Enzymatic Production of Psicose from Glucose

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Abstract

Excess sugar consumption is an undisputed primary cause of type 2 diabetes. Alternative sweeteners have since been developed to counteract this issue, however some products such as artificially produced sweeteners are unfavored due to underlying health impacts and taste profile. In its place, rare sugars as have grown commercial interest as an alternative sweetener and psicose is top candidate. Psicose is a rare sugar with virtually zero calories since it is not metabolized after consumption. Current psicose manufacturing processes, due to thermodynamic limitations, are not cost efficient and produce low psicose yields. This study proposes an alternative pathway in which thermodynamic limitations are overcome to produce higher theoretical yields. Two key enzymes, a phosphatase and epimerase, are required for the proposed pathway. This study screens several enzymes, obtained from computational methods, for positive activity toward specific substrates present in the proposed psicose production pathway.

Introduction

The prevalence of diabetes has considerably increased in the past few decades. It is the one of the most prominent causes of death globally and affects over 400 million people worldwide.[1] It is a well-studied fact that excess sugar intake is the primary cause of type 2 diabetes, or development of insulin resistance. The problem lies in the large amount of sugar consumed through our food produce with added sugars and sweeteners like high fructose corn syrup.

As the threat of diabetes continues to grow, several developments in artificial sweeteners have been produced. In fact, artificial sweeteners have consistently grown in popularity as the primary sugar alternative.[2] Artificial sweeteners can be sweeter than sugars like glucose, while still maintaining a much lower caloric intake. These products are therefore beneficial to those that are at risk for developing diabetes. Unfortunately, the long-term effects of artificial sweeteners may pose a larger threat. Studies have reported that consumption of artificial sweeteners can worsen metabolic disorders and accelerate diabetes development and obesity.[2]

It is imperative to search for a natural, non-artificial, sweetener that can compete not only against the sweetness and flavor of traditional sugars (e.g., glucose and sucrose) but more importantly one that does not have underlying negative health impacts of sugars or artificial sweeteners. Psicose, a natural rare sugar, is a top candidate for an effective alternative sweetener. Psicose has 70% sweetness compared to sucrose but has only 0.2 Kcal/g energy, which means it does not increase caloric intake.[3] And due to its inherently low glycemic index, psicose does not lead to the development of diabetes. Studies on the long-term effects of psicose in rats suggests that there are no adverse health effects from increased psicose intake.[4,5] Psicose is an excellent candidate for an alternative sweetener that can compete against traditional sugars and artificial sweeteners.

Since psicose is produced naturally only in certain fruits at trace amounts, it is essential to develop an efficient psicose production pathway that is low in cost and high in yield. Current industrial standards of psicose production utilize DAEase family of enzymes to catalyze the isomerization reaction between fructose to psicose. With this method, however, they are only able to produce no more than 25% yield.[3] The limitations in yield from conventional psicose production methods stems from the inherent thermodynamics of the pathway. The conversion of fructose to psicose is a reversible reaction by isomerization with an epimerase enzyme, and it can be largely favorable in the forward direction when given a high fructose input. However, as psicose product concentration increases over time, the equilibrium for the reaction tends to shift toward the reactants side and decrease production of psicose. Ultimately, when equilibrium has been reached, the system will contain a mixture of sugars that must still be separated and isolated, adding additional costs and efforts.

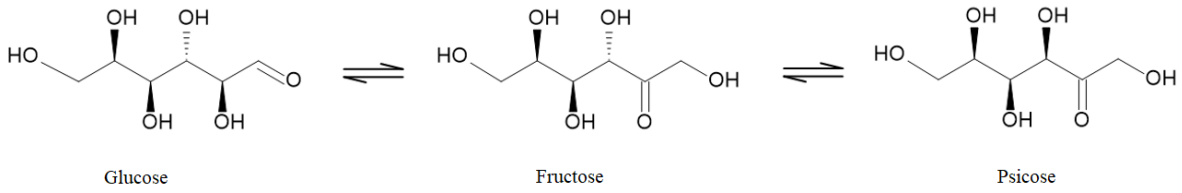


Figure 1. Current industrial standards of enzymatic psicose production. In this pathway, a mixture of sugars: glucose, fructose, and psicose will all be present in the final equilibrium.

Current industrial methods of psicose conversion pathways are not effective for efficient large-scale production of psicose for commercial use, ultimately due to its thermodynamic limitations. This project proposes an alternative pathway, which converts glucose → glucose-6-p ↔ fructose-6-p ↔ psicose-6-p → psicose. A key advantage to this pathway lies in the final irreversible phosphorylation step. Since the conversion of psicose-6-p to psicose cannot go in the

reverse direction, thermodynamic challenges are overcome resulting in higher psicose theoretical yields.

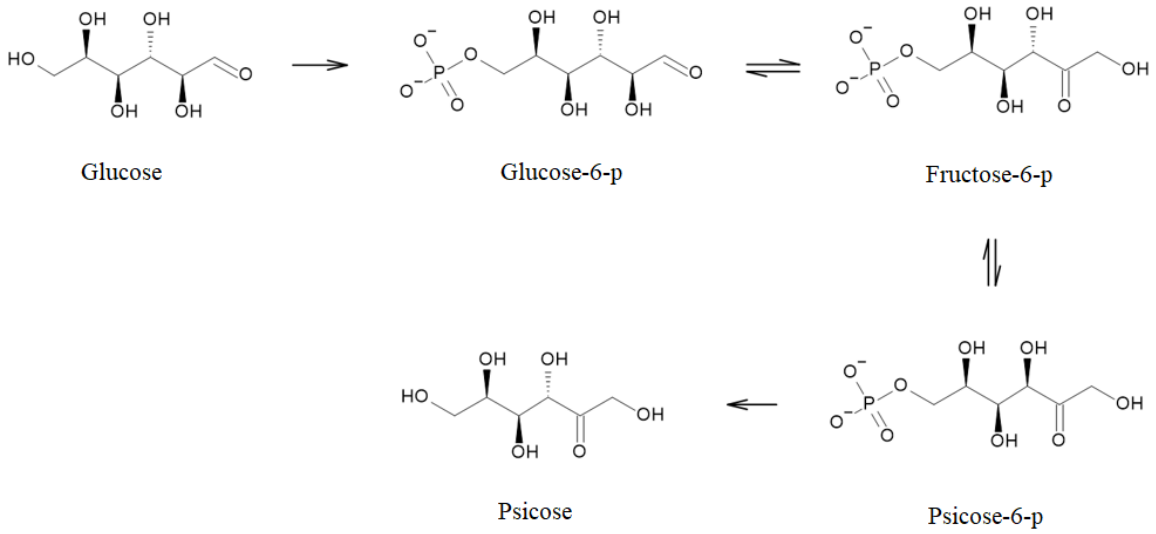


Figure 2. The proposed alternative psicose production pathway. Phosphorylated sugar intermediates are utilized to set up a final irreversible dephosphorylation step, driving reaction flux forward. The first two steps (top row) are key steps involved in glycolysis, and naturally occurs within the cell when applied in-vivo. The final two steps are engineered steps which require epimerase and phosphatase enzymes to be added to the in-vivo process.

The proposed psicose production pathway displayed in figure 2 utilizes a kinetically limited approach, which requires searching for the most efficient and substrate specific enzymes for each step. This project particularly explores epimerase and phosphatase enzymes, which catalyze the two final steps of the pathway. The initial dephosphorylation step and subsequent isomerization (first two steps in figure 2) are accomplished through glycolysis by in-vivo cell metabolism. Ultimately, the best performing enzymes will be implemented into an in-vivo E. coli growth system, ideally upscaled to a commercial level for psicose production.

A psicose-6-phosphate phosphatase enzyme is imperative to the final step (figure 2) of this pathway. The effectiveness of the final dephosphorylation step is key to the pathway's kinetic efficiency, as it separates the reaction flux from the reversible intermediary steps. Furthermore,

the phosphatase enzyme needs to be specific toward psicose-6-p exclusively. Specificity could increase product yield and prevent buildup of intermediates that could leak into other pathways. This project investigates phosphatase specificity by screening activity and comparing amongst different sugars.

Epimerases also play a crucial role in the psicose production pathway proposed for this project, especially for in-vivo applications. The epimerase is responsible for isomerizing fructose-6-p to psicose-6-p, ultimately siphoning fructose-6-p from glycolysis and other competing metabolic pathways. The efficiency of this conversion step is predicted to be beneficial for increasing product yield, as it must outcompete natural cell processes crucial for cell maintenance. Collaborative efforts with the Shota Atsumi Lab aid with solutions to in-vivo application challenges.

This project aims to identify and characterize phosphatase enzymes with specific activity toward psicose-6-p to produce psicose, and epimerase reactions successful at converting fructose-6-p to psicose-6-p. Both of these reactions ultimately contribute as the final two steps of the psicose production pathway. Since enzymes are ultimately crucial to the reaction cascade, it is essential to focus on improving enzyme performance.[6] Successful implementation of substrate specific phosphatase and epimerase enzymes to in-vivo psicose production methods should theoretically increase production yields.

Materials and Methods

Standards, Media, and Buffer Preparation

Glucose-6-phosphate used for sugar quantification standards and assay substrate was obtained from Sigma-Aldridge. Fructose-6-phosphate was obtained from Alfa Aesar. Psicose-6-p was obtained from Toronto Research Chemicals by special order. Phosphorylated sugars were

dissolved in MilliQ Water and stored in -20 degrees in PCR tubes to be thawed once and used later as substrate standards.

Cell culture media was made with Terrific Broth obtained from Fisher Bioreagents**.** Chem comp BL21 cells were made in house. HEPES wash buffer was produced by dissolving solid HEPES, obtained from Fisher Bioreagents, in MilliQ water and adding NaCl. HEPES wash buffer contained 10 mM imidazole, and HEPES elution buffer contained 200 mM imidazole. An additional HEPES buffer containing no imidazole for assay solvent was also made. All HEPES buffers were made at pH 7.5. LCMS solvents included pure acetonitrile and 25 mM ammonium formate dissolved in LCMS grade water.

Phosphorylated Sugar Standardization (LCMS methods)

Phosphorylated sugars such as psicose-6-p are not UV active but are detectable via mass spectroscopy.[8] This project utilizes liquid chromatography mass spectroscopy for phosphorylated sugar detection and analysis. Phosphorylated sugars must first be separated with an appropriate column in order to allow characterization based on respective retention times. Similar polarities in sugar isomers provide a challenge for column separation. A HILIC, or hydrophilic interaction, column known for isomer separation is used for this purpose. A consistent LCMS analysis method to successfully separate and detect phosphorylated sugars was developed for in house instrumentation.

An Agilent Technologies LCMS model 1260 Infinity was employed for phosphorylated sugar detection after separation. The MS was equipped with quadrupole and electron spray ionization. Optimized settings were on SIM negative -3700V and set to filter for m/z 259.

The Shodex HILICpak VT-50 2D column was used to separate glucose-6-p, fructose-6-p, and psicose-6-p before MS detection. Column temperature was set to 60 degrees with flow rate

0.3 mL/min. Solvents used were 80:20, 25 mM ammonium formate:acetonitrile at isocratic concentrations. Although substrates elute at around 5-8 minutes, the total run time was set to 50 minutes to allow column equilibration after column cleaning protocols. Column stabilization is a necessary step for HILIC column methods in order for establishing consistency between polar and nonpolar regions in the column.[9]

Due to the polar nature of HILIC columns, it is also important to note that column performance and accuracy is highly susceptible to unwanted binding of salts, i.e. imidazole, from sample solutions.[10] Desalting or buffer exchange techniques are crucial to combating LCMS analytical inconsistencies such as retention time variability and peak splitting caused by excess salt concentrations.

Computational Methods

Computational methods such as genome mining are employed to explore an effective phosphatase and epimerase enzyme for the psicose pathway. Initially, known genetic sequences of different phosphatases were screened for sequence similarity, and subsequent models were virtually docked with fructose-6-p and psicose-6-p substrates on Rosetta to predict catalytic capabilities and psicose-6-p specificity. Seventeen enzyme targets were ultimately chosen for in vitro testing based on their substrate docking scores, which predicts substrate binding capability of that respective enzyme. Other phosphatases with known or reported hexose dephosphorylation activity were also obtained to test potential psicose-6-p activity.

Epimerase enzymes were obtained through both genome mining and design efforts. Enzyme engineering efforts including directed evolution can increase specificity of an enzyme.[7] First, a known psicose-6-p epimerase, *AlsE*, was used as an initial target enzyme to screen for other epimerases with potential psicose-6-p activity, based on sequence similarity to AlsE. Similar to

phosphatase computational methods, a subset of the genome mined epimerase sequences are docked with fructose-6-p to predict binding capability. Twenty-six epimerase enzymes obtained from genome mining were selected for in-vitro testing. Computational design was also employed to discover active epimerases. Rosetta docking protocols were used to model the AlsE crystal structure with fructose-6-p substrate docked in several geometric poses. The protocol also gave permission to Rosetta to add mutations within the active site area, while conserving the catalytic residues. Subsequent models were then analyzed for good binding interactions. Thirteen AlsE design models were selected for in-vitro activity screening based on reasonable mutations (i.e. new interaction with ligand) and good docking performance. Figure 3 below displays AlsE mutants with their respective DNA sequences.

AlsE Mutant	DNA Sequence
AlsE Des. 1	ATGAAGATCTCGCCAAGTTTAATGTGTATGGACCTTCTGAAGTTCAAGGAGCAAATTGAGTTCATCGACTCGCACGCTGATTATTTTCACATTGACATTATGGACGGGCACTTTGTTCCCAACCTCACTCTGAGCCCATTCTT TGTTTCTCAAGTGAAGAAGTTAGCGACAAAACCACTGGATTGTCACCTGATGGTAACCCGCCCTCAAGATTACATTGCCCAACTTGCACGCGCGGGCGCTGATTTCATCACGCTTCATCCGGAGACGATTAATGGCCAAGC CTTCCGTCTGATTGATGAGATCCGTCGTCACGACATGAAGGTCGGCCTTATTTTGAACCCCGAAACCCCAGTGGAAGCCATGAAGTATTATATCCATAAGGCCGACAAAATTACCGTGATGACCGTCGACCCGGGCTTTGCG GGACAGCCGTTCATTCCCGAGATGTTAGACAAGTTAGCGGAGTTAAAGGCCTGGCGCGGAGCCGCGAGGGTTTAGAGTACGAGATTGAGGTCGATGGTGATTGCAATCAGGCCACTTATGAAAAGCTGATGGCCGCGGGTGCG GACGTGTTTATCGTTGGTACGCGCGGGCTTTTCAATCACGCGGAGAACATCGATGAGGCTTGGCGCATCATGACGGCCCAGATTCTTGCCGCGAAGAGCGAGGTCCAACCTCACGCGAAGACGGCA
AlsE Des. 2	ATGAAGATTTCACCATCACTCAATTGTATGGACCTTCTGAAGTTCAAGGAGCAGATTGAATTTATCGACTCACATGCCGACTACTTTCATATCGACATCATGGATGGTCACTTCGTCCCCTCACTTACGTTATCGCCCTTCTT CGTGTCTCAGGTGAAGAAATTAGCGACTAAGCCGCTGGATTGCCACTTAATGGTGACTCGTCCGCAAGACTACATCGCCCAGCTGGCACGTGCAGGCGCCGACTTCATTACCCTTCATCCGGAGACGATTAACGGCCAGGC ATTCCGCTTGATTGACGAGATCCGTCGCCACGACATGAAGGTTGGTTTGATCCTGAACCCCGAGACGCCTGTTGAGGCCATGAAATACTATATCCACAAGGCCGATAAGATCACTGTGATGACGGTAGACCCGGGCTTTGC GGGACAGCCCTTTATCCCTGAGATGCTGGACAAATTAGCTGAGCTCAAGGCCTGGCGTGAACGCGAGGGACTTGAATATGAAATCGAGGTGGATGGGTCTTGTACTCAAGCCACGTACGAAAAGCTGATGGCAGCGGGTGC GGACGTATTTATTGTGGGCACGTCAGGTTTGTTTAACCATGCGGAGAATATCGATGAGGCATGGCGCATCATGACTGCACAAATTTTGGCGGCAAAATCAGAGGTACAACCCCACGCGAAAACGGCC
AlsE Des. 3	ATGAAAATCAGTCCATCGTTGATGTGCATGGATTTACTTAAATTTAAAGAGCAGATCGAGTTTATTGACAGCCACGCGGATTATTTCCATATCGATATCATGGACGGACATTTTGTACCAAACTTGACGTTGTCTCCGTTCTTT GTAAGTCAAGTCAAGAAGCTGGCCACGAAGCCATTAGATTGTCACCTGATGGTCACCCGCCCGCAGGATTATATTGCTCAACTGGCCCGCGCTGGGGCAGACTTTATTACACTGCACCCCGAAACAATTAATGGGCAAGCG TTTCGTCTCATCGATGAGATCCGTCGCCACGACATGAAGGTCGGTCTTATCTTGAATCCCGAAACGCCGGTAGAGGCAATGAAGTACTACATTCACAAGGCGGATAAGATTACCGTAATGACGATTGACCCGGGCTTTGACG GCCAACCGTTCATCCCTGAGATGCTTGACAAATTGGCAGAGTTAAAGGCGTGGCGCGAGCGCGAAGGGTTAGAATACGAAATTGAAGTGGACGGAGCTTGCAACCAAGCCACTTACGAGAAGCTGATGGCAGCCGGAGCG GACGTGTTTGTTGTAGGTACTAGCGGCCTTTTCAACCACGCCGAGAACATTGATGAAGCCTGGCGCATCATGACTGCCCAAATCTTAGCAGCGAAGTCGGAAGTGCAACCACACGCGAAGACCGCC
AlsE Des. 4	ATGAAGATCTCCCCGAGCCTCATGTGTATGGATCTGCTGAAATTCAAGGAGCAAATCGAGTTCATTGACTCTCATGCAGATTATTTCCATATCGATATCATGGATGGTCATTTCGTACCCAATCTCACGCTCTCTCCATTCTT CGTTAGCCAGGTTAAGAAGCTGGCCACTAAGCCTTTAGATTGTCACCTGATGGTTACACGTCCCCAAGACTATATTGCCCAGCTGGCGCGTGCGGGAGCCGATTTCATCACCCTTCACCCGGAGACTATCAATGGCCAAGC CTTTCGTTTAATCGACGAGATCCGCCGTCACGACATGAAGGTCGGTCTTATCTTGAATCCTGAAACGCCAGTAGAGGCCATGAAGTACTACATCCATAAAGCAGACAAGATTACTGTGATGACTATCGACCCAGGGTTCGAC GGTCAACCGTTCATTCCGGAAATGCTGGACAAGTTAGCGGAGTTGAAGGCATGGCGTGAGGCGTGAAGGCTTGGAATACGAAATTGAAGTGGACGGAGCTTGCAACCAAGCCACGTACGAGAAGCTGATGGCAGCGGGTGCA GACGTATTTGTCGTTGGTACATCTGGGTTATTTAACCATGCAGAGAATATCGACGAGGCGTGGCGTATTATGACAGCGCAAATTCTGGCCGCGAAGTCAGAGGTCCAGCCGCATGCGAAGACGGCA
AlsE Des. 5	ATGAAAATTAGTCCGTCTTTAAATTGCATGGACCTGTTAAAGTTCAAGGAGCAAATCGAGTTCATTGACAGCCACGCAGACTACTTCCATATCGATATTATGGATGGTCACTTTGTGCCAAGCTTAACTTTATCGCCATTCTT TGTCTCGCAAGTTAAGAAGCTGGCGACTAAGCCTCTTGACTGTCACTTGATGGTTACGCGTCCCCAAGGACTATATTGCCCAGCTGGCACGTGCTGGCGCAGACTTCATCACTTTGCACCCAGAGACGATCAATGGTCAGGCC TTCCGTCTCATTGACGAGATTCGCCGTCACGACATGAAGGTGGGACTCATCCTCAATCCTGAGACGCCTGTTGAGGCGATGAAGTATTACATCCACAAGGCCGATAAAATCACGGTCATGACTATCGATCCAGGCTTTGCGG GTCAGCCTTTCATCCCAGAGATGCTGGACAAGCTCGCGGAACTCAAGGCCTGGCGTGAACGCGAAGGCCTGGAATACGAGATTGAGGTGGATGGCGACTGTACACAGGCGACGTACGAGAAGTTGATGGCCGCAGGCGCC GACGTTTTCGTTGTCGGGACGTCAGGCTTGTTTAATCACGCGGAGAATATCGACGAGGCCTGGCGTATTATGACAGCCCAAATCCTCGCCGCGAAGAGTGAGGTACAGCCCCACGCAAAGACTGCA
AlsE Des. 6	ATGAAAATCAGCCCCTCTCTGATGTGCATGGACCTGCTCAAATTTAAGGAACAGATTGAGTTTATCGACAGTCACGCAGACTATTTTCACATTGATATCATGGATGGTCATTTTGTCCCGAGCCTGACCCTTTCACCTTTCTT CGTATCTCAGGTTAAGAAGTTGATCCACAAAACCCCTGGACTGTCACTTGATGGTGACCCGCCCCCAAGACTATATCGCACAGCTTGCACGCGCAGGCGCAGATTTCATCACTCTTCATCCCGAGACTATCAATGGGCAAGC GTTCCGTTTAATCGACGAGATCCGCCGCCATGACATGAAAGTCGGTCTCATCTTAAACCCGGAGACTCCCGTCGAGGCGATGAAGTATTATATCCACAAGGCTGATAAGATCACCGTGATGACCATCGACCCGGGCTTTGG CGGGCAACCGTTCATCCCTGAGATGTTGGACAAGTTAGCCGAATTGAAGGCCTGGCGTGAGCGCGAAGGCTTGGAGTACGAGATTGAGGTGGACGGTGGCTGTAAGCAAGCGACCTACGAGAAATTAATGGCCGCCGGAG CTGATGTCTTTGTAGTGGGAACGAGCGGATTGTTTAACCACGCCGAGAACATCGACGAGGCGTGGCGCATCATGACCGCCCAAATTTTGGCGGCGAAATCAGAAGTACAACCCCACGCTAAGACCGCG
AlsE Des. 7	ATGAAAATTTCGCCCAGCTTAAACTGCATGGATCTTTTAAAATTCAAGGAGCAAATCGAATTTATCGACAGCCACGCAGATTATTTCCACATTGACATTATGGACGGGCACTTTGTGCCGTCCTTAACTCTGTCTCCCTTCTT CGTATCACAGGTCAAGAAGCTGGCAACCAAACCTCTTGATTGTCACTTGATGGTGACGCGTCCACAAGATTACATTGCGCAACTCGCCCGCGCGGGCGCAGATTTTATCACCTTACACCCAGAGACGATTAACGGACAAGC ATTCCGCCTGATTGACGAAATTCGTCGCCACGACATGAAGGTGGGATTGATTCTGAACCCGGAGACTCCTGTTGAAGCAATGAAGTATTATATTCATAAGGCCGACAAGATCACCGTAATGACCATTGACCCAGGATTCAAT GGACAACCATTCATCCCGGAGATGCTCGACAAGTTAGCCGAGCTGAAGGCATGGCGTGAACGTGAGGGACTTGAATATGAGATTGAGGTTGACGGAGACTGTACACAGGCGACGTACGAGAAGTTGATGGCGGCCGGCGC GGACGTATTTGTGGTCGGGACCCGTGGCCTGTTTAACCATGCGGAAAATATCGACGAGGCATGGCGTATTATGACCGCCCAAATTTTGGCGGCGAAATCAGAGGTACAGCCTCATGCGAAGACGGCG
AlsE Des. 8	ATGAAGATCTCCCCGAGCCTTATGTGTATGGACCTGCTGAAGTTTAAGGAACAGATTGAGTTTATTGATAGCCATGCCGATTACTTCCATATCGACATCATGGACGGCCATTTCGTGCCTAATCTTACCCTTAGCCCTTTCTT TGTTAGTCAAGTAAAGAAATTGGCCACTAAACCGCTCGACTGTCACCTGATGGTCACTCGCCCACAAGACTATATCGCACAGTTGGCCCGTGCGGGTGCCGACTTCATTACCCTTCACCCGGAGACGATCAACGGCCAGGC ATTTCGCTTGATTGATGAAATTCGTCGCCACGACATGAAGGTCGGATTAATTCTCAACCCGGAGACCCCTGTGGAAGCTATGAAGTACTACATTCACAAAGCAGACAAGATTACTGTAATGACTGTGGATCCGGGCTTCGAC GGTCAACCATTCATTCCAGAGATGTTAGACAAGCTTGCCGAACTTAAGGCGTGGCGTGAGCGTGAAGGACTGGAATATGAGATCGAAGTAGACGGAGCGTGTAATCAGGCCACGTATGAGAAACTGATGGCGGCGGGAGCG GACGTTTTCATCGTAGGTACCTCGGGGCTCTTCAACCACGCCGAAAATATTGATGAGGCGTGGCGCATTATGACCGCGCAAATCCTGGCAGCAAAGTCCGAGGTACAACCACACGCCAAGACCGCT
AlsE Des. 9	ATGAAGATTTCACCATCCCTTATGTGTATGGACCTCTTGAAGTTCAAAGAGCAGATTGAGTTTATCGATTCGCATGCCGACTACTTCCACATCGACATCATGGATGGCCATTTCGTACCGAATTTAACGTTGTCGCCATTCTT TGTCTCGCAAGTGAAGAAGTTGGCCACTAAGCCACTTGACTGCCACTTGATGGTCACACGTCCGCAAGACTACATCGCACAACTGGCGCGTGCGGGCGCGGATTTTATCACACTGCACCCGGAAACAATCAATGGACAAGC GTTCCGCCTGATCGACGAGATTCGCCGCCACGATATGAAAGTGGGCTTAATCCTTAATCCGGAAACCCCAGTCGAGGCGATGAAATACTACATCCACAAGGCCGACAAAATCACGTTATGACGGTGGACCCCGGTTTTGAT CGGCCAACCGTTTATTCCGGAGATGCTGGACAAGCTTGCAGAATTGAAGGCGTGGCGTGAGCGTGAAGGGTTTAGAGTATGAGATCGAGGTGGATGGAAACTGCAACCAAGCGACCTACGAGAAGCTGATGGCTGCGGGAG CCGACGTGTTCATTGTAGGTACGTCCGGCTTATTTAACCATGCCGAGAACATTGATGAGGCGTGGCGTATTATGACAGCACAAATCTTAGCCGCAAAGAGCGAAGTACAGCCTCACGCTAAGACCGCC
AlsE Des. 10	ATGAAGATTAGCCCCAGTTTGATGTGCATGGACCTTCTGAAATTCAAGGAGCAAATTGAATTTATCGACTCACACGCTGACTATTTCCATATCGACATCATGGACGGCCACTTCGTTCCCAACTTGACCTTGTCTCCATTCTT CGTGTCTCAAGTTAAGAAGCTGGCTACTAAGCCGCTGGATTGTCACCTTATGGTAACACGCCCGCAAGATTATATCGCGCAGCTCGCGCGCGCTGGTGCCGATTTTATCACCTTGCATCCAGAGACCATCAACGGTCAGGC GTTCCGTTTAATCGATGAGATCCGTCGCCACGATATGAAGGTTGGCCTTATCCTGAATCCTGAGACACCGGTCGAGGCCATGAAGTATTACATTCATAAAGCAGACAAAATCACAGTTATGACGGTGGACCCCGGTTTTGAT GGTCAACCATTTATCCCGGAGATGCTTGACAAGCTTGCCGAGCTCAAGGCATGGCGCGAACGCGAGGGCTTAGAATATGAGATCGAGGTTGACGGGCAATGTAACCAAGCCACCTACGAGAAGTTAATGGCGGCCGGGGC CGACGTTTTCGTGGTAGGTACCAGCGGCTTGTTCAACCATGCTGAGAATATTGATGAGGCATGGCGCATTATGACCGCGCAAATCCTGGCAGCGAAGAGTGAAGTTCAACCACACGCAAAGACAGCC
AlsE Des. 11	ATGAAGATTTCTCCTAGTCTTATGTGTATGGATCTTCTGAAGTTCAAAGAGCAAATCGAATTCATTGACTCGCACGCCGATTACTTCCACATTGATATTATGGATGGCCACTTTGTACCAAACCTGACATTGAGCCCGTTCTT CGTAAGCCAGGTCAAGAAACTGGCGACCAAGCCCCTGGACTGTCACTTAATGGTGACGCGTCCGCAGGACTACATCGCCCAATTAGCGCGTGCAGGCGCCGATTTCATCACCCTGCATCCCGAGACGATCAACGGTCAAG CATTTCGTTTGATTGATGAGATTCGTCGCCACGACATGAAGGTAGGGTTGATTCTCAACCCGAGACGCCAGTAGAAGCAATGAAGTACTACATTCACAAAGCGGATAAGATCACCGTAATGACCGTGGACCCGGGGTTCG ATGGGCAACCTTTCATCCCGGAAATGTTGGACAAGCTCGCAGAACTGAAGGCATGGCGCGAGCGTGAGGGGCTGGAGTACGAGATTGAGGTTGACGGACAGACAAACCAGGCCACCTATGAGAAGTTAATGGCCGCTGGA GCAGACGTGTTTGTTGTTGGCACCAGCGGTCTGTTTAATCATGCGGAGAACATTGACGAAGCGTGGCGCATCATGACTGCGCAAATCCTGGCGGCGAAGAGCGAGGTTCAACCGCATGCGAAGACTGCA
AlsE Des. 12	ATGAAAATCTCACCATCACTGAATTGCATGGATCTCCTCAAGTTCAAAGAGCAGATCGAATTTATCGACTCGCACGCCGACTATTTCCACATTGATATTATGGACGGTCATTTTGTTCCGTCTTTAACGCTGAGCCCTTTCTT CGTCAGTCAAGTGAAGAAGTTAGCCACCAAGCCACTTGACTGTCACTTGATGGTCACGCGTCCACAAGACTATATTGCCCAACTGGCACGCGCGGGCGCGGACTTCATCACATTGCATCCAGAGACAATTAACGGTCAAGC CTTTCGCCTGATTGATGAGATTCGTCGTCACGATATGAAAGTCGGACTTATTCTTAACCCTGAGACTCCCGTGGAAGCCATGAAGTACTACATTCACAAGGCGGACAAGATTACTGTAATGACCGTGGACCCGGGGTTCG GGTCAACCATTCATTCCCGAGATGTTAGATAAACTGGCTGAGTTAAAGGCGTGGCGTGAACGCGAGGGGTTAGAGTACGAGATCGAGGTTGATGGTCAAACCAATCAGGCCACATACGAGAAATTGATGGCCGCGGGCGCC GACGTTTTCGTGGTCGGTACCAGCGGTCTGTTCAATCACGCGGAGAATATTGACGAGGCCTGGCGCATCATGACTGCGCAAATCTTAGCGGCCAAGTCGGAAGTCCAACCTCATGCTAAGACTGCA
AlsE Des. 13	ATGAAGATCTCGCCGTCTCTGAATTGTATGGATCTGCTTAAATTCAAGGAGCAAATCGAATTCATCGACAGCCATGCGGACTATTTTCACATCGACATCATGGATGGCCACTTTGTGCCTAATCTGACACTTAGCCCGTTCTT CGTTTCGCAGGTGAAGAAGCTGGCGACCAAGCCTTTGGACTGTCACTTGATGGTGACACGCCCCCAAGATTACATCGCCCAACTGGCGCGCGCCGGCGCAGACTTTATCACTCTCCACCCTGAGACAATTAATGGTCAGGC GTTCCGCCTGATTGACGAAATTCGTCGTCACGACATGAAGGTAGGCCTGATCCTCAATCCAGAGACACCGGTGGAAGCTATGAAGTACTACATCCACAAAGCAGATAAGATTACTGTGATGACTGTGGACCCAGGATTTGA CGGCCAACCATTTATTCCAGAGATGTTAGATAAGTTAGCGGAGTTGAAGGCGTGGCGTGAGCGTGAGGGGCTGGAGTATGAGATCGAGGTAGATGGCCAAACTAATCAAGCAACGTATGAGAAGCTGATGGCGGCCGGTGC GGACGTCTTTGTCGTGGGAACGAGCGGCCTGTTCAATCACGCGGAGAATATCGACGAGGCGTGGCGTATTATGACCGCGCAGATCCTTGCGGCCAAGAGCGAAGTGCAACCTCATGCCAAAACGGCG

Figure 3. DNA sequences for each AlsE mutant design. Sequences displayed here do not include cellular DNA or vector plasmid, rather they are the direct sequences for enzyme expression.

Enzyme Expression

DNA plasmids of selected enzymes from computational efforts were ordered from a DNA manufacturer, Twist Bioscience. Both epimerase and phosphatase were expressed with protocol. The PET29b+ DNA plasmids were first transformed into BL21(DE3) competent cells via heat shock. 2 uL DNA and 20 uL competent cells were combined in a Falcon tube and kept in an ice bath for 20 minutes. The cells were then immersed in a 45 degrees water bath for 90 seconds and removed to rest for 2 minutes. 200 uL of Terrific Broth (TB) was then added and the cells were set on a 250 rpm shaker at 37 degrees for 1 hr to recover. 50 uL of the cells were finally transferred to an agar plate and incubated for 24 hours until colonies were formed.

Overnight cell cultures were started by inoculating 2-4 mL TB with cell colonies from the agar plates. Between 16-24 hrs, the overnight cultures are transferred to larger 500 mL TB media. 500 uL Kanamycin (1:1000) antibiotic is also used in the cultures. After the cells have reached the appropriate point in their growth phase, measured by optical density (OD) between 0.6-1.0 at 600 nm, IPTG is added to induce enzyme expression. After 24 hours, the cells are collected through centrifugation at 4700 RPM for 20 min and stored at -20 degrees before purification.

Protein Purification

Collected cells are first lysed by sonication. 30 mL HEPES wash buffer prepared at pH 7.5 was added to the cells pellet after centrifugation and subsequently sonicated for 15 sec and rested for 30 sec through a 1 min 30 sec cycle. The cells were kept in an ice-water bath during the sonication process to prevent enzyme denaturation. Sonicated cells were then centrifuged at 4700 RPM for 1 hr to separate protein from denser cellular components. Next, target enzymes must be purified through column chromatography. For 500 mL cell cultures, 500 uL Co^{2+} resin (1 mL slurry) was added to a column tube and washed with 5 mL HEPES buffer 3 times. Supernatant

samples from centrifugation were added to respective tubes and washed with 5-15 mL HEPES buffer 3-5 times. Target enzymes were then collected by adding 1.5 mL HEPES elution buffer. Finally, a buffer exchange is necessary to remove imidazole from solution. High salt concentration in the final assay solution will disrupt LCMS analysis by binding to the HILIC column's polar amine groups. Buffer exchange is accomplished by centrifuging purified enzymes in 10 kDa, 2 mL concentration tubes and subsequently diluting with HEPES buffer containing no imidazole. After 3-4 rounds of buffer exchange, imidazole concentration decreases thousand-fold and purified enzymes are ready to proceed with activity assays.

Phosphatase Activity Assay

The goal of phosphatase activity screening is to test for specificity of target phosphatase enzymes toward psicose-6-p exclusively. Three phosphorylated sugars including psicose-6-p were separately tested to determine the specificity of each enzyme. Phosphatase enzymes are tested via an activity assay which establishes and monitors the dephosphorylation step in sugar production (i.e. converting psicose-6-p to psicose). Phosphatase assay components include 0.5 mg/mL purified enzyme, 1 mM phosphorylated sugar (fructose-6-p, psicose-6-p, and glucose-6-p separately), and 5 mM $MgCl_2$ in HEPES buffer at pH 7.5. All assay components are dissolved in HEPES buffer with imidazole removed. Magnesium acts as a metal cofactor and is necessary for enzyme activity. First, 15 uL $MgCl_2$ is added to 25 uL of the purified enzymes in separate PCR tubes and incubated at 37 degrees for 5 min. The reaction is catalyzed by addition of 10 uL sugar. Fructose-6-p, glucose-6-p, and psicose-6-p were individually screened against each phosphatase enzyme in separate tubes. Enzyme reactions are allowed to proceed for 1 hr before being quenched with 150 uL methanol to stop the reaction and crash out protein. Assay samples are then centrifuged at 4700 RPM for 10 min to separate the enzyme before LCMS analysis.

Phosphatase LCMS analysis

Liquid chromatography mass spectroscopy is employed for phosphorylated sugar detection as described earlier. Phosphatase activity is determined by qualitatively analyzing the disappearance of the phosphorylated sugar signal (m/z 259) on the mass spectra. The MS signal for the phosphorylated sugar-substrate decreases in intensity as the substrate diminishes in concentration, a positive indicator for successful dephosphorylation. Enzyme activity, reported later, was organized by level of activity based on relative signal decrease. For example, a 50 percent decrease in peak intensity for psicose-6-p substrate would indicate partial enzyme activity while 100 percent decrease indicates complete activity. A separate sample containing phosphorylated sugar with no added enzyme was used as a negative control. Substrate signal for the negative control sample does not decrease during the reaction time, acting as an indicator of no active dephosphorylation by thermal degradation.

Epimerase Activity Assay

The epimerase activity assay aims to identify enzymes successful at isomerizing fructose-6-p to psicose-6-p. The objective of epimerase activity screening does not prioritize substrate specificity. Rather, the primary aim is to discover an epimerase with better isomerization activity toward F6P than AlsE, the native E. coli epimerase. Therefore, fructose-6-p is the only sugar used as a substrate. Furthermore, in order to compare target epimerase activity to AlsE activity, enzyme concentration and assay run time had to be standardized to allow differences in activity to become observable. For instance, if AlsE completes the reaction within 20 min while another enzyme within 10 min, an assay run time of 1 hr would not successfully resolve the difference in activity since both reactions would have seemingly reached 100 percent completion simultaneously. Therefore, assay conditions were standardized to allow AlsE to reach 25 percent

completion of the equilibrium. Target enzymes screened at the same conditions can then be compared by observing reaction completion of either above or below 25 percent. Assay standardization for LCMS analysis is further elaborated in the Epimerase LCMS analysis section.

Epimerase assay components and protocols are similar to the phosphatase assay. Components include 1 mM fructose-6-p substrate, 5 mM $MgCl_2$, and 0.003 mg/mL purified enzyme in HEPES buffer. 15 uL $MgCl_2$ is first added to 25 uL enzymes in separate PCR tubes and incubated at 37 degrees for 5 min. The reaction is catalyzed by addition of 10 uL fructose-6-p. Enzyme reactions are allowed to proceed for 30 min before being quenched with 150 uL methanol. AlsE was tested alongside target epimerases as a positive control. A Fructose-6-p sample with no added enzyme was used as the negative control. Assay samples are then centrifuged at 4700 RPM for 10 min to separate the enzyme before LCMS analysis.

Epimerase LCMS analysis

Liquid chromatography mass spectroscopy is also required for epimerase activity screening. Fructose-6-p (F6P) and psicose-6-p (F6P) are both separated and detected under the LCMS protocol described earlier. Since the isomerization reaction between F6P and P6P occurs in equilibrium, both sugar signals will be present on the mass spectra after reaction completion. Unlike phosphatase LCMS analysis where signal disappearance is observed for activity, epimerase LCMS analysis determines activity based on formation of an MS signal separate from the F6P signal, an indication of P6P formation.

Increasing levels of signal intensity for the psicose-6-p peak indicates higher concentrations of psicose-6-p as determined by a standard curve. Epimerase assay conditions standardization was necessary, as briefly discussed earlier, in order to successfully observe

differences in activity between the epimerase enzymes. As a recap, if the assay conditions allowed all enzymes to reach 100 percent completion by the time analysis occurs, it would be challenging to elucidate their differences in efficiency. Therefore, conditions were optimized to allow for 25 percent completion by AlsE activity, which then acted as a benchmark to compare activity of the target epimerases.

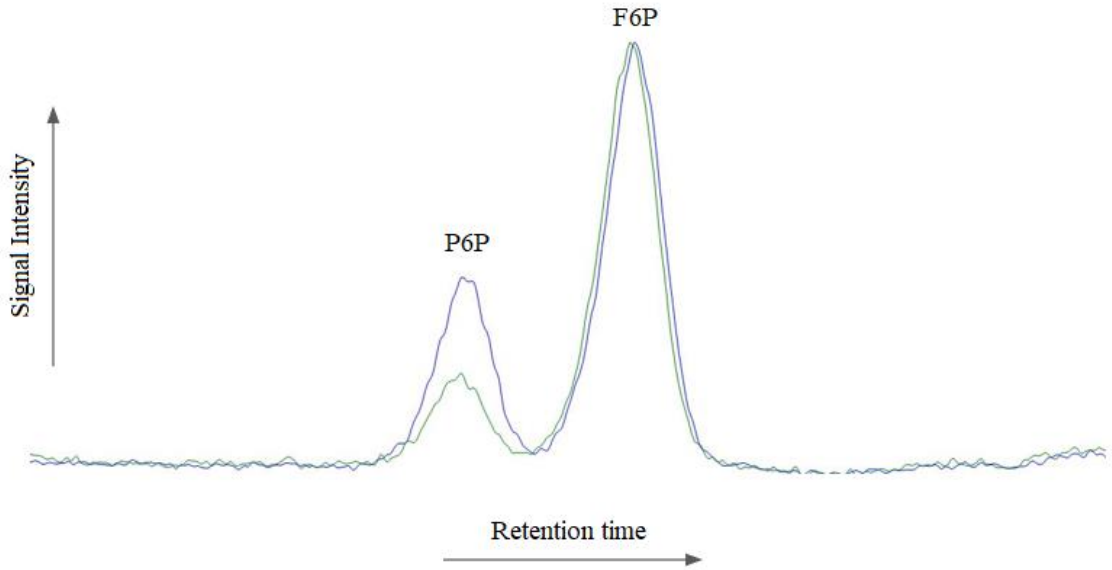


Figure 3. Idealized LCMS spectra displaying both fructose-6-p (F6P) and psicose-6-p (P6P) signals. As established, the signal intensity reflects substrate concentration. The intensity increases as concentration increases. The two different signal levels for P6P illustrates how LCMS methods can qualitatively examine enzyme activity through analyzing MS peaks.

Assay conditions were optimized by first determining the approximate signal intensity of 100 percent AlsE reaction completion. The psicose-6-p signal growth would cease after increasing AlsE concentration above ~0.3 mg/mL or was running the assay longer than 1 hr, indicating 100 percent completion toward the reaction equilibrium. Next, AlsE concentration and run time was systematically decreased until 25 percent completion was established by qualitatively observing the decrease in the LCMS signal. Optimal conditions were determined to be 0.003 mg/mL AlsE with 30 min assay run time. All other epimerases were assayed under the optimal conditions and

resulting psicose-6-p MS signal is compared to AlsE. Ultimately, enzyme targets resulting in higher psicose-6-p MS signal than the established 25 percent mark indicate higher enzyme activity, while lower than 25 percent indicates lower enzyme activity than AlsE.

Results and Discussion

Phosphatase Activity Screening

Phosphatase enzymes are responsible for the dephosphorylation reaction in the final step of the psicose production pathway. As mentioned earlier, phosphorylated sugars are detected via LCMS. As the phosphate group is removed from the sugar by the phosphatase, its signal intensity decreases until it completely diminishes, indicating that all phosphorylated sugar has been converted to the dephosphorylated sugar. Ultimately, decrease in signal intensity occurs as a result of successful dephosphorylation by the phosphatase enzyme. Glucose-6-p, fructose-6-p, and

psicose-6-p were individually tested with various phosphatase targets reported below. All three sugars were tested to determine specificity of enzymes toward psicose-6-p.

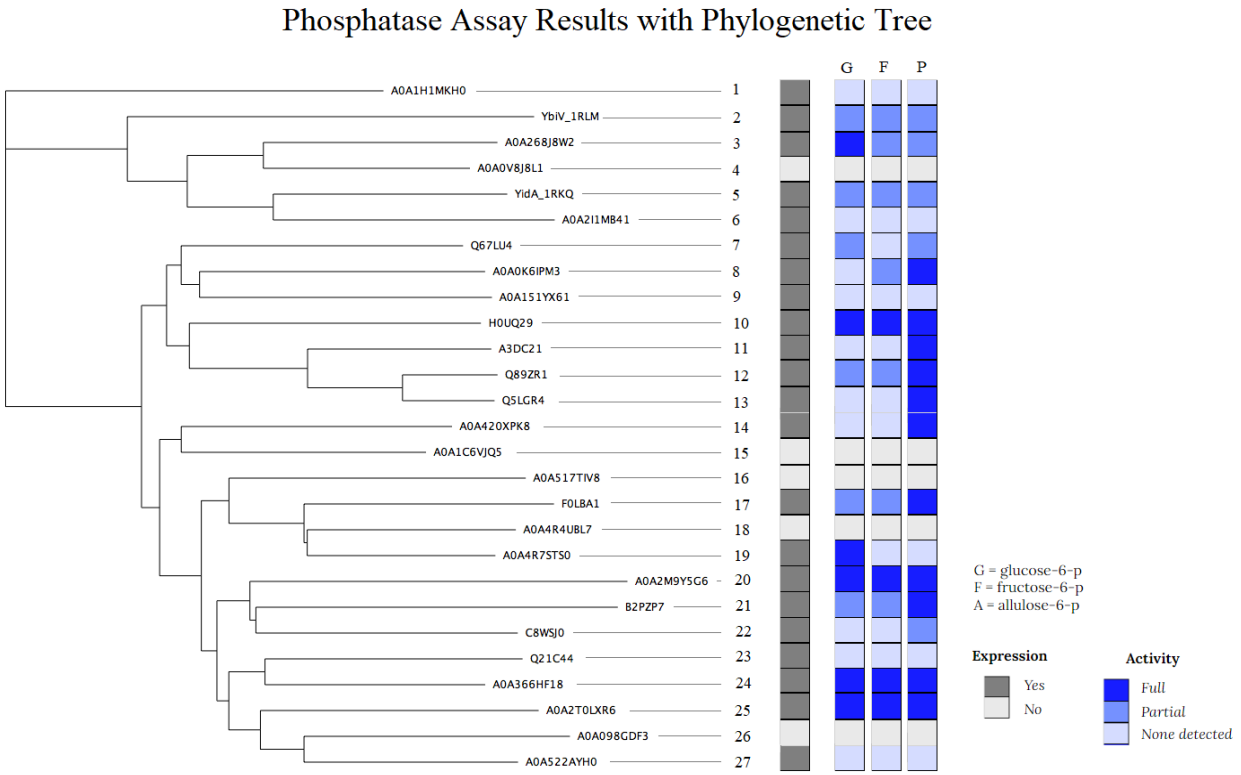


Figure 5. Phosphatase activity results displaying enzyme expression and relative activity levels. Phosphatases reported here were obtained from both genome mining and patent literature.[11] Three columns labeled G, F, and P are used to separate screening results for each phosphorylated sugar.

The results for each phosphatase in figure 5 are organized by glucose-6-p, fructose-6-p, and psicose-6-p columns labeled as G, F, and P respectively. Depicted in varying shades of blue are activity results for the enzyme against a specific sugar substrate. Full, partial, and no activity detected are three reported result options. Full activity is reported when all of the phosphorylated sugar is depleted, as indicated by diminishing MS signals. Quantitative analysis of partial activity was not performed in this study since enzymes performing at higher activity are of more interest. Enzyme expression was also reported in varying shades of gray. Phosphatases which reported as

expressed had a minimum 1 mg/mL enzyme concentration as measured by UV-Vis absorbance at 280 nm.

When comparing enzyme activity between the three substrates, enzymes with "full activity" toward a particular sugar and no activity detected for the other sugars are considered to have "specificity" toward that sugar substrate. Similarly, phosphatases which are promiscuous toward several sugars are reported to have full activity, or at least partial activity for more than one sugar substrate. Phosphatases with specificity toward psicose-6-p are exclusive candidates for psicose production pathway implementation.

Specificity toward psicose-6-p exclusively is important for in-vivo applications with other sugar intermediates present in solution. Promiscuity in phosphatase activity could possibly hinder product yield. Other intermediary sugars such as glucose-6-p or fructose-6-p, present in the equilibrium reactions of psicose production pathway, could become unintentionally dephosphorylated to produce undesired sugar byproducts.

In figure 5 phosphatases 11, 13, 14, and 22 are observed to have total specificity toward psicose-6-p. These enzymes do not have any activity detected for glucose-6-p and fructose-6-p. Although further tests with other phosphorylated sugars could be conducted to screen for higher degree of specificity, for the scope of this project, only three sugar substrates were tested. Glucose-6-p, fructose-6-p, and psicose-6-p are the only sugar intermediates present in the psicose production pathway. Therefore, the psicose-6-p specific phosphatases reported above should not have any activity toward other sugars when implemented in the in-vivo psicose production pathway.

Other phosphatases, such as numbers 8, 12, 17, and 21 are considered to have partial specificity toward psicose-6-p. Although activity assay results indicated that there was full activity

measured for psicose-6-p, these enzymes also displayed partial activity toward glucose-6-p and fructose-6-p. Partial specificity could further be studied to elucidate structure function in specific sugar binding and catalysis. For instance, genetic similarities for specific phosphatases are not obvious from the phylogenetic tree but are observed between enzymes 13 and 14.

Epimerase Activity Screening

The epimerase enzyme catalyzes the second to final step of the psicose production pathway. Fructose-6-p is converted to psicose-6-p through an isomerization reaction, keeping both sugars in equilibrium with each other. Epimerase activity was measured with LCMS through observation of psicose-6-p MS signal formation. Increasing levels of signal intensity indicates higher levels of enzyme activity, reported as "percent completion" toward establishing the equilibrium end-state of the reaction.

Two separate sets of epimerases were expressed and screened for activity. The first round consisted of thirteen AlsE mutant designs and nine epimerases discovered through genome mining. After obtaining the screening results of first round epimerases reported in figure 6, a second round of screening was performed (figure 7). The seventeen epimerases in round two were also obtained from genome mining, however the gene sequence identities were restricted to be more identical to AlsE and increase the probability of better or similar activity as native AlsE.

In the first round of activity screening, enzyme concentrations were trialed at both 0.003 mg/mL and 0.3 mg/mL. As initially mentioned in the Epimerase LCMS Analysis section, assay conditions for reaching 25 percent reaction completion had to be optimized. It was found that 0.003 mg/mL AlsE for a 30 min run time would complete 25 percent of the reaction toward equilibrium. As reported in figure 6 column A, all other epimerases in round 1 did not show any detectable activity at the same optimized assay conditions as AlsE. To attempt detection of activity at higher

enzyme concentrations, assay conditions were modified by increasing enzyme concentration to 0.3 mg/mL while remaining at a 30 min run time. At these concentrations, a range of activity was detected as reported in figure x column B. AlsE was found to have highest levels of activity, strongly outcompeting the other epimerases.

Although none of the enzymes performed at the level of AlsE, the diversity in activity within the genome mined epimerases (#14-22) provided insight to sequence-function relationships. Epimerase A6LQT7 (#15) in figure 6 had higher detected levels of activity than the rest of its cohort. Upon further investigation, A6LQT7 gene sequence similarity was more unique than the other epimerases. Out of the other epimerases, it has the lowest percent similarity to the RPE enzyme family, a general epimerase family, while maintaining an average percent similarity to AlsE. Specifically, the other round 1 epimerases had an active site sequence identity ranging between 65-80 percent similar to RPE. The sequence similarity of A6LQT7 to RPE is only 48 percent. All epimerases, including A6LQT7, have around 50 percent similarity to AlsE.

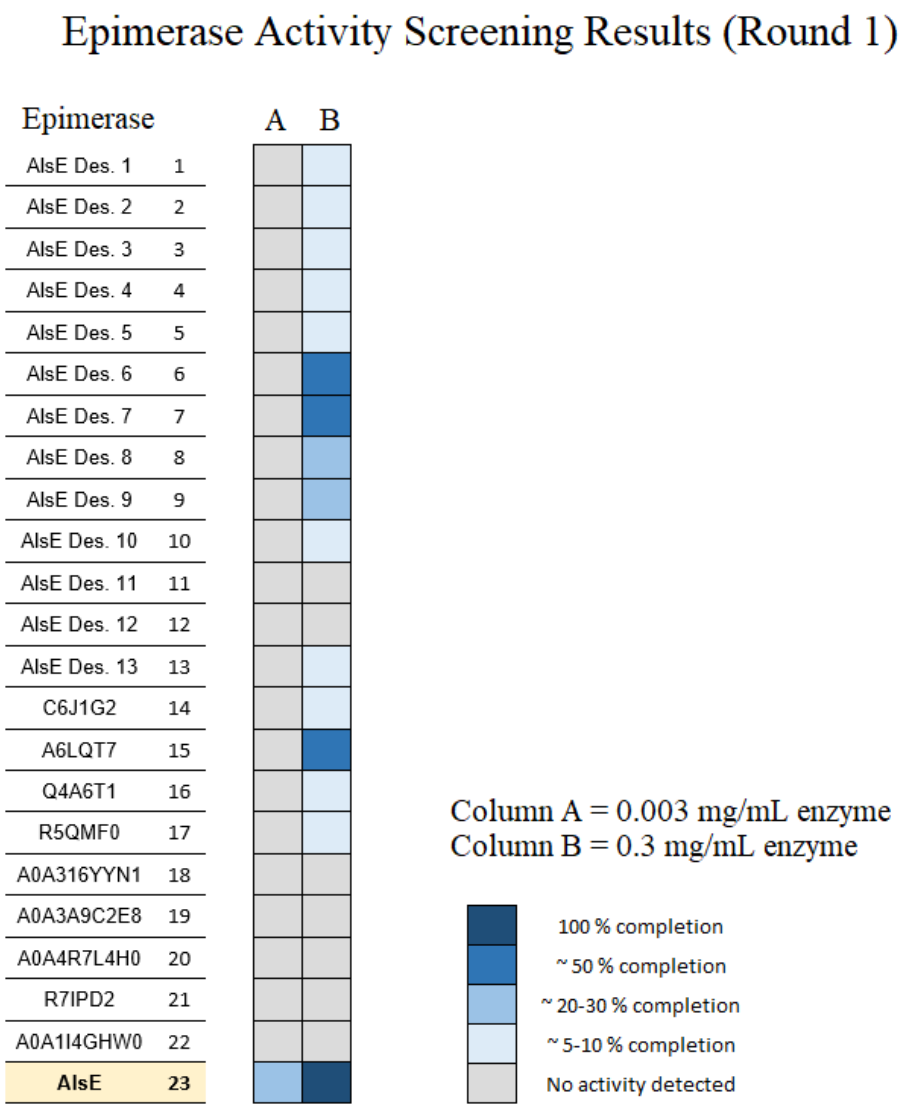


Figure 6. First round of epimerase screening results. Enzymes here were obtained from engineering efforts as well as genome mining efforts. Relative levels of activity are colored in different shades of blue. Darker shades indicate higher reported levels of activity based on "percent completion" toward the equilibrium reaction between f6p and p6p. For example, 100 percent completion would mean that p6p production was no longer observed beyond the 30 min reaction run time.

A second round of activity screening was conducted with a new set of epimerases possessing lower sequence similarity to RPE while maintaining similarity with AlsE, comparable to A6LQT7. Results of round two epimerase activity screening are reported in figure 7 below.

Epimerase Activity Screening Results (Round 2)

Epimerase		% Activity
B0US02	24	20%
A0A1V8XCK1	25	
A0A3A6QSF1	26	10%
A8FDW0	27	<5%
A0A1H0GZN2	28	10%
K1M0G0	29	20%
U2N0A4	**30**	**30%**
5ZFS	31	
UlaE	32	
A0A1Y4S427	33	10%
A0A498CJV5	34	
A0A2Y0BCX5	35	<5%
H1LJ01	36	<5%
A0A374X1X3	37	
A0A0R2C6I5	38	<5%
A0A416EFX4	39	
E7G878	40	10%
AlsE	**23**	**25%**

Assay conditions: 0.003 mg/mL enzyme concentration, 30 min run time

Activity levels are reported in percent (%) completion, referring to completion toward equilibrium. (not percent of product formed)

Figure 7. Second round of epimerase screening results. Enzymes here were obtained exclusively from genome mining efforts refined by round 1 result. Relative levels of activity are colored in different shades of blue as figure 1, but also individually labeled with percent completion. Due to similar activity levels detected amongst the epimerases, more precise values are reported to better distinguish activity levels.

A much higher diversity in activity was observed from second round epimerase screening All samples reported in figure 7 were assayed alongside AlsE at 0.003 mg/mL enzyme concentration for a 30 min run time. One enzyme was reported to have higher activity levels than AlsE. U2N0A4, which was assayed at the same conditions alongside AlsE, had reached around 30 percent completion toward the equilibrium point, the final state of the isomerization reaction. In identical assay conditions, AlsE reported slightly lower levels of activity (25 percent), observed

by a smaller psicose-6-p MS signal formation. Two other samples, #24 and #29, also have similar levels of activity as AlsE, with around 20 percent completion. Eight other epimerases were found to have minimal levels of activity below 10 percent and the rest did not have any detectable activity.

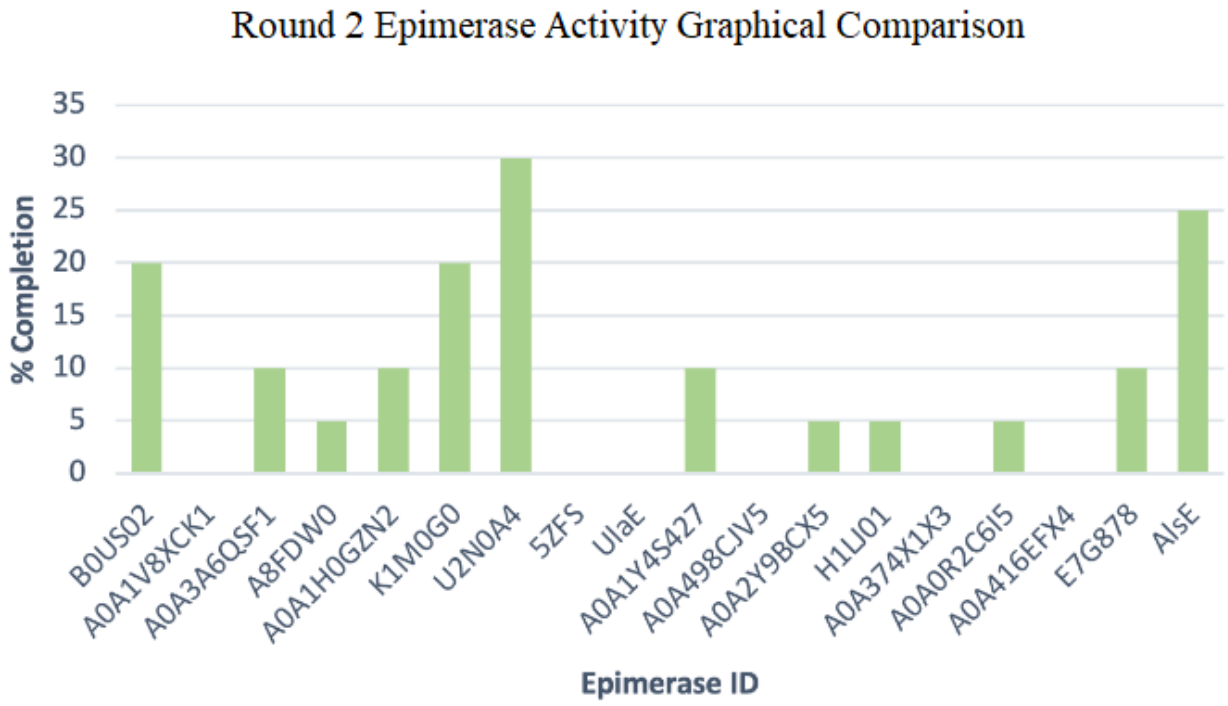


Figure 8. Epimerase activity screening results displayed as a bar graph to visually demonstrate similarities in activity between epimerases and highlight higher performing epimerases.

Figure 8 displays a comparison of enzyme activity between the round 2 epimerases, represented by percent completion. A majority of epimerases do not exceed the 25 percent completion benchmark set by AlsE. Sequence similarity studies are yet to be conducted on second round epimerases. Further studies could explore sequence similarities of higher functioning epimerases to elucidate potential key residues essential for fructose-6-p to psicose-6-p isomerization reactions. Moreover, a deeper understanding of sequence to function relationships

of sugar epimerases could be the beginning of other rare sugar epimerase discoveries, a topic to be explored in the future.

Conclusion

Increase of diabetes due to excess sugar consumption among the global population has sparked interest in alternative sweetener production. Artificial sweeteners have since been developed to substitute traditional sugar. However, dissimilarity in taste to traditional sugar along with other negative outlooks, such as potential adverse health effects, has shifted interest to other alternative sweeteners such as rare sugars. Amongst the rare sugars, psicose is a top candidate due to its high sweetness levels and low caloric content. Current industrial standards of psicose production are not cost effective. Thermodynamics challenges due to the equilibrium established between the isomerization of glucose, fructose, and psicose limits theoretical yields. Furthermore, psicose product must still be separated from the sugar mixture, forcing additional costs.

This study has proposed an alternative psicose production pathway, in which phosphorylated versions of the sugars are isomerized and subsequently dephosphorylated by an irreversible reaction. The advantage of this pathway is the final irreversible dephosphorylation step, in which the psicose product cannot return to the equilibrium state between the sugar isomers. Two key enzymes are necessary for psicose production in an in-vivo E. coli culture, an epimerase and phosphatase. Epimerization occurs between fructose-6-p and psicose-6-p, and phosphatases will dephosphorylate psicose-6-p to produce psicose in the final step of the pathway. In-vivo, the cell will naturally produce fructose-6-p from glucose through the glycolysis pathway.

Computational methods such as genome mining and enzyme engineering were employed to obtain epimerase and phosphatase samples for activity screening. Phosphatases were studied with three sugars: glucose-6-p, fructose-6-p, and psicose-6-p. The specificity of phosphatases to

psicose-6-p were favored and selected for in-vivo experiments conducted by a collaborating lab. Highly specific phosphatases are predicted to benefit psicose production by preventing dephosphorylation of intermediates and side product formation. Epimerases were compared to native E. coli epimerase, AlsE, a known psicose-6-p epimerase. Epimerase activity assays measured fructose-6-p isomerization. High performing enzymes were sent to collaborators for in-vivo testing similar to results from phosphatase screening.

Results have indicated successful discovery and activity screening of several sugar phosphatases and epimerases. Amongst the phosphatases, four were found to have specificity toward psicose-6-p. The epimerase studies have found a few targets with competitive activities compared to AlsE. The enzyme targets reported to have specific and efficient activity are predicted to increase overall psicose production yields when implemented in-vivo.

Further studies of sequence similarities from active enzymes can be explored to discover a wider range of functioning epimerases and phosphatases. A deeper dive into catalytic residues and relationships to reaction mechanisms should also be further studied to explore other rare sugar production prospects. Overall, this study has demonstrated successful in-vitro enzymatic production of psicose by screening the activity of several epimerase and phosphatase targets. The combination of these enzymes is predicted to increase in-vivo psicose production yields, resulting in a more cost-effective commercial psicose manufacturing process.

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