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Delshadi, Rana ***Characterization of Novel Yeasts that Ferment Lactose in Cheese Whey***

Abstract

Cheese whey offers a potential carbon source for ethanol production since it is rich in lactose. Yeast species such as *Kluyveromyces marxianus* can produce ethanol from whey. Here, we enriched for and screened wild yeast strains for their ability to ferment the lactose in whey with the goal of providing new species for industrial adoption. We characterized 11 strains capable of growing on galactose and lactose under mildly acidic conditions. Of these, we chose 3 yeast strains capable of producing gas and lowering the pH. Their fermentation ability was compared to the known fermenters *Saccharomyces cerevisiae* and *K. marxianus*. Lactose concentrations decreased while ethanol yield increased for *S. cerevisiae* and cultivated strain 1-TENH-1 grown in whey containing 25% lactose. In contrast, *K. marxianus* and cultivated strains 3-RMLT-1 and RM-3 showed higher ethanol production in 12%-lactose whey. The maximum ethanol concentration attained was 12%, produced by *S. cerevisiae* grown in 25% lactose, compared to 2.9% by 1-TENH-1 in the same medium. Although we cultivated wild *K. marxianus* strains capable of producing ethanol from lactose, the ethanol yield was relatively low compared to *S. cerevisiae.* These results suggest that although wild yeasts and *K. marxianus* are capable of ethanol production, *S. cerevisiae* is more economically feasible.

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Chapter I: Introduction and Literature Review

As a by-product, cheese whey has a considerable amount of lactose that can be fermented for ethanol production. There are yeast species such as *Kluyveromyces marxianus* that are capable of producing ethanol from cheese whey but unfortunately, this organism is not widely used by the dairy industry. As a result, large volumes of unutilized dairy whey are currently treated as waste. It is important to investigate the parameters and the best condition that optimize cheese whey fermentation.

Fermentation

Fermentation has long been used in the food industry to produce beverages, bread, and dairy products, and in ethanol plants to produce alternative renewable fuel. Anaerobic metabolisms are also widely used to produce pharmaceuticals and industrial chemicals (Beniwal, Saini, Kokkiligadda, & Vij, 2017; Budimir, Jarić, Jaćimović, Genić, & JaćImović, 2011). Respiration and fermentation are two pathways for producing adenosine triphosphate (ATP) from sugars. During glycolysis, sugar is enzymatically oxidized to an organic compound (pyruvate) leading to the net production of 2 ATPs. The liberated electrons are captured by 2 NADHs. The yield of ATP in respiration is 12-14 times higher per glucose molecule oxidized compared to fermentation. In aerobic respiration, pyruvate dehydrogenase transforms pyruvate to acetyl coenzyme A, and via the Krebs cycle, the acetyl coA is completely oxidized to CO_2 (Figure 1). The resulting electrons are transferred by the electron transport chain to O_2 (Piskur, Rozpedowska, Polakova, Merico, & Compagno, 2006). In contrast, fermentation occurs in the absence of oxygen. Without oxygen, the electrons captured by NADH are transferred to pyruvate, creating energy-rich products (ethanol or organic acids) but resulting in little ATP production (Goddard & Greig, 2015). This metabolism requires the enzymes pyruvate

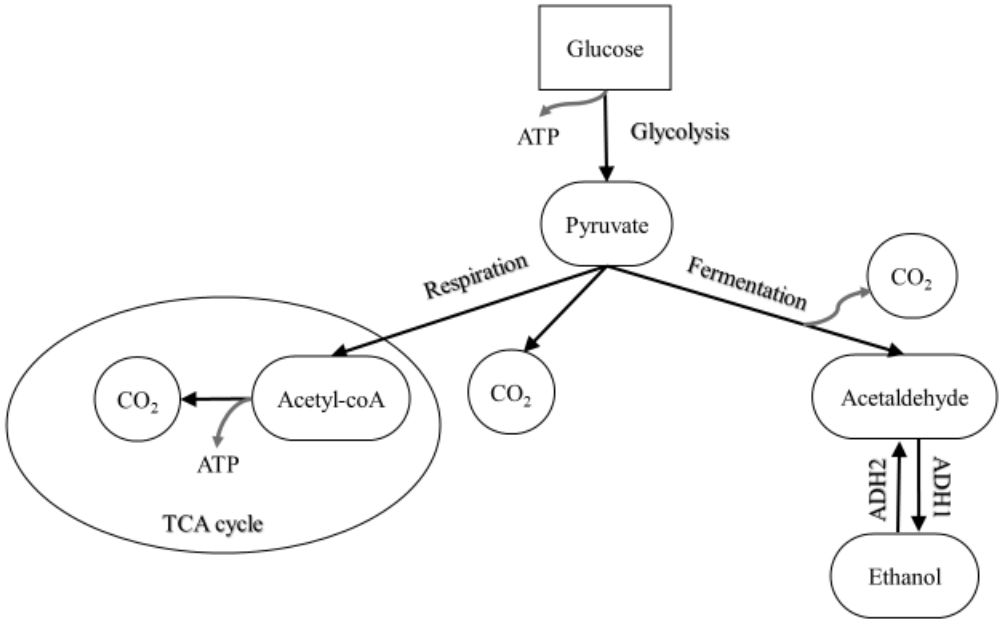

Figure 1. Fermentation and respiration pathways of glucose. ADH1, alcohol dehydrogenase 1; ADH2, alcohol dehydrogenase 2; ATP, adenosine triphosphate; TCA, tricarboxylic acid cycle.

decarboxylase (removal of CO_2 from pyruvate to produce acetaldehyde) and alcohol dehydrogenase (conversion of acetaldehyde to ethanol). The transformation of energy without the involvement of oxygen is called anaerobic fermentation and pyruvate acts as the electron acceptor (Dos Santos, Maria Gonçalves, & Suguimoto, 2014; Piskur et al., 2006). During alcoholic fermentations, microorganisms degrade carbohydrates without completely oxidizing them to CO_2 and energy is not captured by oxidative phosphorylation.

While most fermentations occur under anaerobic conditions, some yeast perform fermentation in the presence of O_2. Aerobic fermentations happen when there is a high amount of available sugar. This phenomenon is called the Crabtree effect and organisms that generate ethanol in the presence of oxygen are Crabtree-positive (Reynders, Rawlings, & Harrison, 1997). Once the sugar concentrations become sufficiently high, Crabtree-positive organisms set their sugar metabolism to fermentation. *S. cerevisiae* is Crabtree-positive while *K. marxianus* is Crabtree-negative (Verduyn et al., 1992). Since ethanol is poisonous to most microorganisms, accumulating ethanol in the presence of oxygen is a strategy that *S. cerevisiae* uses to prevent growth of its competitors (Piskur et al., 2006). Other fermentation products include organic acids (acetate, lactate, propionate and butyrate) and alcohols (butanol, isopropanol and 2,3-butanediol). In addition to the fermentation products, secondary yeast metabolites (amino acids and vitamins) are often used in the biopharmaceutical and chemical industries (Beniwal et al., 2017; Lane & Morrissey, 2010).

Cheese Whey

Cheese whey is a byproduct of cheese manufacturing. During cheese production, the volume of whey is almost nine times more than the volume of cheese (Hadiyanto, Ariyanti, Aini, & Pinundi, 2014). Whey contains 80% of the original milk volume and 20% of the original milk

protein. It also contains nutrients such as 4-5% lactose, 0.4-0.5% lipids, 0.05% lactic acid, citric acid, 0.5% mineral salts, and essential vitamins (Dos Santos, Maria Gonçalves, & Suguimoto 2014; Hadiyanto et al., 2014; Ling 2008; European Dairy Association, 2016). According to the USDA, a total of 1037 million pounds dry whey was produced by US dairies in 2017 (USDA, 2017) and the total dry whey production of Wisconsin was 317,336 pounds in 2016 (USDA, 2016). The annual world production of whey exceeds 160 million tons (Das, Sarkar, Maiti, & Bhattacharjee, 2016). Depending on the milk and type of cheese being produced, the composition of whey varies from sweet (pH 6-7) to acid whey (pH <5) (Grba, Stehlik-Tomas, Stanzer, Vahèi, & Škrlin, 2002). Whey created during production of hard cheeses such as cheddar, mozzarella and Swiss via rennet-coagulation is sweet while whey created during production of cottage cheese obtained via acid-coagulation is acidic (Koushki, Jafari, & Azizi, 2012).

Cheese manufacturers utilize whey in several ways (Figure 2). The first step of whey utilization is to separate proteins by ultrafiltration. This filtered product is called whey permeate and is rich in the sugar lactose, representing more than 70% of whey solids. Permeate has high concentrations of lactose (normally 5%, but up to 25% if the whey was concentrated via reverse osmosis) (Guimarães, Teixeira, & Domingues, 2010). Most whey permeate is currently disposed via sewage treatment or spraying on agricultural fields (Das et al., 2016; Parrondo, Garcia, & Diaz, 2000). Alternatively, whey can be used to produce foods such as whey protein, lactose powder, and animal feed. Whey proteins containing α-lactalbumin, β-lactoglobulin, immunoglobulin, and bovine serum albumin (Koushki et al., 2012) are separated by ultrafiltration and reverse osmosis concentration and are used in the food industry.

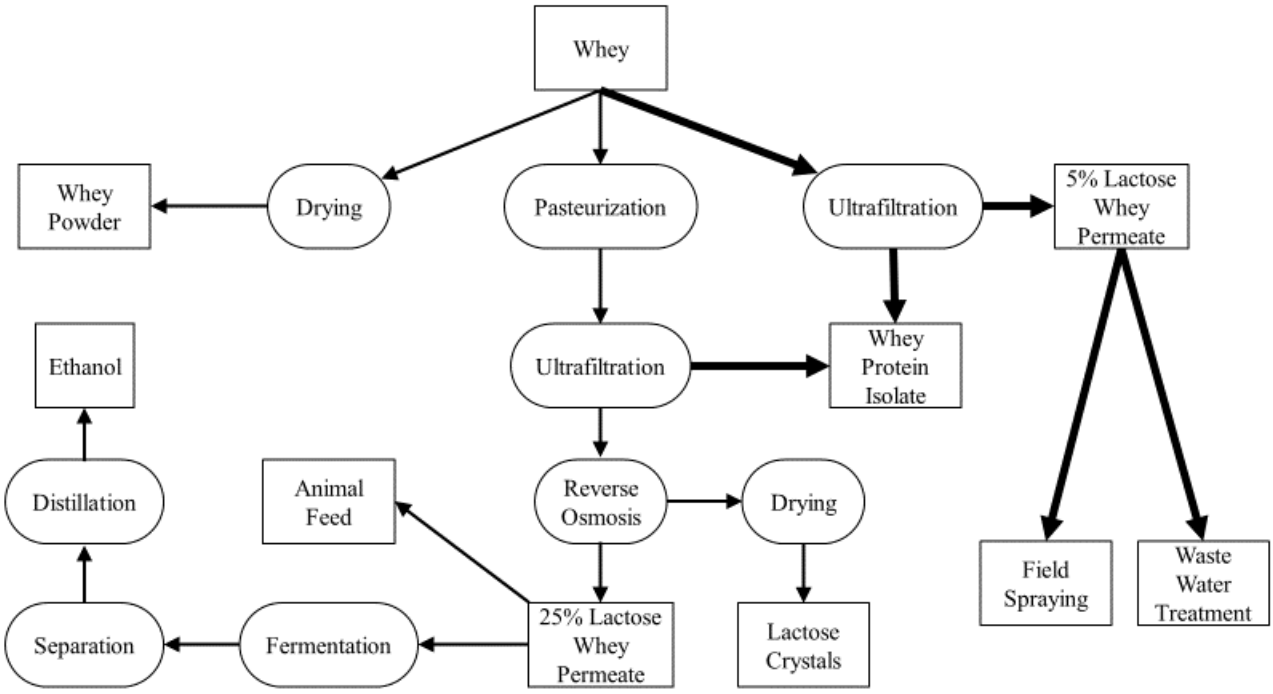

Figure 2. Products of whey and the steps to create ethanol from whey. (Modified from Das et al., 2016; Dos Santos et al., 2014; Ling, 2008) Double thickness denotes a larger relative amount of whey utilization.

Another possible application is to use the lactose in whey as a carbon source for ethanoic fermentation. In this case, the concentrated whey permeate is fermented by yeast that are capable of fermenting lactose. Once fermentation is complete, the liquid is distilled to extract ethanol (Ling, 2008).

Due to market economics, many manufacturers choose to discard the whey as waste by spraying it on fields. Whey has a high polluting load and introducing it into a wastewater treatment system raises the Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) from 30,000 – 50,000 ppm and 60,000-80,000 ppm, respectively. That waste can represent a significant environmental problem (Das et al., 2016). Polluted rivers typically have a BOD of 10-20 ppm (Connor, 2016). The biological technology used to reduce the BOD

and COD of whey by aerobic and anaerobic wastewater treatment is an expensive solution to this environmental problem (Ryan et al., 2013).

Microbes

Yeasts are most widely used in the food and biotechnology industries to produce foods, beverages, and enzymes. They can be classified as aerobic, facultative, or respire-fermentative (Lane & Morrissey, 2010). The common feature of yeasts is their ability to assimilate and acquire energy from different sugars. The goal of this study is to understand how different strains of *K. marxianus* and *S. cerevisiae* ferment the lactose in whey permeate into ethanol (Table 1). These two strains are both classified as respire-fermentative yeasts and they acquire energy either by fermentation or oxidative phosphorylation via the tricarboxylic acid (TCA) cycle (Lane & Morrissey, 2010). Depending on the strain, sugar concentrations and oxygen levels determine the choice between fermentation or respiratory pathways (Piskur et al., 2006; Silveira, Passos, Mantovani, & Passos, 2005).

Table 1

Summary of K. marxianus and S. cerevisiae Characteristics for Different Carbon Sources

	Strains	Glucose	Galactose	Lactose	Sucrose	Maltose	Raffinose	Terehalose	Inulin	Melibiose	Ethanol	Methanol
Fermentation	*S. cerevisiae*	+	V	-	+	V	+	-		V		
	K. marxianus	+	S	V	+	-	+	-	S			
Assimilation	*S. cerevisiae*	+	V	-	+	+	+	+		V	+	-
	K. marxianus	+	S	V	+	-	+	W/-	+		+	-

+, positive; -, negative; V, variable; W/-, weak or negative; S, positive but slow.

Saccharomyces cerevisiae. *Saccharomyces* is a diverse group of yeast strains that includes baker's yeast, wine yeasts, brewer's yeast, and distiller's yeast (Kurtzman & Fell, 1998). This strain has evolved the ability to adapt to a wide diversity of environments (Goddard & Greig, 2015). *S. cerevisiae* has been the leader in fermentation due to its osmotolerant behavior, low cost, and tolerance to high sugar and ethanol concentrations, making it ideal for most ethanolic fermentations (Goddard & Greig, 2015; Mohd Azhar & Abdulla, 2018). When the sugar concentration is high, the Crabtree effect in *S. cerevisiae* pushes the process toward fermentation and ethanol production becomes even higher under high oxygen concentrations (Piskur et al., 2006). However, because *S. cerevisiae* lacks the enzymes lactose permease and β-galactosidase that transport lactose into the cell and hydrolyze it to the monomers glucose and galactose, the vast majority of *Saccharomyces* species are not able to metabolize lactose and produce ethanol. Consequently, it is mandatory to pre-hydrolyze lactose with the enzyme lactase (β-galactosidase) if we are to use *Saccharomyces* to produce ethanol from whey (Beniwal et al., 2017; Dos Santos et al., 2014; Lane & Morrissey, 2010). The cost of this added enzyme must be considered when calculating the economics of ethanol production from whey. Moreover, galactose fermentation in this yeast is slower compared to other sugars such as glucose and fructose since this sugar isn't directly incorporated during glycolysis (Beniwal et al., 2017). Because of these reasons, *Saccharomyces* is not the first choice for fermenting the lactose in cheese whey. Creating genetically engineered lactose-consuming *S. cerevisiae* strains was recently employed to overcome lactose metabolism deficiencies (Das et al., 2016).

Kluyveromyces marxianus. *K. marxianus* is a GRAS (generally recognized as safe) yeast strain that belongs to the group of dairy yeasts long known for their potential in the food industry. *Kluyveromyces* is fully capable of metabolizing lactose as a carbon source due to

presence of the enzymes lactose permease and β-galactosidase. These enzymes transport and hydrolyze lactose into glucose and galactose (Guimarães et al., 2010). As a respiro-fermentative yeast, *K. marxianus* has the ability to acquire energy either via the TCA (tricarboxylic acid cycle) or by fermentation to ethanol under certain conditions (Lane & Morrissey, 2010). *K. marxianus* has extracellular pectinolytic activity and can produce glycerol from lactose but it has lower tolerance to ethanol compared to *S. cerevisiae* (Kurtzman & Fell, 1998).

Types of Fermentation

In industrial applications, two types of fermentation are used, liquid-state and solid-state. In liquid-state fermentation, microorganisms are grown in a liquid medium and environmental conditions such as nutrient concentration, pH, temperature, and other parameters are controlled. In solid-state fermentations, fermentation occurs on solid substrates in the absence of free water, thereby producing less waste (Pandy, 2003). Compared to liquid-state, solid-state fermentations offer less control over environmental conditions. A study showed that enzyme productivity of *Aspergillus niger* was higher using solid-state fermentation than liquid-state fermentation (Viniegra-González et al., 2003).

Types of Processes

Fermentation can be classified as continuous, batch, and fed-batch operation (Grba et al., 2002; Silveira et al., 2005). In continuous fermentation, fresh media is added continuously to the fermenter and spent medium is removed so the organisms are always in log phase. In contrast, batch fermentation happens in a closed vessel for a specific period and under optimal conditions. Widely used in industry, fed-batch mode adds carbon substrates periodically during fermentation. A study showed that fed-batch operation resulted better in β -galactosidase production and offered many other advantages compared to the batch system since it can prevent

decreasing carbon substrate concentrations during fermentation (Rech & Ayub, 2007).

Parameters

Environmental parameters can significantly affect fermentation processes. By controlling environmental variables, successful fermentations can be achieved. The main environmental conditions influencing fermentations are available oxygen, sugar availability, temperature, pH, and nutrient concentrations (Dragone, Mussatto, Almeida e Silva, & Teixeira, 2011; Hadiyanto et al., 2014). Temperature is an important factor in fermentation. Research conducted by Hadiyanto et al. (2014) concluded that a temperature of 30°C resulted in the highest ethanol concentration during whey fermentation with *K. marxianus* compared to 35°C and 40°C while another study reported that *K. marxianus* can produce ethanol from lactose media at temperatures as high as 45°C (Brady et al., 1995). As sugars are fermented and nutrients (N, P) are consumed, nutrient limitations present another concern. Likewise, high concentrations of lactose can prevent fermentation (Guimarães et al., 2010). High ash content accompanies higher whey concentrations and can inhibit yeast growth and cause fermentation disorders (Mahmoud & Kosikowski, 1982). Oxygen should be low enough to enhance fermentation, yet if oxygen concentrations are too high, this causes excessive growth occurs which ends up producing yeast biomass rather than ethanol (Guimarães et al., 2010).

Lactose

Lactose is the dominant carbon source in whey. This disaccharide consists of two monomers, glucose and galactose, connected via a β-1,4 glycosidic bond. The solubility and sweetness of lactose is lower than other sugars. Lactose can be obtained for use in the food industry by drying and crystalizing whey permeate. Bioplastics can also be produced by bioconverting lactose in whey (Ryan et al., 2013). Fermentation is another application to utilize

lactose and produce ethanol (Guimarães et al., 2010). The conversion of lactose into ethanol is not currently economically competitive with cornstarch and other sugars (Guimarães et al., 2010). Before lactose fermentation is economically competitive with corn-based fermentations, we need to better understand the carbon metabolism and discover new yeasts capable of economic lactose fermentation.

Purpose of the Study

To achieve good utilization of lactose from whey, it is important to increase our knowledge of yeast strains capable of utilizing dairy sugars. The present work aims to study the physiology of *K. marxianus* growing on galactose as a carbon source and to optimize the conditions for ethanol production. This study is intended to investigate the possibility of using lactose in whey as a carbon source for vodka production. Here, we cultivated and tested several wild yeast strains including *K. marxianus* with the aim of choosing strains suitable for alcohol production. Our goal is to discover yeast strains capable of fully fermenting lactose by utilizing both glucose and galactose. Several recent studies have investigated the fermentation of whey to ethanol by *Kluyveromyces* (Beniwal et al., 2017; Boudjema, Fazouane-naimi, & Hellal, 2016; Dragone et al., 2011; Hadiyanto et al., 2014; Koushki et al., 2012) but none have compared the ethanol yield of *Kluyveromyces* to *S. cerevisiae* in a whey-to-vodka application. In addition, we aim to provide the resulting yeast to our industrial partner, Copper Crow Distillery, to help them develop their whey-vodka product.

Chapter II: Methodology

Galactose and lactose were procured from Fisher Scientific (Hampton, NH) and Neogen corporation (Lansing, MI). Yeast nitrogen base was purchased from DOT scientific (Burton, MI). YM agar and YM broth were obtained from Difco Laboratories (Fisher Scientific, Hampton, NH). Cheese whey permeates were obtained from Eau Galle Dairy (Eau Galle, WI) and Burnett Dairy Cooperative (Grantsburg, WI). Whey permeate was ultrafiltered and concentrated via reverse osmosis at Burnett Dairy only (Table 2). The HPLC used in this research was a Shimadzu liquid chromatograph (LC-20AB) equipped with a refractive index detector (RID-10A). The HPLC column was BIO-RAD Aminex HPX-87C250 4 mm (Hercules, CA) with a Phenomenex (Torrance, CA) guard column. *K. marxianus* was obtained from the American Type Culture Collection (ATCC 8554 and). *S. cerevisiae* was distiller's active dry yeast (#8147) obtained from Beverage Artisan (Menomonie, WI). The enzyme Hydrolact-W lactase (3000 U/g) was purchased from Enzyme Innovation (Chino, CA).

Enrichment Cultures

The wild microorganisms used in this study were isolated from soil samples collected from diverse environments in West Central WI and included spoiled dairy products. Enrichment cultures were established using different carbon source additions to sterile whey (Table 3). Lactose and galactose were added individually and together to cheese whey do determine the effect of added sugars.

Two grams of soil sample were added to 200 ml of each medium type in 1 pint sterilized Mason jars equipped with air locks. Air locks contained 5% potassium metabisulfite to limit microbial growth in the air lock. Enrichment cultures were incubated at 30°C with shaking (80 rpm). After 48 h, samples were removed from the jars, streaked for isolation on YM agar plates

Table 2

Components of Burnett Dairy Cooperative Whey Permeate Provided by AgSource Laboratories (Marshfield, WI)

Component	Concentration
Lactose	23.69%
Protein	0.83%
Casein Protein	0.46%
Sodium	1770 mg/l
Calcium	1720 mg/l
Cooper	<0.5 mg/l
Iron	<1.5 mg/l
Magnesium	353 mg/l
Manganese	<0.5 mg/l
Zinc	<0.5 mg/l
Lactic Acid	0.6%
Fat	0.01%

to obtain pure cultures, and grown overnight at 30°C. Several passages between solid media were sometimes required to obtain isolated colonies. To determine carbon utilization abilities and observe colony morphology, purified colonies were streaked for isolation on solid media containing YNB augmented with 10% lactose, 10% galactose, 5% lactose and 5% galactose, and

undiluted whey permeate, then incubated overnight at 30°C. Media were the same as during the enrichment culture (Table 3), except solidified with 15% agar. Strains that could grow on galactose and lactose as their sole carbon source were chosen for further experiments.

Table 3

Enrichment Culture Composition

Carbon Source	N and P Source	Diluted
10 g Lactose	6.7 g YNB	1 liter distilled water
10 g Galactose	6.7 g YNB	1 liter distilled water
5 g Galactose + 5 g Lactose	6.7 g YNB	1 liter distilled water
	6.7 g YNB	1 liter whey
	6.7 g YNB	1 liter whey

YNB, yeast nitrogen base; N, nitrogen; P, phosphorous.

Screening Experiment to Reduce the Strains

Selected strains were used to inoculate 10 ml test tubes containing YM broth and Durham tubes for assaying carbon dioxide production (Karki et al., 2017). The pH was measured aseptically using pH paper before inoculation (pH 6), after two days, and after seven days of incubation at 30°C. Gas production was observed after two and seven days of fermentation. From this experiment, 3 strains were selected and streaked on 100% whey plates in two trials. The first trial was incubated at 30°C aerobically and the second trial was incubated at the same temperature under anaerobic conditions using a Becton Dickinson gas-pack (BD, Franklin Lakes, NJ). These same strains were inoculated in 100% sterile whey tubes containing Durham tubes to assay gas production. Strains that could grow anaerobically, reduce the pH, and produce gas were chosen for further experimentation and appear in Table 6.

Molecular Characterization

DNA was extracted from a 250 mg cell pellet using the Ultra Clean Soil DNA Kit (MoBio Laboratories, Inc., Carlsbad, CA). For clone library construction, extracted DNA was diluted 1:25 with sterile water and PCR amplified using the eukaryotic primers 566F (5′ -CAG CAG CCG CGG TAA TTC C- 3′) and 1200R (5′ -CCC GTG TTG AGT CAA ATT AAG C- 3′) (Lekang et al., 2018). This approach yielded 634 bp of information for sequence analysis. Reactions contained 1× PCR buffer (Promega Corp, Madison, WI), 2.5 mM $MgCl_2$, 500 µg BSA/ml, 200 µM each dNTP, 10 pmol each primer, 2.5 U DNA polymerase, and ~200 ng template DNA in a final reaction volume of 25 µl. Reactions were held at 94°C for 2 min, followed by 30 amplification cycles of 90°C for 15 s, 55°C for 15 s, and 72°C for 45 s with a final extension of 72°C for 2 min. PCR products were separated on a 0.8% agarose gel, bands were excised, and amplified DNA was purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corp.) before TA cloning into the pGEM®-T Easy Vector (Promega Corp.) and transforming into *Escherichia coli* JM109 competent cells following the manufacturer's protocols. Plasmid DNA was mini-prepped using the Promega Wizard® plus mini prep kit (Promega Corp.). Plasmids were sequenced using the primers T7 (5′ -TAATACGACTCA CTATAGGG-3′) and SP6 (5′ -CATACGATTTAGGTGAC ACTATAG-3′).

Data Analysis

Consensus sequences were compiled for individual clones using the contig assembly function of the BioEdit software. Full-length consensus sequences for individual clones were compared to other known SSU rRNA gene sequences in the NCBI database using BLAST similarity searches. Phylogenetic affiliations were determined using the classifier program in the Ribosomal Database Project and verified by comparison to nearest GenBank relatives via

BLAST searching. Sequences were analyzed for chimeras using the chimera-check program available through the Ribosomal Database Project. Based on this analysis, none were removed from the library.

Sugar and Ethanol Characterization Experiment

Wild yeast strains 1-TENH-1, RM-3, 3-RMLT-1, and control strains *K. marxianus* and *S. cerevisiae* were inoculated in 10 ml sterile whey permeate and incubated at room temperature. After incubation for 24 hours, 10 ml of each were separately inoculated into 1 pint jars containing either 400 ml of 100% Burnett Dairy whey or 400 ml of 50% whey as described above. The pH of these media was reduced to 5.5 by adding citric acid. Citric acid is an intermediate of the citric acid cycle and can be used as a carbon source for ethanolic fermentation. When interpreting results from these experiments, we must consider that the added sugar (lactose) may therefore not be the sole carbon source. Lactase (24 U) was added to the flask containing *S. cerevisiae* to break down the lactose. Flasks were incubated at room temperature for 24 days (no shaking) and concentrations of lactose, glucose, galactose, and ethanol were determined using high performance liquid chromatography (HPLC). The mobile phase was 0.5 mM sulfuric acid applied at flow rate of 0.6 $ml \cdot min^{-1}$ and an injection volume of 25 µl. The column temperature was 60°C. Samples were aseptically obtained on days 0, 3, 6, 10, 17 and 24 and filtered through 0.45 µm nylon filters before analysis.

Statistical Analysis

The difference in ethanol yield between the two concentrations of whey (100% and 50%) by the strains 1-TENH-1, RM-3, 3-RMLT-1, *K. marxianus* and *S. cerevisiae* of were assessed by a two-sample t-test: assuming unequal variance at a 0.05 confidence interval.

Large Scale Fermentation Experiment

Two large tote containers each containing 1000 l of whey permeate were purchased from Burnett Dairy Cooperative. The brix, pH, and temperature of the whey was measured using a hydrometer, pH meter, and thermometer each day of the experiment. *S. cerevisiae* (Red Star distiller's active dry yeast) was used and the enzyme β-galactosidase (60,000 U) was used to break down the lactose. Tomato paste (12 oz), Fermaid yeast nutrient (25 g, Scott Laboratories, Petaluma CA), diammonium phosphate (50 g), and termamyl SC (An α-amylase, 12000 U, Novoenzymes North America, Franklinton, NC) were added on days 5 and 13. The main difference between the containers was sugar concentration. Lactose (25 kg) was added to the second container on the first day (Table 4). Due to lack of sterile conditions, citric acid (1000 g in the container with added lactose and 3500 g in the container with no added lactose) was added to prevent growth of bacteria and unwanted organisms on first and second days. The experiment was performed at ambient temperature at the Copper Crow Distillery (Red Cliff, WI). Samples were obtained every day of the experiment and were kept frozen (-20°C) until analysis via HPLC.

Distillation

The whey fermentation liquor was transferred to a 1000 l single pot still for distillation and heated using a water jacket. The 2.5 m distillation column was held at 78.5 °C and ethanol passing through a dephlegmator was collected through the condenser. A stripping run resulting in 95 proof alcohol was followed by a finishing run yielding 190 proof that was later diluted with water to 80 proof for final sale.

Table 4

Large-scale Experimental Conditions

Days	Control Container	Added Lactose Container
Day 1		2500 g citric acid
Day 2	1000 g citric acid	1000 g citric acid and 25 kg lactose
Day 3	10 g lactase	10 g lactase
Day 4	10 g lactase	10 g lactase
Day 5	12 oz tomato paste, 350 g red star yeast, 50 g DAP	12 oz tomato paste, 350 g red star yeast, 50 g DAP
Day 13	25 g yeast nutrient	25 g yeast nutrient and 100 ml Termamyl SC

DAP, diammonium phosphate

Chapter III: Results

Physiology of *K. marxianus* and several wild strains isolated from soil samples that were capable of growing on galactose and lactose as carbon sources was studied. Yeast strains that some of them were characterized as *K. marxianus* were capable of fully fermenting lactose in cheese whey. Ethanol production for strains were different in different lactose concentrations and more than 20 gallons of vodka from whey was produced by *S. cerevisiae.*

Wild Yeast Cultivation

The experimental results of the strains obtained from the enrichment cultures are shown in Table 5. 63 strains were isolated from 26 soil samples and purified on YM agar. We obtained 11 strains capable of growing at 30°C (pH 4.5) on the carbon sources galactose, lactose, galactose + lactose, whey, and whey + yeast nutrient broth. Strains with the same microscopic morphology were omitted from further analysis.

Physiological Capabilities of Selected Strains

The initial pH of the YM broth before inoculation of the 11 strains was 6. After 7 days of incubation at 30°C the pH of all strains dropped to between 4 and 5 except for strains 1-PEAL-1 and 4-NSMZ-1 that remained at 6. All strains were capable of producing gas except for 1-PEAL-1, 4-NSMZ-1, and 5-CKAD-1. In these tubes weak gas production was observed in the Durham tubes after 2 days, but gas was not present after 7 days (Table 6). Strains 1-TENH-1, 3-RMLT-1 and RM-3 were selected for further experiments due to their strong gas production after 7 days in whey broth, production of acid, and their ability to grow anaerobically on whey agar (Table 7).

Molecular Characterization

Based on their rRNA sequences, strains 1-TENH-1 (Figure 3) and 3-RMLT-1 were determined to be strains of *K. marxianus*. Strain RM-3 displayed an rRNA sequence identical to

Table 5

Characterization and Growth Behavior of Selected Strains

Strains	Molecular Characterization	Media	Observations	Growth on YM Agar and all Media?	Odor
1-TENH-1	*K. marxianus*	Lactose	Mildly cloudy	Yes	Bread
RM-3	*Barnettozyma. californica*	Galactose	Clear	Yes	Cheese
3-RMLT-1	*K. marxianus*	Lactose, Galactose	Bubbly scum on top, opaque	Yes	Sourdough
1-PEAL-1	*Galactomyces. candidum*	Lactose	Yellow opaque, foam on top	Yes	Sour
2-CV-1	*K. marxianus*	Galactose	Cloudy, foam bubbles on top	Not on Galactose + Lactose	Bread
2-RHKH-2	*K. marxianus*	Galactose	Cloudy, fuzzy white	Yes	Bread
4-NSMZ-1	*E. coli*	Whey	Cloudy, foam on top	Yes	Sour
5-HRTJ-1	*E. coli*	Whey, YNB	Slightly yellow turbid	Yes	Sweet
5-JMKB-2(1)	*Enterobacter. cloacae*	Whey	Yellow turbid	Yes	Sweet
5-JMKB-1	*Enterobacter. xiangfangensis*	Whey	Yellow turbid	Not on Galactose + Lactose	Bitter
5-CKAD-1	*K. marxianus*	Galactose	Turbid	Yes	Sweet

Table 6

pH and Gas Production of 11 Selected Strains

Strains	Gas after 2 days	Gas after 2 days	pH after 2 days	pH after 2 days
1-TENH-1	++	+	6	5
RM-3	++	++	6	4
3-RMLT-1	+	++	6	4
1-PEAL-1	++	-	6	6
2-CV-1	++	+	6	5
2-RHKH-2	+	++	6	4
4-NSMZ-1	+	-	6	6
5-HRTJ-1	+	++	6	5
5-JMKB-2(1)	++	++	6	4
5-JMKB-1	+	++	6	5
5-CKAD-1	++	-	6	4

++, strong; +, weak; -, negative

Barnettozyma californica. Although YM agar was used to select for yeast strains, a few strains of bacteria were also cultivated (Table 5) due to their tolerance to an acidic environment.

Effect of Whey Concentration

The effect of whey concentration on the production of ethanol was investigated. Ethanol production for *S. cerevisiae* reached 3.9 % in the medium containing 50 % Burnett Diary whey diluted in water by day 24, the highest among all strains. During the same time period, *S. cerevisiae* produced 12% ethanol in 100% whey, 3 times greater than in 50% whey (Figure 4). Of the wild strains, strain 1-TENH-1 displayed the highest ethanol production (2.9%) in 100%

Table 7

pH, Gas Production, and Anaerobic Growth Behavior of Selected Strains

		Results on whey agar and broth after 2 days				Results on whey broth after 7 days	
Strain	pH of control	Gas production	pH	Aerobic growth	Anaerobic growth	Gas production	pH
1-TENH-1	6	++	5	++	++	+	3
3-RMLT-1	6	+	5.5	++	+	++	4.5
K. marxianus	6	++	5	++	+	++	4
RM-3	6	+	5	++	+	++	5

++: strong, +: weak, -: negative

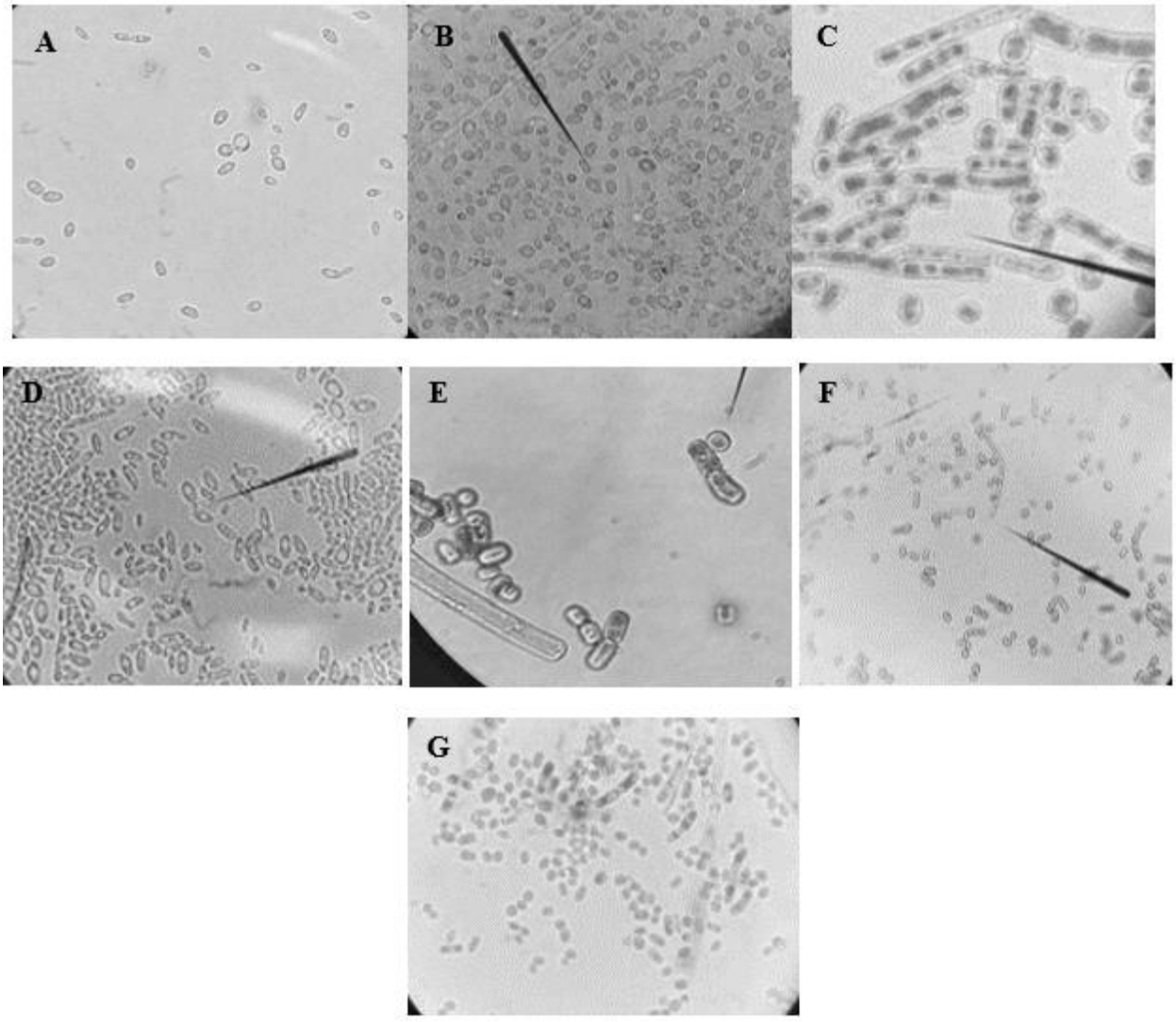

Figure 3. Microscopic characteristics (400 x magnification) of isolated organisms cultivated on YM Agar. **A**, 1-TENH-1; **B**, 5-CKAD-1; **C**, 2-RHKH-2; **D**, 5-JMKB-1; **E**, 5-HRTJ-1; **F**, 1-PEAL-1; **G**, 4-NSMZ-1.

whey but *K. marxianus* produced more ethanol in 50% whey after 24 days. Strains 3-RMLT-1 and RM-3 produced ethanol, but the concentrations were relatively low compared to other strains (Table 8). The difference between the ethanol yield of all strains grown in 50% versus 100% whey was not significant (p= 0.45). No ethanol formation was observed in control flasks for both concentrations.

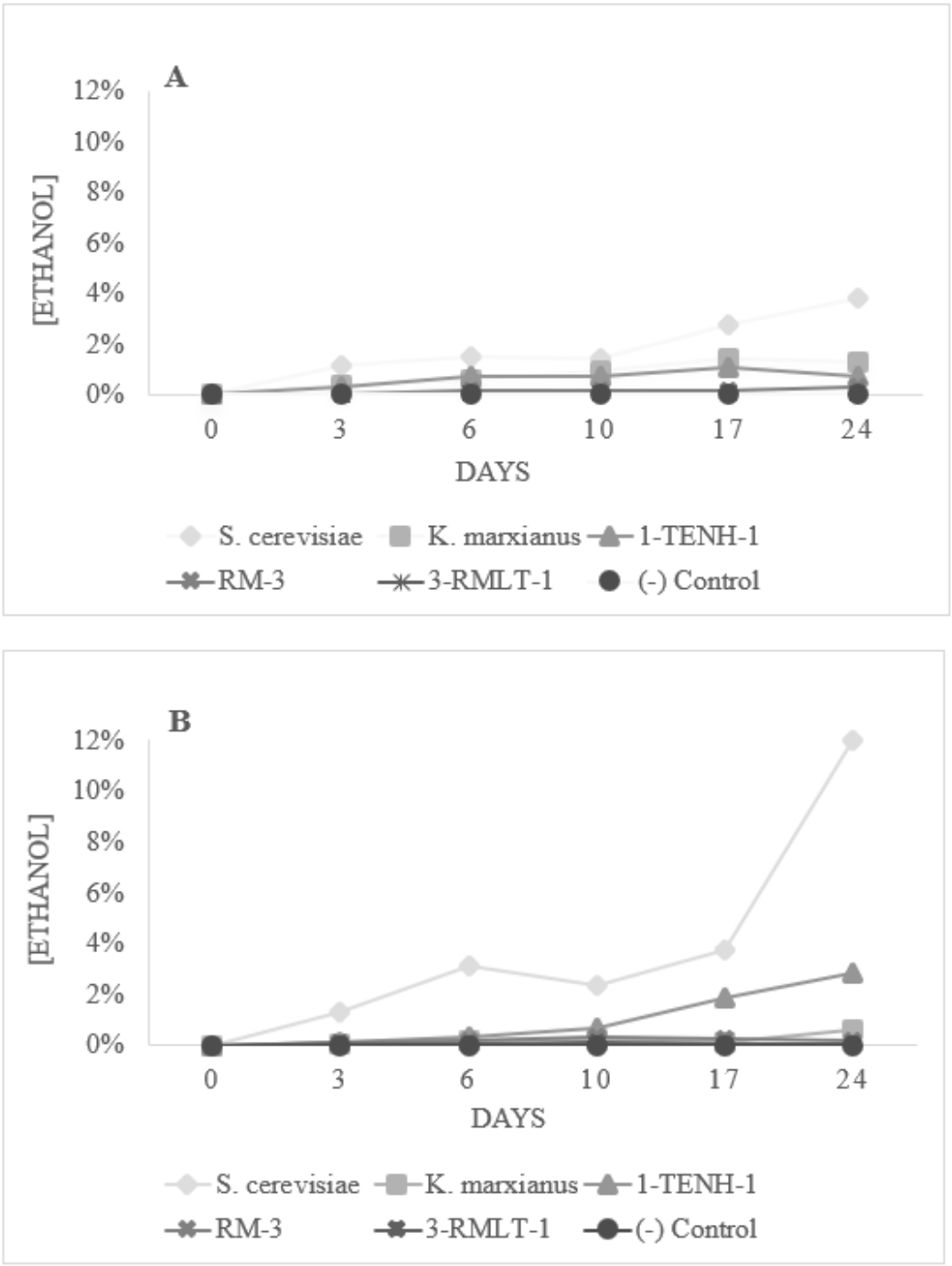

Figure 4. Ethanol production for all strains compared to *S. cerevisiae* and *K. marxianus* in differing lactose concentrations during 24 days of fermentation. **A**, 50% Whey; **B**, 100% Whey.

Table 8

Ethanol Produced by Each Strain After 24 Days

Strains	50% whey	100% whey
S. cerevisiae	3.9	12
K. marxianus	1.3	0.6
1-TENH-1	0.8	2.9
RM-3	0.3	0.2
3-RMLT-1	0.1	0.1

All selected wild strains were capable of fermenting galactose (Figure 5). The *S. cerevisiae* culture showed little galactose on day 0, but this sugar increased by day 3, then gradually decreased. The *K. marxianus* (1-TENH-1 and 3-RMLT-1) and *B. californica* (RM-3) strains displayed low galactose concentration from day 0. Due to incomplete separation of these sugars by HPLC we failed to measure sugar concentrations for some days and their data are not displayed.

Large-scale Lactose Addition Experiment

Since the totes were moved from ambient to indoor conditions, the initial temperature for both 1000 l totes was 6.6°C and it sharply increased until day 7. During days 7 to 16, the temperature remained relatively constant (Figure 6). The pH decreased slightly from 6.8 to 5.7 in both containers. The hydrometer displayed the same relative density of 13% until day 4 and after that it decreased to 7% in the last day. No ethanol data was obtained for days 7, 8, 11, 12, and 14 in the container with added lactose due to the thickness of a floating mass of protein that didn't allow the hydrometer to float. Brix, a measure of sugar content, held stationary until day 5, but after that it decreased from 22.2 to 17.5 in the control container and 15 in the container

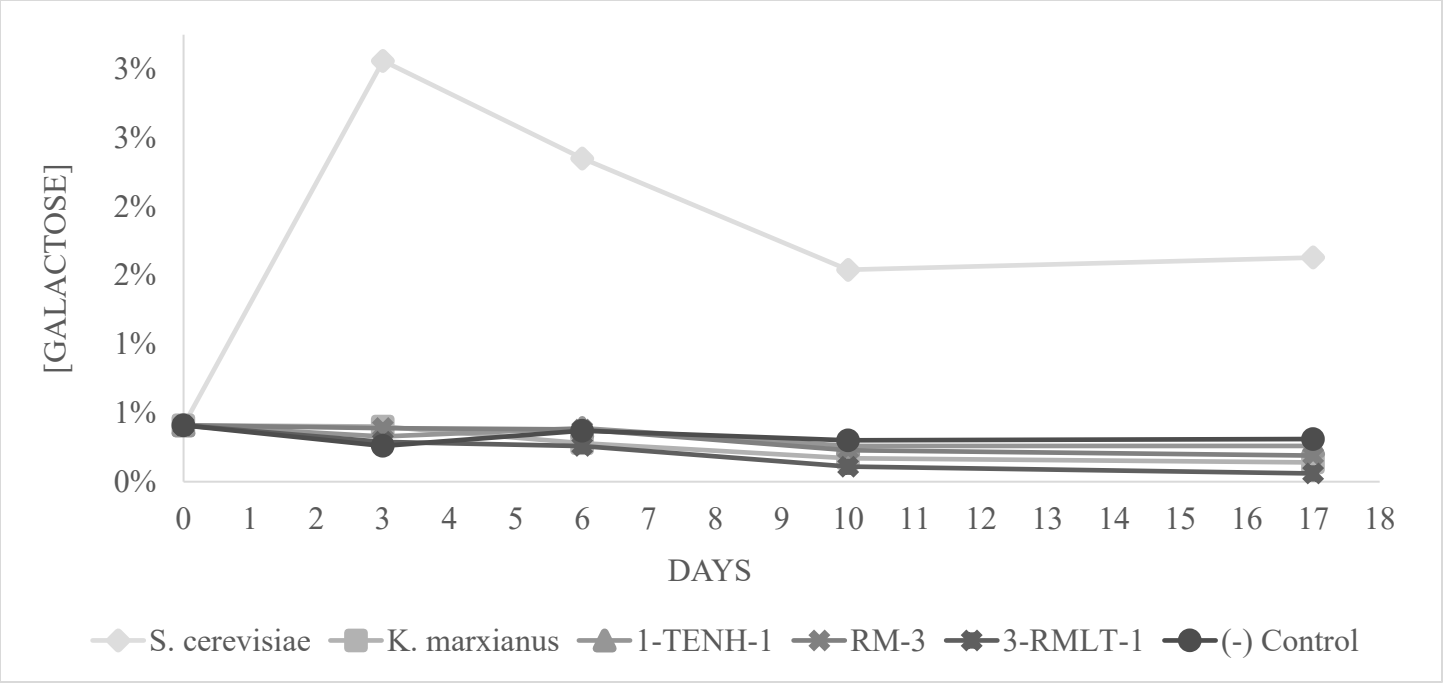

Figure 5. Galactose concentrations in 50% whey during 17 days of fermentation.

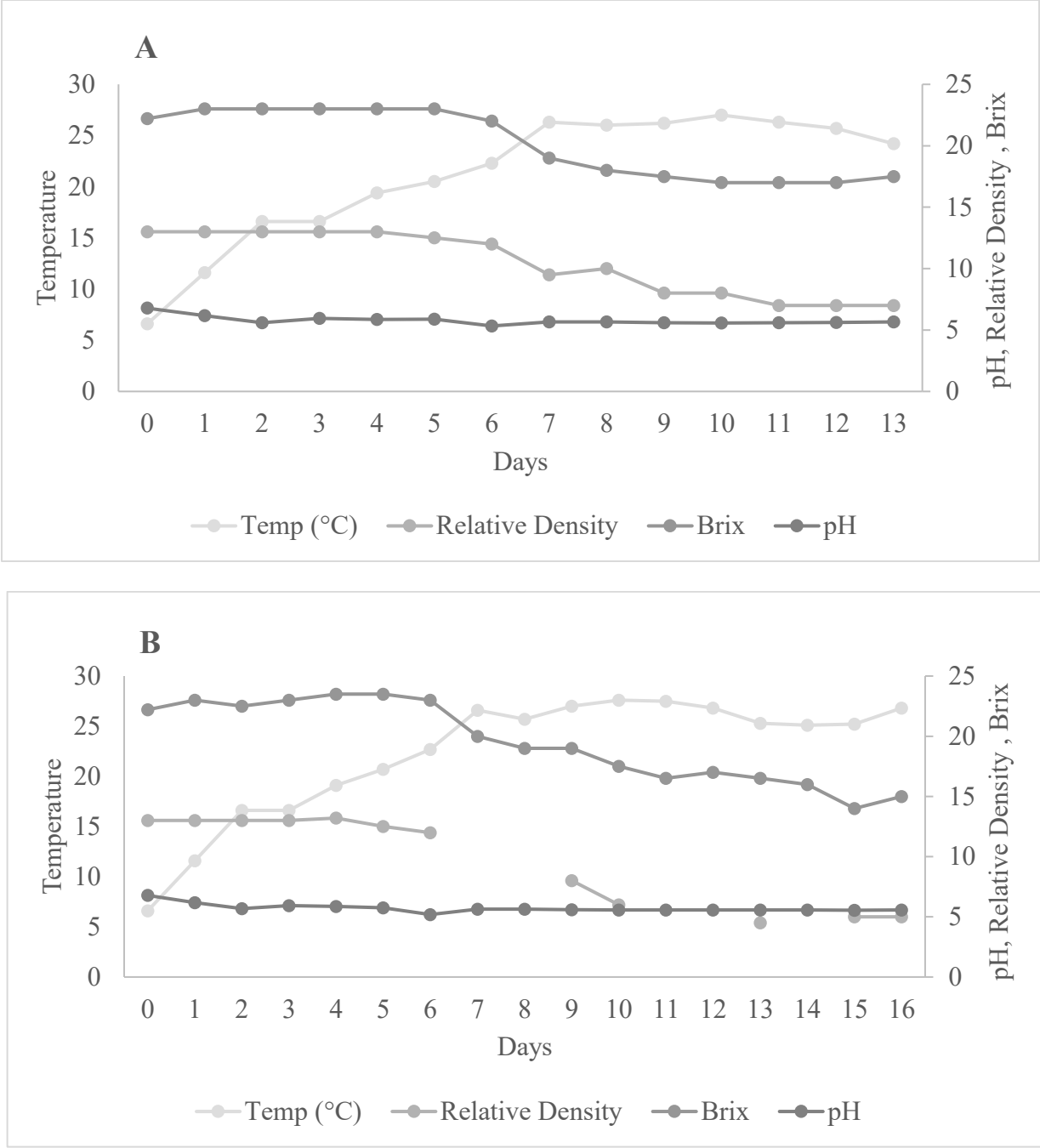

Figure 6. Data from temperature, ethanol, Brix, and pH of the control and added lactose containers. **A**, Control; **B**, Added Lactose.

with added lactose. The time of fermentation was longer in the container with added lactose (17 days) compared to (14 days) for the control container. We failed to measure sugar concentrations on days 4 to 7 due to HPLC difficulties (Figure 7). Lactose concentrations

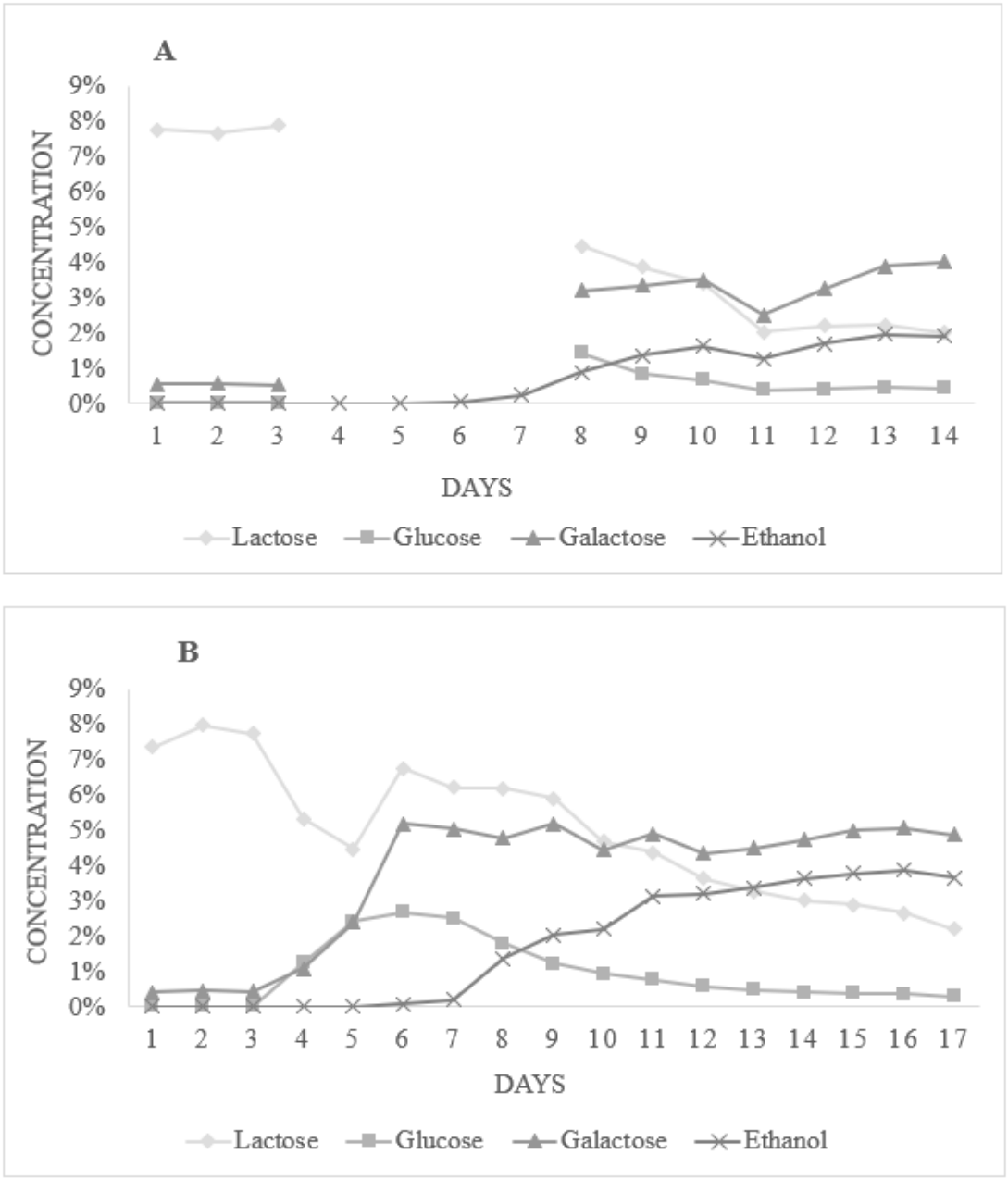

Figure 7. Ethanol and sugar concentrations of the control container and added lactose container during fermentation. **A**, Control; **B**, Lactose Added.

decreased during fermentation and remained at 2% in the control container until day 14. In the container with added lactose, lactose decreased to 2.2% on day 17. Glucose concentrations were less than 0.05% at the beginning for both containers and remained constant at the same level until day 3, then started to increase. Glucose concentrations began to decrease on day 6 until it reached less than 0.5% in both containers. Galactose concentrations were ~0.5% on the first three days but sharply increased from day 4 until reaching 4% in the control container and 4.8% in the container with added lactose. Yeast in both containers started to produce ethanol on day 6. Ethanol concentrations for the control and the container with added lactose were 1.9% and 3.6%, respectively. Upon distillation, 22.7 gallons of 95% proof ethanol was obtained from the control container, and 27 gallons of 112% proof ethanol was obtained from container with added lactose.

Chapter IV: Discussion, Conclusion and Recommendations

This study pursued using cheese whey as an alternative carbon source for fermentation. Cheese whey for ethanol production was affected by many variables such as type of strain, lactose concentration, enzymatic activities, pH, and temperature. The final outcomes helped our understanding of fermentation mechanisms and maximizing ethanol production from cheese whey, a common waste stream in the dairy State of Wisconsin

Research Summary

Although *K. marxianus* and wild *K. marxianus* strain 1-TENH-1 could produce ethanol from both 100% and 50% whey at room temperature, the level of ethanol produced by *K. marxianus* was relatively low compared to *S. cerevisiae*. This only occurred because we added the lactase to the *Saccharomyces* cultures to break down lactose into the monomers galactose and glucose. This could be due to environmental factors such as low temperature or oxygen levels. The best conversion of whey to ethanol by *Kluyveromyces fragilis* was measured at 34°C (Parrondo et al., 2000). Our wild strains may also be poisoned by high ethanol concentrations. Although 1-TENH-1 and 3-RMLT-1 are both *K. marxianus* strains, all behaved differently in different concentrations of whey. High whey concentrations negatively affected ethanol production of most *K. marxianus* strains, but it increased the ethanol produced by strain 1-TENH-1. Previous studies by Silveira et al. (2005) showed that *K. marxianus* grown in high lactose and under low oxygen levels had maximal conversion of lactose to ethanol. In another study, lactose utilization was more rapid under aerobic conditions (Mahmoud & Kosikowski, 1982). High lactose concentrations favored ethanol production by *S. cerevisiae* as well, while this strain showed minimal utilization of galactose. This fermentation requires the addition of lactase enzyme to cleave the lactose molecule. Other studies show that glucose was a preferred

substrate over galactose and that galactose utilization takes more time (Nguyen, Ra, Sunwoo, Jeong, & Kim, 2017). However, the *S. cerevisiae* strain KL17 was shown to be capable of fermenting galactose even in the presence of glucose (Kim et al., 2014). Another wild strain of *S. cerevisiae* isolated from grapes was able to ferment galactose to 15% ethanol (Mohd Azhar & Abdulla, 2018). Sugar concentration plays a key role in the behavior of this yeast. When sugar concentrations are high, *S. cerevisiae* switches to fermentative metabolism (Silveira et al., 2005). All strains of *K. marxianus* and strain RM-3 (*Barnettozyma californica*) were capable of permease and lactase enzyme activity resulting in the production of monosaccharides and the full fermentation of lactose, including galactose utilization. In a similar study to our work, ethanol production from lactose media was relatively low and it was suggested that the low ethanol amount was due to the inaccessibility of lactose to the produced β-galactosidase (Brady et al., 1995). In another study, the enzyme activity of *K. marxianus* decreased when sugar concentration was increased in media containing galactose and lactose. The maximum activity of β-galactosidase was obtained at low lactose concentrations (Martins, Jr, Simões, & Jr, 2002). Based on the data obtained from our large-scale experiment, *S. cerevisiae* successfully fermented whey to ethanol, but only when we added the enzyme lactase. We also showed that ethanol production increases with increasing lactose concentration. Many changes occurred around day 6 of fermentation since at this time, ethanol appears, sugars reach the highest amount, and brix and the relative density of liquid begins to decrease.

Future Status of Whey Fermentation

The whey to ethanol industry has significant room for growth. There were two plants in the United States; one in Corona, California and another in Melrose, Minnesota in 2008. Together, they produced 8 million gallons of fuel ethanol per year (Ling, 2008). However, there

is no longer record of these plants, and they have likely closed or are producing another product. There are other whey to ethanol plants in New Zealand, home to the largest whey to ethanol plant in the world (5 million gallons annually) (Ling, 2008), proving the possibility of this technology. Unfortunately, the application of this industry is not widespread enough. Production of ethanol from whey is generally not economically feasible because the concentration of lactose in whey permeate is relatively low. The low lactose content results in low ethanol production. Concentrating whey via reverse osmosis or adding lactose may overcome the problem. However, high lactose concentrations cause lactose intolerance of some strains of *K. marxianus* and may make the distillation process too expensive. Fermentation must be rapid to maximize ethanol production but *K. marxianus* does not ferment as well at room temperature than at higher temperatures, thereby requiring costly energy input to raise the fermentation temperature. From our data, *S. cerevisiae* is more promising for ethanol production from whey permeate than using *K. marxianus*, even when with the added cost of needing to add an enzyme to break down the lactose is considered.

Economic Considerations

While previous studies demonstrate the economic benefits of ethanol production from whey, there are several challenges that limit its widespread adoption as an industrial process. Many factors determine the economic feasibility of an operation such as whey permeate price, initial cost of set up, technical expertise, and whey transportation. It is necessary to balance the operation costs and prices of ethanol produced from whey to make this industry economically feasible. One way to overcome these economic difficulties is to set up small scale ethanol plants and distilleries close to cheese and dairy production factories (Das et al., 2016). Another way is to market the product as potable ethanol or spirit drinks that command a much higher final price

than fuel ethanol. Turning whey into vodka makes it possible to save carbohydrate-containing foods such as grains, potato and corn that are important for human nutrition and also decreases costs associated with enzymes used for starch hydrolysis (Jin, Parashar, Mason, & Bressler, 2016). The final price of ethanol is affected by raw materials used in the fermentation. Starch sources such as sugar beets and corn are climate-dependent (Budimir et al., 2011). According to Antonov et al. (1978), up to 35 tons of grain and 12 tons of potatoes would be saved in producing one ton of ethanol from whey. If we consider a perfect whey fermentation, it would take 12.29 pounds of lactose to produce a gallon of ethanol (Ling, 2008). To make whey to ethanol economically feasible, it is important to balance production costs and product price. According to previous study, 1 pound of lactose would yield 0.538 pound of ethanol and it would take 12.29 pounds of lactose to produce a gallon of ethanol. If the lactose is completely consumed during fermentation and ethanol conversion is 100 percent of the theoretical yield, the cost of whey to ethanol is estimated between $1.60-1.85 per gallon. That value is relatively high compared to other carbon sources (Ling, 2008). According to USDA, total estimated costs of ethanol production from corn and molasses were $1.03 and $1.27 per gallon. Both estimates are lower than whey-based ethanol. Ethanol production costs from sugar beets, sugar cane, and raw sugar are $2.35, $2.40, and $3.48 per gallon (USDA, 2006) that are not economically feasible compared to whey-based vodka. If we consider all ethanol plants close to sugar sources and ignore the transportation expenses, the main differences in costs would be feedstock preparation and processing costs that are relatively lower in whey.

The major conclusion is that whey vodka may be economically feasible if we minimize transportation costs by building the plant close to the source, provide the whey permeate at a low cost compared to other sugars, and use suitable yeast strains.

References

2017AgStats_web.pdf. (n.d.). Retrieved from https://www.nass.usda.gov/Statistics_by_State/Wisconsin/Publications/Annual_Statistical_Bulletin/2017AgStats_web.pdf

Beniwal, A., Saini, P., Kokkiligadda, A., & Vij, S. (2017). Physiological growth and galactose utilization by dairy yeast Kluyveromyces marxianus in mixed sugars and whey during fermentation. *3 Biotech*, *7*(5), 349.

Boudjema, K., Fazouane-naimi, F., & Hellal, A. (2016). Isolation, identification of yeast strains producing bioethanol and improvement of bioethanol production on cheese whey / Biyoetanol üreten maya türlerinin izolasyonu, tanılaması ve peynir altı suyunda biyoetanol üretiminin sağlıklaştırılması. *Turkish Journal of Biochemistry*, *41*(3), 157–166.

Brady, D., Marchant, R., McHale, L., & McHale, A. P. (1995). Isolation and partial characterization of P-galactosidase activity produced by a thermotolerant strain of Kluyveromyces marxianus during growth on lactose-containing media. *Enzyme Microb. Technol., 17*.

Budimir, N. J., Jarić, M. S., Jaćimović, B. M., Genić, S. B., & JaćImović, N. B. (2011). Rectified Ethanol Production Cost Analysis. *Thermal Science*, *15*(2), 281–292.

Dairy Products 2017 Summary 04/26/2018. (2017). *Dairy Products*, 58. United States Department of Agriculture National Agricultural Statistics Service. ISSN: 1057-784X. www.nass.usda.gov

Das, B., Sarkar, S., Maiti, S., & Bhattacharjee, S. (2016). Studies on production of ethanol from cheese whey using Kluyveromyces marxianus. *Materials Today: Proceedings*, *3*(10), 3253–3257.

Dos Santos, L., Maria Gonçalves, C., & Suguimoto, H. (2014). Fermentation process and physicochemical treatment applied to dairy industry: an approach for waste management (production and use) and new perspectives in the transformation of these residues in value-added products. *Journal of Biotechnology and Biodiversity*, *5*, 1.

Dragone, G., Mussatto, S. I., Almeida e Silva, J. B., & Teixeira, J. A. (2011). Optimal fermentation conditions for maximizing the ethanol production by Kluyveromyces fragilis from cheese whey powder. *Biomass and Bioenergy*, *35*(5), 1977–1982.

EDA_EWPA_Economic_Report_2016.pdf. (n.d.). Retrieved from http://eda.euromilk.org/fileadmin/user_upload/Public_Documents/Facts_and_Figures/EDA_EWPA_Economic_Report_2016.pdf

EthanolSugarFeasibilityReport3.pdf. (n.d.). Retrieved from https://www.usda.gov/oce/reports/energy/EthanolSugarFeasibilityReport3.pdf

Goddard, M. R., & Greig, D. (2015). Saccharomyces cerevisiae: a nomadic yeast with no niche? *FEMS Yeast Research*, *15*(3).

Grba, S., Stehlik-Tomas, V., Stanzer, D., Vahèi, N., & Škrlin, A. (2002). Selection of Yeast Strain Kluyveromyces marxianus for Alcohol and Biomass Production on Whey. *Chem. Biochem. Eng. Q.*, 4.

Guimarães, P. M. R., Teixeira, J. A., & Domingues, L. (2010). Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. *Biotechnology Advances*, *28*(3), 375–384.

Hadiyanto, Ariyanti, D., Aini, A. P., & Pinundi, D. S. (2014). Optimization of Ethanol Production from Whey Through Fed-batch Fermentation Using Kluyveromyces Marxianus. *Energy Procedia*, *47*, 108–112.

Jin, Y., Parashar, A., Mason, B., & Bressler, D. C. (2016). Simultaneous hydrolysis and co-fermentation of whey lactose with wheat for ethanol production. *Bioresource Technology*, *221*, 616–624.

Karki, T. B., Timilsina, P. M., Yadav, A., Pandey, G. R., Joshi, Y., Bhujel, S., … Neupane, K. (2017). Selection and Characterization of Potential Baker's Yeast from Indigenous Resources of Nepal. *Biotechnology Research International*, *2017*.

Kim, J. H., Ryu, J., Huh, I. Y., Hong, S.-K., Kang, H. A., & Chang, Y. K. (2014). Ethanol production from galactose by a newly isolated Saccharomyces cerevisiae KL17. *Bioprocess and Biosystems Engineering*, *37*(9), 1871–1878.

Koushki, M., Jafari, M., & Azizi, M. (2012). Comparison of ethanol production from cheese whey permeate by two yeast strains. *Journal of Food Science and Technology*, *49*(5),

Lane, M. M., & Morrissey, J. P. (2010). Kluyveromyces marxianus: A yeast emerging from its sister's shadow. *Fungal Biology Reviews*, *24*(1), 17–26.

Lekang, K., Hadziavdic, K., Sandnes Skaar, K., Jonassen, I., Thompson, E. M., & Troedsson, C. (2018). Development and testing of an 18S rRNA phylogenetic microarray for marine sediments. *Journal of Microbiological Methods*, *154*, 95–106.

Ling, K. C. (2008). Whey to Ethanol. *Rural Cooperatives; Washington*, *75*(2), 10–11.

Mahmoud, M. M., & Kosikowski, F. V. (1982). Alcohol and Single Cell Protein Production by Kluyveromyces in Concentrated Whey Permeates with Reduced Ash. *Journal of Dairy Science*, *65*(11), 2082–2087.

Martins, D. B. G., Jr, C. G. de S., Simões, D. A., & Jr, M. A. de M. (2002). The β-Galactosidase Activity in Kluyveromyces marxianus CBS6556 Decreases by High Concentrations of Galactose. *Current Microbiology*, *44*(5), 379–382.

Michael, P., Ryan. Gary, Walsh. (2016). The biotechnological potential of whey. *Environmental Science and Bio/Technology 15*(3), 479-498

Mohd Azhar, S. H., & Abdulla, R. (2018). Bioethanol production from galactose by immobilized wild-type Saccharomyces cerevisiae. *Biocatalysis and Agricultural Biotechnology*, *14*, 457–465.

Nguyen, T. H., Ra, C. H., Sunwoo, I., Jeong, G.-T., & Kim, S.-K. (2017). Bioethanol production from Gracilaria verrucosa using Saccharomyces cerevisiae adapted to NaCl or galactose. *Bioprocess and Biosystems Engineering*, *40*(4), 529–536.

Pandy_solid-state-fermentn2003.pdf. (n.d.). Retrieved from http://www.davidmoore.org.uk/21st_Century_Guidebook_to_Fungi_PLATINUM/REPRINT_collection/Pandy_solid-state-fermentn2003.pdf

Parrondo, J., Garcia, L. A., & Diaz, M. (2000). Production of an Alcoholic Beverage by Fermentation of Whey Permeate with Kluyveromyces fragilis I: Primary Metabolism. *Journal of the Institute of Brewing*, *106*(6), 367–375.

Piskur, J., Rozpedowska, E., Polakova, S., Merico, A., & Compagno, C. (2006). How did Saccharomyces evolve to become a good brewer? *Trends in Genetics*, *22*(4), 183–186.

Rech, R., & Ayub, M. A. Z. (2007). Simplified feeding strategies for fed-batch cultivation of Kluyveromyces marxianus in cheese whey. *Process Biochemistry*, *42*(5), 873–877.

Reynders, M. B., Rawlings, D. E., & Harrison, S. T. L. (1997). Demonstration of the Crabtree effect in Phaffia rhodozyma during continuous and fed-batch cultivation. 5.*Biotechnology Letters, 19*(6). doi: 10.1023/A:1018341421122

Silveira, W. B., Passos, F. J. V., Mantovani, H. C., & Passos, F. M. L. (2005). Ethanol production from cheese whey permeate by Kluyveromyces marxianus UFV-3: A flux analysis of oxido-reductive metabolism as a function of lactose concentration and oxygen levels. *Enzyme and Microbial Technology*, *36*(7), 930–936.

Verduyn et al. (1992). Effect of benzoic acid on metabolic fluxes in yeas. Retrieved from tps://pdfs.semanticscholar.org/54ea/1ad123b5d1102d757fd8f934078a28281c97.pdf.

Viniegra-González, G., Favela-Torres, E., Aguilar, C. N., Rómero-Gomez, S. de J., Díaz-Godínez, G., & Augur, C. (2003). Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochemical Engineering Journal*, *13*(2–3), 157–167.