The Mechanism of the Enzymatic Synthesis of 2,4-hydroxymethylfurfural phosphate

Caroline Brown

University of North Georgia, carolineabigail@outlook.com

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The Mechanism of the Enzymatic Synthesis of 2,4-hydroxymethyfuural phosphate

A Thesis Submitted to
the Faculty of the University of North Georgia
In Partial Fulfillment
Of the Requirements for the Degree
Bachelor of Science in Chemistry with concentration in Biochemistry
With Honors

Caroline Abigail Brown
Spring 2019
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The Center for Undergraduate Research and Creative Activities for funding to complete this research.

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Abstract
This project stems from the desire to explore and eventually harness the enzymatic mechanism of 4-(hydroxymethyl)-2-furan-carboxaldehyde-phosphate synthase (MfnB). The enzyme of interest, MfnB, is found most prominently in methanogens and some bacteria and can catalyze five or more separate chemical transformations in a single active site. This singular enzyme takes two molecules of glyceraldehyde-3-phosphate and creates a furan-containing compound, 4-(hydroxymethyl)-2-furan-carboxaldehyde-phosphate (4-HMF-P). Furan-containing compounds have been highlighted for their high potential in the production of biofuels and biomaterial compounds; thus, research efforts seek to create a more efficient synthesis process for furan compounds. The broad industrial applications of MfnB could usher in a new era for the synthesis of furan compounds to be used in the generation of liquid fuel or other valuable biomaterials via enzyme-catalyzed reactions. Despite the initial characterization of MfnB and the identification of Schiff base-forming lysine, the detailed mechanism of MfnB remains speculative. Our team seeks to understand the details of the catalytic mechanism utilizing a site-directed mutagenesis, comprehensive kinetic evaluation, and structural study.
Introduction
In an attempt to focus research efforts for chemical opportunities from biorefinery carbohydrates, the Department of Energy (DOE) released a “Top 10 + 4” list of the most promising platform chemicals.\(^1\) A platform chemical is a versatile molecule that can be chemically converted into a variety of value-added chemicals. Platform chemicals are crucial to the biorefinery concept which is an industrial term used to describe the conversion of biomass into various valuable chemicals through a variety of chemical transformations.

**Figure 1.** Outline of the biorefinery concept.

Economically speaking, platform chemicals are crucial to the success of biorefinery. If one compound can be converted into numerous compounds, the conversion process will be much more efficient and profitable than if a variety of compounds were to needed to produce a variety of products. The platform chemical makes the industrial conversion of biomass to valuable chemicals much more streamlined. The DOE list identifies underdeveloped target molecules which have potential to be used as platform molecules in an attempt to catalyze research focused
specifically on these molecules which could turn profit and thus make the research efforts a worthy investment.

Working at the intersection of biology and chemistry, this research focuses efforts on 2,4-hydroxymethylfurfural (2,4-HMF) derivatives and their potential to be used as a platform and precursor molecule. Furan-containing compounds were left off the original DOE list due to problems with yields and selective conversion processes but were added to the list when it was revised in 2010 as methods for the dehydration of sugars to furans had greatly improved.² The specific benefit of 2,4-HMF is that it functions as a precursor molecule to a variety of value-added chemicals including a biofuel (2,4-dimethylfuran), liquid crystals (2,4-furandicarboxylic acid), crosslinking copolymers (EOEFCA), and pharmaceutical products (2,4-bis(hydroxymethyl)furan).

![Chemical structures of platform molecule 2,4-HMF and its valuable conversion products.](image)

**Figure 2.** Chemical structures of platform molecule 2,4-HMF and its valuable conversion products.

Due to the resonance of the furan ring, the 2,4-HMF isomer is more difficult to synthesize than the 2,5-isomer which limits its viability for large-scale production process. The current method utilizes organic synthesis which uses harsh conditions and results in problems
with selectivity. On the industrial level, glycerol is converted to glyceraldehyde (GLYD) or dihydroxyacetone (DHA) through a selective biocatalyzed or metal-catalyzed oxidation respectively.\textsuperscript{4-5} A base catalyzed condensation then converts GLYD and DHA to a ketohexose and an acid catalyzed dehydration reaction then converts the ketohexose to a final mixture of 5- and 4-HMF (Figure 3).\textsuperscript{6}

![Figure 3](image)

**Figure 3.** Current organic synthesis pathway for isomeric mixture of HMF.

It has been demonstrated that DHA condensed with DHA produces the 4- isomer while DHA condensed with GLYD produces the 5- isomer meaning that the batch method produces an isomeric mixture of HMF. As the 4- isomer is desired as the precursor to numerous valuable chemicals, the low selectivity of the current synthetic method poses a problem for industrial use.

As an alternative to the current organic synthesis methods, this research presents a potential biochemical approach to synthesize 2,4-HMF and its derivatives. A recently discovered and novel enzyme, 4-(Hydroxymethyl)-2-furancarboxaldehyde-phosphate synthase (MfnB), has potential to be used in the biochemical synthesis of 2,4-HMF.

Novel enzyme MfnB was discovered in 2014 when it was isolated from the methanogenic archaebacteria, *Methanocaldococcus jannaschii.*\textsuperscript{7} Methanogens partake in a biological process known as methanogenesis to produce methane gas from carbon dioxide through metabolic processes. In the methanogenesis pathway, methanofuran is a coenzyme
necessary for the reduction of carbon dioxide to methane. Enzyme MfnB exists as the first step in the synthesis of methanofuran within the metabolic processes of *M. jannaschii* where MfnB takes two molecules of glyceraldehyde-3-phosphate (Ga3P) and converts them into 2,4-HMF-phosphate (2,4-HMF-P).  

\[
\text{Ga3P} \rightarrow \text{H}_2\text{O} + \text{P}_4
\]

**Figure 4.** The first step in the biosynthetic pathway of methanofuran in *M. jannaschii*.

Previous study has elucidated that MfnB functions using a single active site but takes the molecules of Ga3P through a series of chemical transformations including dephosphorylation, isomerization, aldol condensation, cyclization, and dehydration (Figure 5). This reaction scheme makes MfnB unique in that it performs five separate chemical reactions using one active site where typical enzymes perform only one reaction; however, the detailed mechanism of the reactions taking place at the active site is still unknown. This gap in knowledge is due to the difficulty in identification of the catalytic intermediates of the reaction. Currently, the wild-type MfnB has been successfully crystallized; however, there is no structural evidence with respect to the catalytic intermediates. Due to the activity of the conversion of substrate to product in the wild-enzyme, it is very difficult for co-crystallization of the catalytic intermediates in the active
site. Thus, one aim of this project is to generate mutant enzymes with limited or no activity in hopes that trapping the catalytic intermediates will become possible.

**Figure 5.** Proposed mechanism for the catalytic activity of enzyme MfnB.

As shown above, the two molecules of Ga3P bind at two binding sites within the active site of the enzyme. Mass spectrometry studies have shown that at binding site I, lysine 27 forms a Schiff base with the triose sugar allowing for a dephosphorylation reaction to occur.\(^8\) Although the role of one amino acid residue in this complex mechanism is known, there is no evidence to support how the other reaction steps are catalyzed within the active site.
The defining step in this series of reactions is the aldol condensation reaction which catalyzes a new C-C bond and turns the two 3 carbon molecules into one 6 carbon molecule. Upon comparison with the enzyme deoxyribose-5-phosphate aldolase (DERA), a class 1 aldolase that is also able to catalyze an aldol condensation reaction, no distinct tertiary structural deviations were evident.⁸

![Figure 6. Structural comparison of MfnB (left) and Class I aldolase DERA (right).](image)

Both enzymes have an α/β barrel (TIM) structure and both active sites shared four conserved amino acids: Two aspartic acid residues and two lysine residues (Figure 6).⁸ Because the structure-function relationship for enzymes is so strong, the stark structural similarities between the enzymes indicates that their functions should also be very similar; however, DERA can catalyze only one reaction, an aldol condensation, while MfnB can catalyze five distinct chemical transformations.

To probe the active site of MfnB, generate mutant enzymes with limited and/or no catalytic activity, and ultimately determine a detailed catalytic mechanism, a site-directed mutagenesis method combined with a kinetic study of the mutant enzymes was used. After
structural evaluation of the MfnB active site along with a sequence alignment, a set of 14 residues residing at or near the active site were chosen for mutation. The sequence alignment was used to track the evolutionary history of enzyme MfnB. Residues which are strictly or highly conserved within the enzyme structure have a high likelihood of being catalytically important as they have not been changed throughout evolution. As the goal of this study is to determine which amino acids are catalytically important and thus generate a detailed mechanism, it follows that by changing amino acids deemed to be catalytically important it would be expected the mutant enzyme would have no activity. This means that mutants with no or very limited activity are of interest as this limited activity signifies the enzyme cannot function in their absence thus implicating them in the catalytic mechanism.

By designing a primer to carry the mutation, the amino acid sequence can be changed using polymerase chain reaction (PCR). Primers for use in PCR are typically designed in a way so that the DNA sequence is complementary to the desired binding site. This ensures specific binding and results in replication of the desired DNA sequence. The process of site-directed mutagenesis utilizes primers that have been designed to carry a mutation where the point of mutation will not bind to the existing DNA sequence while the rest of the primer sequence exhibits complementarity so base pairing can occur. The DNA can then be replicated through the first round of PCR. When the DNA denatures to begin the second cycle, the daughter strand will contain the mutation which is then propagated by replication through approximately 30 PCR cycles. At the end of the process, the majority of the DNA population contains the desired mutation, and the remaining parent DNA can be easily degraded through enzyme digestion.
Figure 7. Site-directed mutagenesis converting a serine residue to an alanine residue. Base pairs in blue signify the desired mutation point, and base pairs in red show the inherent mismatch of the mutagenesis process.

The mutations are then confirmed through sequencing. Once confirmed, the target mutant enzyme is recombinantly expressed on a large scale, purified, and quantified. A kinetics study was then conducted using Michaelis-Menten enzyme kinetics to determine the turnover number \(k_{cat}\) and Michaelis constant \(K_M\) which in turn allowed for calculation of catalytic activity \(k_{cat}/K_M\).

In this experiment, UV/vis spectroscopy was used to track the progress of the reaction as the substrate does not exhibit UV/vis absorbance; however, the product does exhibit absorbance at 280 nm. The velocity of reaction was determined for each mutant enzyme at a variety of
substrate concentrations, and consequently, $V_{\text{max}}$ was determined. The catalytic activity can then be calculated and compared for each mutant enzyme. In theory, the activity of the mutant should correlate directly to the importance of the original amino acid residue in the enzymatic mechanism giving clues as to which amino acids are catalytically important.

Thinking in a broader context, there is potential to construct an *in vitro* pathway where enzyme MfnB is paired directly with the first 4 enzymes of glycolysis to convert glucose, a readily available feedstock, into 4, HMF-P, a valuable precursor to a platform molecule. This single-pot synthesis would have broad implications in the field of biorefinery in that it would offer an efficient biochemical synthesis for a valuable platform molecule. Additionally, as the research is being conducted at an undergraduate teaching institute, there is potential for classroom development in that this project can be adapted for a classroom laboratory setting exposing students to interdisciplinary work in the field of molecular biology.
Methods
For simplicity, this study was divided into two main categories: molecular biology and enzymology. Both sections were composed of a variety of individual processes outlined below in Figure 8.

**Figure 8.** The general workflow of the experimental methods.

The two categories had distinct goals with molecular biology being used to introduce a mutation to the amino acid of interest and enzymology to characterize the generated mutant.

**Molecular Biology**

*Sequence Alignment*

The amino acid sequence for protein MfnB was obtained from the Protein Data Bank (PDB) and aligned to a variety of homologous enzymes from the same protein family. Amino acids that were strictly or highly conserved throughout the alignment were noted for further exploration as conservation is indicative of evolutionary importance. Traditionally, if a residue does not change throughout the evolutionary history of the enzyme, then the residue has a high chance of being implicated in the catalytic mechanism. The amino acid residues chosen for mutation were as follows: serine-6 (Ser 6/S6), aspartate-25 (Asp25/D25), lysine-27 (Lys27/K27), serine-33
(Ser33/S33), serine-56 (Ser56/S56), tyrosine-83 (Tyr83,Y83), lysine-85 (Lys85/K85), methionine-149 (Met149/M149), aspartate-151 (Asp151/D151), lysine-155 (Lys155/K155), serine-188 (Ser188/S188), arginine-217 (Arg-217/R217, aspartate-216-arginine-217-asparagine-218 (Δ216-218) for a total of 14 potential mutants. These residues were chosen specifically because they are both highly conserved and at or near the active site of MfnB meaning they could be important for catalysis. The active site of enzyme MfnB is shown below.

Figure 9. Active site of enzyme MfnB. Active site residues chosen for mutation are highlighted.

Primer Design

To introduce mutations to the amino acids of interest, primer pairs were designed for use in polymerase chain reaction (PCR). Typically, point mutations were introduced; however, in some cases, more than one base pair was changed in order to introduce a restriction site for mutation detection. A palindromic sequence was introduced near the mutation which is used for detection
of the mutation via restriction digest. A primer is a piece of DNA sequence between 25 and 35 nucleotides. The primer length and annealing temperature are both optimized to provide specific
binding to the template DNA. The length is optimized as a shorter primer would share sequence similarity with many portions of the parent DNA while a longer primer has a higher chance of internal complementarity resulting in self-binding. The annealing temperature is near the middle of a temperature range that has been optimized for the pfu polymerase (55°C-80°C). The program A Protein Editor (APE) was used to check primer design and ensure that any changes made to the nucleotide sequence did not affect the phenotype displayed by the enzyme in any place other than the desired mutation. The table of all primers designed can be found in Supplemental Information Figure S1.

Primer Preparation

Primers were synthesized from Invitrogen and received in a lyophilized form. Primer tubes were spun down at high speed using a table top centrifuge, and 100 µL deionized, autoclaved H₂O was added to each tube. Primers were then diluted using 2 µL of primer and 118 µL deionized, sterile H₂O. It was then necessary to take an absorbance measurement to determine the concentration of the primers. They were then diluted accordingly to achieve a working concentration of 100 ng/µL.

Polymerase Chain Reaction

PCR was carried out at a total volume of 50 µL. To a sterile, dome-capped, PCR tube, the following was added: 40 µL deionized, sterile H₂O, 5.0 µL 10x pfu buffer, 1.0 µL 10mM deoxynucleotide triphosphates (dNTPs), 1 µL pfu polymerase, 1.0 µL plasmid DNA template⁸.

⁸ For this experiment, the pet-41(a)+ plasmid was used with gene insert MJ1099 was used as template DNA
1.0 µL forward primer, 1.0 µL reverse primer with the primers used carrying the desired mutation for each individual reaction. A negative control was also included using the same reagents listed above leaving out the pfu polymerase.

The following parameters were used for the thermal cycler:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>2</td>
</tr>
<tr>
<td>2 Melting</td>
<td>95</td>
<td>0.5</td>
</tr>
<tr>
<td>3 Annealing</td>
<td>61</td>
<td>0.5</td>
</tr>
<tr>
<td>4 Extension</td>
<td>72</td>
<td>6</td>
</tr>
</tbody>
</table>

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeat back to step 2 for 30 cycles</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

After PCR, the products were run out on a 1% agarose gel to confirm the reaction was successful as described below.

**Gel Electrophoresis**

A mass of 0.50 g agarose was added to 50 mL 1X TAE (tris base, acetic acid, ethylenediaminetetraacetic acid) buffer and microwaved for approximately one minute to completely solubilize. The solution was then poured into a gel casting mold, a comb was inserted, and was allowed to solidify forming a 1% agarose gel. After solidification, the gel was transferred to a running chamber and submerged in 1X TAE buffer. The comb was removed. A volume of 2 µL loading dye was mixed with 10 µL PCR product and loaded into the wells of the gel. The running chamber was connected to a power supply and the gel was run at 120V for one hour. To visualize the DNA, the gel was soaked in a bath of ethidium bromide and visualized using UV light.
**DpnI Digestion**

To degrade the parent DNA, which does not carry the desired mutation, enzyme DpnI was used for digestion. DpnI was used specifically because it only degrades methylated DNA which makes it specific for the parent DNA as methylation is a post-translational modification not introduced during the PCR process. After PCR, 1 µL enzyme DpnI was added to each reaction tube to digest any methylated DNA, ensuring that only DNA with the desired mutation was remaining. The tubes were incubated at 37 °C for one hour.

**Ligation**

The resulting PCR product is linear; however, plasmid DNA should be circular in order to be taken up and expressed by the E. coli cells. Ligation, using the enzyme ligase, is used to circularize the PCR product. The following reagents were added to a sterile microcentrifuge tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Each reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water (Sterile)</td>
<td>3 µl</td>
</tr>
<tr>
<td>DNA from PCR</td>
<td>5 µl</td>
</tr>
<tr>
<td>T4 Ligase</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 x T4 ligase buffer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

The tubes were then incubated for two hours at room temperature. After ligation it was important to proceed directly to transformation for best reaction efficiency.

**Transformation**

In order to introduce exogenous DNA, in this case the plasmid DNA carrying the desired mutation, to host cells for replication, the process of transformation was used. Sterile microcentrifuge tubes were chilled on ice. Supercompetent XL1-Blue (purchased from Agilent)
cells were thawed on ice, resuspended, and 50 μL aliquoted into each pre-chilled tube. A volume of 2 μL ligation product was added to each tube and allowed to incubate with the cells for 20 minutes on ice. The cells were heat-shocked at 42 °C for 40 seconds and immediately allowed to recover in the ice bath for 2 minutes. Then, 500 μL pre-warmed super optimal broth with catabolite repression (SOC) medium was added to each tube and the cells were incubated at 37°C for one hour with shaking at 250 rpm. Cells were spun-down using a high-speed centrifuge, approximately 300 μL SOC was removed, the cells were resuspended, and plated on LB agar plates infused with the antibiotic kanamycin (KAN). The plates were incubated at 37 °C overnight.

**Liquid Culture**

After successful transformation, colonies were transferred to liquid culture for growth and proliferation. A sterile test tube was filled with 2 mL sterile LB broth and 2 μL KAN. Colonies were picked from LB agar plates and placed into LB solution. The tubes were incubated overnight at 37°C with shaking at 250 rpm.

**Plasmid Purification**

To extract the plasmid DNA, a Quiagen Miniprep Kit was used. All buffers are pre-made, and the buffer compositions can be found in Table S2. The 2 mL cultures were transferred to a sterile microcentrifuge tube and spun down at 13,000 rpm for 3 minutes with a pellet forming at the bottom of the tube. The supernatant LB was poured off and each pellet was resuspended in 250 μL buffer P1. A volume of 250 μL buffer P2 was added to each tube and then gently inverted 5 times. A volume of 350 μL buffer N3 was added to each tube and gently inverted 5 times. The tubes were then spun-down at 13,000 rpm for 10 minutes. The supernatant was transferred to a miniprep column, and the columns were centrifuge are 13,000 rpm for one minute. The column
was washed with a volume of 750 μL buffer EB and centrifuged at 13,000 rpm for two minutes with the flow through being discarded after each minute. The miniprep column was transferred
to a sterile microcentrifuge tube and the DNA was eluted in 50 μL sterile, deionized H₂O through centrifugation.

**Restriction Digest**

To determine if the mutation had been successfully introduced, a restriction digest was used. The following reagents were mixed in a microcentrifuge tube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutsmart Buffer</td>
<td>1 μL</td>
</tr>
<tr>
<td>Enzyme of Choice(^{b})</td>
<td>1 μL</td>
</tr>
<tr>
<td>H₂O</td>
<td>3</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>5 μL</td>
</tr>
</tbody>
</table>

The tubes were then incubated at 37 °C for one hour. The resulting solutions were run out on a 1% agarose gel as described above. This process was used to check if a mutation was successfully introduced to the plasmid DNA.

**Sequencing**

All samples confirmed through restriction digest were sent to the DNA Facility at Iowa State University for sequencing. If the correct mutations were confirmed through sequencing, the plasmid was then transformed to supercompetent BL21-DE3 (purchased from Agilent) expression cells. If the mutation was not confirmed through sequencing, new colonies were picked from the transformation plates.

\(^{b}\) The enzyme used is specific to the mutant that is being checked. Table S1 contains a comprehensive list of enzymes used to detect each mutation.
**Protein Kinetics**

*Transformation of Expression Host*

Sterile microcentrifuge tubes were chilled on ice. Supercompetent BL21-DE3 cells were thawed on ice, resuspended, and 50 μL aliquoted into each pre-chilled tube. A volume of 1 μL plasmid carrying desired mutant MfnB was added to each tube and allowed to incubate with the cells for 20 minutes on ice. The cells were heat-shocked at 42 °C for 20 seconds and immediately allowed to recover in the ice bath for 2 minutes. Then, 500 μL pre-warmed super optimal broth with catabolite repression (SOC) medium was added to each tube and the cells were incubated at 37°C for one hour with shaking at 250 rpm. Cells were spun-down using a high-speed centrifuge, approximately 300 μL SOC was removed, the cells were resuspended, and plated on LB agar plates infused with antibiotics kanamycin and chloramphenicol. The plates were incubated at 37 °C overnight.

*Inducement of Expression Host*

A 2 mL LB culture was started for each mutant transformed to BL21-DE3. After approximately 18 hours, the 2 mL culture was transferred to a 50 mL LB culture and incubated with shaking at 37 °C for approximately 18 hours. The 50 mL culture was then transferred to a 500 mL culture and monitored until the OD600 reached a value of 0.6. At this point, 500 μL 1M IPTG was added to induce expression of MfnB. The culture was incubated at 37 °C with shaking for 4 hours and the solution was spun down, the cells pelleted, and the supernatant discarded. The pellets were then resuspended in approximately 15 mL phosphate buffer and sonicated to lyse cells. The solution was then centrifuged at high speed to remove cellular debris.

*Protein Purification*

After cell lysis, large amount of the target protein were present in the supernatant (visualized by SDS-PAGE gel); however, there is also a large amount of unimportant cellular proteins. To
separate protein of interest, MfnB, affinity chromatography was used. Specifically, because MfnB was subcloned to a vector containing a 6-Histidine tag (is this a reference?), nickel resin was used to bind and purify the target protein. Histidine has two nitrogen atoms in the imidazole ring that both have a lone pair of electrons that are capable of coordinating to the iron resin forming a complex that allows the tagged protein to be separated from all other cellular proteins.

The supernatant from cell lysis (approximately 15 mL) was incubated with 5 mL nickel bead resin for one hour at room temperature with slow, consistent shaking. After incubation, the resin was resuspended and transferred to a filtration column. The resin was allowed to settle and flow through was collected. The resin was then washed with approximately 10 mL of three wash buffers each with increasing concentration of imidazole (Table 1). The resulting fractions were run on an SDS-PAGE gel to determine which fraction contained MfnB.

![Table 1. Composition of buffers used for protein purification.](image)

<table>
<thead>
<tr>
<th>Wash Buffer</th>
<th>Elution 1 Buffer</th>
<th>Elution 2 Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Na₂HPO₄</td>
<td>50 mM Na₂HPO₄</td>
<td>50 mM Na₂HPO₄</td>
</tr>
<tr>
<td>250 mM NaCl</td>
<td>250 mM NaCl</td>
<td>250 mM NaCl</td>
</tr>
<tr>
<td>10 mM imidazole</td>
<td>50 mM imidazole</td>
<td>150 mM imidazole</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>pH 7.0</td>
<td>pH 7.0</td>
</tr>
</tbody>
</table>

**SDS PAGE Electrophoresis**

An SDS-PAGE gel was made using the following reagents
All reagents for the running gel were combined saving ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) for last. Once APS and TEMED were added, the running gel solution was transferred immediately to a gel casting rig. A thin layer of butanol was then layered on top of the running gel and it was left to solidify. All reagents for the stacking gel were combined except for APS and TEMED. After solidification, the butanol was decanted and the gel was rinsed with H2O. The APS and TEMED were added to the stacking solution, the solution was transferred immediately to the gel casting rig, a comb was inserted, and the gel solidified. After solidification, the gel was transferred to the running chamber and covered with 1X SDS running buffer. A volume of 10 μL SDS loading buffer was mixed with 10 μL protein sample, heated to 90 °C for 10 minutes and loaded to the gel. The gel was run at 100 V for approximately one hour and protein was visualized using Coomassie staining.

**Buffer Exchange**

To remove excess imidazole from the protein solution and to get the protein in the correct buffer for the kinetic study, buffer exchange was performed. A desalting column was drained, filled with extraction buffer\(^c\) and allowed to drain. A volume of 3 mL of the fraction from purification containing the protein, was poured into the column and allowed to drain. A volume of 4 mL

<table>
<thead>
<tr>
<th></th>
<th>Stacking Gel</th>
<th>Running Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>3 mL</td>
<td>1.8 mL</td>
</tr>
<tr>
<td>0.5M Tris-HCl</td>
<td>(pH 6.8) 1.25 mL</td>
<td>(pH 8.8) 3 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05 mL</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>Acrylamide Bis (30%)</td>
<td>0.67 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>0.05 mL</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>Tetramethylethylenediamine</td>
<td>0.005 mL</td>
<td>0.010 mL</td>
</tr>
</tbody>
</table>

\(^c\) Composition of extraction buffer is as follows: 50 mmol HEPES, 5 mmol KCl, 15 mmol MgCl₂ (pH 7.01)
extraction buffer was added to the column, allowed to flow through, and collected in a centrifuge tube. This process was repeated until the entire volume of protein solution was put through the column. The resulting solution was stored at 4 °C until a Bradford assay was performed.

**Bradford Assay**

To establish the concentration of purified protein, a Bradford Assay was conducted. A series of BSA standards were generated ranging in concentration from 0-1500 µg/mL. A 30 µL aliquot of each standard and each unknown protein was combined with 1.5 mL Bradford reagent, mixed thoroughly, and allowed to react for 10 minutes. The absorbance of each solution was taken at a wavelength of 595 nm. A calibration curve was built from the standards and the unknown protein concentrations were calculated accordingly. The protein solutions were aliquoted into microcentrifuge tubes and stored at -20 °C for future use.

**Kinetic Analysis**

A kinetic study utilizing UV/vis spectroscopy was conducted to calculate the catalytic activity of MfnB mutants. Because, HMF-P, absorbs UV at a wavelength of 280 nm and the substrate, Ga3P does not, the change in absorbance at 280 nm was used to track the progress of the reaction. A constant amount of enzyme (4 µg) was incubated with varying amounts of substrate and allowed to react over a period of 5 minutes at 70°C. This temperature was used because enzyme MfnB is a thermostable enzyme as it is isolated from a hyperthermophile. The initial and final absorbances from the most linear portion of the curve were recorded for each substrate concentration, and the slope of the line, or the rate of the reaction, was calculated. A control measurement was taken using just the enzyme and buffer to account for natural instrument fluctuations, and the experimental slope was corrected using this measurement. The corrected
rate was then converted into units of 1/s by dividing out enzyme concentration and the resulting values were plotted on a Michaelis-Menten graph to determine $k_{cat}$, $K_M$, and $k_{cat}/K_M$ (catalytic efficiency).
Data and Results
**Figure 8.** Sequence alignment of MfnB with homologous enzymes.
### Kinetic Parameters at °C

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_M$ (μM)</th>
<th>$k_{\text{cat}}/K_M$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild-type</strong></td>
<td>0.041 ± 0.002</td>
<td>21 ± 4</td>
<td>2.0 × 10$^3$</td>
</tr>
<tr>
<td><strong>D25N</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>K27R</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>K85R</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>D151N</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>K155A</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>S33A</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>S6A</strong></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>M149A</strong></td>
<td>0.0048289 ± 0.0004</td>
<td>9.0 ± 3.9</td>
<td>5.36 × 10$^2$</td>
</tr>
<tr>
<td><strong>S188A</strong></td>
<td>0.066 ± 0.004</td>
<td>18 ± 5</td>
<td>3.6 × 10$^3$</td>
</tr>
<tr>
<td><strong>Y83A</strong></td>
<td>0.000192 ± 1.80e-05</td>
<td>19.8 ± 8</td>
<td>9.7</td>
</tr>
<tr>
<td><strong>R217A</strong></td>
<td>0.00204 ± 0.0003</td>
<td>8.2 ± 6.2</td>
<td>2.49 × 10$^2$</td>
</tr>
<tr>
<td><strong>Δ216-218</strong></td>
<td>0.000166 ± 8.03e-06</td>
<td>3.9 ± 1.1</td>
<td>42.6</td>
</tr>
</tbody>
</table>

**Table 2.** Results of the kinetic study comparing catalytic activity of wild type MfnB to mutant enzymes. ND = not determined indicating no activity.
Discussion
Of the 14 amino acid residues that were initially identified as strictly or highly conserved, a total of 12 mutants were successfully generated and the activities determined. After completion of the kinetic study, each of the twelve mutants was sorted into three categories: no activity, limited activity, and increased activity (Table 3).

<table>
<thead>
<tr>
<th>No Activity</th>
<th>Decreased Activity</th>
<th>Increased Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>D25N</td>
<td>Y83A</td>
<td></td>
</tr>
<tr>
<td>K27R</td>
<td>R217A</td>
<td></td>
</tr>
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<tr>
<td>K155A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S33A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.** Summary of activities of mutant enzymes with respect to wild type activity.

Because the amino acid residues chosen for mutation were strictly or highly conserved, it was hypothesized that by changing the residue, the enzyme would lose most, or all, function. This assertion assumes that the consistency of an amino acid through the evolutionary history means that the residue is important to enzyme function and changing the amino acid results in a non-functional enzyme which would be detrimental to the organism allowing natural selection to occur within the population. Using this logic, the one mutant with increased activity was a surprising result. Upon examination of the active site structure of MfnB, the mutated residue resulting in increased activity appears to interact with a phosphate molecule in Ga-3P (previously, you used Ga3P) binding site I where a phosphate elimination reaction occurs. It is hypothesized that changing the amino acid serine to something much smaller, such as alanine, encourages the release of phosphate increasing the efficiency of the enzyme; however, further testing will be needed to support or refute this claim.
With a total of twelve mutants generated and characterized, the focus of the project now shifts to the mutants which fall into the category of limited or no activity. The next step is a collaboration with a protein crystallographer in hopes of trapping the substrate in the active site of the enzyme. Currently, using the wild type enzyme, the catalytic intermediates cannot be crystallized due to the fast catalytic activity. The limited and no catalytic activity mutants hopefully alleviate this problem making it possible to crystallize the enzyme in a way to capture a crystal structure of the amino acid residues interacting with the catalytic intermediate. This structure will give insight to the detailed mechanism of MfnB as it will give a clear picture of which residues are directly interacting with the substrate lending themselves to catalytic importance.
Conclusion and Future Works
As a result of this study, a total of 12 mutant enzymes were generated and characterized using a site-directed mutagenesis approach paired with a kinetic analysis. Of these 12 mutants, 7 were determined to have no activity, 4 were determined to have limited activity, and one mutant was determined to have an increased activity. These results are promising as the limited activity and no activity mutants indicate which amino acids appear to be most important to the enzymatic mechanism. Because 11 mutants resulted in limited or no catalytic activity, they are good candidates for crystallographic studies. Moving forward, a crystallography experiment will be conducted using one of the limited or no activity mutants. Once crystallographic studies are conducted, there will be a much better understanding of the active site dynamics for MfnB, and a detailed mechanism can be generated where specific amino acids are catalytically responsible for each reaction step.

Furthermore, a well-developed understanding of enzyme MfnB will allow it to be better utilized in industry. Ultimately, MfnB can be paired with the first four enzymes of glycolysis to construct an *in vitro* pathway where glucose, a readily available feedstock from biomass, can be converted directly to 4-HMF-P using a single-pot synthesis. The efficient biochemical production of a valuable chemical in a single process has exciting implications for the biorefinery industry.

As an added value, because this research was conducted at a teaching institute, it was developed to be accessible to undergraduate students. The process of mutant generation and characterization through site-directed mutagenesis and kinetic analysis was refined over many iterations throughout the past two years. Ultimately, this project has been transformed into an upper-level laboratory course designed specifically to give undergraduate students an in-depth experience with the scientific research process. As the project is highly interdisciplinary in that it requires students to utilize techniques drawn from genetics, organic chemistry, biochemistry, and
microbiology, students develop critical thinking and logical analysis skills that are crucial to a successful scientific career.
References


## Supplementary Data

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Sequence</th>
<th>Tm</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
</table>
| S6 → A   | **Sense:** GAAGAAGCGAAGAAGCCATTGCTGGTGG  
**Antisense:** AACGTCATTGCCGCGACTAGTGGATCA | 66 | SpeI |
| D25 → N  | **Sense:** gttAAAACCCCAAGGAAGGGCTCACTGGGT  
**Antisense:** gttGATAATGTCAGCACCACAGCAATGGC | 66 | AcII |
| K27 → R  | **Sense:** PHOS-CGATGtaacgaACCCAAGGAGAAATTCA  
**Antisense:** PHOS-ATAATTGCAGCACCACACCAATGGTCTT | 66 | SnaBI |
| S33 → A  | **Sense:** GAAGAAACCCGAAGGAAGGagCtCTGGGTG  
**Antisense:** ACATCGATAATGTCAGCACCACAGCAATGG | 66 | SacI |
| Y83 → A  | **Sense:** GCCTGTACGGTGTCAAGAACTATTACCAGGCA  
**Antisense:** CAACTTTGATagcATCGGCgCCAGAAATAGCT | 66 | SfoI |
| K85 → R  | **Sense:** CCGATTATATCAgAGTaGGCCTGTACGGTGTCA  
**Antisense:** CACCAGAAAATAGCTGCACCACAGGCG | 65 | StuI |
| M149 → A | **Sense:** CAGGTTGCGACGTCGACgctctagaTAC  
**Antisense:** CATTGCCGCACGCTCAGCTGACTTGCACCAGC | 66 | XbaI |
| D151 → N | **Sense:** ---Phos- GCGACGTGcaaatgCTGaataCCGCGC  
**Antisense:** -phos-ACACCTGCACCGCGCGATTTGTTT | 66 | Nb.BsrDII |
| K155 → A | **Sense:** CTGGATACCGCaATTgcAGACGGCAAGA  
**Antisense:** CATTGCCGACGTCGCAACCTGCATCG | 66 | Mfel |
| S188 → A | **Sense:** GCACCTGGCTggcgccATTAAAAAGG  
**Antisense:** CACCAGAAAATAGCTGCACCACAGGCG | 65 | SfoI |
| Δ216-218 | **Sense:** AATGGGTCTGATCGACCGCGAACTGGT  
**Antisense:** ACCtCCTtTaCAAGCTGCACCACGGA | 66 | |
| R217 → A | **Sense:** GTGATgcACACggcgcgATACGGCAACCTGCATCG  
**Antisense:** GCACCTGGCTggcgccATTAAAAAGG | 66 | EagI |

**Table S1.** Primer pairs used to introduce desired mutations and restriction enzymes used to detect mutations.

<table>
<thead>
<tr>
<th>Buffer P1</th>
<th>Buffer P2</th>
<th>Buffer N3</th>
<th>Buffer PE</th>
</tr>
</thead>
</table>
| 50 mM Tris-HCl pH 8.0  
10 mM EDTA  
100 μg/ml RNaseA | 200 mM NaOH  
1% SDS | 4.2 M Gu-HCl (Guanidine hydrochloride)  
0.9 M potassium acetate pH 4.8 | 8 mM Tris-HCl pH 7.5  
80% ethanol  
80 mM NaCl |

**Table S2.** Buffer Compositions from Quiagen MiniPrep Plasmid Purification Kit.