CONVERTING LACTOSE TO ALLULOSE USING CELL-FREE PROTEIN SYNTHESIS: CONTRIBUTING TO BETTER DIABETES MANAGEMENT

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Professional Master in Food Science

by

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ABSTRACT

Background According to the American Diabetes Association (ADA), individuals with diabetes should limit their milk consumption to no more than 1 cup a day. This is due to the presence of lactose in milk, a sugar that can increase blood glucose levels. However, this restriction can affect the quality of life of milk lovers who may miss out on milk's nutritional benefits. To solve this problem, many milk substitutes have emerged in the market, such as almond, rice, and soy milk. Most provide 75% less protein than cow's milk, while soy milk (12.5% less protein) is controversial. A recent emerging technology, cell-free protein synthesis (CFPS/TXTL), may allow the synthesis of a series of enzymes needed to make dairy more diabetic-friendly. The enzyme synthesis method has the potential to decrease production costs due to higher yield and easier purification. **Purpose** The main aim of this project is to convert milk lactose into allulose with enzymes produced using CFPS/TXTL, thus providing a better milk option for individuals with diabetes. Milk with allulose, a rare sugar, may be superior to lactose-free milk because it is less sweet, has fewer calories, does not cause blood glucose spikes, and may be considered a functional food. **Methods** The first step focuses on converting lactose into glucose and galactose using lactase enzyme. This enzyme was first synthesized in the lab using a plasmid containing the gene for lactase (LacZ) as template for TXTL. Following an 18-hour incubation, the lactase enzyme produced was quantified using Qubit™ 4 Fluorometer. The efficiency of the cell-free generated lactase was then compared against commercially prepared powdered lactase using an ONPG-inspired protocol. After the conversion of lactose into glucose and galactose was achieved, the prospect of implementing an additional enzymatic cascade to further convert these substrates into allulose was considered. However, due to the temporal limitations inherent in this

study, the exploration of the supplementary enzymatic cascade was not attempted. **Results** The Qubit fluorometer recorded concentrations of 3.66 mg/mL, 4.75 mg/mL, and 3.62 mg/mL in the experimental samples, suggesting the presence of protein in the form of lactase enzyme. The ONPG-based test showed an enzyme that is equally if not more effective than its commercially manufactured equivalent. **Discussion** The TXTL or Cell-Free Protein Synthesis (CFPS) method yielded high quantities of the lactase enzyme which has in some instances shown to have equal if not higher efficiency than commercial powdered lactase. **Conclusion** Knowing that the production of lactase using CFPS hasn't been attempted before, the results are promising. The emerging cell-free protein synthesis technology has succeeded in generating a substantial yield of an enzyme, sometime surpassing its commercially produced equivalent. However, further research is needed to achieve the comprehensive complete transformation of lactose into the intended final product, allulose.

BIOGRAPHICAL SKETCH

Joy Khalil is a dietitian and a food safety specialist. She is currently in the final stages of completing her professional master's degree in food science, with a concentration in business, at Cornell University. Alongside her expertise in food and health, she's deeply intrigued by entrepreneurship, business development, and sustainability.

Joy's passion for health, longevity, and disease prevention has guided her academic journey. This led her to embark on the allulose cascade project using cell-free protein synthesis aimed at improving diabetes management. This project uniquely aligns with her three core interests:

1. Health: Joy's objective is to create a diabetes-friendly milk product that contributes to better diabetes management.
2. Innovation: By incorporating emerging technology, such as cell-free protein synthesis, Joy emphasizes her commitment to exploring innovative approaches to health challenges.
3. Sustainability: The project's use of cell-free protein synthesis underlines Joy's belief in sustainable practices, as this method efficiently produces protein in an environmentally conscious manner.

In summary, Joy Khalil's academic journey reflects her dedication to health, innovation, and sustainability. Through her allulose cascade project using cell-free protein synthesis, she showcases her interdisciplinary approach and potential to make meaningful contributions in her chosen field.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to the individuals who have contributed to the realization of this research project. First and foremost, my sincere appreciation goes to my advisor, Dr. Sam Nugen, for his valuable guidance, unwavering support, and insightful feedback that have greatly influenced the course of this study. I extend my gratitude to PhD candidate, David Parker, for his technical assistance and collaborative efforts throughout this project. Additionally, I would like to thank all the participants who generously contributed their time and resources, making this study possible.

A special thank you goes to Cornell University for providing me with the opportunity to pursue my master's degree and delve into this fascinating field of study.

Lastly, I would like to thank my supportive family and cherished friends. Their unwavering encouragement and understanding during the ups and downs of this journey have been my rock.

Sincerely,

Joy

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Health Benefits of Milk

Milk constitutes an essential topping for morning cereal, serves as delicious addition to coffee, and functions as an ideal post-workout recovery beverage. Essentially, it emerges as a crucial ally for athletes and a fundamental component of a healthy lifestyle. The 2020-2025 Dietary Guidelines for Americans (DGA) recommend three servings of dairy products per day for all populations above the age of two as part of a 2,000-calorie "Healthy U.S.-Style Dietary Pattern," and as part of "Healthy Vegetarian Dietary Pattern" (1). The DGA underline that there are different types of healthy eating patterns which depend on regular daily intake of dairy products, mainly low-fat and fat-free dairy.

When consumed according to the national guidelines, milk and dairy products supply crucial nutrients at all stages of the life cycle (2).

For young children, dairy products such as milk, provide a great source of energy and protein, and supply a wide range of micronutrients of which calcium, a crucial mineral for strong bones and teeth (3). Studies conducted in Europe reported that milk proportionally benefits the diets of young children more than adults'(4). However, evidence from the literature suggests otherwise. Data gleaned from cross-sectional studies (5) and intervention studies (6,7) has shown that milk consumption in childhood and adolescence had a positive effect on bone mineral content and bone mineral density. Moreover, a few research studies indicated an association between the consumption of milk and milk products throughout adolescence and neutral or decreased adiposity (8,9).

Dairy products continue to add value to the life cycle, supplying calcium and other key nutrients to the diet of pregnant women. Moderate milk consumption compared to no or low intakes

during pregnancy was shown to be positively associated with fetal growth and normal infant birth weight in healthy, Western populations (10).

Finally, numerous studies highlight the advantages of milk and dairy products in diets of older adults, and emphasize how, when combined with exercise, can increase muscle mass and function, thus reducing the risk of sarcopenia and spinal fractures (11,12).

Similarly, milk consumption after a workout has been shown to beneficially impact both acute recovery and chronic training adaptation. Milk is approximately isotonic (osmolality of 280-290 mosmol/kg) with a mixture of high-quality protein, carbohydrate, water and micronutrients (particularly sodium). Due to its composition, milk consumption post-exercise has been shown to enhance post-exercise muscle protein synthesis and rehydration, contribute to post-exercise glycogen resynthesis, and attenuate post-exercise muscle soreness/function losses. For these aspects of recovery, milk is comparable, cheaper, and sometimes outperforms commercially available sports drinks (James et al., 2018).

Nutrient in milk	Health benefit
Protein (whey and casein)	Helps build and repair tissue. Helps maintain a healthy immune system.
Fat	May help reduce the risk of obesity, atherosclerosis, and cancer due to the presence of conjugated fatty acids (CLA), a fatty acid naturally found in ruminant food products including milk (14).
Carbohydrates (lactose)	Known to facilitate mineral absorption, mainly calcium, magnesium, and manganese. This is due to a drop in pH resulting from the fermentation of lactose in the large intestine, which increases the solubility of calcium and other minerals (15).
Calcium	Helps build and maintain strong bones and teeth.
Vitamin B2 (Riboflavin)	Helps your body use carbohydrates, fats, and protein for fuel.
Vitamin B12	Helps with normal blood function, helps keep the nervous system healthy.
Vitamin D	Helps build and maintain strong bones and teeth. Helps maintain a healthy immune system.
Vitamin A	Helps skin and eyes healthy; helps promote growth. Helps maintain a healthy immune system.
Potassium	Helps maintain healthy blood pressure and supports heart health. Helps regulate body fluid balance and helps maintain normal muscle function.
Phosphorus	Helps build and maintain strong bones and teeth, supports tissue growth.
Niacin	Used in energy metabolism in the body.
Pantothenic acid	Helps your body use carbohydrates, fats, and protein for fuel.
Zinc	Helps maintain a healthy immune system, helps support normal growth and development and helps maintain healthy skin.
Selenium	Helps maintain a healthy immune system, helps regulate metabolism and helps protect healthy cells from damage.
Iodine	Necessary for proper bone and brain development during pregnancy and infancy; linked to cognitive function in childhood.

Table 1. The health benefits of nutrients found in an 8-oz serving of cow's milk.

Diabetes & Milk Consumption

According to the American Diabetes Association (ADA), individuals with diabetes should limit their milk consumption to no more than 1 cup per day. This is due to the presence of lactose in milk, a sugar that can increase blood glucose levels. Moreover, fat is another component individuals with diabetes should look out for. In fact, diabetes puts people at risk of

cardiovascular diseases and worsens with weight gain, thus lower-fat milk options are usually recommended by the ADA to limit the consumption of saturated fatty acids (SFA) and calories.

However, a restriction of no more than 1 cup of milk a day can affect the quality of life of milk lovers who may miss out on its nutritional benefits. An intake higher than 4-8 oz (varies from one individual to another) might cause blood sugar spikes, which can have many complications and negative consequences on health.

In an attempt to solve this problem, many milk alternatives have emerged in the market, such as almond, rice, and soy milk. Following is a nutritional comparison between cow's milk and the most popular low-carb and unsweetened alternative milk options.

Milk type	**Calories**	**Fat**	**Carbs**	**Fiber**	**Protein**	**Calcium**
Whole cow's milk	149	8g	12g	0g	8g	276 mg
Skim cow's milk	91	0.61g	12g	0g	9g	316 mg
Soy milk	79	4.01g	4.01g	1g	7g	300 mg
Almond milk	39	2.88g	1.52g	0.5-1g (brand)	1.55g	516 mg
Rice milk (unsweetened)	113	2.33g	22g	0.7g	0.67g	283 mg
Flax milk (unsweetened, no protein added)	24	2.5g	1.02g	0g (brand)	0g	300 mg

USDA Food Composition Database

Milk Alternatives Compared to Cow's Milk

Upon analyzing the nutritional profiles of various milk varieties, it becomes evident that plant-based milks (mainly almond, rice, and flax based) have one characteristic drawback: lower protein content than cow's milk with less than 2g of proteins per serving (around 75% less protein than cow's milk). Moreover, with 22g of carbohydrates per serving, rice milk is not to be recommended for people with diabetes.

This observation directs our attention to soy milk. Soy milk has been one of the most popular milk substitutes because its nutritional makeup closely resembles that of cow's milk. It might be the optimal high-protein milk alternative. 1 cup of soy milk provides about 7g of protein and 300 mg of calcium, compared to cow milk's 8g and similar calcium content per cup (12.5% less protein than cow's milk).

The downside for soy milk is the controversy surrounding it. Media speculation and research suggest potential health risks of soy such as increasing breast cancer risk and reducing testosterone levels in men, which could lead some consumers to avoid it altogether.

Consequently, altering the composition of cow's milk seems to be the ideal option for people on restricted and/or controlled carbohydrate diets, mainly people with diabetes. Moreover, while current research on the effect of milk alternatives on Type 2 Diabetes Mellitus (T2DM) is not available, many studies have evaluated the effect of dairy consumption on T2DM.

Dr. Giosuè (2022) conducted a review of 13 existent meta-analyses to study the risk of developing type 2 diabetes by eating different animal-based foods, including dairy products (16). It was found that increasing low-fat dairy consumption or replacing high-fat dairy with low-fat products was generally associated with a lower risk of diabetes. 200 g/day of milk decreased the risk by 10%, and low-fat dairy by 3%.

An Innovative Approach

The remaining aspect of potential market competition to be considered is lactose-free milk. Rare sugar-based milk will have a competitive advantage because it will be less sweet than lactose-free milk, will have less calories per cup, will not cause blood glucose spikes, and it might be considered a functional food because some rare sugars, such as allulose, might add additional health benefits to food products. However, the mechanisms that give allulose its functional properties are not yet fully understood.

A recent emerging technology, cell-free protein synthesis (TXTL), may allow the synthesis of a series of enzymes needed to make dairy more diabetic-friendly. The enzyme synthesis method has the potential to decrease production costs due to higher yield and easier purification. The main aim of this project is to convert milk lactose into allulose with enzymes produced using TXTL, thus providing a better milk option for individuals with diabetes. The justification for selecting allulose as the preferred rare sugar will be shortly explained.

Rare Sugars as a Sugar Alternative in Milk

According to the International Society of Rare Sugars, rare sugars have been defined as monosaccharides and their derivatives that hardly exist in nature. Two rare sugars have so far gained the “Generally Recognized as Safe (GRAS)” status: D-psicose (PSI, commonly known as allulose) and D-tagatose (TAG). Both are currently being used in the food industry for the manufacturing of biscuits, chocolate, jam, protein bars, soft drinks, and commercial sweetener blends, among other products.

PSI provides 0.4 kcal/g and is 70% as sweet as sucrose while TAG provides 1.5 kcal/g and is 92% as sweet as sucrose.

Because of its lower calorie contribution, only PSI got exempted from “total sugars” and “added sugars” figures on nutrition labeling in the USA. The FDA did not exempt TAG from these requirements and denied Bonumose LLC’s petition for exemption in May 2022, stating that TAG provides more empty calories than PSI (17).

For these stated reasons and regulations, the emphasis of this paper will be on the use of PSI, commonly allulose, as a sugar alternative in milk.

A scoping review by Smith et al. (18) examined the metabolic impacts of rare sugars and how they are interlinked. Results are summarized in the mapping diagram below (Figure 1).

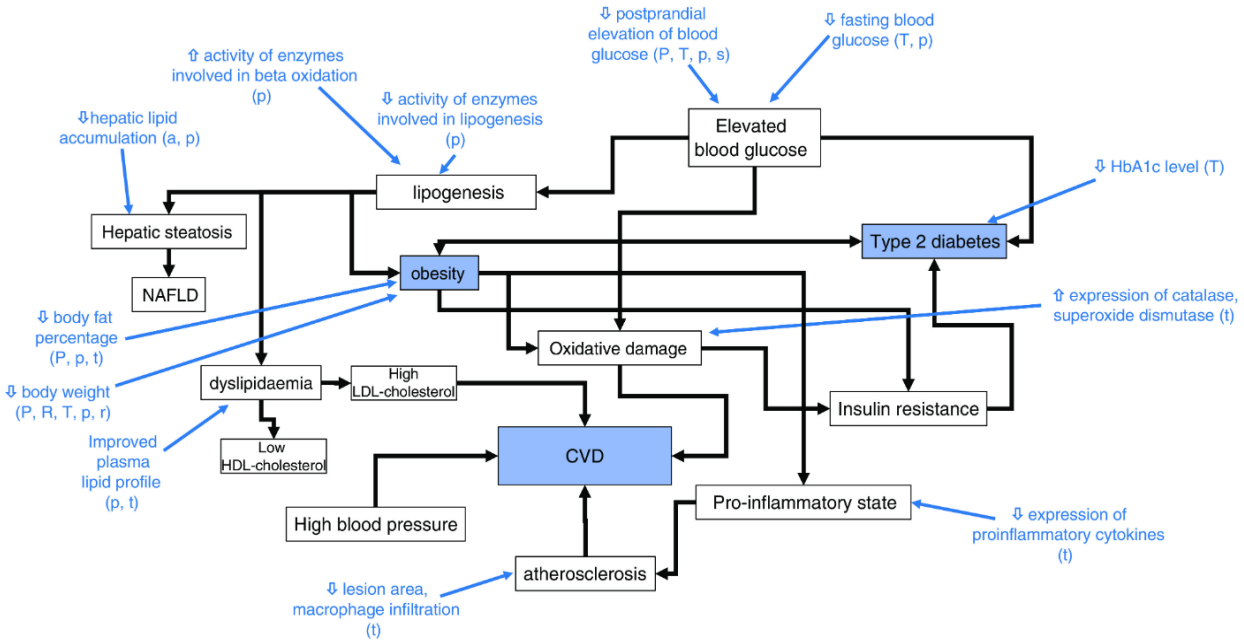


Figure 1. Mapping diagram showing the health benefits of rare sugars and how they are interlinked. Blue text indicates actions of rare sugars demonstrated in at least one study included in this review. Letters in brackets indicate the rare sugars involved, with capital letters denoting human studies and lower-case letters denoting animal studies: A/a – allose, P/p – psicose, S/s – sorbose, T/t – tagatose.

Health Benefits of PSI / Allulose

The reported in vivo effects of allulose consumption in humans included improved glycemic control and reductions in body weight and body fat. Animal studies showed similar effects. In addition, animal trials suggested that allulose consumption can reduce LDL-cholesterol and total cholesterol, decrease fasting blood glucose, reduce hepatic lipid accumulation, alter the gut microbiome, and improve inflammatory and oxidative status.

Consequently, rare sugar consumption, specifically D-psicose, could decrease the risk of many chronic diseases including T2DM, obesity, CVD, and NAFLD (non-alcoholic fatty liver disease). However, the precise mechanisms of action are not yet fully understood.

Potential Use of Allulose in Milk as Sugar Alternative

PSI is currently being used as a sweetener in the industry for soft drinks, protein bars, cookies, and more. It occurs naturally in small quantities in food products, particularly selected bakery products, sweets, and fruits (Oshima et al., 2006). It is odorless, white or almost white, and non-hygroscopic. Its cost is now estimated at $7/kg, comparable with erythritol. As previously mentioned, it provides 0.4 kcal/g and is 70% less sweet than sucrose. Although it is less sweet than sugar, it has similar sensory characteristics (temporal sweetness profile and sweetness quality). Another consideration for the use of allulose in functional foods or in the reformulation of foods is the safety of its long-term intake. Both PSI and TAG, as previously discussed, are considered GRAS. Through tolerance testing studies in healthy individuals, Han et al. (19) found that the maximum single dose of PSI that resulted in no severe GI symptoms was 0.4 g/kg BW while Hayashi et al. (20) had no evidence of toxicity with a single dose of PSI at 0.5-0.6 g/kg BW.

Property	Value
CAS	No. 551-68-8
Molecular formula	$C_6H_{12}O_6$
Molecular weight	180.16 g/mol
Physical form	Solid white crystalline powder
Odor	None
Optical rotation	$[\alpha]^{20}/D$ = E85° (*c* = 1, H_2O)
Melting temperature	96°C
Solubility	291 g/100 ml (25°C)
Caloric value	0.4 kcal/g

Table 2. Physical and chemical properties of D-allulose

Metabolism of D-Allulose in the body

The chemical name of allulose is D-ribo-2-ketohexose. It is a C-3 epimer of D-fructose. This difference in structure prevents the body from metabolizing allulose the same way it processes fructose. Allulose belongs to the non-digestible carbohydrate category. It is absorbed in the small intestine and passed into the colon where it's fermented by bacteria, thus producing short chain fatty acids (SCFAs). On one hand, these SCFAs may cause abdominal discomfort, flatulence, and diarrhea. However, on the other hand, they benefit the microbiome and help support diverse cellular processes, including those governing the goblet cells, leading to a mature mucus layer, consequently establishing a fully functional barrier against enteric pathogens (21).
Moreover, being unmetabolized by the body, allulose doesn't seem to raise blood sugar or insulin levels, which makes it ideal for people with diabetes (22).

Production of Rare Sugars, specifically D-Allulose

A. D-fructose to D-Allulose

D-allulose is produced through the isomerization of D-fructose under the catalysis of DTEase family enzymes. DTEase family enzymes include DTEases such as D-Tagatose-3-epimerases, D-psicose 3-epimerases (DAEases), and ketose 3-epimerase.

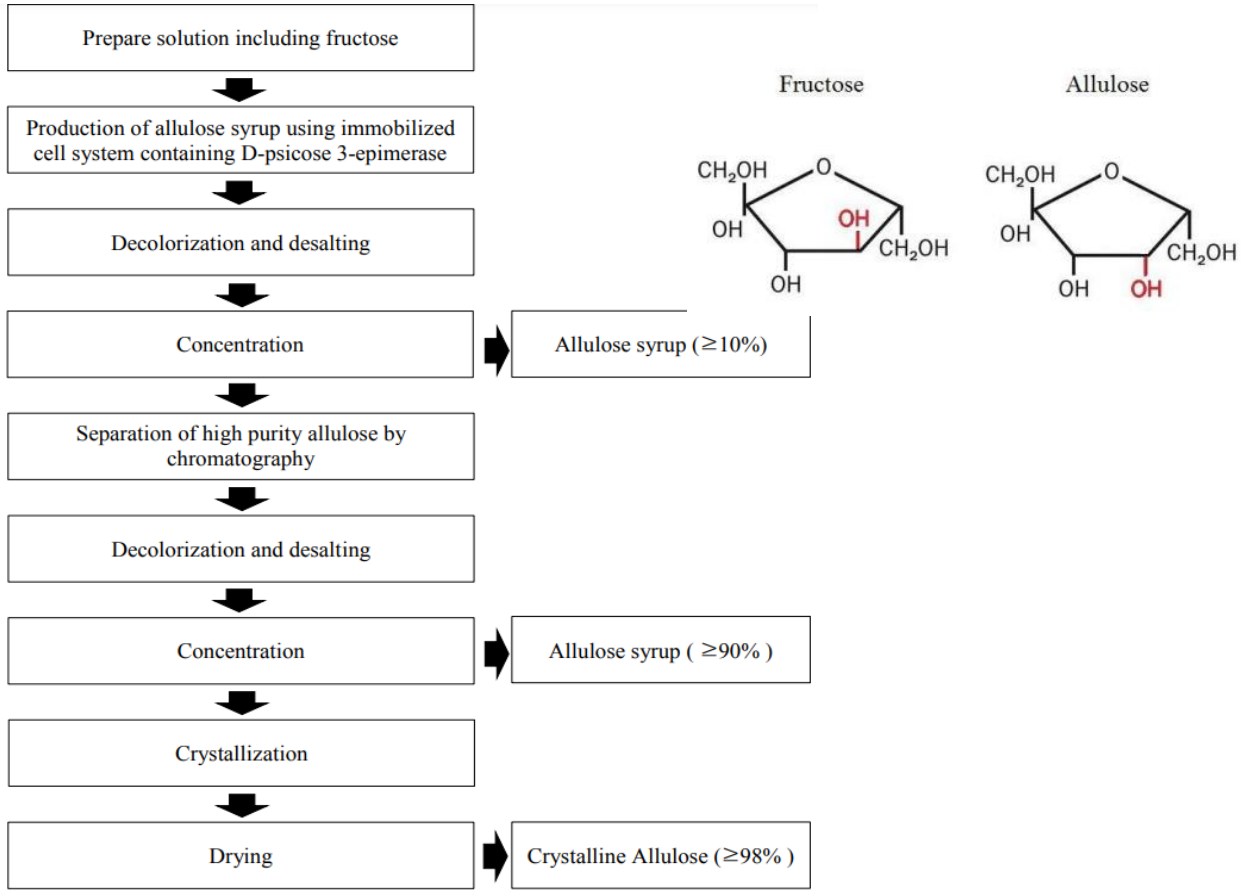


Figure 2. Flow Diagram of the Manufacturing Process of D-allulose from D-fructose

The majority of the enzymes in the DTEase family have been identified and isolated from bacteria. The E.coli expression system was found to have low cost and high expression efficiency. First, the DTEase family enzymes are overexpressed in soluble form in E. coli. Then, the recombined DTEase enzymes are separated and purified through affinity chromatography.

The purified recombinant DTEase enzymes can subsequently be used to catalyze the production of D-allulose in a bioreactor via immobilization approach (23).

Since E. coli is a high adoption platform for cell free protein synthesis (Gregorio et al., 2018) and it can be used to produce most of the DTEase family enzymes (Takeshita et al., 2000), it might be the way to go for the synthesis of D-allulose.

B. From bovine-sourced lactose

D-allulose can also be produced from bovine-sourced lactose. The idea entails a dual bioconversion of glucose into allulose and galactose into tagatose, which produces a novel, rare sugar blend initially made from bovine-sourced lactose, which constitutes a major component of waste streams in the dairy industry (patent pending).

Advantages: very low-calorie count, significant "bulking effect", low production cost, sustainable as it makes use of waste streams.

C. From milk lactose

The main aim of this paper is to convert milk lactose into allulose to produce the best milk option for diabetic people. Evidence from the literature suggests that so far, enzymes have been identified to convert D-fructose to D-allulose, D-glucose to D-allulose (24), and D-galactose to D-tagatose (25).

The rare sugar of interest in this study is D-allulose as it has a lower calorie density and is exempted by the FDA from labeling requirements under "added sugars" and "total sugars", unlike tagatose.

1. Break down lactose into its monosaccharides - glucose and galactose -through the addition of lactase enzyme (lactose-free milk production process).
2. Convert D-glucose to D-allulose.
3. Convert D-galactose to D-allulose.

Step 1: Lactose breakdown into glucose and galactose

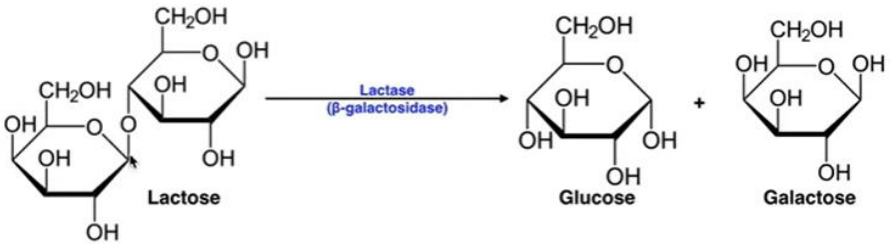


Figure 3. Chemical reaction for the breakdown of lactose in milk

Step 2: Conversion of D-glucose to D-allulose

Logically, D-glucose could be converted to D-fructose using various enzymes including D-glucose isomerase (GIase), then D-fructose could follow the approved mechanism to get converted to D-allulose using D-tagatose 3-epimerase (DPEase).

However, Zhang et al. (2017) discovered an innovative way to convert D-glucose to D-allulose in a one step process that co-expressed the GIase from Acidothermus cellulolyticus and the DPEase from Dorea sp. CAG.

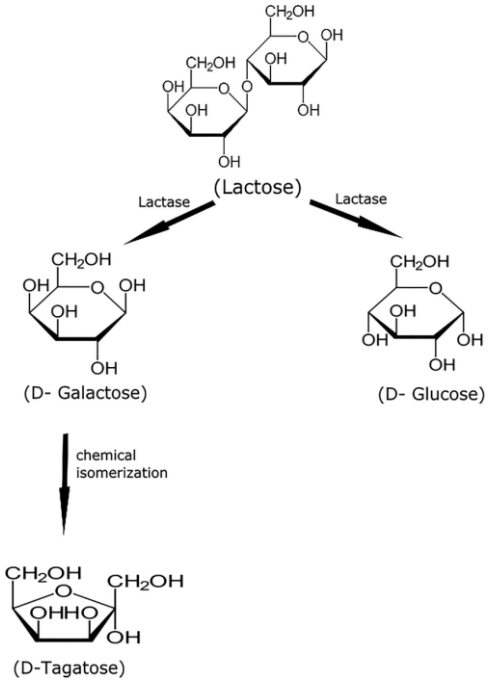
pETDuet-Dosp-DPE/Acce-GI, a co-expression plasmid, was created and introduced into Escherichia coli BL21 (DE3) cells. Maximum catalytic activity was observed in the recombinant co-expression cells at pH 6.5 and 75 °C. Less than 60 °C caused these cells to become thermostable. The catalytic activity was 10.8 times higher with the addition of Co2+. When the reaction equilibrium was attained, the ratios of D-glucose, D-fructose, and D-allulose were approximately 6.5:7:3.

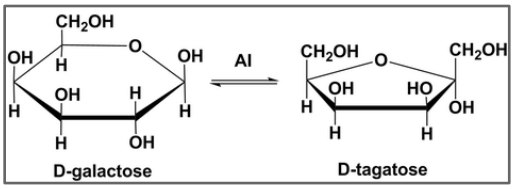
Step 3: Conversion of D-galactose to D-allulose

D-galactose could be converted to D-glucose, then D-glucose could go through step 2 and yield D-allulose.

Scientists were able to convert D-galactose into D-tagatose (which is not the rare sugar of interest in this paper), thus discovered a way to convert lactose into a sweetening mixture comprising lactose, glucose, galactose, fructose and tagatose.

D-galactose to D-tagatose was done using the versatile L-arabinose isomerase enzyme from Thermoanaerobacter mathranii, which was produced heterologously in E. coli.





As for the direct conversion of lactose, it was successfully done in a single reactor using both a thermostable beta-galactosidase and thermostable L-arabinose isomerase. The two enzymes were also combined with a commercially available glucose isomerase for the conversion of lactose into a sweetening mixture comprising lactose, glucose, galactose, fructose, and tagatose.

Methodology and Results

Due to the time limitations of this study, lactase enzyme was the only enzyme synthesized using TXTL/ myTXTL® kit, an emerging technology for Cell-Free Protein Synthesis (CFPS). This kit typically includes a mixture of essential components such as ribosomes, amino acids, energy sources, and other cellular machinery needed for protein synthesis without the complexities and constraints associated with cellular growth and regulation. Figure 4 illustrates the mechanics of cell-free protein synthesis in an open system. (A) Upon the addition of circular or linear template DNA to the myTXTL Master Mix, cell-free gene expression starts immediately. Produced target proteins are readily accessible for rapid downstream processing due to the absence of cell membrane and compartmentalization. (B) The myTXTL system supports gene expression regulated by different promoters including inducible and constitutive endogenous E. coli promoters as well as phage promoters, such as the T7 system (26).

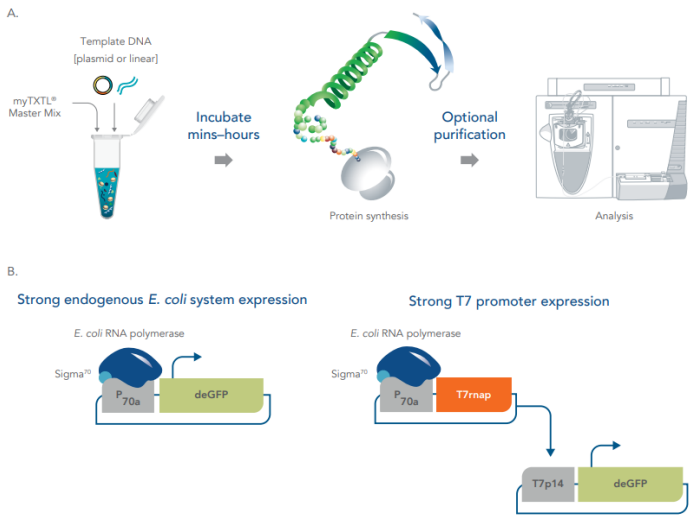


Figure 4. Mechanics of cell-free protein synthesis in myTXTL

As the first step, a 6.5 Kb; 959.1 ng/µL plasmid containing the gene for lactase was synthesized in Dr. Nugen's laboratory at Cornell University. The plasmid will be denoted "LacZ". LacZ was expressed in E. Coli and incubated. A polymerase chain reaction (PCR) was also performed to amplify the plasmid which was then characterized on gel electrophoresis (Figure 5) and sent for sequencing.

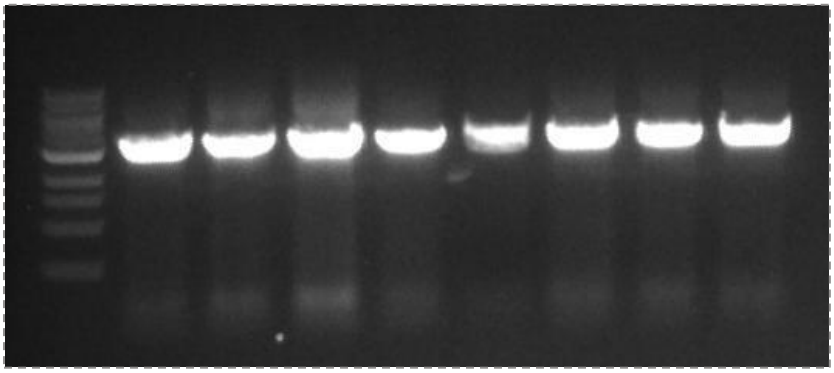


Figure 5. LacZ plasmid characterized on gel electrophoresis.

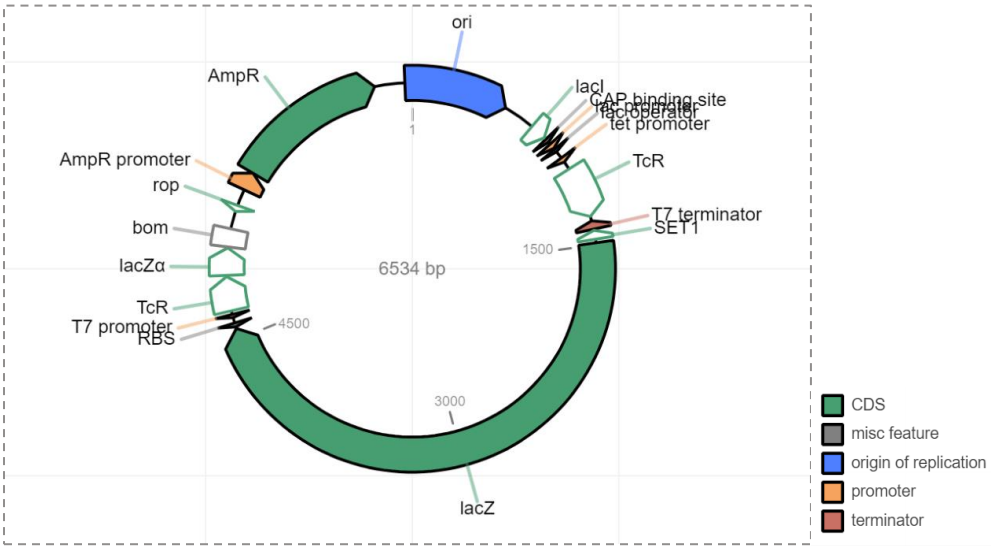


Figure 6. LacZ sequencing results

The sequencing results showed LacZ backwards (Figure 6). Consequently, new primers to fix the plasmid were ordered and the process was successfully repeated. The subsequent day, the cell-free protein synthesis was initiated following a modified protocol, tailored for the assay.

A container filled with ice was used to ease the careful defrosting process, which entailed thawing each tube containing Sigma70 Master Mix, p70a_deGFP plasmid, and p70a_T7RNA polymerase plasmid. This 15-to-20-minute thawing process was handled carefully to assure accuracy. As part of the preliminary stage, LacZ plasmid was also defrosted. The experimental setup entailed setting the Biotek plate reader to a pre-warmed temperature of 37°C after defrosting. Then, 9 Eppendorf tubes, divided into three unique sets of experimental, positive control, and negative control tubes, were prepared to ensure statistically significant results. The pipetting scheme is summarized in table 3.

Components	**Experimental tube**	**Positive control**	**Negative control**
LacZ Plasmid 6.5 kb	1 μL (5 nM) with 1:1 dilution	–	–
p70a_T7RNA polymerase	1 μL (0.1 nM)	–	1 μL
deGFP Positive Control Plasmid 3.2kb	–	2.4 μL (5 nM) with 1:1 dilution	–
Master Mix	27 μL	27 μL	27 μL
Nuclease-free water	7 μL	6.6 μL	9 μL
Total	36 μL	36 μL	36 μL

Table 3. Pipetting scheme for LacZ expression

To ensure accuracy, the MasterMix and Eppendorfs were vortexed before and after pipetting. During the pipetting procedure, conscientious efforts were made to avoid bubble and foam

formation to mitigate the potential for inaccuracies. Subsequently, the MasterMix was returned to -80C freezer at the earliest convenience to optimize preservation conditions and the tubes were incubated for 18 hours. The following day, the amount of lactase produced post incubation was quantified using a Qubit™ 4 Fluorometer. The results are summarized in table 4.

Sample	Quantity
Standard 1 (also taken as blank)	23.60 RFU
Standard 2	1633.55 RFU
LacZ 1	3.66 mg/ml
LacZ 2	4.75 mg/ml
LacZ 3	3.62 mg/ml
Neg 1	4.41mg/ml
Neg 2	4.91 mg/ml
Neg 3	4.38 mg/ml
DeGFP 1	13 mg/ml
DeGFP 2	5277.76 RFU (too HIGH)
DeGFP 3	4928.32 RFU (too HIGH)

Table 4. Protein quantification using Qubit fluorometer. Standard 1,2: samples needed as per Qubit protocol. LacZ 1,2,3: samples the three experimental tubes. Neg 1,2,3: samples from the three negative control tubes. DeGFP 1,2,3: samples from the three positive control tubes.

As a subsequent step, the purpose is to measure the efficiency and concentration of the lactase produced through cell-free protein synthesis and compare it to that of commercially available powdered lactase. A tailored protocol was developed, inspired by the ONPG test procedure. First, the Biotek plate reader was set up and pre-warmed to 37C (for optimal enzyme activity).

Then, ONPG was freshly prepared as 36 mg of ONPG were dissolved in 9 mL of phosphate buffer to reach a final concentration of 4 mg/mL in 0.1M phosphate buffer pH 7.0.

Subsequently, 2 buffers were needed: a Z buffer and a phosphate buffer. The phosphate buffer is stable at room temperature and does not need to be made fresh each time; consequently, a pre-made phosphate buffer was used from the lab with a pH of 7.2. The Z buffer, on the other hand, was freshly prepared. For a 50 ml volume, the following constituents were combined: 0.80g $Na_2HPO_4.7H_2O$ (0.06M), 0.28g $NaH_2PO_4.H_2O$ (0.04M), 0.5 mL 1M KCl (0.01M), 0.05 mL 1M $MgSO_4$ (0.001M), 0.135 mL b -mercaptoethanol (BME) (0.05M), and H_2O to dissolve all the salt. The pH was then adjusted to 7.0. It's important to note that the BME should be added right before the start of the pipetting procedure, under the hood.

The following steps were time sensitive due to their manual execution, with the start of the reaction occurring immediately upon initiation of pipetting. The pipetting scheme is detailed in table 5 while the mise en place is illustrated in Figure 7.

Experimental test tubes (3)	**Positive control tubes (3)**	**Negative control tubes (3)**
2 µL from TXTL experiment experimental tubes + 798 µL Z buffer (BME included) + 200 µL phosphate buffer (ONPG included)	12 mg of pure commercial powdered lactase from asp. Flavus were dissolved in 150 µL of Z buffer (BME included) => 50 µL in each of the 3 tubes + 750 µL Z buffer under hood + 200 µL phosphate with ONPG	2 µL from the negative controls used in TXTL + 798 µL Z buffer (BME included) + 200 µL phosphate buffer (ONPG included)
Total = 1 mL each	Total = 1 mL each	Total = 1 mL each

Table 5. Pipetting scheme

Figure 7. Pipetting mise en place for measuring the efficacy of lactase enzyme produced.

After this step, a volume of 12 µL was meticulously pipetted from each respective tube into designated wells of a 96-well plate, following the arrangement illustrated in Figure 8. It is noteworthy that, for enhanced precision and data fidelity, the positive control and LacZ samples underwent serial dilution at factors of 10 and 100. Once all samples were pipetted, the plate was securely covered with a lid and the Biotek reading was initiated with a specific wavelength parameter of 420 nm. The results are depicted in Figures 9 and 10 for visual reference and analysis.

	1	2	3	4	5	6	7	8	9
A	Neg	Neg	Neg						
B									
C									
D	BGal:1 4	BGal:1 4	BGal:1 4	BGal:2 0.4	BGal:2 0.4	BGal:2 0.4	BGal:3 0.04	BGal:3 0.04	BGal:3 0.04
E									
F									
G	LacZ1	LacZ1	LacZ1	LacZ2	LacZ2	LacZ2	LacZ3	LacZ3	LacZ3
H									

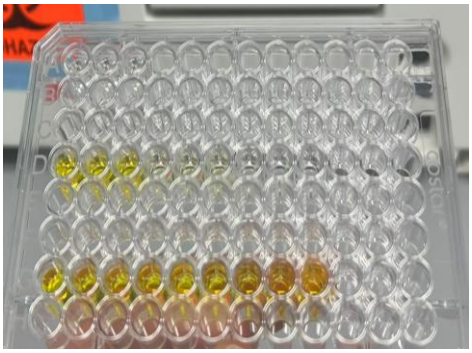


Figure 8. Pipetting arrangement into a 96-well plate to run through Biotek

Well ID	Well	Conc/Dil	420	[Concentration]	Count	Mean	Std Dev	CV (%)
Neg	A1		0.063	0.01	3	0.008	0.002	20.834
	A2		0.06	0.006				
	A3		0.062	0.009				
BGal	D1	4	3.514	4.141	3	4	0.136	3.388
	D2	4	3.386	3.988				
	D3	4	3.288	3.87				
	D4	0.4	0.404	0.418	3	0.401	0.015	3.792
	D5	0.4	0.387	0.397				
	D6	0.4	0.379	0.389				
	D7	0.04	0.097	0.051	3	0.039	0.011	27.642
	D8	0.04	0.081	0.032				
	D9	0.04	0.082	0.033				
LacZ1	G1		2.816	3.305	3	3.068	0.205	6.679
	G2		2.518	2.949				
	G3		2.52	2.951				
LacZ2	G4		3.618	>4.198	2	3.926	0.022	0.556
	G5		3.321	3.91				
	G6		3.347	3.941				
LacZ3	G7		3.577	>4.198	2	4.049	0.001	0.027
	G8		3.438	4.05				
	G9		3.436	4.048				

Figure 9. Results from Biotek reading showing the concentration of protein in each sample.

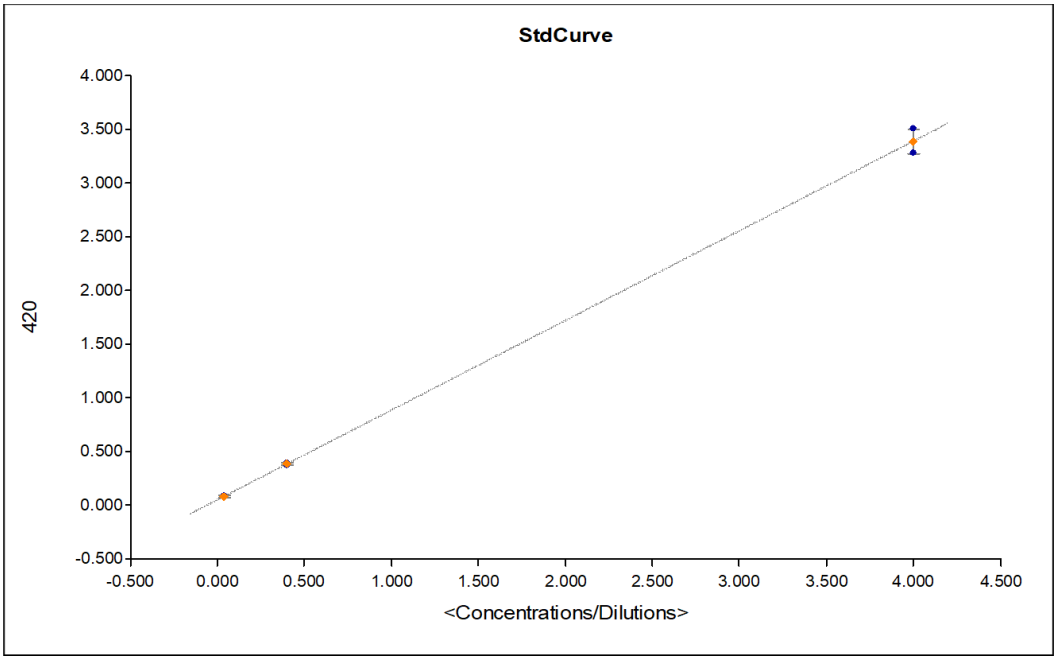


Figure 10. Standard curve illustrating the concentration of lactase produced.

Discussion

The results from the Qubit™ 4 Fluorometer indicate the presence and quantities of protein within the samples tested. Standard 1 is used as a blank reference to account for background fluorescence. Standard 2 serves as a known protein concentration for calibration. The higher fluorescence value of Standard 2 (1633.55 RFU) compared to Standard 1 (23.60 RFU) indicates that the fluorescence readings correspond to increasing protein concentrations. As for the experimental samples (LacZ 1, LacZ 2, and LacZ 3), the recorded concentrations (3.66 mg/ml, 4.75 mg/ml, and 3.62 mg/ml) suggest the presence of protein in the form of lactase enzyme in these tubes. Ideally, the negative controls are typically meant to confirm the absence of the protein of interest, in this case, lactase. However, the recorded concentrations (4.41 mg/ml, 4.91 mg/ml, and 4.38 mg/ml) are unexpectedly high, which could indicate potential issues with the negative controls or the quantification process. As for the positive control samples (deGFP 1, deGFP 2, and deGFP 3), they should contain a significant amount of the protein of interest. deGFP 1 has a concentration of 13 mg/ml, suggesting the presence of the control protein at the expected level. deGFP 2 and deGFP 3, however, exhibit extremely high fluorescence values (5277.76 RFU and 4928.32 RFU, respectively), considered "too high." This could indicate potential issues with these samples, such as overestimation of protein content or technical errors. In summary, the results do suggest the presence of protein, particularly lactase enzyme in the experimental samples and the control protein (DeGFP) in deGFP 1. However, the unexpected high values in the negative controls (Neg 1, Neg 2, Neg 3) and the extremely high values in deGFP 2 and deGFP 3 raise concerns about potential technical issues or experimental anomalies that should be further investigated and addressed.

From the ONPG test results, it can be inferred that the TXTL method yielded high quantities of the enzyme and has in some instances shown to have equal if not higher efficiency than commercial powdered lactase.

Conclusion

In conclusion, this research marks a significant step towards the utilization of cell-free protein synthesis (CFPS) as a tool for the conversion of lactose into allulose, with prominent implications for diabetes management. Innovative research into lactase manufacturing using the TXTL technique has produced encouraging results, demonstrating the enormous potential of this cutting-edge technology. The results highlight CFPS's capacity to generate enzymes with a level of effectiveness that is comparable to or even greater than that achieved by traditional industrial techniques. Nonetheless, further investigation is needed to advance the progress towards achieving a comprehensive conversion of lactose to the intended final product, allulose.

It is important to note that the ramifications of this research go beyond the laboratory. These discoveries have the potential to considerably contribute to better diabetes management. Allulose, as a low-calorie sugar alternative, has the potential to help diabetics manage their blood glucose levels and reduce their overall sugar intake. Furthermore, applying cell-free protein synthesis to industrial production has evident transformative potential. This approach could revolutionize the manufacturing of diabetes-targeted products, offering a more sustainable and flexible alternative to conventional methods.

As we advance, it is critical to acknowledge the interdisciplinary nature of this study, which spans the fields of biotechnology, health sciences, and industrial innovation. Collaboration among researchers, industry partners, and medical professionals is essential for realizing the full potential of CFPS-generated allulose and its implications for diabetes care. This research

establishes a precedent for the prospective combination of cutting-edge technology and health-focused solutions, paving the way for a healthier, more sustainable future.

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