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Utilizing Isothermal Titration Calorimetry for Measuring Beta-Galactosidase Activity in Liquid Dairy Products

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Master of Science

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ABSTRACT

Utilizing Isothermal Titration Calorimetry for Measuring Beta-Galactosidase Activity in Liquid Dairy Products

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This research explores Isothermal Titration Calorimetry as a method for measuring beta-galactosidase activity directly and continuously in milk, sweet whey, sweet whey permeate, acid whey, and acid whey permeate. Beta-galactosidase in various concentrations was injected into each of the liquid dairy products spiked with lactose to verify if the heat rate from the enzymatic reaction could be observed. In addition, a consistent concentration of beta-galactosidase was injected into various concentrations of lactose in the products, to observe the heat rates from the enzymatic reaction. There was exothermic activity that never returned to baseline demonstrated in milk, sweet whey, and sweet whey permeate with beta-galactosidase from *Kluyveromyces lactis* in runs done in the isothermal titration calorimeter. The baseline was approximately 3-9 uJ/s above the control's baseline at the end of the runs. The exothermic activity ranged from approximately 2-10 uJ/s and did not return to baseline when beta-galactosidase concentrations were varied and lactose concentrations remained the same. The exothermic heat rate was approximately 3-7 uJ/s when lactose concentrations were varied and enzyme concentrations remained the same. With runs with increasing lactose concentrations, there was no corresponding increase in the exothermal reaction indicating saturation of the enzyme. There was a short exothermic reaction(s), ranging from approximately 3-26 uJ/s, demonstrated when varying concentrations of beta-galactosidase from *Aspergillus oryzae* in acid whey and acid whey permeate were injected into a consistent concentration of lactose in acid whey and acid whey permeate. There was a pattern of increasing heat with increasing concentrations of enzyme, with some of these differences being statistically significant. There was also a short exothermic reaction(s), ranging from approximately 2-17 uJ/s, demonstrated when a consistent concentration of beta-galactosidase from *Aspergillus oryzae* was injected into varying concentrations of lactose. There was a pattern of increasing heat rate with increasing concentrations of lactose, with some of these differences being statistically significant. This research demonstrates that ITC is a useful method for measuring residual beta-galactosidase and/or residual lactose in liquid dairy products. This research leads to further understanding of how enzymes and substrates interact directly in the food matrix, rather than in an isolated environment.

Keywords: whey, milk, beta-galactosidase, isothermal titration calorimeter, enzyme activity

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LITERATURE REVIEW

Bovine Milk

Cow's milk is a complex food matrix that provides all nutrients for growth in the neonate period of a cow's life. Milk contains proteins, fat, lactose, and minerals (1). The lipids are emulsified globules that are coated with a membrane and the proteins are micelles in a colloidal dispersion. The proximate composition of cow's milk is in TABLE 1 (2). The composition of types of proteins in cow's milk is in TABLE 2 (3, 4).

Table 1: Composition of Bovine Milk

Composition	**WT% (wwb)**
Protein	3.4%
Casein	2.8%
Fat	3.7%
Lactose	4.6%
Ash	0.7%

Table 2: Composition of Proteins in Milk

Protein	**WT% (wwb)**
αs1-casein	1.2
αs2-casein	0.3
β-casein	1.0
κ-casein	0.35
γ-casein	0.12
α-lactalbumin	0.12
β-lactoglobulin	0.32
Serum Albumin	0.04
Immunoglobulins	0.08
Proteose-peptone fraction	0.1

Milk is generally pasteurized to kill harmful bacteria for the safety of consumers. During pasteurization, raw chilled milk passes between two stainless steel plates that heat the milk to 161°F for 15 seconds. The milk is then quickly cooled back to 39°F (5). Milk can also be

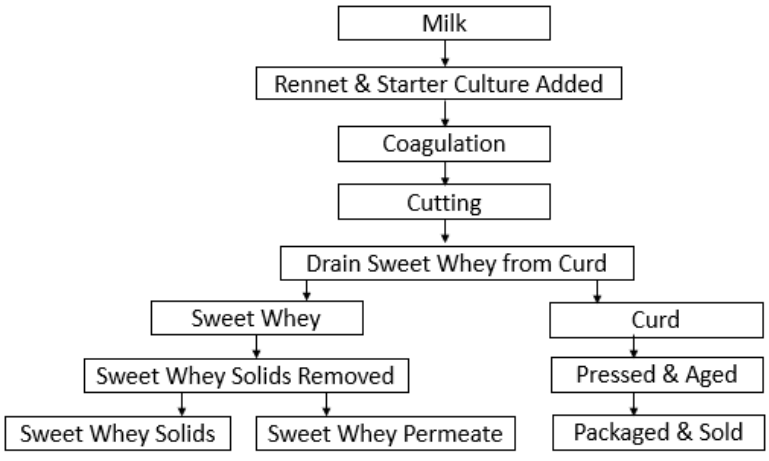
sterilized to create shelf-stable milk by ultra-high temperature pasteurization in which milk is heated to 280-300°F for 2-6 seconds. The sterilized milk is placed in sterile packaging in a sterile environment. Sterilization allows the unopened milk to last about six months (6). Milk can be consumed as a fluid, or can be made into many other products. Examples include cheese, yogurt, Greek yogurt, cream cheese, sour cream, and butter.

Whey & Whey Permeate

Dairy foods are popular among consumers throughout the world. A common byproduct of the dairy industry is whey. Whey is a high protein and high lactose watery thin liquid obtained by separating the coagulum from whole milk, cream, or skim milk during cheese and yogurt making (7). Whey was discovered around 3000 years ago when calves' stomachs, which contain the enzyme chymosin, also known as rennet, were used to store and transport milk. The result was curds and whey which provided the beginnings of cheese making (8).

Hard cheese is made is by adding rennet and starter culture to milk, which causes coagulation. After coagulation, the curd is cut and the sweet whey is drained from the curd. The curd is then pressed and aged prior to being packaged and sold (Figure 1). For every pound of hard cheese that is made, nine pounds of sweet whey is leftover (9). Given that in the U.S. in 2017, 12.7 billion pounds of cheese was produced (10), 114 billion pounds

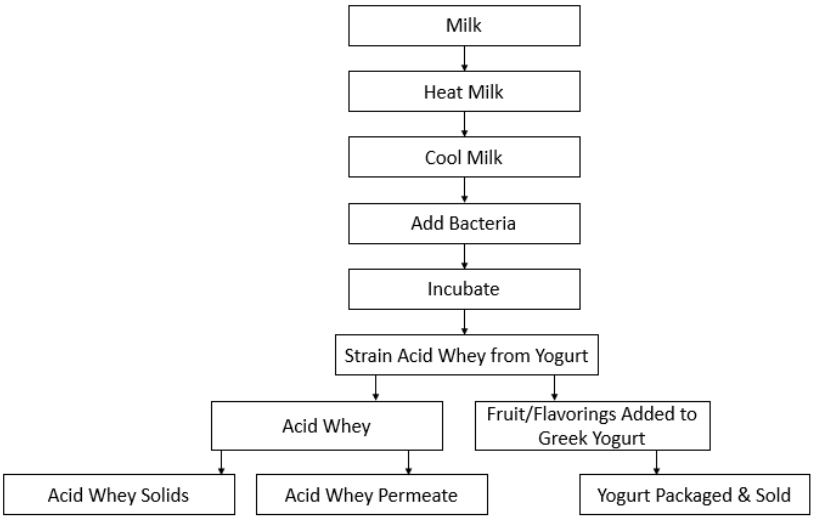
Figure 1



or 52 billion kilograms of sweet whey was the byproduct. There is a need for research to increase the opportunities and efficiencies of whey.

Greek yogurt is made by heating and then cooling milk. Bacteria is added to the milk followed by a period of incubation. Acid whey is then strained from the yogurt. Fruit and or/flavorings are added to the Greek yogurt prior to being packaged and sold (Figure 2). The strained whey can be utilized for protein content, and far more whey permeate (whey after protein removed) remains than can currently be used. Only about 50% of residual whey is being recycled for the production of value-added products in the food and chemical industries (11).

Figure 2



For every pound of Greek yogurt that is produced, three pounds of whey is leftover (12), which provides plenty of need for innovation. Today, due to the popularity of cheese and strained yogurts (such as Greek or Icelandic yogurts), quantities of whey being produced continue to grow at a rate of >2% per year (13). In 2006, the estimated worldwide production of whey was 190 x 10^9 kilograms per year (14).

Throughout most of history, whey was discarded and sometimes viewed as a noxious byproduct (14). However, in the 17th, 18th, and early 19th centuries, whey was valued as a fashionable drink, a medicinal agent that was effective against various ailments, and even as a

liquid suitable for bathing. During these years when whey was a fashionable drink, "whey houses" existed, similar to a bistro or coffee shop today, where they served whey porridge, whey soup, whey tea, and whey butter (15). Bathing in whey was popular at health spas based on its presumed skin healing and topical health-promoting properties (16). Currently, there are still a few whey spas that can be found in Europe (17).

The protein from whey is often filtered off for use in other products that can be sold independently, such as whey protein powder or can be added to other products, such as bars. After the protein has been removed, whey permeate remains. In 2015, the US produced an estimated 475,000 kilograms of whey permeate, which is a 48% increase from 2010 (18). This presents an opportunity for innovation to use the whey permeate.

Because disposal of whey permeate presents some serious challenges, industry requires innovation for use of whey permeate. Whey permeate is a pollutant to the environment primarily due to the lactose, which is a large part of the composition of whey permeate. On a dry weight basis, sweet whey permeate, the byproduct of cheese making, is composed of 86% lactose, 2.4% lactic acid, and 8.8% ash. On a dry weight basis, acid whey permeate, the byproduct of strained yogurt and soft cheese making, is composed of 74% lactose, 7.5% lactic acid, and 9.7% ash (19). Whey permeate has a Biochemical Oxygen Demand (BOD) of 30-50 g/L (20), with lactose responsible for 90% of the BOD. The BOD of whey permeate is higher because the lactose becomes more concentrated when the protein is removed (21). These high BOD values have led to an increase in regulations aimed to protect wildlife and the environment. These increased measures to protect the environment have led to an increase in costs to industry for disposal of the toxic waste and provides opportunities for innovation.

Utilizing whey has been the focus of much research and innovation, and progress has been made. Historically, whey has been viewed as a waste stream with need of disposal in the most economical ways because of its lack of perceived value. Whey has been discharged into rivers, lakes, and the ocean, but the high polluting power of whey makes the disposal unethical and is now restricted in many parts of the world. Whey has been (and is currently) sprayed onto fields as a fertilizer, but the smells and salts produced have caused problems. Whey has been sent to the municipal sewage system, but with the BOD of whey being around 175-fold higher than typical sewage effluent, it can easily overload the system and is expensive or prohibited. Whey has been (and can be) sold as animal feed for a low return (8). Whey has made progress from being a nuisance to being more valuable primarily because of the protein content. Whey protein is popular among consumers because of its high biological value (BV), which is a measure of how well and how quickly the body can utilize the protein consumed. In fact, the BV is 15% higher than egg protein which was the former benchmark (8). Whey proteins are available that have undergone different processing with the result of varying compositions and levels of protein. Examples are whey protein concentrate, whey protein isolate, and whey protein hydrolysate.

Industry has made progress in making use of whey permeate (deproteinized whey) as well, with plenty of room remaining for innovation and improvement. The carbohydrate lactose is a major source of value in the whey permeate. The lactose can be used as a source of carbohydrate in infant formulas and as an excipient in food processing and pharmaceuticals. In the confectionary industry lactose can serve as the reducing sugar for maillard browning and to adsorb dyes and flavors. In the pharmaceutical industry, about 70% of tablets have lactose as the carrier for drugs because it is not sweet and makes good quality tablets (22). It has been

demonstrated that whey permeate can be used directly as a substrate for the growth of different microorganisms to obtain products such as ethanol, lactic acid, citric acid, biogas, and more (23), although not all are commercially viable at this time. Again, with only about 50% of residual whey being recycled for the production of value-added products in the food and chemical industries, there is room for further research and innovation on the utilization of all components of sweet and acid whey (11).

Enzymes of the Dairy Industry

Enzymes are proteins that decrease the activation energy for a chemical reaction and increase the speed of a chemical reaction without being consumed by the reaction. Enzymes are highly specific, typically only catalyzing one type of reaction with one substrate or class of substrates (24). Enzyme activity can be optimized through pH and temperature alterations and sometimes by the addition or removal of co-factors. A reaction between an enzyme and substrate is either endothermic, requiring energy from the environment, or exothermic, expelling energy into the environment. The amount of heat required or released can be increased or decreased based on alterations to the concentration of substrate.

There are many enzymes that are important for the dairy industry. They play a role in food processing, safety, and flavor profile development through their activity. TABLE 3 is a summary of enzymes utilized in the dairy industry and their function.

Table 3: Enzymes of the Dairy Industry

Enzyme	**Function**
Rennet	Curdles milk in the first phase of cheese processing (25).
Protease	Decreases the time required for cheese aging, changes milk proteins to decrease the allergic effects of cow milk products in infant foods (26).
Lactase (beta-galactosidase)	Increases solubility and sweet flavor. Hydrolyzes lactose into glucose and galactose for lactose-free products (27).
Lipase	Improvement of cheese flavors and separates milk fat (27).
Catalase	Converts hydrogen peroxide into water and oxygen during cheese making so that milk for cheeses, such as Swiss, does not need to be thermally pasteurized (27, 28).
Transglutaminase	Improves functional properties of yogurt made from goat milk by enhancing gel stability and reducing syneresis (27, 29).
Lysozyme	Lysozyme lowers the quantity of bacteria in milk without influencing *Bifidobacterium* in fermented milks (30).

Beta-galactosidase

In the 1880's and 1890's, many enzymes were described. Beta-galactosidase, more commonly known as lactase, is an enzyme that hydrolyzes lactose to glucose and galactose. In 1889, Martinus Willem Beijerinch, of the Netherlands, was the first scientist to document a lactose hydrolyzing enzyme (beta-galactosidase) (31). Due to some ambiguity with the results of his bioassay, a few years later the results were credited to Fischer (32). Beta-galactosidase is a tetramer with 222-point symmetry (33). The 1,023-amino-acid polypeptide chain (34, 35) folds into five domains with an extended segment at the amino terminus (33).

Beta-galactosidase was originally discovered to be produced by yeast (the word enzyme literally means "in yeast" in Greek) species *S. kefyr* and *S. tyrocola* (31). Now it is known that

beta-galactosidase is produced by a wide variety of microorganisms, including yeasts, fungi, and bacteria. Beta-galactosidase is also made by many plants and mammals (36). Beta-galactosidase from different sources have different characteristics, such as pH and temperature optimums. Beta-galactosidase in industry is primarily obtained from the yeast, *Kluyveromyces,* and the fungus, *Aspergillus (37).* In industry, the most commonly used sources of beta-galactosidase are microbial because of the higher productivity, which leads to decreased costs. Basically, the source of the enzyme is chosen based on the required reaction conditions (38). For example, fungal beta-galactosidase generally has a pH optimum from 2.5-5.4, which makes them most effective in acidic environments, such as acid whey. Beta-galactosidases from bacterial sources have been used for lactose hydrolysis because of the ease of fermentation, high enzyme activity, and good enzyme stability (39).

Beta-galactosidase from *Aspergillus Oryzae* has an optimal pH of 4.75 and optimal temperature of 60°C, which makes it more suitable for use in liquid dairy products, such as acid whey and acid whey permeate (40). The optimal pH and temperature of beta-galactosidase from *Kluyveromyces lactis* is 6.5-7.0 and 35-45°C, respectively, which makes it more suitable for use in milk, sweet whey, and sweet whey permeate (41).

Beta-galactosidase from different sources are intracellular or extracellular. In some fungi the beta-galactosidase activity has been found to be extracellular, while beta-galactosidases from yeast and bacteria are generally intracellular (42). *Streptococcus pneumoniae* has been found to have β-1,4-galactosidase and β-1,3-galactosidase on the cell surface rather than intracellularly. The genes responsible for the enzyme being extracellular in *Streptococcus pneumoniae* are BgaA and BgaC (43).

Beta-galactosidase is a valuable enzyme in the food industry because it hydrolyzes lactose. Beta-galactosidase is used to create lactose free dairy products. Lactose intolerance means that a person lacks beta-galactosidase production in the jejunum. Beta-galactosidase is generally present in the intestines during the breast-feeding period because lactose is the primary source of carbohydrates for infants. In most individuals, beta-galactosidase production decreases at some point after weaning and creates symptoms of lactose intolerance (38). Prior to approximately 10,000 years ago, everyone became lactose intolerant after weaning. However, due to genetic mutation, certain groups of people, all raising cattle or camels, from Northern Europe, East Africa, and the Middle East developed the ability to produce beta-galactosidase for life (44).

When the body is unable to digest or hydrolyze lactose due to a lack of beta-galactosidase, the lactose increases fluid secretion in the small intestine. Lactose passes into the colon where microbes convert it into short-chain fatty acids, carbon dioxide, hydrogen, and methane (45). This leads to symptoms such as flatulence, diarrhea, bloating, and cramps when lactose is consumed. Lactose intolerance affects about 70% of the world's population (38).

Beta-galactosidase can also be used to make beta-galactooligosacharrides (GOS). GOS are a prebiotic produced by transglycosylation during the hydrolysis of lactose (38). GOS and prebiotics in general have been demonstrated to have positive health effects, such as stool improvement, mineral absorption, weight management, and allergy alleviation (46). GOS improves intestinal flora, immunity, reduces blood fat, and helps the body resist tumors and aging (47). Health benefits of yogurt can be improved if beta-galactosidase is incorporated into the milk while producing yogurt because the GOS content of yogurt can be increased up to 30x (48). Due to these health benefits, GOS are ideal for use in functional foods. GOS can be used

as a sweetener and, compared to lactose and other larger sugar molecules, are less likely to cause cavities, have a lower caloric value, and lower sweetness profile (49).

In addition to the health benefits, GOS have characteristics that improve sensory and functional aspects. Because lactose is hygroscopic, it undergoes crystallization in food products (38). Beta-galactosidase eliminates lactose crystallization in such products. Crystallization is particularly of concern in frozen desserts and confectionaries where lactose has a gritty texture that affects sensory characteristics. In yogurts and desserts, GOS can be used as a replacement for sugar, leading to improved texture and mouthfeel, and increased fiber. In bakery applications, GOS can be used as a sugar replacement and to increase fiber. Moisture retention of the product also increases (46). GOS have similar applications in baby food, fillings, confectionaries, and sauces (50).

The long-term outlook for GOS looks good with the global market size projected to reach $1.58 billion by 2025, which represents a growth rate of 9.6%. GOS used to augment infant formula is anticipated to drive this growth (51). The unit price for lactose is approximately $0.80/kg (52), while after the transformation, the GOS price is approximately $5.80/kg (53). Beta-galactosidase could be used in industrial synthesis of GOS and provides an excellent increase in value for the nuisance of lactose in whey permeate.

Uses for beta-galactosidase expand outside of the food industry. Beta-galactosidase enzymes are used to create pills that people with lactose intolerance can consume during ingestion of dairy products to alleviate the effects of lactose intolerance. Beta-galactosidase can be used as the catalyst during fermentation of lactose to produce ethanol (54). In the case of low-price bulk products, like ethanol, the cost of beta-galactosidase is the problem that limits the economic feasibility of the fermentation process (55).

Research is needed to gain a better understanding of beta-galactosidase activity directly in the food matrix where it acts differently than in an isolated and controlled environment.

Other Methods for Measuring Enzymes of the Dairy Industry

There are many different methods available to measure enzyme activity. The challenge with most of these methods is that the enzyme needs to be isolated and purified in preparation for the assay; the enzyme cannot remain in the food matrix. Purification of the enzyme can be done with gel filtration, affinity chromatography, salting out, liquid chromatography, with organic solvents, column chromatography, and ion exchange (56). The food matrix will have an effect on how the enzyme will perform because the food matrix will contain other ingredients, including proteins that would interfere with many assays for measuring enzyme activity. In addition, during processing, packaging, and storage, the food can undergo changes in pH and/or temperature, and there could be interactions with salts that could increase or decrease enzyme activity (57). The food matrix has an effect when measuring enzyme activity using the ITC as well, but with ITC directly measuring heat changes, the heat change that occurs with interactions between the enzyme and substrate will still occur and be measurable.

In a clinical study, beta-galactosidase present in yogurt was indirectly measured by measuring breath hydrogen (58). Because people who are lactose intolerant do not make beta-galactosidase (or makes less), they will expel Hydrogen in their breath after consuming lactose as the body ferments the lactose in the colon (59). With the hydrogen being measured, this can determine how much beta-galactosidase is in the yogurt.

Some methods for measuring enzyme activity are direct and some are indirect, some are continuous and some discontinuous. When deciding what type of assay to perform when measuring enzyme activity, it is important to consider the goals of performing the assay and the capabilities of the method being used. With a direct continuous assay, absorbance, fluorescence, pH, optical rotation, conductivity, enthalpy, viscosity, or volume can all be used as methods for measuring enzyme activity (60). Spectrophotometric assays appear to be the most commonly used because they are accurate, reliable, and relatively cheap (61). A fluorometric assay can be used when a more sensitive assay is needed. The K_m value of an enzyme is the amount of substrate is takes for an enzyme to reach half of the enzymatic rate velocity (62). A more sensitive assay is needed when a limited amount of the enzyme is available or with a low K_m value for its substrates. With impure preparations or solutions that contain absorbing and/or particulate matter, optical assays may be difficult to perform with accuracy (60). Therefore, a food, such as milk or whey would not be a good candidate for an optical assay.

In an indirect assay, a substrate is directly modified by the enzyme, but the signal is produced by an interaction or reaction with another reagent (61). An indirect assay can be discontinuous or continuous. For a discontinuous indirect assay, radiochemical assays and liquid chromatographic systems can be used. The discontinuous assays provide less information about the nature of the reaction because snapshots are taken of the reaction, rather than a continuous flow of data that would demonstrate anomalous behavior. It is also more difficult to obtain an initial rate and to see any deviations from the initial linear phase. For a continuous indirect assay, the reaction is monitored continuously, rather than taking snapshots. They are less prone to errors compared to a discontinuous assay. A coupled assay is another type of indirect assay in which an additional enzyme is used to catalyze a reaction and yield a compound that can be

directly measured. Until the reaction reaches a steady-state velocity, the coupled assay will not provide an accurate measurement of enzyme activity (60).

The most commons assay for measuring beta-galactosidase is an indirect and colorimetric assay using a spectrophotometer and the substrate ortho-nitro-phenyl-galactoside (ONPG). ONPG has a similar structure to lactose, except it is made of galactose and ortho-nitrophenol, rather than galactose and glucose. When hydrolysis occurs, the ortho-nitrophenol turns yellow and has an absorbance at 420nm, which is then measured in the spectrophotometer and used to calculate enzyme activity (63).

Isothermal Titration Calorimetry

ITC directly measures the heat rate of a reaction. In an ITC, there are two cells that hold liquids. One cell, the reference cell, holds water and remains at the temperature that the user sets. The other cell is called the sample cell. In the case of measuring enzymatic activity, the solution containing the dissolved substrate (or enzyme) is placed in the sample cell. The solution with the enzyme (or substrate, if the enzyme solution is in the sample cell) is placed in the syringe and injected into the sample cell at the desired time. The rate of energy for the sample cell to remain at the same temperature as the reference cell is measured, creating the heat rate data in real-time.

ITC technology is primarily used in the pharmaceutical industry to measure binding affinities for ligands to proteins and is considered the gold standard for measuring molecular interactions, allowing researchers to better understand the chemistry and physics of these interactions. ITC allows for the studying of macromolecular complex formations, interaction

stoichiometries, thermodynamic correlations to binding interface areas, and the conformational changes that occur through interaction (64).

In the food industry, ITC has recently been used to better understand starch-fatty acid interactions (65). ITC has also been used to provide valuable information regarding droplet-droplet interactions in flocculated emulsions (66), such as are present in agrochemicals, biological fluids, cosmetics, explosives, petrochemicals, and pharmaceuticals (67). In addition, ITC has been used to study tannin-protein interactions, which has been a complex subject pertaining to human health (68). Thus far, the ITC has been largely underutilized in the food industry and shows high potential for furthering understanding of enzymes in food matrices.

ITC has notable positive characteristics, for measuring enzyme activity in real-time and continuously. There is no coupled or colorimetric assay needed. The ITC can provide kinetic and thermodynamic data simultaneously, and the assay is relatively simple and hands off. Once the calorimeter is cleaned, the solutions with substrate and enzyme are placed in the instrument and then the operator can do other things while the experiment is run and data is recorded. Because the ITC measures changes in heat rate, it can be used with liquids that are not translucent and/or have different colors or sediment. This is unique compared to most enzyme assays.

While the ITC is a powerful tool for measuring enzyme activity, a drawback in a complex system, such as milk, is that there are many endothermic and exothermic reactions taking place because of molecular interactions that occur due to dilution, e.g.; micelle formation and dissociation, conformational changes, aggregation, and phase transitions (69), which makes it hard to distinguish the enzyme-catalyzed reaction. Alternatively, the ITC has the ability to provide valuable information about the food matrix and the complex interactions involving food

components, such as proteins, carbohydrates, lipids, minerals, vitamins, minerals, and surfactants (69) in a unique manner. There are models of ITCs that hold more in the sample cell, which also makes them more sensitive.

The ITC is a unique tool that could be used to measure enzyme activity directly in milk, whey, and whey permeates. This could promote further research in the dairy industry to use the largely wasted carbohydrate lactose in whey permeate. The ITC could be used to measure the stability and activity of isolated enzymes or enzymes present in a matrix or interactions between enzymes and other proteins.

Hypothesis and Objectives:

Hypothesis: Isothermal Titration Calorimetry can be used as a method for distinguishing beta-galactosidase concentrations in liquid dairy products with varying lactose concentrations.

Objectives:

1. Compare various concentrations of beta-galactosidase injected into a consistent concentration of lactose in each liquid dairy product.
2. Compare a consistent concentration of beta-galactosidase injected into varying concentrations of lactose in each liquid dairy product.
3. Compare beta-galactosidase from *Aspergillus oryzae* in high acid liquid dairy products and beta-galactosidase from *Kluyveromyces lactis* in low acid liquid dairy products.

MATERIALS AND METHODS

Materials and Chemicals

Beta-galactosidase from *Kluveromyces lactis* (≥2600 units/g) was purchased from Sigma (St. Louis, MO, USA). Beta-galactosidase from *Aspergillus Oryzae* (>5000 U/g) was purchased from MP Biomedicals LLC (Solon, OH, USA). The Nano-Isothermal Titration Calorimeter was manufactured by TA Instruments. The lactose is D-Lactose, anhydrous, 98%, obtained from BeanTown Chemical (Hudson, NH, USA). Acid whey and acid whey permeate was donated by the Danone plant in West Jordan, UT. The sweet whey and sweet whey permeate was donated by Glanbia in Twin Falls, ID. The shelf-stable milk was purchased from Natrel (St. Paul, MN, USA).

Methods

For lower pH liquids (acid whey and acid whey permeate), beta-galactosidase from *Aspergillus oryzae* was weighed out and placed in 1mL of the liquid of choice (acid whey or acid whey permeate) and centrifuged. Lactose was also weighed out and placed in another 1mL microfuge tube with 1mL of the same type of liquid. After the ITC was properly cleaned, 350uL of lactose solution was used to rinse the sample cell of the ITC. Then, 350uL of lactose solution was placed in the ITC. The ITC temperature was set to 60°C and the experiment was run.

The same procedure as above was followed for higher pH liquids (sweet whey, sweet whey permeate, and milk), except the source of beta-galactosidase was *Kluyveromyces lactis* and the ITC was run at 40°C.

All runs in the ITC were done in triplicate.

Statistics

For the statistical analysis, a one-way ANOVA followed by Tukey's Multiple Comparison Test was performed using GraphPad Prism version 9.2.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

Statistical comparisons were performed at different time points on the data depending on the source of beta-galactosidase. In buffer, the heat of injection resolved within 40 seconds. Therefore, the data points needed to be collected after 40 seconds. Because the reactions vary significantly in length, depending on the source of beta-galactosidase, it seemed appropriate to collect data points from different lengths of time after injection depending on the source of the enzyme. After visually comparing the runs, it was determined that the curves flattened, became consistent, and there appeared to be no residual heat from the heat of injection at 600 seconds for runs with beta-galactosidase from *Aspergillus oryzae*. Therefore, 600 seconds was used as the time point to run a comparison test to find statistical differences (α=0.05). In runs done with beta-galactosidase from *Kluyveromyces lactis,* the reaction was much quicker. Therefore, 50 seconds was chosen because the heat of injection would have resolved and near the maximum heat of the reaction could be measured.

RESULTS AND DISCUSSION

This research is valuable because it promotes further research for the use of whey and lactose through an effective method to measure lactose hydrolysis directly in milk, whey, and whey permeates without the need of isolation and purification of the enzyme. ITC can be used as a method to measure the reactions of other enzymes important to the food industry, directly in their food matrices, where they behave differently than when enzyme and substrate are isolated and purified.

Milk

When beta-galactosidase in milk is injected into the lactose and milk solution, there is a linear pattern of increasing heat rates over time with increasing concentrations of beta-galactosidase (figure 3). Beta-galactosidase was injected following 1500 seconds of instrument equilibration. At 600 seconds after injection, which is 2100(s) on the x-axis, mean heat rates were collected. The 600 second time point after injection was chosen because it is when the reaction had become steady. This time point was chosen for all runs in milk, sweet whey, and sweet whey permeate.

The mean heat rate for the control at 600 seconds is 0.18 uJ/s, for the 444.4 ug/mL of beta-galactosidase is 2.88 uJ/s, for the 888.9 ug/mL of beta-galactosidase is 6.09 uJ/s, and for the 1333.3 ug/mL of beta-galactosidase is 7.72 uJ/s. All p-values are significant (figure 4). Runs done in milk were the only runs that had statistical significance between each of the concentrations of enzyme.

Figure 3. Heat rates over time of varying concentrations of beta-galactosidase from Kluyveromyces lactis in milk spiked with 500mM lactose, pH= 6.37

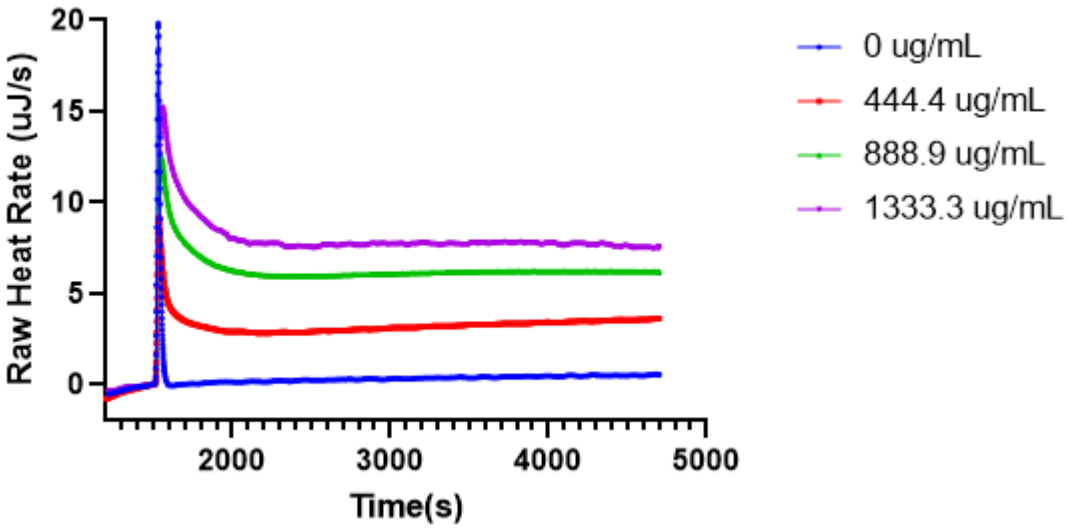
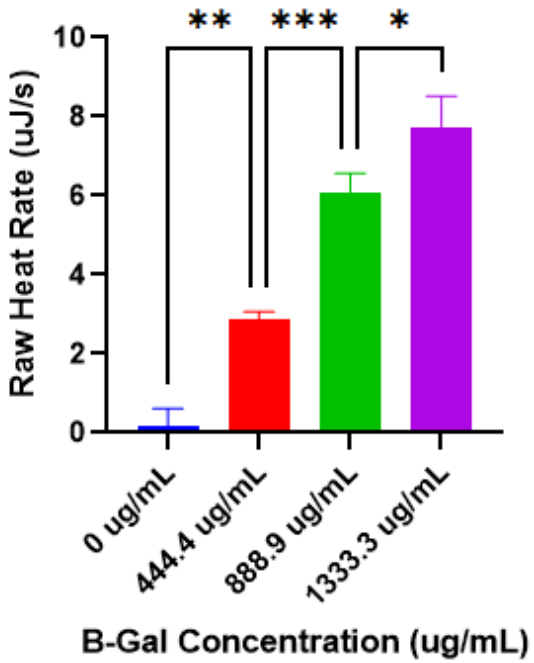


Figure 4. Heat rates of varying concentrations of beta-galactosidase from Kluyveromyces lactis in milk spiked with 500mM lactose at 600(s) after injection, pH=6.37



When a consistent beta-galactosidase concentration in milk is injected into various concentrations of lactose in milk, there appears to be a pattern of increasing heat rates with increasing concentrations of lactose (figure 5). However, all p-values are >0.05 (figure 6), demonstrating no statistical significance. The top three lines with 100mM, 300mM, and 500mM lactose concentrations, are mostly level after the peak of injection and/or peak from an initial secondary reaction resolves, demonstrating the enzyme is saturated or nearly saturated and/or there is a secondary reaction or change of state. The bottom control line is not saturated, demonstrated by the decreasing heat rate.

Figure 5. Heat rates over time of 1.11 mg/mL beta-galactosidase from Kluyveromyces lactis in milk with varying concentrations of lactose, pH= 6.37

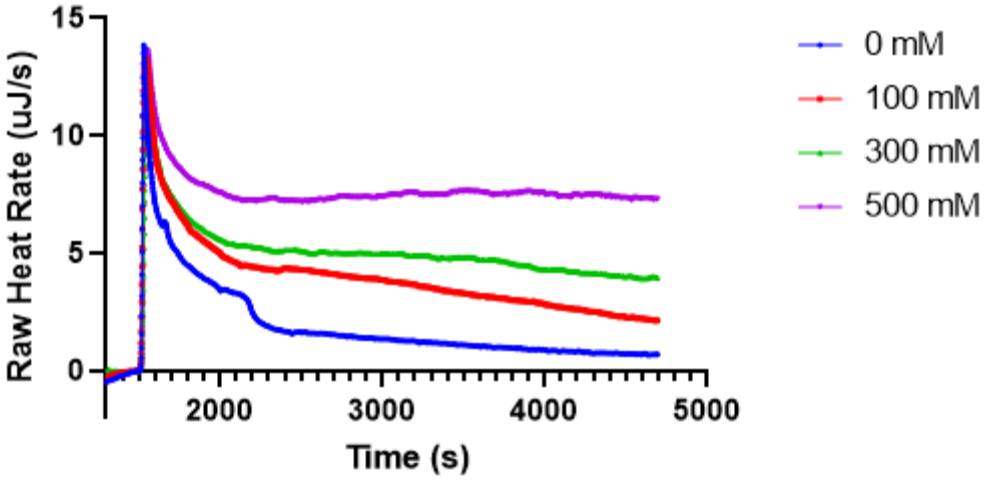
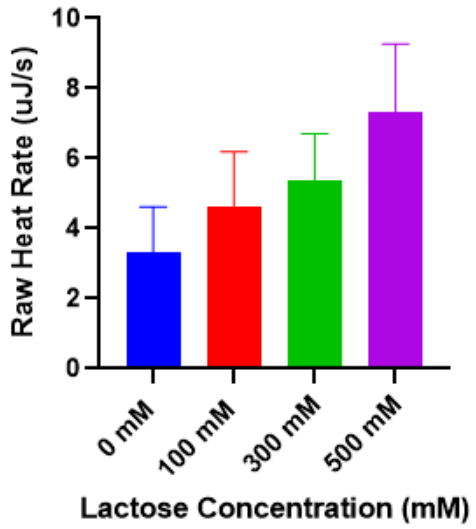


Figure 6. Heat rates of 1.11 mg/mL beta-galactosidase from Kluyveromyces lactis in milk with varying concentrations of lactose at 600(s) after injection, pH=6.37



Sweet Whey

As shown in figure 7, when beta-galactosidase in sweet whey is injected into the lactose in sweet whey solution, there appears to be a linear pattern of increasingly exothermic heat rates with increasing concentrations of beta-galactosidase. At 600 seconds after injection, the mean heat rate for the control is -0.16 uJ/s, for the 444.4 ug/mL of beta-galactosidase is 4.25 uJ/s, for the 888.9 ug/mL of beta-galactosidase is 5.4 uJ/s, and for the 1333.3 ug/mL of beta-galactosidase is 9.72 uJ/s. However, the p-value is >0.05 in all comparisons, except 0 ug/mL vs. 1333.3 ug/mL, which had a p-value of 0.0023 (figure 8). There is an initial heat of injection at 1500 seconds and within about 600 seconds, the heat rate levels out and remains relatively steady throughout the remainder of the run. This heat rate appears to be a slow and steady reaction between the lactose and beta-galactosidase. However, from the literature and from a series of runs done in the ITC with beta-galactosidase from *Aspergillus oryzae*, we know the reaction from beta-galactosidase to be endothermic and much faster (figure 9). The heat from injection of

beta-galactosidase in buffer only lasts about 40 seconds, which is much shorter than in most runs performed in the liquid dairy products, with the exception of some of the varying lactose concentrations in acid whey and acid whey permeate with beta-galactosidase from *Aspergillus oryzae.* The cause of the unexpected longer and exothermic reaction is unknown. There appears to be a secondary reaction that is maintaining the heat rate or potentially a change of state caused by the enzymatic reaction. For example, it is possible that as the lactose is hydrolyzed into glucose and galactose, there is an increase in viscosity.

In the literature, a study was found where the ITC measured the enzyme activity when milk was injected into an acetate buffer with beta-galactosidase and milk spiked with lactose was injected into an acetate buffer with beta-galactosidase. The injections both resulted in an endothermic reaction, similar to those in figure 9 (70). However, when both the lactose and enzyme are in the liquid dairy products, the reaction demonstrated exothermic heat.

In research on the synthesis of GOS, based on ITC data with acetate buffer with pH 4.8 as the solution, it was also concluded that lactose hydrolysis is endothermic with beta-galactosidase from *Aspergillus oryzae*. The synthesis of galacto-oligosaccharides was determined to be endothermic, and the hydrolysis of galacto-oligosaccharides was determined to be exothermic. It is possible that the exothermic nature of these runs in all the liquid dairy products is due, at least in part, to the hydrolysis of galacto-oligosaccharides (71).

In addition, when GOS are produced in industry, beta-galactosidase shows higher transgalactosylation activity with high concentrations of lactose (71). The concentrations used in the present research were near or above the solubility limits of the solutions. One method to form GOS uses high concentrations of lactose in low acid solutions to increase GOS formation. Optimal GOS formation conditions could contribute to why the ITC data looks different in

solutions of different pH values (72). It has also been found that during GOS formation, covalent intermediates may form (73, 74). Reactions with and formation of these intermediates has the potential of changing the heat rates of the ITC data. Also important to note, is that many different types of GOS products can be formed through hydrolysis, which could also cause our results to look different in different solutions and with different sources of beta-galactosidase being used.

Also evident by the control lines on all of the graphs with beta-galactosidase from *Kluyveromyces lactis* varying lactose (figures 5, 10, and 14), is that there is enough lactose present naturally within the fluid dairy products to saturate the enzyme without the additional lactose.

Figure 7. Heat rates over time for varying concentrations of beta-galactosidase from Kluyveromyces lactis in sweet whey spiked with 500mM lactose, pH=6.51

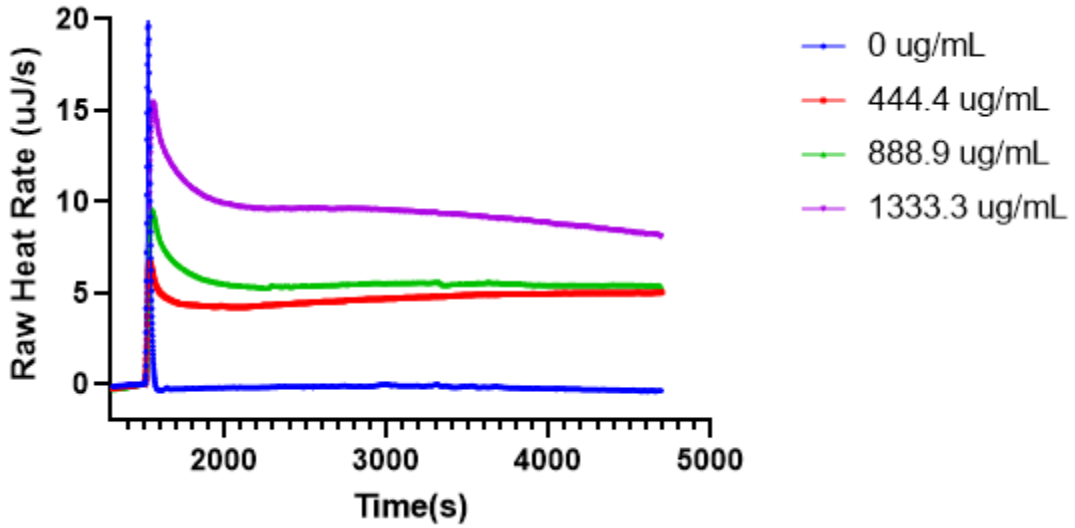


Figure 8. Heat rates of beta-galactosidase for varying concentrations of beta-galactosidase from Kluyveromyces lactis in sweet whey spiked with 500mM lactose at 600(s) after injection, pH=6.51

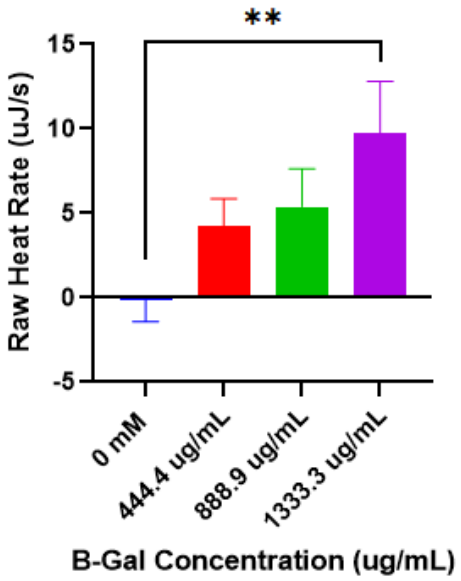
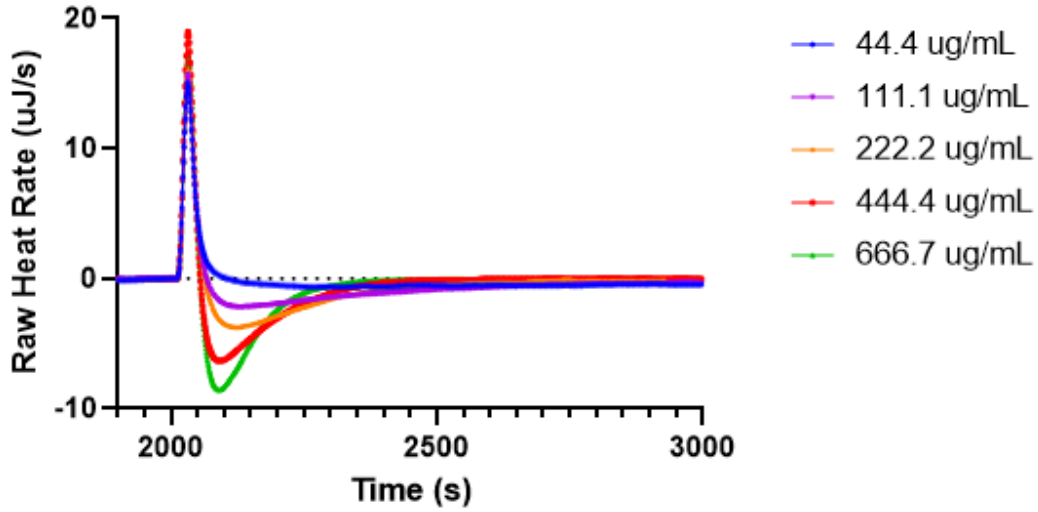


Figure 9: Heat rate over time of 450mM lactose injected into various concentrations of beta-galactosidase from Aspergillus oryzae in sodium acetate buffer, pH=4.6



As shown in figure 10, when a consistent beta-galactosidase concentration in sweet whey is injected into various concentrations of lactose and sweet whey, the difference in concentrations appears much tighter than with varying concentrations of enzyme. Upon visual observance, there appears to be a linear pattern of increasingly exothermic reactions with increasing concentrations of lactose. At about 700 seconds after injection, or 2200 seconds on the x-axis, the heat rate of the control begins to drop at a much higher rate than the solutions spiked with lactose. It is unknown why this would be the case with this run. It is interesting to note, that the control lines in both runs with beta-galactosidase from *Kluyveromyces lactis* and varying the concentrations of lactose in milk (Figure 5) and sweet whey (Figure 10), have a notable drop at about 2200 seconds. Because these runs were done in triplicate, for further observation, each individual run was compared, and two out of the three runs making up the averaged data (in both milk and sweet whey), appear to have drops in raw heat rates around 2200 seconds. It is unknown why there is this pattern, but is potentially due to the higher concentrations of fat in milk and sweet whey.

All p-values were >0.05, showing no statistical difference between the different concentrations of lactose (figure 11). The top three lines, with lactose concentrations of 100mM, 300mM, and 500mM demonstrate the enzyme is saturated or there is a secondary reaction or change of state. The control line is likely not saturated because the heat rate is gradually decreasing. The statistical data would have been different in figure 11 if a later time point had been chosen to compare heat rates.

Figure 10. Heat rate over time of 1.11 mg/mL beta-galactosidase from Kluyveromyces lactis in sweet whey with varying concentrations of lactose, pH= 6.51

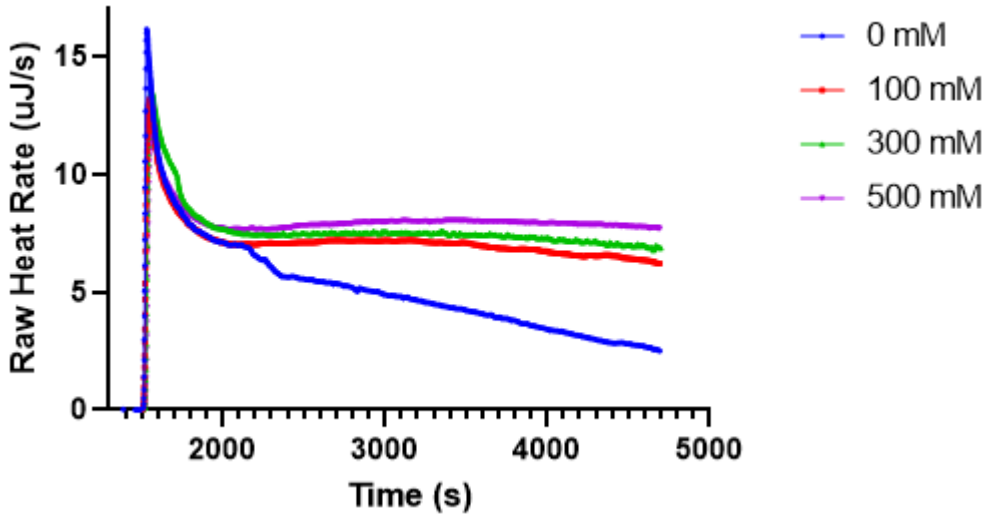
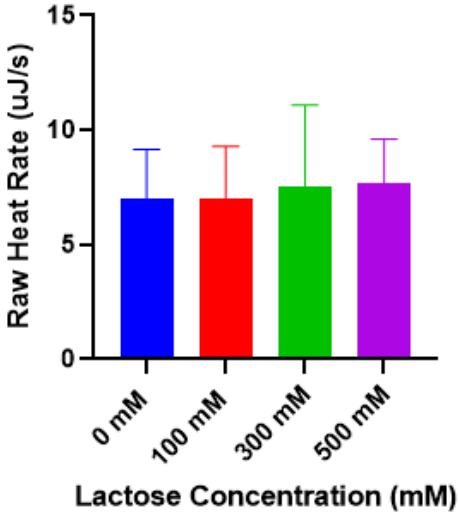


Figure 11. Heat rate over time of 1.11 mg/mL beta-galactosidase from Kluyveromyces lactis in sweet whey with varying concentrations of lactose at 600(s) after injection, pH=6.51



Sweet Whey Permeate

Upon visual inspection, the graph of varying concentrations of beta-galactosidase in sweet whey permeate (figure 12) appear similar to the same types of runs in sweet whey. When beta-galactosidase in sweet whey permeate is injected into the lactose and sweet whey permeate solution, there appears to be a linear pattern of increasing exothermic heat rates with increasing concentrations of beta-galactosidase. However, there is more statistical significance in the variation of heat rates between enzyme concentrations in sweet whey permeate than in sweet whey. At 600 seconds after injection, the mean heat rate for the control is 0.31 uJ/s, for the 444.4 ug/mL of beta-galactosidase is 2.27 uJ/s, for the 888.9 ug/mL of beta-galactosidase is 3.63 uJ/s, and for the 1333.3 ug/mL of beta-galactosidase is 5.61 uJ/s. The p-value of 0 ug/mL vs. 444.4 ug/mL is >0.05, 0 ug/mL vs. 888.9 ug/mL has a p-value of 0.03, 0 ug/mL vs. 1333.3 ug/mL has a p-value of 0.002, 444.4 ug/mL vs. 888.9 ug/mL has a p-value of 0.51, 444.4 ug/mL vs 1333.3 ug/mL has a p-value of 0.03, and 888.9 ug/mL vs. 1333.3 ug/mL has a p-value of 0.23 (figure 13). In all runs, there is an initial heat of injection at 1500 seconds and within about 600 seconds, the heat rate levels out and remains relatively steady throughout the remainder of the run. There appears to be a slow and steady reaction between the lactose and beta-galactosidase. But, as stated previously, there is likely a secondary reaction and/or change of state causing the appearance of a steady exothermic reaction, rather than a short endothermic reaction.

Although the pattern of heat rate appears similar in sweet whey (figure 7) and sweet whey permeate (figure 12), it can be noted that the scales are different and there is a lower heat rate in the sweet whey permeate. The lower heat rate is likely due to the protein being removed during processing of the sweet whey permeate which allows for less interactions to create heat.

Figure 12. Heat rates over time for varying concentrations of beta-galactosidase from Kluyveromyces lactis in sweet whey permeate spiked with 500mM lactose, pH= 6.39

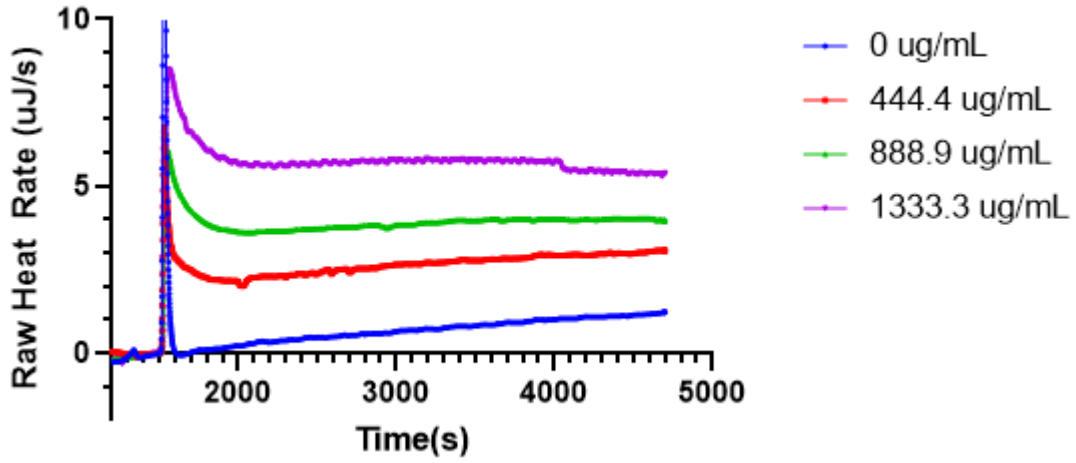
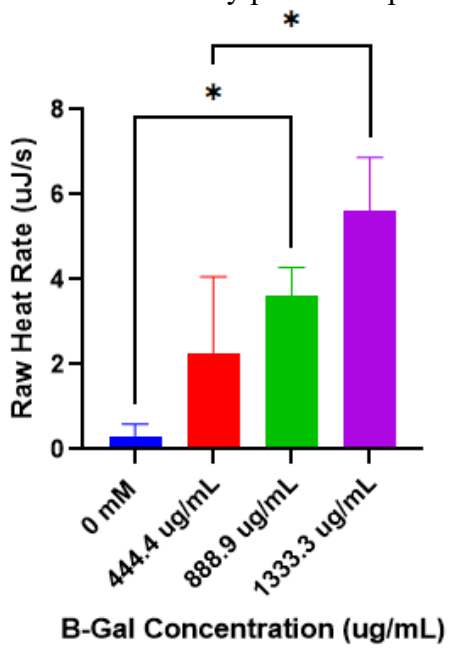


Figure 13. Heat rates of varying concentrations of beta-galactosidase from Kluyveromyces lactis in sweet whey permeate spiked with 500mM lactose at 600(s) after injection, pH=6.39



As demonstrated in figure 14, when a consistent beta-galactosidase concentration in sweet whey permeate is injected into increasing concentrations of lactose in sweet whey permeate, the difference in concentrations again, appears tighter than with varying

concentrations of enzyme. Upon visual observance, there appears to be a linear pattern of increasingly exothermic reactions with increasing concentrations of lactose. Although this data has a definite pattern, it is noisier than other runs. The noise is likely due to mechanical noise. Mechanical noise happens occasionally and does not invalidate the overall results of the data.

As with sweet whey with varying concentrations of lactose, with sweet whey permeate all p-values were >0.05, showing no statistical difference between the different concentrations of lactose (figure 15). All lines show us the enzyme is saturated and/or there is a secondary reaction or change of state.

Figure 14. Heat rates over time of 1.11 mg/mL beta-galactosidase from Kluyveromyces lactis in sweet whey permeate with varying concentrations of lactose, pH= 6.39

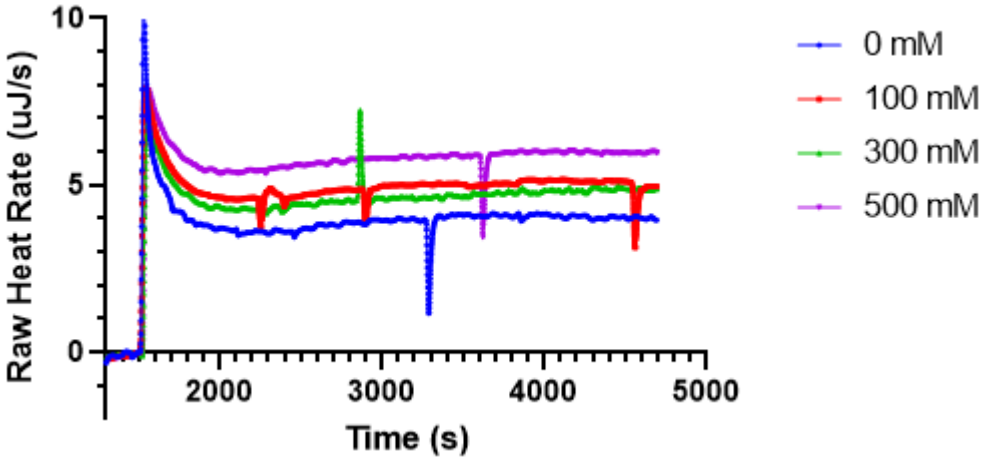
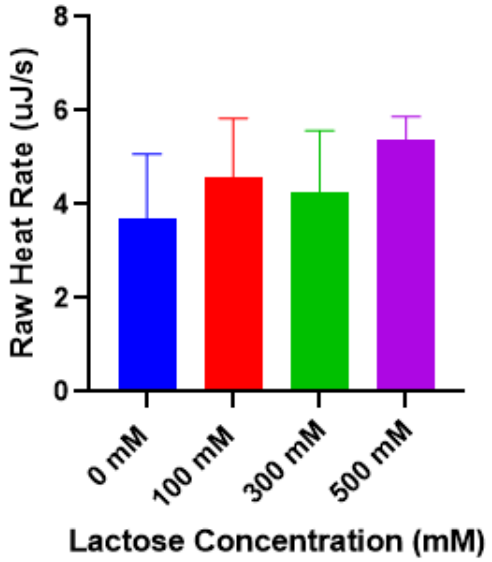


Figure 15. Heat rates over time of 1.11 mg/mL beta-galactosidase from Kluyveromyces lactis in sweet whey permeate with varying concentrations of lactose at 600(s) after injection, pH=6.39



Acid Whey

As demonstrated in figure 16, when beta-galactosidase from *Aspergillus oryzae* in acid whey is injected into the lactose and acid whey solution, there appears to be a linear pattern of increasingly exothermic heat rates with increasing concentrations of beta-galactosidase. Beta-galactosidase was injected at 1500 seconds in all runs done with acid whey. 1500 seconds gave sufficient time for the ITC to equilibrate. At 50 seconds after injection, which is 1550(s) on the x-axis, mean heat rates were collected. This time was chosen so the heat of injection wouldn't be contributing to the data, but the peak of the reaction could be statistically analyzed.

At 50 seconds after injection, the mean heat rate for the control is -0.79 uJ/s, for the 444.4 ug/mL of beta-galactosidase is 3.02 uJ/s, for the 888.9 ug/mL of beta-galactosidase is 6.94 uJ/s, and for the 1333.3 ug/mL of beta-galactosidase is 8.41 uJ/s. However, the p-value is >0.05 in all comparisons, except 0 ug/mL vs. 888.9 ug/mL, which has a p-value of 0.0034 and 0 ug/mL vs

1333.3 ug/mL, which has a p-value of 0.0011, and 444.4 ug/mL vs 1333.3 ug/mL, which has a p-value of 0.0263 (figure 17). There is an initial large heat of injection at 1500 seconds and any reactions appear to be complete around 3400 seconds, which is much faster than with beta-galactosidase from *Kluyveromyces lactis* in sweet whey products, where there is no clear end of reaction, even after 4700 seconds.

The reaction in the acid whey and acid whey permeates resolving quickly may be due to the nature of the beta-galactosidase from *Aspergillus oryzae* versus beta-galactosidase from *Kluyveromyces lactis.* In the information provided by the manufacturers, beta-galactosidase from *Kluyveromyces lactis* has an enzyme activity rate of ≥2600 U/g and the beta-galactosidase from *Aspergillus oryzae* has an activity rate of >5000 U/g. And from the literature, the percentage of lactose hydrolysis is generally much higher in beta-galactosidase from *Kluyveromyces lactis*, at 75-100%, compared to that of beta-galactosidase from *Aspergillus oryzae*, which is generally 41-50% (75). This could be why the runs with beta-galactosidase from *Aspergillus oryzae* don't completely resolve during the duration of the runs in the ITC, and have a higher area under the curve in comparison to the runs with beta-galactosidase from *Kluyveromyces lactis.*

The most common use for ITC is for measuring binding affinities by measuring changes in heat. In research from Deaville et al., they measured the binding affinities of tannins to proteins. They noted that the heat rate and therefore, binding affinity changed based on the molecular weight of the molecules involved in the interaction (76). Although, the current project is measuring beta-galactosidase activity, not binding affinity, because of the diversity of molecules contained in the matrices of these liquid dairy products, it is possible that some of the

change in heat rate in the various dairy matrices is due to the size of the molecules that make up the compositions interacting with the enzyme or the products of the enzymatic reaction.

It can also be noted that the pH optimum of beta-galactosidase from *Aspergillus oryzae* is 4.75 (40) and with the acid whey having a pH of 4.16 and the acid whey permeate 4.03, there is a chance that with these slightly sub-optimal conditions, the reaction could potentially end more quickly. Additionally, the optimal temperature for the Aspergillus oryzae is 60ºC, which is the temperature these reactions were run and prolonged exposure to this temperature may cause the enzyme to unfold, thus limiting the catalytic activity.

In the literature, beta-galactosidase from *Kluyveromyces lactis* is shown to have a higher rate of hydrolysis than beta-galactosidase from *Aspergillus oryzae* (77). From the data gathered thus far, it cannot be determined if the results from ITC supports this because all of the runs with beta-galactosidase from *Kluyveromyces lactis* appear to have heat being produced from a secondary reaction or a change of state.

Figure 16. Heat rates over time of varying concentrations of beta-galactosidase from Aspergillus oryzae in acid whey spiked with 500mM lactose, pH=4.16

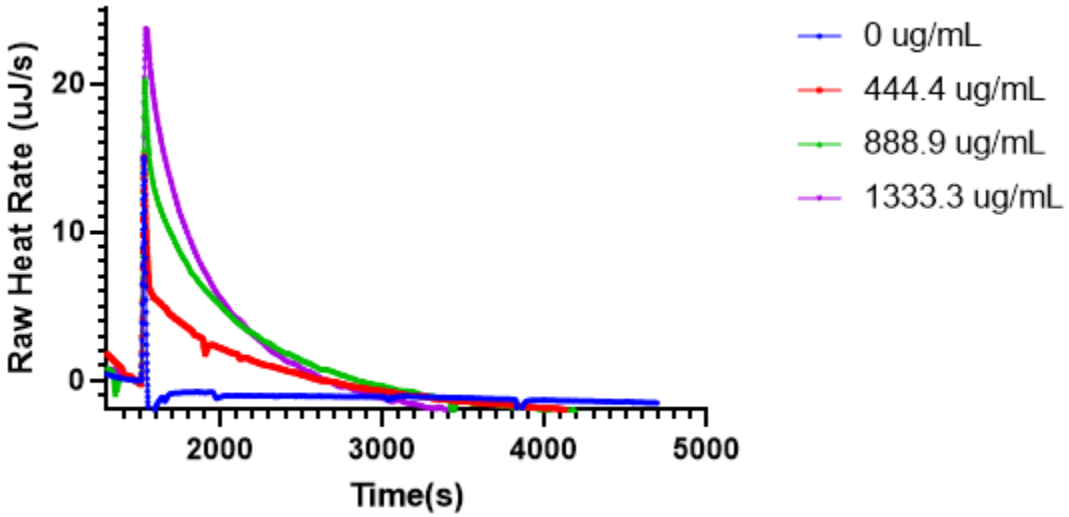
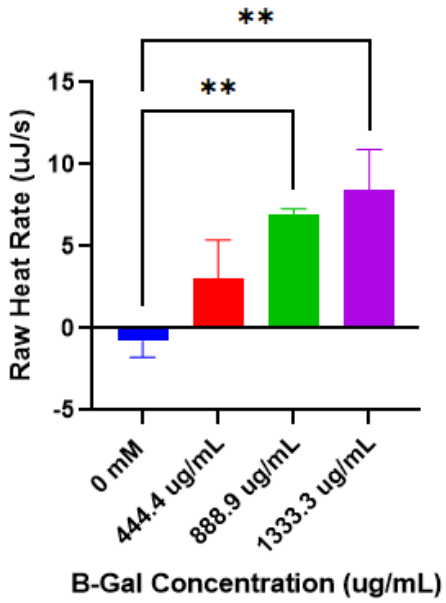


Figure 17. Heat rates of varying concentrations of beta-galactosidase from Aspergillus oryzae in acid whey spiked with 500mM lactose at 50(s) after injection, pH=4.16



As shown in figure 18, when a consistent beta-galactosidase concentration in acid whey is injected into various concentrations of lactose and acid whey, upon visual and statistical observation, there is no consistent pattern. However, at 50 seconds after injection, there is a pattern of increasing heat rates with increasing concentrations of lactose. At 50 seconds after injection, the mean heat rate for the control is -0.88 uJ/s, for the 100 mM lactose is 1.26 uJ/s, for the 200 mM lactose is 10.26 uJ/s, and for the 300 mM lactose is 17.35 uJ/s. All p-values were significant, except 0mM vs. 100mM. The p-value for 0 mM vs. 300 mM is 0.0039, 0 mM vs. 500mM is 0.0001, 100 mM vs. 300 mM is 0.0135, 100 mM vs. 500 mM is 0.0003, and 300mM vs. 500 mM is 0.0453 (figure 19). The control line increases in heat rate rather than remaining flat as would be expected. The runs making up this data were done in triplicate as the others were and were even run a fourth time about a month later to verify. Results were similar. It appears that beta-galactosidase from *Aspergillus oryzae* does not stay stable for long. It is

possible that the beta-galactosidase from *Aspergillus oryzae* is more sensitive to a sub-optimal pH, which causes the instability of the enzyme. The pH of the acid whey was 4.16 with the optimal being 4.75. However, this minor difference from the optimal pH is unlikely to make this dramatic of an impact. During preliminary testing, milk runs were done with beta-galactosidase from *Aspergillus oryzae* and generally maintained enzyme activity (Figure 20), even though the pH of milk is 6.37 and the optimal pH for beta-galactosidase from *Aspergillus oryzae* is 4.75. This demonstrates that the inconsistent data is likely to be due to the matrix of acid whey, rather than the pH. Another possibility is that temperature settings were not optimal for the runs in acid whey. For the acid whey runs, the ITC was set to 60°C to optimize enzyme activity from *Aspergillus oryzae*, based on the literature. For the preliminary runs of beta-galactosidase from *Aspergillus oryzae* in milk, the temperature was set to 37°C. However, because all the runs with beta-galactosidase from *Kluyveromyces lactis* have somewhat predictable results and were run at 60°C, it is unlikely the temperature is causing the inconsistent results in acid whey and acid whey permeate. (Note- The runs for Figure 20 are not shown in triplicate as they were preliminary runs.)

The data for figure 21 with beta-galactosidase from *Kluyveromyces lactis* in acid whey being injected into acid whey with and without additional lactose was collected after Figures 18 and 20 had been created in an attempt to verify how beta-galactosidase from *Kluyveromyces lactis* responds in a solution with a pH that is not optimal. It is demonstrated in figure 21 that no pattern of enzyme activity is demonstrated in runs with either 888.9 ug/mL or 1333.3 ug/mL concentration of beta-galactosidase from *Kluyveromyces lactis*. There are three possible conclusions from this data. Beta-galactosidase from *Kluyveromyces lactis* is capable of maintaining enzyme activity without the optimal pH and beta-galactosidase from *Aspergillus*

oryzae is not or something about the nature or matrix of acid whey is preventing the enzyme from maintaining stability with beta-galactosidase from *Aspergillus oryzae* or the temperature of 60°C, from the literature, is not appropriate for the experiments only in acid whey and acid whey permeate solutions, with the most likely conclusion being because of the acid whey matrix.

Figure 18. Heat rates over time of 1.11 mg/mL beta-galactosidase from Aspergillus oryzae in acid whey with varying concentrations of lactose at 600(s) after injection, pH= 4.16

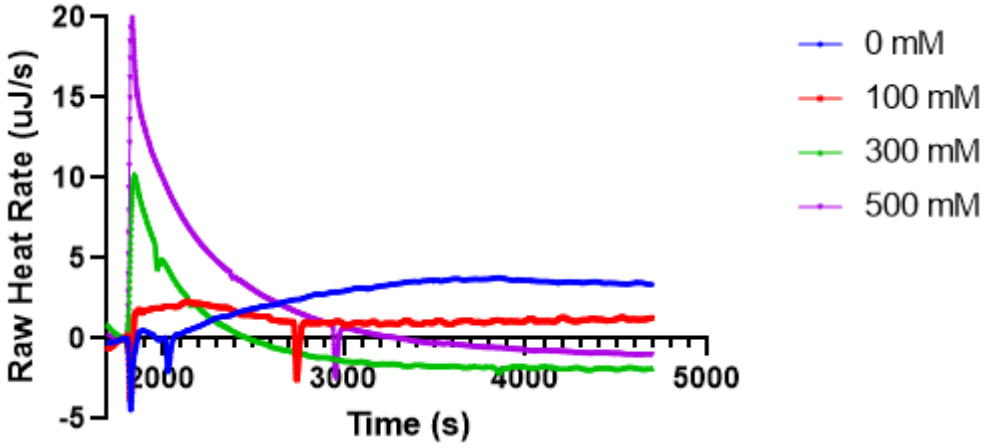


Figure 19. Heat rates of 1.11 mg/mL beta-galactosidase from Aspergillus oryzae in acid whey with varying concentrations of lactose at 50(s) after injection, pH=4.16

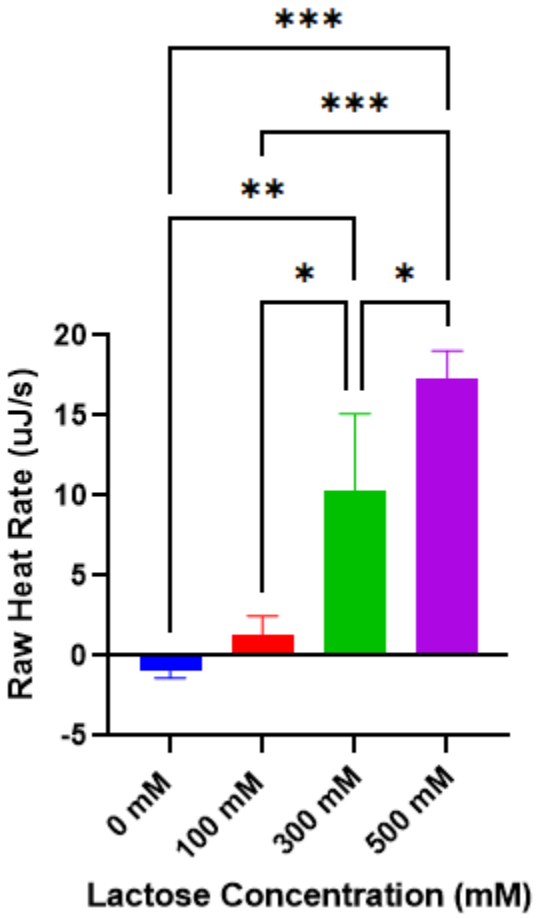


Figure 20: Heat rates over time of 0.667 mg/mL beta-galactosidase from Aspergillus oryzae in milk spiked with lactose, pH=6.37

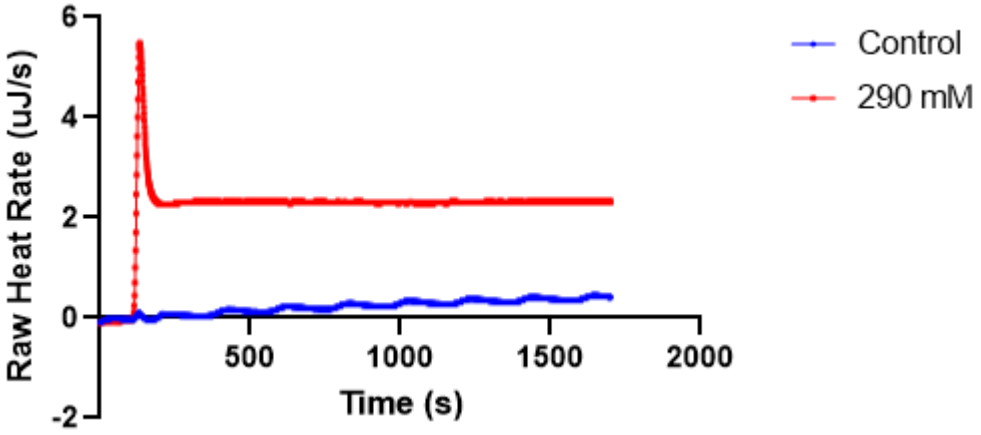
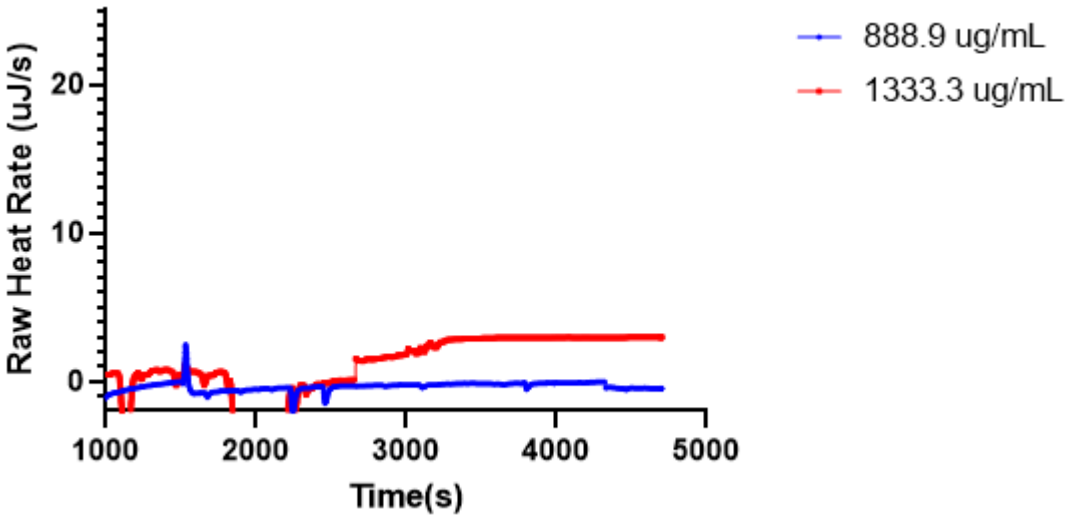


Figure 21: Heat rates over time of varying concentrations of beta-galactosidase from Kluyveromyces lactis in acid whey spiked with 500mM lactose, pH=4.16



Acid Whey Permeate

As demonstrated in figure 22, when beta-galactosidase in acid whey permeate is injected into the lactose and acid whey permeate solution, there is a linear pattern with increasing concentrations of beta-galactosidase injected into the lactose and acid whey permeate solution at

50 seconds after injection or 1850 seconds on the x-axis, with only some of the values being significant. Beta-galactosidase was injected at 1500 seconds in all runs done with acid whey. For runs in acid whey permeate, 1800 seconds gave sufficient time for the ITC to equilibrate. At 50 seconds after injection, which is 1850(s) on the x-axis, mean heat rates were collected. 50 seconds after injection was the time was chosen so the heat of injection wouldn't be contributing to the data, but the peak of the reaction could be statistically analyzed.

At 50 seconds after injection, the mean heat rate for the control is -0.60 uJ/s, for the 444.4 ug/mL of beta-galactosidase is 7.27 uJ/s, for the 888.9 ug/mL of beta-galactosidase is 22.03 uJ/s, and for the 1333.3 ug/mL of beta-galactosidase is 29.31 uJ/s. 0 ug/mL vs. 889 ug/mL has a p-value of 0.0230. 0 ug/mL vs 1333.3 ug/mL has a p-value of 0.0048 (figure 23). 444.5 ug/mL vs. 1333.3 ug/mL has a p-value of 0.0263. The remaining comparison have p-values greater than 0.05. The heats of injection of beta-galactosidase from *Aspergillus oryzae* are larger in acid whey permeate than any other runs performed. There is a linear pattern of increasing heats of injection with increasing concentrations of enzyme, which is surprising because generally there is a larger peak of injection when there are more interactions in the solution, largely from proteins. Since acid whey permeate has already had the protein removed, the expectation would be that the peaks from injection in both whey permeate solutions would be smaller than in the whey solutions, such as was the case in the runs with sweet whey permeates with beta-galactosidase from *Kluyveromyces lactis* in comparison with those in sweet whey.

After the heat from the injection and reaction, the heat rate lowers quickly. Because of the series of runs with beta-galactosidase from *Aspergillus oryzae* in sodium acetate buffer (Figure 9) that show that in a simple buffer, the heat of injection resolves after only about 40 seconds. It is evident that at least one quick and small reaction is taking place.

Figure 22. Heat rates over time of varying concentrations of beta-galactosidase from Aspergillus oryzae in acid whey permeate spiked with 500mM lactose, pH= 4.03

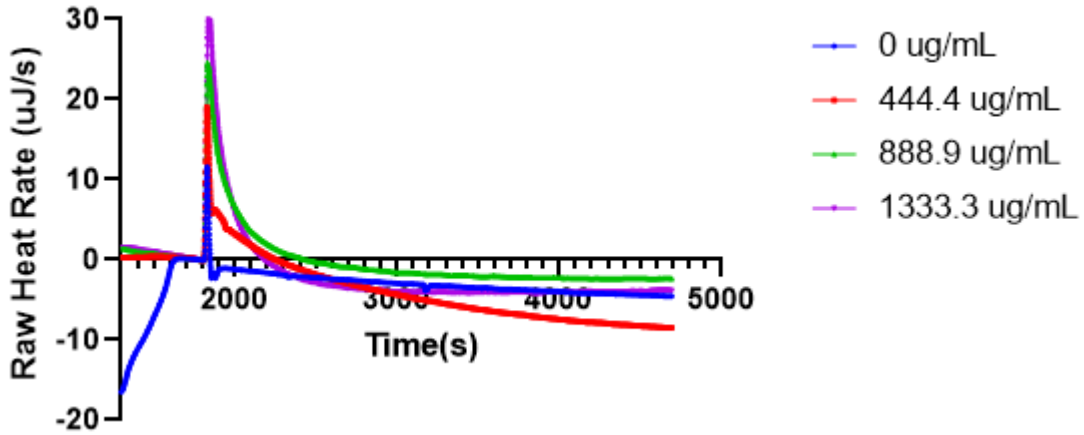
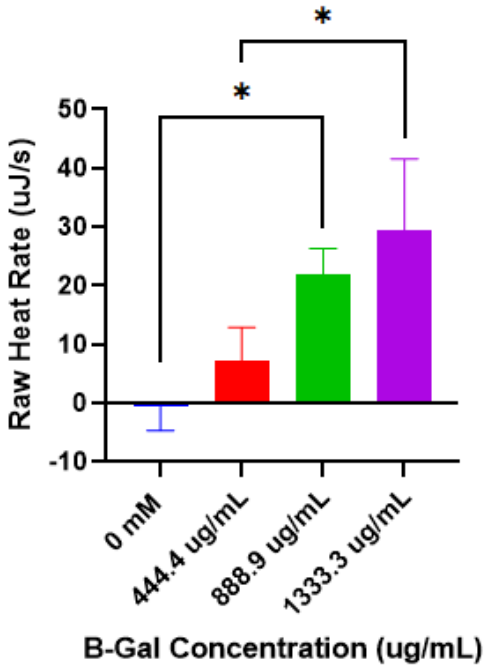


Figure 23. Heat rates of varying concentrations of beta-galactosidase from Aspergillus oryzae in acid whey permeate spiked with 500mM lactose at 50(s) after injection, pH=4.03



As demonstrated in figure 24, when a consistent beta-galactosidase concentration in acid whey permeate is injected into various concentrations of lactose and acid whey permeate, upon

visual observation, there is no pattern. However, at 50 seconds after injection, the mean heat rate for the control is 0.60 uJ/s, for the 100 mM lactose is 3.08 uJ/s, for the 200 mM lactose is 8.06 uJ/s, and for the 300 mM lactose is 15.26 uJ/s. All p-values were significant, except 0mM vs. 100mM (figure 25). All p-values are >0.05, except 0mM vs. 300mM has a p-value of 0.003, 100mM vs. 300mM has a p-value of 0.027, and 300mM vs. 500mM has a p-value of 0.03.

Upon first inspection, due to the location of the control, the reactions appear to be endothermic, rather than exothermic, but there is no linear pattern with increasing concentrations of lactose. All concentrations have an initial heat of injection and then appear endothermic. The endothermic nature of these reactions is suspected to be anomalous because of the lack of pattern. Figure 24 appears very similar to Figure 18 with the data appearing random with a lack of conclusiveness as to if the reactions are endothermic or exothermic. As stated previously, this could be due to the pH being sub-optimal, but with the optimal pH being 4.75 and the pH of acid whey permeate being 4.03, the sub-optimal pH is unlikely to make this large of a difference in the data and is more likely due to the acid whey permeate matrix and quick reaction that experiments with beta-galactosidase from *Aspergillus oryzae* exhibit.

Figure 24. Heat rates over time of 1.11 mg/mL beta-galactosidase from Aspergillus oryzae in acid whey permeate with varying concentrations of lactose, pH= 4.03.

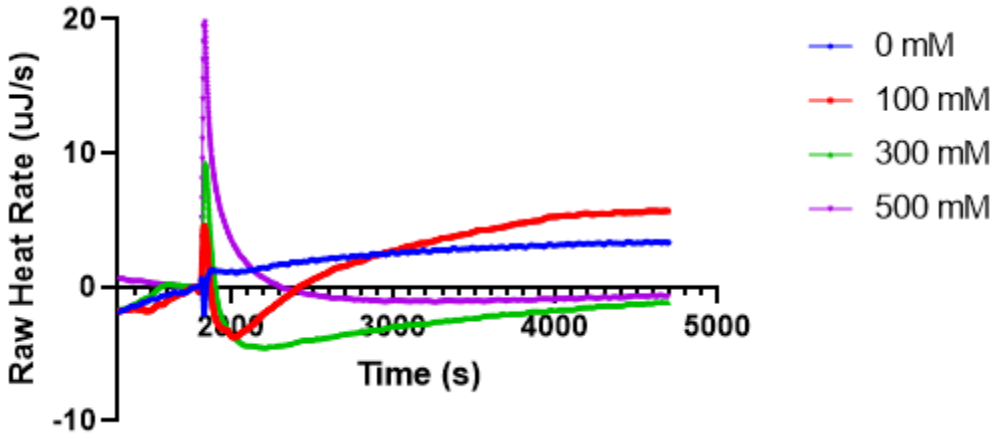
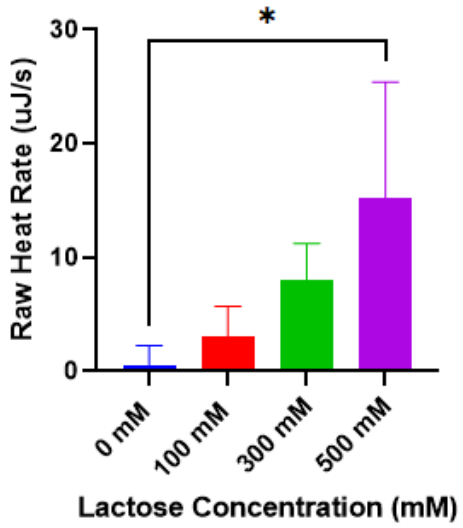


Figure 25. Heat rates of 1.11 mg/mL beta-galactosidase from Aspergillus oryzae in acid whey permeate with varying concentrations of lactose at 600(s) after injection, pH=4.03



Conclusion

There was consistent exothermic activity demonstrated in milk, sweet whey, and sweet whey permeate with beta-galactosidase from *Kluyveromyces lactis* in runs done in the ITC. This was true when beta-galactosidase concentrations were varied and lactose concentrations remained the same and when lactose concentrations were varied and enzyme concentrations remained the same. The cause of the exothermic nature of the reactions in milk, sweet whey, and sweet whey permeate is unknown, but potentially due to the formation and hydrolysis of galacto-oligosaccharides or glucose and galactose binding to another protein.

There was a quick exothermic reaction(s) demonstrated when varying concentrations of beta-galactosidase from *Aspergillus oryzae* in acid whey and acid whey permeate were injected into a consistent concentration of lactose in acid whey and acid whey permeate. There was a pattern of increasing heat with increasing concentrations of enzyme, with some of these differences being statistically significant.

There was also a shorter exothermic reaction(s) demonstrated when a consistent concentration of beta-galactosidase from *Aspergillus oryzae* was injected into varying concentrations of lactose. There was a pattern of increasing heat with increasing concentrations of lactose, with some of these differences being statistically significant.

Using ITC as a method for measuring beta-galactosidase activity and other enzyme activity in dairy fluids or other dairy products merits more research and exploration. These results are promising, and further research would be valuable for understanding enzyme activity directly in the food matrix of dairy products.

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APPENDIX A

The original primary hypothesis for this project was that the chosen Lactic Acid Bacteria (LAB) secrete beta-galactosidase extracellularly and will increasingly secrete beta-galactosidase extracellularly with supplementation of MRS broth with 1%, 2%, and 3% of the carbohydrate lactose. The chosen LAB strains were representative strains of *Lactobacillus plantarum, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus bulgaricus, Streptococcus lactis, Lactobacillus helveticus,* and *Streptococcus thermophilus.*

This hypothesis was shown to be invalid for the following strains: *Lactobacillus acidophilus, Streptococcus thermophilus, and Lactobacillus helveticus.* LAB do not secrete beta-galactosidase extracellularly. With supplementation of lactose in the broth, the LAB do not secrete beta-galactosidase extracellularly either. It is assumed that this is representative of the remaining strains of LAB. It was also discovered during this process that the nano-ITC was not sensitive enough to measure the beta-galactosidase activity, even when the cells were lysed. Therefore, the research moved from testing the original hypothesis to testing the capabilities of the ITC, specifically for the dairy industry. The below background information was excluded from the primary thesis since the goals of the project changed.

Background-Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) are a group of bacteria categorized because of their similar characteristics. They are agents of fermentation involved in the production of many foods, including yogurt, cheese, cultured butter, sour cream, sausage, cucumber pickles, olives, and sauerkraut. They contribute to the taste and texture of these fermented foods and inhibit food

spoilage bacteria (78). LAB are Gram-positive, non-sporeforming cocci, coccobacilli, or rods. All LAB grow anaerobically, but they can grow in the presence of oxygen and therefore are considered aerotolerant anaerobes (78). Sugars are the carbon and energy source for LAB (79). They are generally considered to be beneficial microorganisms and some strains are even health-promoting (probiotic). However, some genera contain species or strains that are known to be pathogenic, such as Streptococcus, Lactococcus, Enterococcus, and Carnobacterium (80).

The history of LAB is rich and interesting with advancements to our understanding of the benefits and functionality of LAB coming from all around the world. As early as 8,000 to 5,000 BC, the people of North Africa began to consume naturally acidified fermented milk, cheese, and other dairy products (81). In Ancient India between 6,000-4,000 BC, yogurt (dahi), cheese, and sour cream were processed (82). In 1,000 BC, Chinese people began to make kimchi, which is salted and fermented vegetables (83). In 1780, Carl Scheele, a Swedish medical chemist, first isolated and identified lactic acid from soured milk and described the chemical properties (84). LAB were viewed under a microscope for the first time by a French scientist, Pasteur, in 1857, while studying rancidity in wine drinks. He linked lactic acid production to microorganisms (85). In 1880, a commercial lactic acid bacteria starter was produced (82). From this time forward, many species have been isolated, manufactured, sold, studied, and characterized. LAB have had a tremendous impact on food, culture, and health throughout the world.

Many LAB contain the enzyme beta-galactosidase, which hydrolyzes lactose into glucose and galactose. In all LAB, following hydrolysis, fermentation takes place. LAB can either be homofermentative, meaning they ferment glucose (or other carbohydrates, depending on the strain) with lactic acid as the primary byproduct or they can be heterofermentative, meaning they ferment glucose (or other carbohydrates, depending on the strain) with lactic acid, ethanol/acetic

acid, and carbon dioxide as byproducts. *Lactobacillus acidophilus, Streptococcus thermophilus, Streptococcus lactis, Lactobacillus bulgaricus,* and *Lactobacillus helveticus* are homofermentative. *Lactobacillus plantarum, Lactobacillus casei,* and *Lactobacillus fermentum* are heterofermentative (86). *Lactobacillus bulgaricus, Lactobacillus lactis,* and *Lactobacillus acidophilus* strains metabolize only the glucose and release the galactose outside of the cell (87). *Streptococcus lactis, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus fermentum,* and *Lactobacillus helveticus* metabolize both glucose and galactose (87-91).

LAB that are homofermentative, ferment via Glycolysis or the Embden-Mayerhof pathway, which is generalized in *Figure 26.* LAB that are heterofermentative ferment via the phosphoketolase pathway (pentose phosphate pathway), which is generalized in *Figure 27*. In some LAB strains, sugars other than glucose, such as galactose, can enter the pathways at the level of glucose-6-phosphate or fructose-6-phosphate after isomerization and/or phosphorylation (78).

Figure 26: Homofermentation

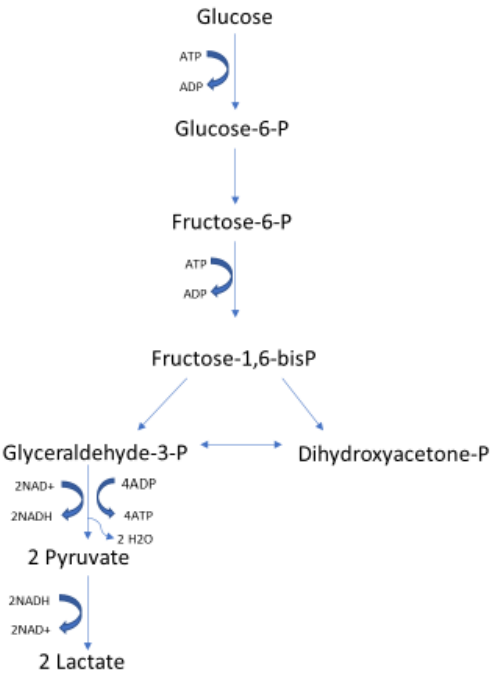
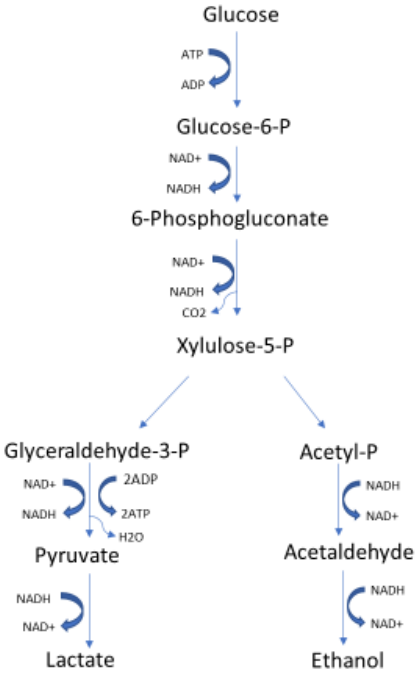


Figure 27: Heterofermentation



For this research, eight representative strains of LAB that have more than one study demonstrating any beta-galactosidase activity within the cell were chosen in order to have a foundation on which to build in finding extracellular beta-galactosidase activity since there is little published research about extracellular beta-galactosidase activity in LAB. If beta-galactosidase is already known to be produced intracellularly by the specific LAB strain, then the odds are more likely that the beta-galactosidase will be secreted. The best growth conditions for these strains were provided by the manufacturer. Although conditions for optimal cell cultivation and conditions for optimal enzyme activity do not match exactly, there is a strong correlation (92). This is why the methods for this research includes alterations in temperature and pH if any enzyme activity is demonstrated.

If any activity is demonstrated with either buffer, the temperature will be increased in 5°C increments up to 50°C to measure the temperature where optimal enzyme activity occurs.

The pH with either buffer will also be adjusted between 4.6-6.5 pH to achieve optimization. Ideas for these temperatures and pH values were obtained from a study that Wierzbicki and Kosikowski (93) did that found optimal pH and temperature values for beta-galactosidase produced from numerous LAB.

It should be noted that the literature involving the release of extracellular beta-galactosidase from LAB does not always agree. Thongaram, Hoeflinger, Chow, and Miller noted that *Lactobacillus acidophilus NCFM* is released extracellularly(94). However, Montanari et.al found that *Lactobacillus plantarum* and *Lactobacillus brevis* did not release beta-galactosidase extracellularly, but the cell autolysed and the cell wall was broken, which was demonstrated with scanning electron microscope images(95). Carevic et. al found no extracellular beta-galactosidase activity in *Lb. acidophilus ATCC 4356, Lb. rhamnosus ATCC 7469, Lb. reuteri ATCC 23271, Lb. helveticus ATCC 15009,* and *Lb. delbreuckii* subspecies *bulgaricus ATCC 11842* (96).

Steps Taken to Reject Original Hypothesis

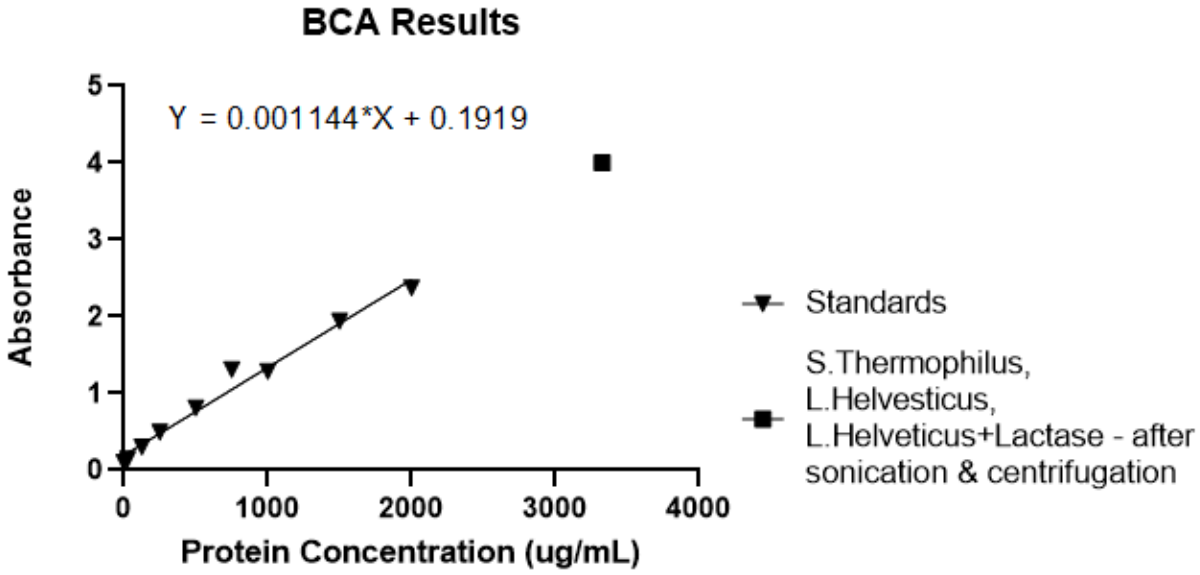
Initially, the exponential growth phase of *S. Thermophilus* and *L.Helveticus* were discovered through literature review and measuring the optical density after growing in an anaerobic environment at 40°C (97). The goal was to measure enzyme activity during the exponential phase. However, enzyme activity was measured during many different phases of growth to ensure there was no enzyme activity measured during any of the phases of cell growth, with the ITC. After centrifugation, no beta-galactosidase activity was measured in the supernatant on the ITC during any of the runs.

After no beta-galactosidase activity was seen on the ITC, it was decided to lyse the cells. It is known that these strains of LAB contain beta-galactosidase intracellularly. It was desired to see if the ITC could measure enzymatic activity from beta-galactosidase, to ensure that if there was any beta-galactosidase secreted, the ITC was sensitive enough to measure the heat from the reaction.

In order to lyse the cells, sonication was attempted at first with various lengths of pulses and intensities. The broth containing the bacteria was centrifuged and the supernatant was placed in the ITC. No enzyme activity was found in *S. Thermophilus* or *L.Helveticus*. As sonication is known to lyse cells, and beta-galactosidase is known to be intracellular in LAB, it was decided to explore a colorimetric assay to compare the sensitivities of a colorimetric assay to a calorimetric assay for measuring beta-galactosidase activity. No beta-galactosidase activity was found in the sonicated and centrifuged supernatant of *L.Helveticus* using the colorimetric assay either.

Since enzymes are proteins and there would be more proteins than just beta-galactosidase in the supernatant, it was determined to test for any protein in the supernatant using a Bicinchoninic Acid Assay (BCA). Protein was found in all of the centrifuged supernatants. The supernatants of *L. Helveticus* grown with additional lactose in the broth, *S.Thermophilus,* and *L.Helveticus* were all tested. Their absorbances were all found to be 4.0, demonstrating that there was more protein than the assay could measure, which is demonstrated in the figure below.

Figure 28: BCA results of supernatant after sonication and centrifugation

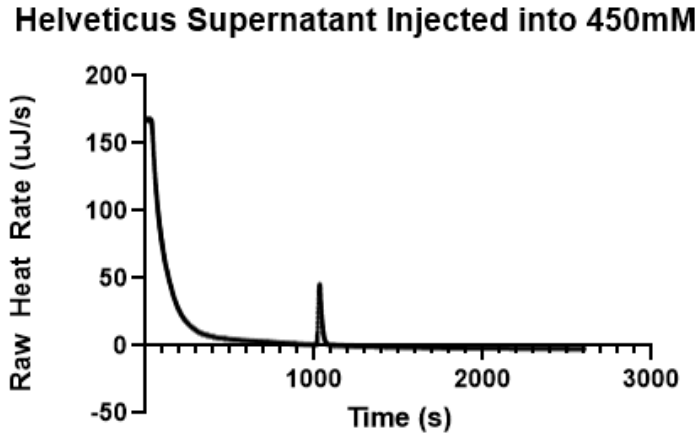


The article with the most helpful data and methods that was used for reference, found beta-galactosidase secreted from *Lactobacillus Acidophilus* (94). It was decided to pursue this strain of LAB. After consulting with the company that provided the *Lactobacillus Acidophilus,* it was decided to change the growing conditions of the bacteria and use an incubator with 5% CO2, rather than an anaerobic chamber. In addition, 3% lactose was added to the broth used to grow each of the strains, which at the time were *L. Acidophilus, S. Thermophilus,* and *L.Helveticus.*

In an attempt to ensure the cells were lysed, after the strains were grown under the above conditions and centrifuged, the supernatant was removed, 5mL of sodium phosphate buffer was added to the pellet, then vortexed and taken through various combinations of freeze/thaw cycles, sonication, chemical lysing, and vortexing with sand to compare efficacy of the lysing methods with a colorimetric assay. Vortexing sand with the pellet and buffer proved to be the most effective for lysing the cell to release the beta-galactosidase. Following vortexing with sand, the

supernatant was tested. A beta-galactosidase colorimetric assay was performed and beta-galactosidase was detected. A run on the ITC was done and no enzyme activity was detected. Through this research, two conclusions were made. First, *L.Acidophilus, L.Helveticus,* and *S. Thermophilus* do not secrete beta-galactosidase. This is likely representative of all LAB. Second, the nano-ITC is not sensitive enough to detect beta-galactosidase inherent in LAB, which is demonstrated in the figure below. There is only a peak where there is heat created through the injection, but no endothermic or exothermic activity is otherwise present.

Figure 29:



It was decided to explore measuring lysozyme activity in the ITC since there is plenty of literature available on this enzyme and there was already a bottle of the enzyme in the lab. A colorimetric lysozyme assay was performed. The colorimetric assay was successful and enzyme activity was demonstrated. By error, 1000x the amount of lysozyme was placed in the ITC for an experiment to measure enzyme activity. This accident finally demonstrated enzyme activity in the ITC. It was determined that the capabilities of the ITC with beta-galactosidase, the original enzyme of interest, should be explored in various liquids pertinent to the dairy industry which do not allow for direct measurement with a colorimetric assay.

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