days, then MRS at pH 5.0 for 15 days, MRS at pH 4.5 for 20 days, MRS at pH 4.2 for 25 days, and finally MRS at pH 4.0 for 30 days. During the 100-day adaptive evolution process samples were collected approximately every five days and preserved at -80°C as glycerol stocks, with occasional PCR screening of *mutS* as described to verify culture purity during the experiment.

At the end of the adaptive evolution process all four cultures were streaked onto MRS plates at pH 4.0 titrated with 85% lactic acid, and incubated in BBL GasPak Systems with GasPak EZ Anaerobe Container System with indicator (Becton, Dickinson and Company, Dublin, Ie) at 37°C for 20 days. After incubation, the seven largest colonies from each culture were collected and subject to growth studies in MRS broth at pH 4.0 (see below), and the isolate from each adaptive evolution culture with the most rapid growth rate at pH 4.0 was selected for further characterization.

Growth at pH 4.0

Working cultures of the seven isolates collected from each of the final four adaptive evolution cultures were prepared by a 1% (v/v) inoculation of 1 ml MRS broth from glycerol stocks, with incubation at 37°C for 17 h. Working cultures of *L. casei* ATCC 334, 334-AE, 334-MAE, 12A, 12A-AE, 12A-MAE, 12A:Δ*hpk*, 12A:Δ*pstB*, 12A:Δ*ndh*, 12A:Δ*hpk*/Δ*pstB*, 12A:Δ*hpk*/Δ*ndh*, 12A:Δ*ndh*/Δ*pstB*, and 12A:Δ*hpk*/Δ*pstB*/Δ*ndh* were prepared from glycerol stocks followed by successive 1% (v/v) transfer in 10 ml MRS and 1 ml MRS, respectively, with incubation at 37°C for 17 h. Cells were harvested from 1 ml cultures by centrifugation (14 x 10^3 rpm, 5 min), washed once with 1 ml PBS, and suspended in 1 ml MRS, adjusted to pH 4.0. These suspensions were used to inoculate

(1%, v/v) 0.2 ml MRS broth, pH 4.0, in Costar 96 well flat bottom assay plates with a low evaporation lid (Corning Inc., Kennebunk, ME). At least three wells were left un-inoculated to serve as blanks throughout the growth curve experiments. The outer wells of the 96 well plates contained 0.2 ml sterile dH_2O. After inoculation, the plates were capped with radiation sterilized adhesive seals (Nunc, Roskilde, Dk) and incubated at 37°C. The OD_{600} was measured every 24 h for 17 d using a SpectraMax Plus 384 microplate reader (Molecular Devices LLC., Sunnyvale, CA) at 37°C with 1 min auto-mixing before each measurement. Growth curves of *L. casei* strains were assembled using data from a minimum of two independent biological-experimental replicates.

The terminal pH and D/L-lactic acid concentration was measured after the 17 d experiment for each *L. casei* strain tested, and compared to the measured blank values. At the end of the growth experiment, cultures were transferred from wells to 1.7 ml microtubes and centrifuged at 14 x 10^3 rpm for 10 min. The supernatant was transferred to 0.5 ml PCR tubes and stored at -20°C until the assay was performed. At that time, the solution was thawed and the pH was measured using an Orion 3 Star benchtop pH meter (ThermoFisher, Waltham, MA) with a Easyferm Plus K8 160 pH probe (Hamilton Robotics, Reno, NV). Quantitative D/L-lactic acid analyses were performed using the R-Biopharm AG (Darmstadt, Germany) D/L-lactic acid UV-method test kit as directed by supplier, except that the total assay volume was reduced from 1 to 0.5 ml while maintaining the proportions of reagents as described in manufacturer's instructions. A Ryan-Einot-Gabriel-Welch q-test (REGWQ) for multiple pairwise comparisons was used to identify statistically significant differences in pH values and lactic acid yields from each treatment [60].

Restoration of *mutS*

The *mutS* lesion in the representative isolate from *L. casei* 12A:Δ*mutS* and 334:Δ*mutS* was repaired to restore DNA replication fidelity. A 4.8 Kb fragment spanning 1 Kb upstream to 1.2 Kb downstream of *mutS* was obtained by Phusion PCR of *L. casei* 12A genomic DNA with the primers *mut*5'-f x *mut*3'-r, with *Xba*I and *Xma*I linkers respectively, and cloned into *E. coli* DH5α using pGEMT, and in *E. coli* EC1000 using pBS1 as previously described [58]. The recombinant plasmid, pBS1:*mutS*, was transformed into *L. casei* 12A:Δ*mutS* and 334:Δ*mutS*, and screening of *L. casei* Ery^R colonies for merodiploids, non-selective passage and screening for the presence of the restored *mutS* gene was performed essentially as described for construction of knockout mutants. DNA sequencing from the 5'-flanking DNA upstream of *mutS* to a point downstream, 2.3 Kb, using primers *mut*1 and *mut4*, from the 3'-flanking DNA downstream of *mutS* to a point upstream, 3 Kb, using primers *mut5* and *mut*7, and an overlapping 2.9 Kb region, across *mutS* using primers *mut6* and *mut*3 was used to confirm complete restoration of *mutS* in the genome of each strain.

Acid resistance

Working cultures of *L. casei* strains were prepared as described for growth studies, then inoculated (1%, v/v) into 15 ml MRS broth and incubated at 37°C for 11 h, until cells reached early stationary phase. Cells were harvested by centrifugation (9×10^3 *g*, 10 min, 25°C), washed once with 15 ml 0.1% Bacto peptone then suspended in 1.45 ml Bacto peptone (approximately 10^{10} CFU/ml). 0.1 ml aliquot of the cell suspension was transferred to 10 ml MRS broth without dextrose at pH 3.0, 2.5, and 2.0 (adjusted with

HCl) and incubated for 1 h at 37°C. Before and after the acid challenge, cells were serially diluted and plated on MRS agar, incubated for 48 h at 37°C, and the difference in cell count was used to calculate percent survival. Two independent biological-experimental replicates of the acid resistance experiment were conducted. A REGWQ test for multiple pairwise comparisons was used to identify statistically significant differences in the percent survival at each pH value.

Cell morphology

Morphology of *L. casei* 12A, 12A-MAE, 12A:Δ*ndh*/Δ*pstB*, and 12A:Δ*hpk*/Δ*pstB* were investigated by SEM. Working cultures were prepared as described for adaptive evolution. Batch cultures in 1 L MRS with 3% glucose broth in Sartorius Biostat B-plus dual controlled biofermenters (Sartorius AG, Goettingen, De) were prepared using a 1% (v/v) inoculum of working cultures standardized to an OD_{600} of 1.0 in MRS. Cultures were incubated at 37°C with an agitation rate of 100 rpm, and when pH 3.8 was reached it was maintained at that level by automatic addition of 15% (v/v) NH_4OH. A 1 ml sample of cells was collected after 30 h, centrifuged at 800 rpm for 25 min, and the supernatant was discarded. For biological fixation of the samples, 0.75 ml buffered gluteraldehyde (2% gluteraldehyde, 0.1M HEPES) was added to the cell pellets, followed by vortexing and incubation overnight at RT. Cells were transferred to glass slides (120 mm) coated with lysine, rinsed with 0.1M HEPES three times for 5 min, serially dehydrated with ethanol (2 x for 10 min in 50%, 2 x for 10 min in 70%, 2 x for 10 min in 95%, then 3 x for 15 min in 100% ethanol). After rinsing, the slides were chemically dried with ethanol (100%):HMDS solutions (2:1 for 15 min, 1:1 for 15 min, 1:2 for 15

min, and 0:1 3 x for 15 min). The HMDS in the final step was removed by evaporation overnight at room temperature, then the fixed cells were mounted onto aluminum stubs and Au/Pd sputter coated with an EMS-150 ES carbon/metal coater (Electron Microscopy Sciences, Hatfield, PA) as a service by the USU Microscopy Core Facility (MCF). Cells were imaged and photographed using a FEI Quanta FEG 650 scanning electron microscope (Hillsboro, OR) under high vacuum.

For analysis of cell size, ImageJ [61] was used to measure the width and length of 35 individual *L. casei* 12A, 12A-MAE, 12A:Δ*ndh*/Δ*pstB*, and 12A:Δ*hpk*/Δ*pstB* cells, using methods described by Kokkinos, et al. [62]. A REGWQ for multiple pairwise comparisons was used to identify statistically significant differences in cell length and width from each treatment.

Whole genome resequencing

Genomic DNA samples was extracted from *L. casei* 12A-AE, 12A-MAE, 334-AE, and 334-MAE using the MasterPure Gram Positive DNA Purification Kit (Epicentre, Madison, WI), following the provided procedure, with the addition of 5 U/ml mutanolysin from *Streptomyces globisporus* ATCC21553 (Sigma) to the Ready-Lyse Lysozyme solution step (37°C, 1 h). Genomic DNA samples were sequenced as service by the Utah State University Center for Integrated Biosystems (CIB) using an Ion Torrent System (Life Technologies, Carlsbad, CA). Sequence contigs were assembled against the respective wt *L. casei* ATCC 334 and 12A reference genomes (GenBank accession # CP000423 and CP006690, respectively) and analyzed for single-nucleotide polymorphisms (SNPs), insertions and deletions, and copy number variations using the

Ion Reporter software, as a service by the USU CIB. Identified mutations were referenced against 12A population sequencing data with regions previously determined to be hypervariable in the *L. casei* wt genome (unpublished data), and were excluded from the mutational analysis if they occurred within these regions. Mutations with a coverage depth greater than 10 and a frequency greater than 90% were considered as genuine. Mutations occurring within annotated open reading frames (ORFs) were subsequently analyzed for their putative effect on translation. Six nonsense mutations identified in *L. casei* 12A-MAE (in *hypI, srtA, pstB, hypII, hpk*, and *ndh* genes) were verified in *L. casei* 12A-MAE and in day-100 *L. casei* 12A:Δ*mutS* culture by sequencing PCR products generated with the primers *hypI*-f x *hypI*-r, *srtA*-f x *srtA*-r, *pstB*-f x *pstB*-r, *hypII*-f x *hypII*-r, *3hpk*-f x *3hpk*-r, and *ndh*-f x *ndh*-r (Table 5-2).

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CHAPTER 6

SUMMARY AND CONCLUSIONS

It was the hypothesis of this study that *Lactobacillus casei* can be engineered to grow on de-oiled algal biomass (algal cake, AC), and effectively ferment this substrate into lactic acid under low pH conditions. To explore this hypothesis, AC from algal strain USU080 generated as a waste by-product from biodiesel production was utilized as a substrate in fermentations with *L. casei* 12A. With no previous research on the suitability of USU080 as a substrate for *L. casei*, it was important to establish baseline conditions for fermentation and lactic acid production. To accomplish this, growth and lactic acid production by *L. casei* 12A on USU080 AC solutions supplemented with a multi-vitamin mixture, casamino acids and/or glucose was measured. Those experiments revealed carbohydrate availability was the primary limiting factor for growth and lactic acid production by *L. casei*. Thus, optimized growth and production of lactic acid required enhancement of both carbohydrate and amino acid availability.

To address these nutrient limitations, we explored the potential to increase carbohydrate availability by enzymatic digestion with α-amylase and cellulase (endo-1,4-β-D-glucanase), and amino acid availability by treatment with pepsin, a nonspecific protease. Significant increases in growth and lactic acid production were observed when the AC was digested with any of the three enzymes, but the largest increase in lactic acid production (0.075 g lactic acid per g AC) occurred when the AC was digested with all three enzymes. Thus, the nutrient limitations of AC can be effectively addressed by enzymatic hydrolysis of amylose, cellulose, and polypeptides.

Since cellulase and α-amylase treatment was effective for improving carbohydrate availability in AC, we explored heterologous expression of these enzymes by *L. casei* as a means for increasing growth and lactic acid production in AC without the need for enzyme addition. Codon optimized cassettes containing a *Clostridium thermocellum* cellulase gene encoding endo-1,4-β-glucanase E (CelE) or a *Streptococcus bovis* gene for α-amylase (AmyA) were cloned separately into *L. casei* 12A. Functional *celE* expression from *L. casei* 12A was not detected, but expression of *amyA* was attained. Furthermore, AmyA activity was significantly increased when the gene was placed under control of an originally designed synthetic promoter (p11) for *Lactobacillus plantarum*. Nonetheless, activity levels were insufficient to significantly increase lactic acid production from AC by *L. casei*. Thus, substantial optimization of *celE* and *amyA* expression in *L. casei* 12A would be needed to obtain activities required for reducing AC fermentation costs.

To improve lactic acid production at low pH, we developed a system for transient inactivation of the DNA mismatch repair (MMR) protein MutS in *L.* casei and coupled it to adaptive laboratory evolution with selection for lactic acid resistance at low pH. The *mutS* gene was deleted in its entirety from the genome of *L. casei* 12A and ATCC334, and the wild-type (wt) parental strains and their Δ*mutS* derivatives were sequentially passaged through decreasing pH and increasing lactic acid concentrations over a 100-day period. Restoration of *mutS* at the end of the adaptive evolution process was used to stop hypermutagenicity and the adapted strains were analyzed for genotypic and phenotypic changes.

Growth studies revealed an increased ability to grow and produce lactic acid at pH 4.0 by all four adapted strains, with the greatest increases observed in the adapted Δ*mutS* derivatives. Further investigation of the 12A adapted Δ*mutS* derivative revealed an increased ability to survive at pH 2.5. Additionally, morphological changes were observed, with a significant decrease in the cell volume and a rougher cell appearance. Genome sequence analysis of the adapted strains confirmed decreased DNA replication fidelity through transient MutS inactivation resulted in increased adaptability by the 12A and ATCC334 Δ*mutS* derivatives. However, mutations were identified in additional genes associated with DNA replication fidelity in the adapted ATCC 334 Δ*mutS* derivative, resulting in a greater increase in hypermutability during the adaptive evolution process. This increased hypermutability likely contributed to greater adaptability to grow and produce lactic acid at low pH. However, restoration of *mutS* in this strain would not be expected to restore replication fidelity, and this outcome illustrates one potential risks of using MutS as a target for inducing transient hypermutation.

Genome sequence analysis of the adapted *L. casei* 12A derivatives identified 19 and 62 potential genotypic changes responsible for the observed increased acid tolerant phenotypes of the 12A wt adapted and Δ*mutS* adapted derivatives, respectively. Three nonsense mutations identified in the *L. casei* 12A Δ*mutS* adapted derivative were chosen for further investigations. These nonsense mutations were located within a NADH dehydrogenase (*ndh*), a phosphate transport ATP-binding protein PstB (*pstB*), and a two-component signal transduction system (TCS) quorum-sensing histidine kinase (*hpk*). Inactivation of these three genes by gene deletions, individually and in combination, revealed that inactivation of *pstB* and *ndh* together increased the growth and lactic acid

production of *L. casei* 12A at pH 4.0, and inactivation of *hpk* and *pstB* together increased the ability of *L. casei* 12A to survive at pH 2.5.

In conclusion, this study demonstrated that AC is a viable substrate for production of lactic acid by *L. casei*. However, optimized growth and lactic acid production requires carbohydrate and amino acid availability to be increased, which can be achieved by enzymatic hydrolysis. While we were successful in engineering α-amylase expression in *L. casei* 12A, substantial optimization is needed for practical expression in *L. casei.* Finally, we successfully developed *L. casei* ATCC 334 and 12A strains that were better able to grow and ferment carbohydrates into lactic acid at low pH by combining transient inactivation of MutS with adaptive evolution. That work also identified three specific genes (*ndh*, *pstB*, and *hpk*) that contribute to lactic acid resistance in *L. casei*.

Since heterologous enzyme expression by *L.* casei was not optimal, future work on AC fermentations should investigate on-site or integrated approaches for α-amylase and cellulase production, and/or co-fermentations with efficient enzyme producing microorganisms. These approaches would alleviate the need for enzyme addition to increase available carbohydrates in AC fermentations for production of value added chemicals (e.g. lactic acid). Additionally, similar approaches should be investigated for expression/production of a non-specific proteases to further enhance growth and co-production of value added chemicals. Future fermentations of AC should investigate and incorporate *L. casei* 12A-AE, 12A-MAE, and/or 12A:Δ*ndh*/Δ*pstB* for the production of lactic acid under low pH conditions. Development of an inducible hypermutation system may provide a more effective method for selecting strains with industrially important properties via adaptive laboratory evolution. More detailed

investigation of *pstB*, *hpk*, *ndh*, and other genes affected by mutations identified in the *L. casei* 12A acid adapted derivatives may provide additional insight for increasing growth and lactic acid production in fermentations at low pH. Lastly, transcriptomic analysis of the acid adapted derivatives and the parental wt could reveal changes in gene expression that allow the acid adapted cells to better grow and produce lactic acid at low pH.

APPENDICES

APPENDIX A

DATA NOT INCLUDED IN TEXT

Table A-1 Monosaccharide$_1$ composition of USU080 algal cake (AC) determined using acid hydrolysis with subsequent high-performance anion exchange chromatography coupled with pulsed amperometric detection.

Monosaccharide	**$\mu g_1/mg_{AC}$**	**Composition of AC (%)**
Galactosamine	0.3	0.03
Glucosamine	10.6	1.06
Galactose	10.7	1.07
Glucose	37.9	3.79
Mannose	7.8	0.78
Total:	67.3	6.73

Table A-2 Amino acid (aa) composition of USU080 algal cake (AC) determined using acid hydrolysis with subsequent reverse phase high pressure liquid chromatography.

Amino Acid	**$\mu g_{aa}/mg_{AC}$**	**Composition of AC (%)**
Asx	27.3	2.73
Glx	29.0	2.9
Serine	11.7	1.17
Histidine	4.8	0.48
Glycine	13.2	1.32
Threonine	13.4	1.34
Arginine	22.2	2.22
Alanine	19.8	1.98
Tyrosine	6.0	0.6
Valine	15.6	1.56
Methionine	4.5	0.45
Phenylalanine	15.2	1.52
Isoleucine	11.1	1.11
Leucine	23.3	2.33
Lysine	14.6	1.46
Proline	11.9	1.19
Total:	243.7	24.37

Asx = Asparagine and Aspartate, Glx = Glutamine and Glutamate.

Table A-3 DNA sequences for pDW2:*celE*-I, pDW2:*amyA*-I, Pp11, and pDW2:*amyA*-II. With the sequences for the promoter underlined, ribosomal binding site bolded, start codon underlined, bolded and italicized, and the terminator italicized.

Sequences
pDW2:*celE*-I TCTAGAGGGTTTCCAACAAAGTGCAACCTTTTTTCAGCAGAGGTTACTATGTT TTTAATTATTTTTTTGCTTTGCGCTGTCCAAAAGAAAATGATTTTTCGAATTAA TATAATAAACAGTAACATTACCGTTTAGTTTGTTATAATGTTTTATTTCAAGC CAAATAGGAAGGGGGAGCTGTAAGAATAAAGTTGATTGATGCAAGAAAAGA GGATTTAAAGTAAAAGATTATTGTTTTTTGTAAAGGATTTTTGTAAA**GGAGA** GGGTAAT***GTG***AAGAAGATCGTCTCATTGGTTTGCGTCTTGGTTATGTTGGTTT CCATCTTGGGCAGCTTCTCCGTTGTTGCTGCTTCACCTGTCAAGGGCTTCCAG GTCAGCGGCACCAAGTTGTTAGACGCCAGCGGTAACGAATTGGTCATGCGTG GTATGCGTGACATTTCAGCTATTGACTTAGTTAAAGAAATTAAAATTGGTTGG AACTTAGGTAACACTTTAGATGCTCCTACTGAAACTGCTTGGGGTAACCCACG TACTACTAAAGCTATGATTGAAAAAGTTCGTGAAATGGGCTTCAACGCTGTTC GTGTTCCAGTTACTTGGGACACTCACATTGGTCCTGCTCCAGACTATAAAATT GATGAAGCTTGGTTAAACCGTGTTGAAGAAGTCGTTAACTATGTTTTAGATTG TGGCATGTATGCTATTATTAACTTACATCACGATAACACTTGGATTATTCCTA CTTATGCTAACGAACAACGTAGCAAAGAAAAATTAGTTAAAGTTTGGGAACA AATTGCTACTCGTTTCAAAGATTATGACGATCATTTATTATTCGAAACTATGA ACGAACCTCGTGAAGTTGGTTCCCCAATGGAATGGATGGGTGGTACTTATGA AAACCGTGATGTTATTAACCGTTTCAACTTAGCTGTTGTTAACACTATTCGTG CTTCTGGTGGCAACAACGACAAACGTTTCATTTTAGTTCCTACTAACGCTGCT ACCGGTTTAGATGTTGCTTTAAACGATTTAGTTATTCCAAACAACGACTCACG TGTTATTGTTAGCATTCACGCTTATTCCCCATATTTCTTCGCTATGGACGTTAA CGGTACTTCTTATTGGGGCTCAGACTATGATAAAGCTTCTTTAACTTCTGAAT TAGATGCTATTTATAACCGTTTCGTTAAAAACGGTCGTGCTGTTATTATTGGT GAATTCGGTACTATTGACAAAAACAACTTAAGCTCACGTGTTGCTCACGCTG AACACTATGCTCGTGAAGCTGTTTCTCGTGGTATTGCTGTTTTCTGGTGGGAC AACGGTTATTATAACCCTGGTGATGCTGAAACTTATGCTTTATTAAACCGTAA AACTTTAAGCTGGTATTATCCAGAAATTGTTCAAGCTTTAATGCGTGGCGCTG GTGTTGAACCATTAGTTTCACCTACTCCAACTCCTACTTTAATGCCAACTCCA AGCCCAACTGTTACTGCTAACATTTTATATGGTGATGTTAACGGCGACGGTAA GATTAACTCCACTGACTGTACTATGTTAAAACGTTACATCTTGCGTGGTATCG AAGAATTTCCATCACCTAGCGGTATCATTGCCGCCGACGTCAACGCCGATTTA AAAATCAACTCTACCGATTTAGTCTTGATGAAGAAATACTTATTACGTTCCAT TGATAAATTCCCAGCTGAAGATAGCCAAACTCCAGATGAAGATAACCCTGGC ATTTTATATAACGGTCGTTTCGATTTCTCAGATCCTAACGGCCCAAAATGTGC CTGGTCTGGCTCTAACGTTGAATTAAACTTCTATGGTACCGAAGCTTCAGTTA CTATTAAATCCGGTGGTGAAAACTGGTTCCAAGCTATTGTTGACGGTAACCCA TTACCTCCATTCTCTGTTAACGCTACTACTTCAACTGTTAAATTAGTTAGCGG*

* Table continues on next page.

TTTAGCTGAAGGTGCTCATCACTTAGTTTTATGGAAACGTACTGAAGCCTCTT
TAGGCGAAGTTCAATTCTTAGGTTTCGACTTCGGTTCAGGTAAATTATTAGCT
GCTCCTAAACCATTAGAACGTAAAATTGAATTCATTGGCGACTCCATTACTTG
TGCTTATGGTAACGAAGGTACTTCTAAAGAACAAAGCTTCACTCCAAAAAAC
GAAAACTCCTATATGTCTTATGCTGCTATTACTGCTCGTAACTTAAACGCTTC
TGCTAACATGATTGCTTGGTCCGGTATTGGTTTAACTATGAACTATGGTGGTG
CTCCTGGTCCATTAATTATGGATCGTTATCCATATACTTTACCATATTCAGGTG
TTCGTTGGGATTTCTCCAAATATGTTCCACAAGTTGTTGTTATTAACTTAGGTA
CTAACGATTTCAGCACTTCTTTCGCTGACAAAACTAAATTCGTTACTGCTTAT
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TACTGAAGTTGTTAATGACTGTAACCGTTCTGGTGATTTAAAAGTTTATTTCG
TTGAATTCCCACAACAAGATGGTTCCACTGGTTATGGTGAAGACTGGCATCCT
AGCATTGCTACTCACCAATTAATGGCTGAACGTTTAACTGCTGAAATTAAAA
ACAAATTAGGCTGGTAATAATACTAGGCC*TTATAAATAAAAATCACCTTTTAGAG*
*GTGGTTTTTTTATTTATAA*ACTGCA

pDW2:*amyA*-I
TCTAGATTTCCAATATACCAGAGAAAGCGGGTGCGAACTTACTGAAAATGGT
GTAACATGAGATTCTGTAAACAGGCAGAAGACTGTCAAATCATAAAAGC**GA**
GGTATTCTCTGAT***ATG***ACCTTCCAGAACAAGGTCAACTTGAAGAAGAAGATG
AAGAAGTCCTTGGGCAGCTTATTGATCTTGACCGCCATCGGCGCCGGCGGCTT
GGTCCAGGTCAAAGTCGTTAACGCCGACGAACAAGTTAGCATGAAAGACGGC
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GATGCTGTTTTGAATCATACCACCTCAGATTATAACCAAATCTCACAAGAAAT
CAAGAACATCCCAAACTGGACCCATGGCAACACCTTGATCTCAGATTGGCAT
AACCGTTATGATGTTACCCAAAACGCTTTGTTGACATTGTATGATTGGAACAC
CCAAAACGAATATGTTCAACAATATTTGTTGTCATATTTGAAGCAAGCTGTTG
CTGATGGTGCCGATGGCTTCCGTTATGATGCCGCTAAGCATATCGAATTGCCA
GGCGAATATGGTTCAAATTTCTGGAACGTTATCTTGAACAACGGCTCAGAATT
CCAATATGGCGAAATCTTGCAAGATGATGTTTCAAACGACGCTGGCTACGGC
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GAAGGATCGTCATATCTCAGCTGGCAACTTGATGAACTATCAAGTTTCAGGC
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TGGCGGCGGCAACGGCACCCGTTTCCCAGGCCAATCACAAATCGGCGATGCT
GGCAGCAACTTGTATAAGGATGCTACCGTTACCGCTGTTAACAAGTTCCATA
ACGCTATGGTTGGCGAATCAGAATATTTGCGTAATCCAGGCGGTGACGAACA*

* Table continues on next page.

GGTTGCTATGATCGAACGTGGCACCAAGGGCGCTGTTATCGTTAACTTGGTTG
ACGGCGATAAACAAATCAACTCAGAAACCAACTTGGCTGATGGCACCTATAC
CGATAAGGTTTCAGGCCGTCAATTCAACGTTTCAAACGGCCGTATCACCGGCT
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GCTCAAGTTTCAGTTGATGGCTATAAGGAAGGCGATAACTCAATCTCAAAGG
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CTGACGGTCAATCCACCACAAAGACCTATACCTTCACCATGAAGGATCCATC
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TTACGCTGAAGAAGGTGTCAAGGTCATTTTCACAAACAACCAAGGCTCACAA
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TTGGTCAAGTCCGGCAACTACAACAAGGACGGCTTGAAGTAATAATACTAGG
CC*TTATAAATAAAAATCACCTTTTAGAGGTGGTTTTTTTATTTATAA*CTGCA

Pp11
CGGTCTAGAAGCGCTATAGTTGTTGACAGAATGGACATACTATGATATATTGT
TGCTATAGCGTACTTAGCTGGCCAGCATATATGTATTCTATAAAATACTATTA
CAA**GGAG**ATTTTAGAT***ATG***ACCTTCCAGAACAAGGTCAACTTGAAGAAGAAG
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pDW2:*amyA*-II
CGGTCTAGAAGCGCTATAGTTGTTGACAGAATGGACATACTATGATATATTGT
TGCTATAGCGTACTTAGCTGGCCAGCATATATGTATTCTATAAAATACTATTA
CAA**GGAG**ATTTTAGAT***ATG***ACCTTCCAGAACAAGGTCAACTTGAAGAAGAAG
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GCTTGGTCCAGGTCAAAGTCGTTAACGCCGACGAACAAGTTAGCATGAAAGA
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CGTTGATGCTGTTTTGAATCATACCACCTCAGATTAT*AACCAAATCTCACAA
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GGCATAACCGTTATGATGTTACCCAAAACGCTTTGTTGACATTGTATGATTGG
AACACCCAAAACGAATATGTTCAACAATATTTGTTGTCATATTTGAAGCAAG

* Table continues on next page.

CTGTTGCTGATGGTGCCGATGGCTTCCGTTATGATGCCGCTAAGCATATCGAA
TTGCCAGGCGAATATGGTTCAAATTTCTGGAACGTTATCTTGAACAACGGCTC
AGAATTCCAATATGGCGAAATCTTGCAAGATGATGTTTCAAACGACGCTGGC
TACGGCAAGTTGATGTCAATCACCGCTTCAAACTATGGCCAAAAGATCCGTT
CAGCTTTGAAGGATCGTCATATCTCAGCTGGCAACTTGATGAACTATCAAGTT
TCAGGCGTTGACGCTGCCAACTTGGTTACCTGGGTTGAATCACATGATAACTA
TGCTAACGATGATCAAGAATCAACCTGGATGAACGATTCAGATATCGGCTTG
GGCTGGGCTATGATCACCGCTCGTGCTAAGGGCACCCCATTGTTCTTCTCACG
TCCAGTTGGCGGCGGCAACGGCACCCGTTTCCCAGGCCAATCACAAATCGGC
GATGCTGGCAGCAACTTGTATAAGGATGCTACCGTTACCGCTGTTAACAAGTT
CCATAACGCTATGGTTGGCGAATCAGAATATTTGCGTAATCCAGGCGGTGAC
GAACAGGTTGCTATGATCGAACGTGGCACCAAGGGCGCTGTTATCGTTAACT
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CACAATATCCACAAAACGAAGGCTTCGATTTCAAGGCTGAAGGCTTGTATTC
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GATAACCCTGGTGGCTGGGATTCAGCTAACGCTTATTTGTATTATGGCAACCC
AGTTCAATATCCATTGGGCGTTTGGCCAGGTACACAAATGACCAAGGATGAT
GCTGGCAACTTCTATTTGGATTTGCCAGAAGAATATGCTGATGTTAACGCTAA
GATCATCTTCAACCAGCCAGGCACCTCCAACCAGTACCCATACAGCGAAGGC
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G

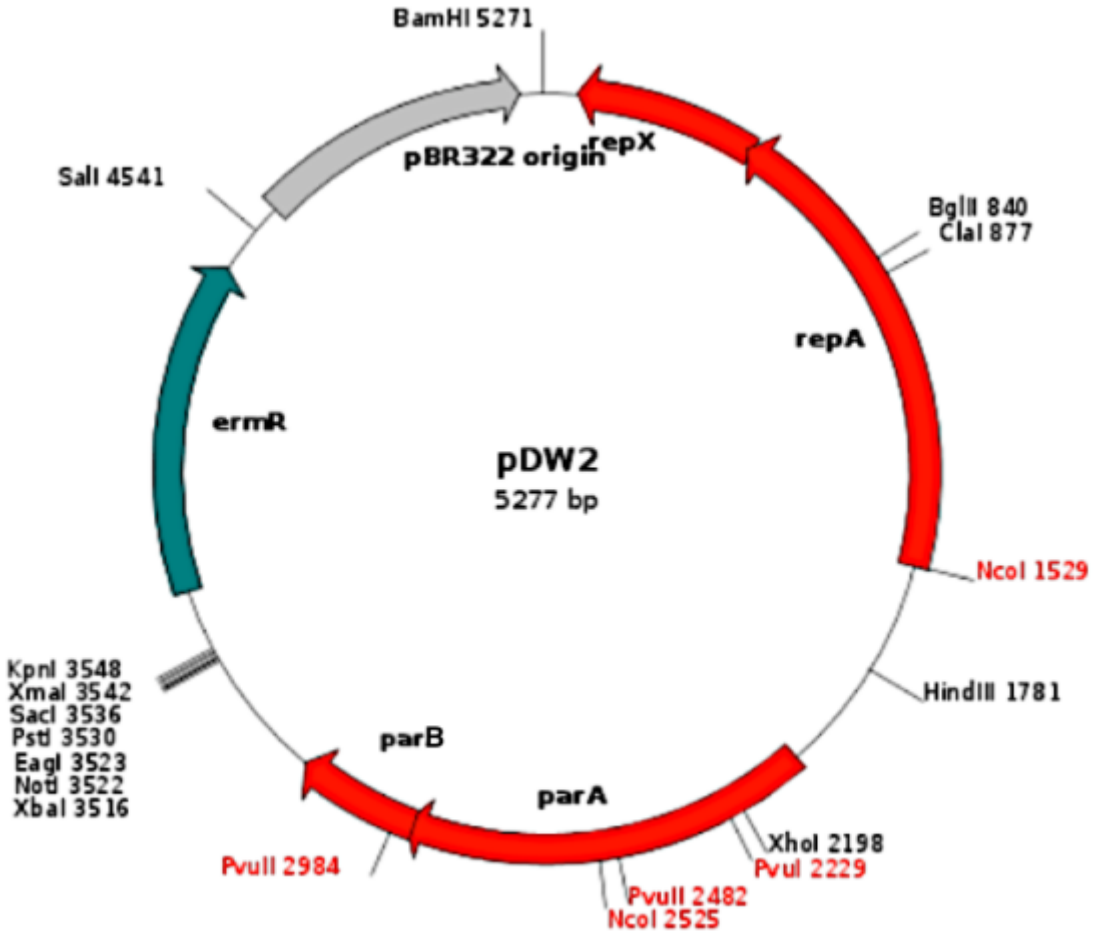

Fig. A-1 Physical map of *Lactobacillus casei* cloning vector pDW2. erm^R = erythromycin resistance gene; parA,B and repA,X replication regions from *L. casei* ATCC334; pBR322 replication origin from *Escherichia coli*.

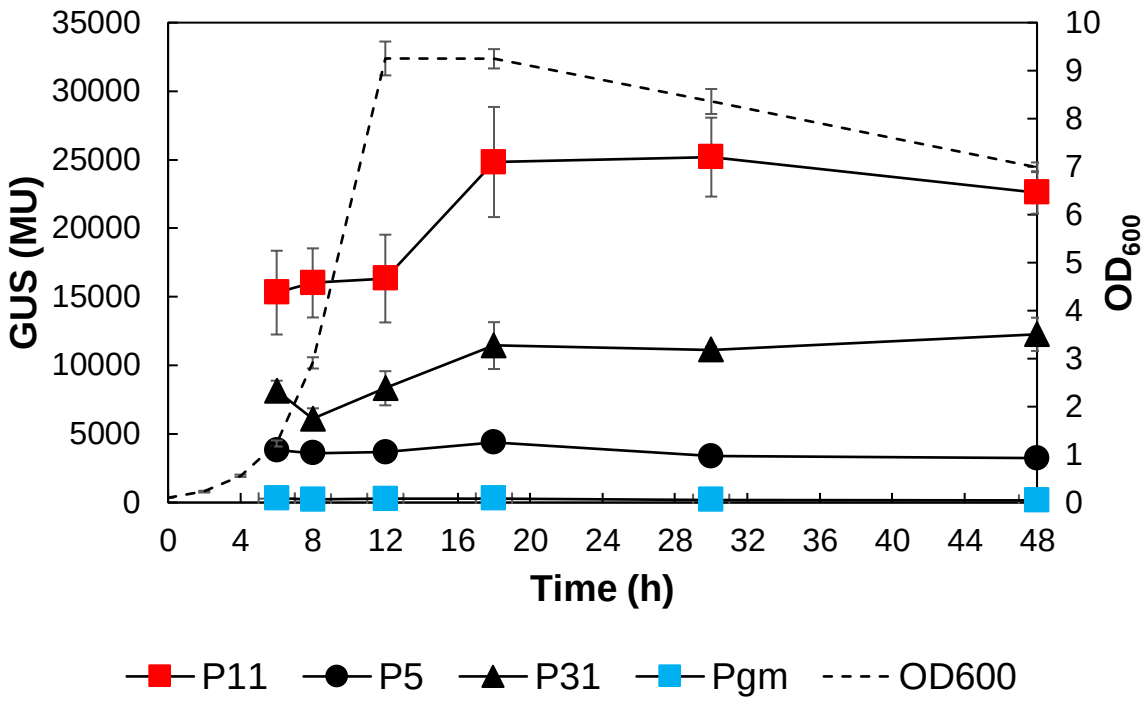

Fig. A-2 The optical density of *Lactobacillus casei* 12A, and ß-glucronidase activity under control of the synthetic *Lactobacillus plantarum* P11, P5 and P31 promoters, and the *L. casei* ATCC334 constitutive phosphoglyceromutase (Pgm) promoter.

Table A-4 Mutations identified in the *Lactobacillus casei* 334-MAE genome within DNA repair enzymes and proteins, the number of mutations within the open reading frame of the protein, and the resulting amino acid (aa) changes.

DNA Repair Protein/Enzyme	# of Mutations	aa Changes
DNA recombination and repair protein RecF	7	A to V, N to D
Single-stranded DNA-binding protein	4	
Methylated-DNA--protein-cysteine methyltransferase (EC 2.1.1.63)	3	S to P
Endonuclease III (EC 4.2.99.18)	7	
DinG family ATP-dependent helicase CPE1197	14	V to I, V to I
DNA-3-methyladenine glycosylase (EC 3.2.2.20)	5	N to S, M to T, T to I
MutS-related protein, family 1	27	A to S, M to L, V to A, W to R, T to A, S to P
Exonuclease SbcD	7	ET to KQ
Exonuclease SbcC	32	K to E, V to A, S to T, V to A, D to A, V to A
A/G-specific adenine glycosylase (EC 3.2.2.-)	6	M to I, A to T, V to L
SOS-response repressor and protease LexA (EC 3.4.21.88)	3	
DNA polymerase IV (EC 2.7.7.7)	9	G to A, A to G
Recombination inhibitory protein MutS2	21	T to S, K to E, S to P
RecA protein	10	R to C, S to A, CP to GR, P to Q
Excinuclease ABC subunit B	39	AQ to DR, D to N, R to H
Excinuclease ABC subunit A	17	K to E, E to D
Uracil-DNA glycosylase, family 1	2	
Exodeoxyribonuclease III (EC 3.1.11.2)	5	D to E
ATP-dependent DNA helicase UvrD/PcrA	22	Q to K, K to E, T to N, A to D
DNA ligase (EC 6.5.1.2)	18	Q to R, G to A, I to L*

* Table continues on next page.

ATP-dependent DNA helicase RecQ	26	V to A
DNA repair protein RadC	4	
Excinuclease ABC subunit A paralog of unknown function	25	L to F, A to T, T to A, D to G
RecD-like DNA helicase YrrC	31	R to Q, D to E, F to Y, S to G
Excinuclease ABC subunit C	14	R to K
ATP-dependent DNA helicase RecQ	6	V to A
RecU Holliday junction resolvase	2	
Endonuclease III (EC 4.2.99.18)	4	V to I
DinG family ATP-dependent helicase YoaA	32	N to D, S to P, H to L
DNA recombination and repair protein RecO	11	H to Q
Endonuclease IV (EC 3.1.21.2)	19	M to T, S to N, E to K, Q to K, A to E
DNA-3-methyladenine glycosylase II (EC 3.2.2.21)	7	L to F, Truncated protein
Single-stranded-DNA-specific exonuclease RecJ	38	E to A, N to G, K to N, T to A, V to A, A to T, T to A (E to A, N to G, K to N, R to L, Truncated protein
DNA repair protein RecN	6	T to P, S to G
Exodeoxyribonuclease VII small subunit (EC 3.1.11.6)	1	
Exodeoxyribonuclease VII large subunit (EC 3.1.11.6)	7	T to A, D to E, K to E
Formamidopyrimidine-DNA glycosylase (EC 3.2.2.23)	5	S to V
DNA polymerase I (EC 2.7.7.7)	28	V to A, D to E, D to N, E to A
3'->5' exoribonuclease Bsu YhaM	1	*

* Table continues on next page.

RecD-like DNA helicase YrrC	43	ATG to GTG, V to I, V to I, H to R, F to L, D to E, K to Q, A to R, R to S, H to P, Truncated protein
DNA-3-methyladenine glycosylase (EC 3.2.2.20)	4	K to N, D to G
DNA mismatch repair protein MutL	9	
Recombination protein RecR	2	
DNA repair protein RadA	5	T to A
Excinuclease ABC subunit A paralog of unknown function	17	L to P, R to Q, S to A, G to A
RecA protein	7	Q to E
DNA-3-methyladenine glycosylase (EC 3.2.2.20)	2	
DNA-3-methyladenine glycosylase (EC 3.2.2.20)	8	Q to K

Table A-5 Mutations identified in the *Lactobacillus casei* 12A-AE genome. The table shows location of the mutation (NCBI accession number CP006690), a description of the location, the reference bp (Ref) in the 12A parental wild type strain, the identified bp variation/mutation (Var) in the 12A-AE strain, and any resulting amino acid (aa) changes to the corresponding open reading frame. (-) = deletion, (>) = insertion

Location	Description	Ref	Var	aa Changes
128948	Lead, cadmium, zinc and mercury transporting ATPase; Copper-translocating P-type ATPase	A	-	Truncated Protein
502422	Orf2 protein	G	T	A to S
564350	hypothetical protein	C	-	aa seq change and loss of stop codon
797677	Upstream of Small Subunit Ribosomal RNA; ssuRNA; SSU rRNA	A	G	
896367	Upstream of Organic hydroperoxide resistance transcriptional regulator	T	C	
914258	Upstream of Maltose operon transcriptional repressor MalR, LacI family	>	T	
948234	Upstream of Transcriptional regulator, MerR family, near polyamine transporter	C	-	
1425556	Lipoprotein signal peptidase (EC 3.4.23.36)	C	T	Silent
1533236	Upstream of Mobile element protein and FIG00747381: hypothetical protein	>	T	
1772910	upstream of Magnesium and cobalt efflux protein CorC	A	-	
1938478	GTP-binding protein HflX	C	T	Silent
1964037	Mobile element protein	C	G	Silent
2062112	FIG00746766: hypothetical protein	A	-	Truncated protein
2081950	Cyclopropane-fatty-acyl-phospholipid synthase (EC 2.1.1.79)	G	T	A to D
2156978	lincomycin-resistance protein	G	-	Truncated protein
2194896	Glutamine amidotransferase class-I (EC 6.3.5.2)	T	C	D to G
2529086	Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	G	A	A to V
2693957	Transcriptional antiterminator, BglG family	A	G	Q to R
2756064	L-alanine-DL-glutamate epimerase	C	-	Truncated protein

Table A-6 Mutations identified in the *Lactobacillus casei* 12A-MAE genome. The table shows location of the mutation (NCBI accession number CP006690), a description of the location, the reference bp (Ref) in the 12A parental wild type strain, the identified bp variation/mutation (Var) in the 12A-MAE strain, and any resulting amino acid (aa) changes to the corresponding open reading frames. (-) = deletion, (>) = insertion.

Location	Description	Ref	Var	aa Changes
4768	Alpha-galactosidase (EC 3.2.1.22)	TGT	CAC	H to C
8473	DNA gyrase subunit A (EC 5.99.1.3)	T	C	V to A
113330	LSU ribosomal protein L9p	C	A	A to D
138627	ABC transporter, ATP-binding protein	A	G	M to V
158029	Upstream of Xanthine/uracil/thiamine/ascorbate permease family protein and Uncharacterized protein UPF0344	A	-	
197813	Upstream of Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	T	-	
209900	Beta-propeller domains of methanol dehydrogenase type	G	A	D to N
222316	Predicted dinucleotide-binding enzyme	T	C	V to A
233205	DNA-binding response regulator, OmpR family	G	A	V to M
240965	Upstream of Major myo-inositol transporter IolT and Transcriptional repressor of the myo-inositol catabolic operon DeoR family	A	G	
283991	Branched-chain amino acid ABC transporter, amino acid-binding protein (TC 3.A.1.4.1)	T	C	no aa change
392696	internalin, putative (LPXTG motif)	A	G	no aa change
396539	**Hypothetical membrane protein-1**	**T**	**-**	**Truncated protein**
463511	Conjugated bile salt hydrolase related amidase	G	A	G to S
486182	Glutamine synthetase type I (EC 6.3.1.2)	A	G	Q to R
504137	**Sortase A, LPXTG specific**	**A**	**-**	**Truncated protein**
555160	Mobile element protein	A	T	no aa change
575492	Dicarboxylate/amino acid:cation (Na+ or H+) symporter	T	C	V to A
620654	Galactokinase (EC 2.7.1.6)	G	A	A to T*

* Table continues on next page.

627793	FIG00744482: hypothetical protein	G	A	no aa change
673801	**NADH dehydrogenase (EC 1.6.99.3)**	**A**	**-**	**Truncated protein**
753549	FIG028593: membrane protein	T	C	T to A
860830	Peptide chain release factor 2	A	G	no aa change
865965	Phosphate regulon sensor protein PhoR (SphS) (EC 2.7.13.3)	C	T	no aa change
870305	**Phosphate transport ATP-binding protein PstB (TC 3.A.1.7.1)**	**A**	**-**	**Truncated protein**
894527	FIG002813: LPPG:FO 2-phospho-L-lactate transferase like, CofD-like	A	G	N to S
948358	Upstream of Transcriptional regulator, MerR family, near polyamine transporter	G	T	
980860	Alpha-galactosidase (EC 3.2.1.22)	G	A	G to D
1051949	Xanthine permease	A	G	M to V
1052108	Xanthine permease	G	A	E to K
1172193	Upstream of NrdR-regulated deoxyribonucleotide transporter, PnuC-like	A	-	
1281775	Late competence protein ComEC, DNA transport	T	C	no aa change
1285891	FIG00743830: hypothetical protein	A	G	T to A
1306448	GTP-binding protein Obg	G	A	no aa change
1380992	Tyrosine recombinase XerC	G	A	no aa change
1420601	Dihydroorotase (EC 3.5.2.3)	T	C	T to A
1463668	ATP-dependent nuclease, subunit B	G	A	A to V
1508842	FIG00745194: hypothetical protein	T	C	no aa change
1547620	Membrane-associated zinc metalloprotease	G	A	L to F
1550154	Upstream of Undecaprenyl diphosphate synthase (EC 2.5.1.31)	G	A	
1743460	Bacitracin export permease protein BceB	A	G	no aa change
1879522	permease of the major facilitator superfamily	C	T	V to I
1898910	FIG00746301: hypothetical protein	G	A	A to V
1995422	Downstream of Flagellar hook-length control protein FliK and Branched-chain amino acid aminotransferase (EC 2.6.1.42)	>	A	
2057753	Upstream of FIG00752836: hypothetical protein and downstream of Alpha-galactosidase (EC 3.2.1.22)	A	G	
2061824	FIG00746766: hypothetical protein	A	G	no aa change*

* Table continues on next page.

2075532	PTS system, sucrose-specific IIB component (EC 2.7.1.69) / PTS system, sucrose-specific IIC component (EC 2.7.1.69) / PTS system, sucrose-specific IIA component (EC 2.7.1.69)	G	T	no aa change
2143845	**Hypothetical membrane protein-2**	**T**	**-**	**Truncated protein**
2236395	L-O-lysylphosphatidylglycerol synthase (EC 2.3.2.3)	G	A	R to C
2307831	Aminopeptidase C (EC 3.4.22.40)	C	T	no aa change
2352269	**Two-component quorum-sensing regulatory system, sensor histidine kinase**	**A**	**-**	**Truncated protein**
2365997	Fumarate hydratase class II (EC 4.2.1.2)	C	T	no aa change
2489781	Transcription-repair coupling factor	A	G	F to L
2588785	Aryl-alcohol dehydrogenase related enzyme	G	A	T to I
2599350	FIG00742144: hypothetical protein	T	C	M to T
2625490	PTS system, galactitol-specific IIC component (EC 2.7.1.69)	A	G	V to A
2661480	Upstream of PTS system, maltose and glucose-specific IIC component (EC 2.7.1.69) / PTS system, maltose and glucose-specific IIB component (EC 2.7.1.69)	C	A	
2726332	Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases	T	C	T to A
2726450	Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases	G	A	no aa change
2848064	Malate Na(+) symporter	A	G	no aa change
2886594	periplasmic component of efflux system	A	G	no aa change
2889068	ABC transporter, permease protein	G	C	M to I

APPENDIX B

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CURRICULUM VITAE

Tom Overbeck
Doctoral Candidate

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Education

Ph.D. 2017
Utah State University, Logan, UT
Major: Food Science with an emphasis on physiology and genetics of lactic acid bacteria.
Advisor: Jeff Broadbent
Dissertation: "Strategies for increased lactic acid production from algal cake fermentations at low pH by *Lactobacillus casei*"

BS 2012
Weber State University, Ogden, UT
Major: Microbiology
Minor: Chemistry

Publications

1. **Overbeck T**, Steele JL, Broadbent JR. 2016. Fermentation of de-oiled algal biomass by *Lactobacillus casei* for production of lactic acid. Bioprocess Biosyst Eng. 39:1817-1823.
2. **Overbeck T**, Hughes JE, Welker DE, Steele JL, Broadbent JR. 2017. Development of a transient *mutS*-based hypermutation system for adaptive evolution of *Lactobacillus casei* to low pH. Manuscript submitted to Appl Environ Microbiol.

Laboratory Experience

Graduate Research Assistant 2012 – Present
Department of Food Science
Utah State University

- My primary responsibilities involve conducting fermentations with lactic acid bacteria, identifying limitations for growth and production of desired end-products, and developing methodologies to address identified limitations to increase production of end-products under sub-optimal conditions.
- Responsible for purchasing and maintaining laboratory supplies and equipment.

Laboratory Skills

Microbiology

- Expertise in physiology of lactic acid bacteria, ability to relate microbial genetics and physiology, batch and fed-batch fermentations, common microbiological skills, enzymology, and experience with scanning electron microscopy.

Molecular Biology

- Expertise in genetics of lactic acid bacteria, genomic and plasmid DNA isolation, RNA isolation, genetic engineering and genome editing in bacteria, PCR, DNA sequence analysis, and experience with metabolic flux analysis.

Statistics

- Proficient in Statistical Analysis Software (SAS), experimental design, and statistical methods.

Advanced Training or Short Courses

Field-Emission Scanning Electron Microscope (FE-SEM) 2016
Microscopy Core Facility
Utah State University
Hands on user training for the field-emission scanning electron microscope FEI Quanta FEG 650.

Microbial Fermentation- Development & Scale-Up 2012
Center for Integrated Biosystems
Utah State University
Certificate Awarded
Laboratory work supported by lectures and discussions in microbial fermentation from bench scale to pilot scale. Designed to provide the skills and knowledge for design and optimization of fermentation processes in batch and fed-batch cultures.

Leadership Experience

Graduate Research Assistant 2012 – Present
Department of Food Science
Utah State University

- Responsible for training and supervising undergraduate researchers in the laboratory of Dr. Jeff R. Broadbent.

United States Army 2004 – 2008
2/505th Parachute Infantry Regiment, 82nd Airborne Division
Fort Bragg, North Carolina

- Served as a Team Leader from 2006 – 2007, responsible for supervising two individuals.

- Served as a Squad Leader from 2007 – 2008, responsible for supervising six individuals.

Awards and Honors

US Department of Energy Early Career Development Travel Award	2016
Dr. Niranjan R. Gandhi and Mrs. Josephine N. Gandhi Competitive Assistantship	2016
Dr. Niranjan R. Gandhi and Mrs. Josephine N. Gandhi Competitive Assistantship	2013
Dr. Niranjan R. Gandhi and Mrs. Josephine N. Gandhi Competitive Assistantship	2012
Bronze Star with Valor	2006

Professional Proceedings

1. **Overbeck T**, Hughes JE, Welker DE, Steele JL, Broadbent JR. 2016. An adaptive evolution approach utilizing reversible *mutS* inactivation for development of *Lactobacillus casei* strains with enhanced lactic acid tolerance at low pH. Oral presentation at the Society for Industrial Microbiology and Biotechnology Ann. Mtg., July 24-28, New Orleans, LA.
2. Broadbent JR, **Overbeck T**, Heidenreich J, Vinay-Lara E, Phrommao E, Steele JL. 2015. Enhancing the value of biofuels derived from algae and perennial grass feedstocks through integration of lactic acid fermentation. Poster at USDA NIFA Sustainable Bioenergy Project Director Meeting, November 3-5, Denver, CO.
3. Gertsch S, Nielsen C, Torgensen A, **Overbeck T**, Putman R, Paskett M, Cook A, Kerns T, Spencer C, Miller C. 2015. LactoWare. Oral and Poster presentations at the International Genetic Engineered Machine Giant Jamboree, September 24-28, Boston, MA.
4. **Overbeck T**, Steele JL, Broadbent. 2014. Optimization of lactic acid production by *Lactobacillus casei* from algae cake using a biorefinery approach. Poster presentation at the 11th Symposium on Lactic Acid Bacteria, August 31-September 4, Egmond aan Zee, Netherlands.
5. **Overbeck T**, Engelhart E, Jee-Hwan O, Phrommao E, Steele JL, Broadbent JR. 2013. Fermentation of Algal Carcass Material by *Lactobacillus casei*. Poster presentation at the 35th Symposium on Biotechnology for Fuels and Chemicals, April 29-May 2, Portland, OR.
6. Broadbent JR, Oberg TS, **Overbeck T**, Ward RE, Welker DE, Hughes JE, Vinay-Lara E, Reed J, Steele JL. 2012. Enhancing the value of biofuels derived from algae and perennial grass feedstocks through integration of lactic acid fermentation. Poster at USDA NIFA Sustainable Bioenergy Project Director Meeting.
7. Vandenberg K, **Overbeck T**. 2012. Comparison of 5-aminolevulinate synthase of the Shemin pathway in alpha-proteobacteria and eukaryotes. Oral presentation at the American Society for Microbiology Intermountain Branch Meeting, April 7, Pocatello, ID.