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Investigation of an enzymatic cascade for the production of 5-hydroxymethylfurfurylamine

A preliminary study

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1. Abstract

Biocatalysis is a promising alternative to chemical synthesis routes for high value chemicals which considers the sustainability and environmental aspect. In this study the feasibility of utilizing an enzymatic cascade for the production of 5-hydroxymethylfurfurylamine (HMFA) was explored. HMFA is a compound with diverse applications in industries such as agriculture and pharmaceuticals. The cascade consists of two main reactions, the first of which involves the decarboxylation of lysine using a lysine decarboxylase to produce cadaverine. The cadaverine produced will then be utilized as an amine donor in the second reaction, which involves the use of a transaminase derived from *Silicibacter pomeroyi* (SpTA) together with 5-hydroxymethylfurfural (HMF). This cascade considers the principals of green chemistry such as milder reaction conditions and less waste, hence aiming to reduce the environmental impact. Although there were challenges preventing the completion of the enzymatic cascade, valuable insights were gained. The contribution of this study sheds light on the intricate reaction mechanisms and some of the key difficulties with enzyme immobilisation. While the intended cascade was not finalized, the lessons learned will provide for new perspectives and potential future advancements in biocatalysis.

2. Keywords

Biocatalysis, *Silicibacter pomeroyi*, EziG carriers, 5-hydroxymethylfurfurylamine, transaminase, enzymatic cascade, HPLC, Green chemistry, Cadaverine, Smart Donors

3. Sammanfattning

Biokatalys medför ett alternativt tillvägagångsätt för att kunna utforska och utveckla kemiskt syntetiserade vägar för produktionen av eftertraktade kemikalier, där hållbarhet och miljön tas till beaktan. I denna studie undersöktes potentialen av en enzymatisk kaskad för produktion av 5-hydroximetylfurfurylamin (HMFA). HMFA är en förening med tillämpningar inom flera industrier som till exempel jordbruks- och läkemedelsindustrin. Den enzymatiska kaskaden består av två reaktioner, varav den första involverar dekarboxylering av lysin med användning av lysindekarboxylas för att producera en så kallad ”*smart amindonotor*” kadaverin. Den andra reaktionen i kaskaden består utav att ett transaminas från Silicibacter pomeroyi (SpTA) som konverterarar 5-hydroximetylfurfuryl (HMF) till HMFA med hjälp av det framkallade kadaverinet från den första reaktionen i kaskaden. En enzymatisk kaskad tillåter för mildare reaktionsförhållande, mindre avfall och energisnål användning som därmed minskar miljöpåverkan samtidigt som det beaktar några av dem 12 principerna av grönkemi. Det uppstod utmaningar som hindrade slutförandet av den enzymatiska kaskaden, men trots detta erhölls värdefulla insikter. Denna studie belyser de invecklade reaktionsmekanismerna och några av de svårigheterna med immobilisering av enzym via EziG carriers. Trots att den avsedda kaskaden inte slutfördes, gav lärdomarna nya perspektiv samt potentiella områden att fortsätta undersöka för framtida framsteg inom biokatalys.

4. Introduction

Biocatalysis is a promising approach to accelerate chemical reactions by utilizing enzymes derived from plants, animals, and microorganisms. [1]
Enzymes have a high catalytic activity and have the ability to combine chemo-, regio-, and stereoselective activity in their reactions with other molecules making them versatile. Recent advancements in biocatalytic research have led to an increased understanding of enzyme structure and functional activity, resulting in enhanced stability, and substrate specificity and has provided applications in the pharmaceutical, chemical, food, and agriculture industries. [2]

Furfurals have become a subject of growing interest in recent years due to their potential applications as feedstocks for the production of biofuels and chemicals. Among these, 5-hydroxymethylfurfural (5-HMF) is considered a high value platform chemical. The development of improved catalytic processes has made furfurals more readily available and commercially viable. [29] One of the valuable chemicals derived from 5-HMF is 5-hydroxymethylfurfurylamine (HMFA), which has attracted significant attention in recent years due to its numerous potential applications in various industries, including food, pharmaceuticals, agriculture, and renewable energy. However, the synthesis of amines such as HMFA is challenging due to the sensitivity of the furan ring in reductive conditions, which can lead to the formation of undesired amine by-products in secondary or tertiary structures. Additionally, the use of reducing agents can generate waste, which is not ideal from a green chemistry perspective. Therefore, researchers have turned to alternative methods, such as the use of transaminases, to synthesize these compounds. [30]

4.1 Transaminases

The transfer of an amino group from an amino donor to a carbonyl aldehyde acceptor is performed by the transaminase enzyme in a transamination reaction. This reaction also involves the cofactor, Pyridoxal-5′-phosphate (PLP) and transaminases belong to the fold type I and IV of the PLP-dependent enzymes. [3] These enzymes play critical roles not only in biosynthesis, but also in the degradation of amino sugars, polyamines, and alkaloids. [6]

Transaminases can be divided into two subgroups depending on the position of the amino group of the amine donor. Alpha-transaminases (α-TAs) catalyse the transfer of amino groups located on the alpha carbon of an amino acid, while omega-transaminases (ω-TAs) can transfer amino groups that are not on the alpha position. ω-TA can also transfer amine molecules with no carboxylic groups such as alpha-methylbenzylamine (MBA) to the amino acceptor. [5] ω-TA are of particular interest because they are versatile and have been used in enzymatic routes for the production of high-value amines such as chiral amines. [6]

Transaminases possess varying specificities for binding amino donors, meaning there are different amino donors that fit better for certain transaminases. In organisms, they promote the redistribution of nitrogen between amino acids and oxoacids involved in protein metabolism and gluconeogenesis. [7]

4.2 Transaminase Cofactor

In all transaminase reactions, the cofactor PLP is a requisite component, despite variations in substrate specificity across enzymes. Typically, transaminases consist of two identical subunits, each containing an active site where PLP binds covalently in a Schiff-base linkage to the amino group of the catalytic lysine. The *phosphate group binding cup* is where the phosphate anchor of PLP coordinates with water molecules and amino acid residues from both subunits. [8] The remarkable versatility of PLP-dependent enzymes arises from their ability to participate in a wide range of chemical reactions, making them key players in a diverse array of biological processes. The PLP-dependent enzymes have the ability to interact with amino acids allowing for transfers of amino group as mentioned earlier, but also decarboxylation, conversion between L and D-amino acids and elimination including replacement of chemical groups attached to the beta and alpha carbon. These various actions are possible through the ability of PLP to covalently bind to the substrate and operate as an electrophilic catalyst in which also stabilizes the different carbanionic reaction intermediates. [4]

4.3 Transaminase Mechanism

Transaminases catalyse in a ping-pong bi-bi reaction mechanism, consisting of a first and second half reaction as seen in figure 1. In the first half reaction, the amine containing substrate donates its amine group to the PLP cofactor bound to the transaminase (E-PLP),

leading to a conversion from PLP to intermediate E-PMP and the corresponding keto acid is produced. Then in the second half reaction, the amino acceptor takes the amino group from the E-PMP and produces the corresponding amino acid and in the process regenerates E-PLP. [9]

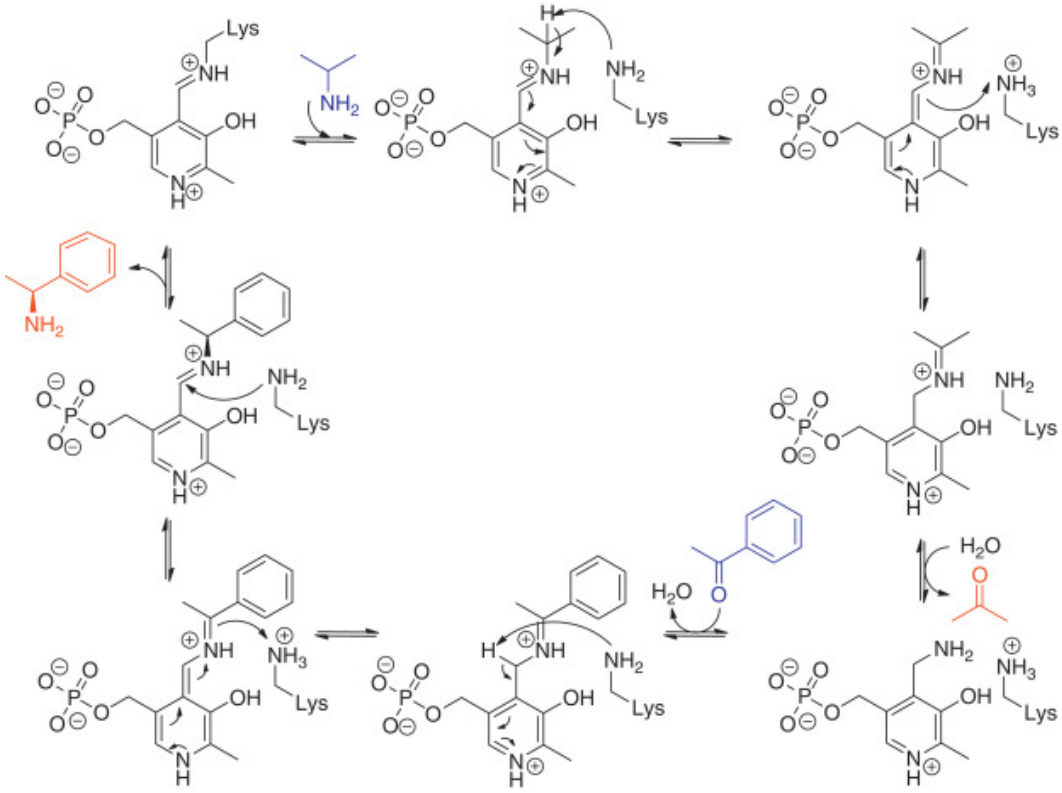

Figure 1. The scheme illustrates a ping-pong bi-bi reaction mechanism including two half-reactions done by transaminases, in which the first half-reaction forms pyridoxamine-5'-phosphate (PMP), a keto product, and a free lysine. In the second half-reaction, PMP reacts with an amino acceptor to create a quinonoid and water. The catalytic lysine then extracts a proton from the quinonoid to produce a chiral amine, while the coenzyme PLP is attached to the lysine residue. [8]

4.4 Lysine Decarboxylase

The lysine decarboxylase (LDC) enzyme plays a crucial role in the maintenance of pH homeostasis and the biosynthesis of cadaverine. Cadaverine, an important industrial platform chemical, is synthesized through the decarboxylation of lysine. Since cadaverine has a role in different applications such as in the production of polyamides, chelating agents, and

additives, it underscores the importance of being able to synthesis cadaverine through biobased production from renewable resources. [25] The biobased production of cadaverine through the usage of LDC performing the decarboxylation of cadaverine, offers an alternative to conventional methods from an environmental and economic perspective. However, several challenges have been encountered in the production process of cadaverine. One major issue pertains to the increase in pH levels in the medium when LDC consumes a proton during the reaction. The increase in medium pH reduces enzyme activity, and ultimately leads to a decrease in the yield of cadaverine. [26] To circumvent this challenge, an enzyme that remains unaffected by the pH increase could be utilized. Additionally, the continuous addition of cofactor PLP has been identified as a factor that increases enzyme activity and subsequently provides high yield of cadaverine. However, this contributes with additional costs when continuously adding more PLP to the reactions. [10]

4.5 Enzyme Cascades

The benefits of enzymatic reactions in comparison to classical chemical synthesis are the high substrate specificity, regio- and stereoselectivity but without the time consuming and costly separation step for the intermediates and products that are otherwise associated with chemical synthesis. [27] Incorporating in vitro enzyme cascades allows for synthesizing complex molecules without having to isolate intermediates and can help with shifting unfavourable equilibria. Some of the key parameters that determine the efficiency of an enzymatic cascade involves the concentration of the final product, the yield obtained through isolation, the reaction rate, and the step and atom economy. These parameters serve as indicators of the productivity and are primarily affected by the enzyme activity and the equilibrium constants, but also by other factors such as the solvent system, substrate and enzyme concentrations, temperature, pH and interactions between the components. [11]

Enzymatic cascades can be constructed through two approaches: pre-existing natural reaction pathways or conducting retrosynthetic experiments. In the former method, metabolic pathways found in organisms can serve as templates and be adapted for in vitro multi-enzymatic reactions. Retrosynthesis involves working backward from the target molecules to identify the necessary bond formations and subsequently determining the suitable precursors and intermediates. This approach allows for the creation of customized pathways specifically designed to produce unique chemical compounds. However, designing reaction cascades

without an existing template can be a complex task. The cascades are categorized as sequential (one after another) or parallel/simultaneous (occurring concurrently), with or without isolation steps in between. For multi-step reactions, there can be more than one isolation step needed and each step is implemented separately since it typically requires removal of, for instance, the enzyme or intermediates in attempt to prevent undesired reactions and formations of by-products. [11]

4.6 Immobilization of Enzymes

Enzyme immobilization allows for the reuse of biocatalysts, leading to reducing cost of these relatively expensive compounds. [12] Immobilized enzymes are more robust and resistant to environmental changes.[28] Additionally, their heterogeneity can allow for easy recovery of enzymes and products, continuous operation of enzymatic processes, quick termination of reactions, and a wider range of bioreactor designs. [13] Although enzymes have potential applications in a wide range of chemical industries, their biological nature poses challenges for industrial use. Enzymes are typically soluble and susceptible to inhibition by substrates, products, and other components. They also exhibit moderate stability and may not possess optimal catalytic properties for non-physiological substrates. Enzyme immobilisation can be a way to solve these challenges through increased control over reactions and product contamination. [12] Without the ability to be reused, the economic viability of enzymes would be compromised. However, maintaining the structural stability of the catalysts during biochemical reactions presents significant challenges. [14]

There are different immobilization techniques in which can improve enzyme limitations such as factors related to purity, stability, activity as well as specificity and selectivity.[12] Enzymes can be made insoluble and recyclable through immobilization methods, which can be classified into physical and chemical approaches, in which physical methods involves adsorption and entrapment, and chemical methods include covalent binding and crosslinking. [15] The most widely used methods includes using solid supports that bind through covalent linkages or physical bonding. [12] Enzyme adsorption occurs through hydrophobic interactions and salt linkages, providing protection against aggregation and proteolysis. Researchers have explored eco-friendly supports like coconut fibres, microcrystalline cellulose, kaolin, and micro/mesoporous materials for enzyme immobilization. [14]

There is also an increasing usage of methods that do not include a solid support but immobilize the enzyme through enzyme crosslinked aggregates. The enzyme is aggregated and then immobilized by chemical crosslinking. Another similar method involves the crystallisation of pure enzymes and subsequent chemical crosslinking. [12]
The efficiency of the immobilized enzyme depends on how compatible the enzyme is with the chosen immobilization support while also taking into account the enzymes ability to catalyse its target reaction while remaining immobilized. [15]

Another method of immobilization is affinity adsorption, in which the enzyme is tethered to a support material. This is made possible through ionic interaction or covalent binding. The support material are often coated with polymeric structures with varying functionalities including epoxides and amines. Immobilized metal ion affinity binding is a method used for enzyme immobilisation where the metal ions are used as chelating agents by connecting the support matrix to the chelating metal ions through spacer molecules, in which the specific amino acid residue on the polypeptide chain of the enzyme is coordinated with. More specifically the interaction occurs between the polyhistidine-tag (His-tag) of the enzyme and the chelating metal ion. Controlled pore glass carriers (CPG) have shown to be more efficient due to their larger surface area allowing for more enzymes to be immobilised. [17]

Ezig carriers are a hybrid of CPG material (EziG immobilisation) where the support material has been coated with functionalized polymers that provides selective binding and also allowing to be used as an enzyme purification method from cell lysate. For these EziG carriers the metal ion used is Fe^{3+} due to its lower impact on the environment and provides an increased binding stability. EziG carriers come in three types with different surface properties: Opal with a hydrophilic surface, Coral with a hydrophobic surface and Amber with semi-hydrophobic surface. [16] The three carriers differ in pore size distribution due to their polymer coating, in which for Opal is 500 Å and for Amber and Coral 300 Å. [17]

The aim of this study is to investigate the feasibility of an enzymatic cascade for the production of 5-hydroxymethylfurfurylamine. The cascade consists of two main reactions, the first of which involves the decarboxylation of lysine using lysine decarboxylase (LDC) to produce cadaverine. The cadaverine produced will then be utilized as an amine donor in the second reaction, which involves the use of the transaminase derived from *Silicibacter*

pomeroyi, together with 5-hydroxymethylfurfural (HMF) as illustrated in Figure 2. The reaction will be carried out using EziG immobilised enzyme.

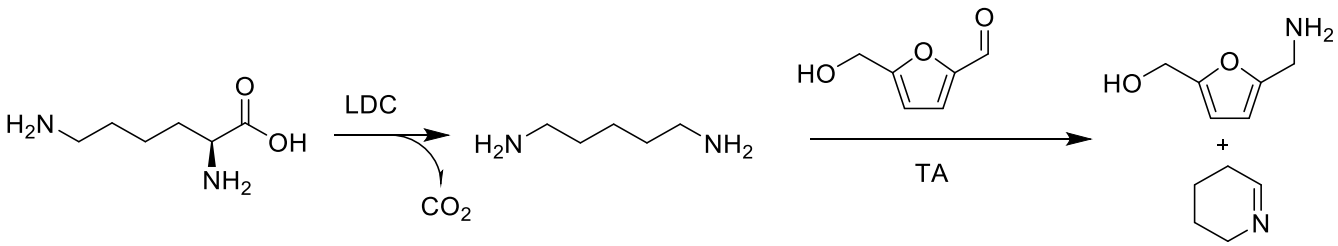

Figure 2. The reaction shows the lysine molecule to the left being decarboxylated by LDC producing cadaverine and by product carbon dioxide. The cadaverine is then used as the amine donor by the transaminase together with an HMF molecule to produce HMFA and a cyclic by product.

5. Methods

5.1 Inoculation and Overnight Culture Preparation

5 ml of lysogeny broth (LB) medium containing ampicillin was inoculated with a glycerol stock of BL21(DE3) cells containing the TA-plasmid. The culture was incubated at 37 degrees Celsius at 200 rpm for 18 h. Terrific broth (TB) medium containing ampicillin was inoculated with 1% (*v/v*) of the overnight culture and incubated at 37 degrees Celsius at 200 rpm until an optical density (OD_{600}) of 600-800 was reached. Once the desired optical density was achieved, induction was performed using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The bacterial culture was incubated at 20 degrees Celsius and 200 rpm for 18 h. The bacterial culture was centrifuged at 4000 rpm at 4 degrees Celsius for a duration of 1h to separate the supernatant and the cell pellet. The supernatant was autoclaved, while the pellet was resuspended in lysis buffer. The resuspended cells were lysed using sonication with 1.5 seconds intervals at 40 % amplitude for one minute, repeated 5x, followed by a centrifugation at 4000 rpm, 45 min to collect the supernatant lysate. Both the supernatant lysate and the pellet were stored in the freezer.

5.2 Enzyme Purification

Enzyme purification was conducted using Ni-NTA His-trap columns binding to the polyhistidin-tag specific to the transaminase. The purification procedure involved a series of washing steps. The following buffers were used: Buffer A: 50 mM HEPES, 300 mM NaCl, pH 7.5; Buffer B: 50 mM HEPES, 300 mM NaCl, 0.5 M imidazole, pH 7.5. Initially, the column was washed with 100 mL distilled water. A second wash was performed using 50 mL of 98 % buffer A and 2% buffer B solution. 5 mL of the lysate was then loaded onto the column. Following the sample loading, the column was washed with 25 mL of 98 % buffer A and 2% buffer B solution, and the resulting flow-through was collected. This was followed by an additional wash step with 25 mL of a 90 % buffer A and 10% buffer B solution, and the corresponding flow-through was collected. Finally, the enzyme was eluted with 10 mL of 50% buffer A and 50% buffer B. The column was subjected to a further washing step using 100 mL of a 2% buffer B solution, and the resulting flow-through was collected.

5.3 SDS PAGE

SDS-PAGE was performed to verify the presence and purity of the transaminase. The procedure involved the preparation of the gel and loading of the samples. Initially, 7.5 µL of a protein ladder (PageRuler Prestained Protein Ladder, Thermo Fisher) was added to a well on the gel. In an Eppendorf tube, 2 µL of the enzyme sample, 2 µL of loading buffer, and 6 µL of distilled water were combined and incubated at 70 degrees Celsius for 10 minutes before it was loaded into the wells. After running the gel at 200 V, the target protein band, typically located around 50 kDa, was examined for sufficient concentration. To visualize the protein bands, the gel was stained overnight in a Coomassie blue staining solution. The following morning, the gel was destained by placing it on a shaking table in a destaining solution composed of 30% ethanol and 10% acetic acid in water. The destaining solution was changed at regular intervals of approximately 30 minutes for the removal of excess stain.

5.4 Bradford Assay

To determine the concentration of proteins, a Bradford Assay was performed. A standard concentration curve was generated using Bovine Serum Albumin (BSA) as the reference protein. A stock solution of 1 mg/mL BSA in 50 mM pH 8 HEPES buffer was prepared, which served as the basis for dilutions. The stock solution was diluted to concentrations of 0.8, 0.6, 0.4, and 0.2 mg/mL using 50 mM pH 8 HEPES buffer, resulting in a final volume of 1 mL for each dilution. From each BSA dilution, 50 µL was transferred to a cuvette. Followed by the addition of 2.5 mL of the Bradford dye reagent, prepared by combining 1 part Bradford dye reagent (BioRad) with 5 parts MilliQ water. The cuvette was carefully inverted to ensure thorough mixing and incubated at room temperature for five minutes to facilitate colour development. Following the incubation, the absorbance of the solutions was measured at 595 nm using a spectrophotometer. Based on the obtained absorbance values and the corresponding BSA concentrations, a calibration curve and a protein standard equation were generated. This was achieved by plotting the absorbance values against the corresponding concentrations. The protein concentration of the samples was determined in the same way.

5.5 Nanodrop

An alternative method to determining the enzyme concentration was performed through Nanodrop spectrophotometry. The absorbance was measured of a drop of 2-3 µL of enzyme solution.

5.6 Activity assay of free enzyme

The acetophenone activity assay was conducted to evaluate the catalytic performance of the free transaminase, the assay quantifies the production of acetophenone resulting from the transamination reaction involving *S*-(α)-methylbenzylamine as the amine donor and sodium pyruvate as the acceptor. The assay involved a reaction mixture comprising 2.5 mM *S*-(α)-methylbenzylamine, 2.5 mM sodium pyruvate, and 5 µL of a 1 mg/ml enzyme solution containing 0,1 mM PLP in 50 mM pH 8 HEPES buffer. The reaction mixture was added and inverted, ensuring proper mixing of the constituents. The absorbance of the reaction mixture was monitored at a wavelength of 245 nm using a spectrophotometer. Measurements were taken every second for a duration of 3 minutes. By tracking the increase in absorbance over time, the enzymatic activity of the free enzyme could be calculated. [18]

5.7 HMFA/HMF standard curve

To determine the 5-(hydroxymethyl)furfurylamine (HMFA) concentration, a standard curve was created using a 10 mM HMFA stock solution and diluting it to 2,4,6 and 8 mM using 100 mM pH 9 CHES buffer. Then 1 ml samples were added into separate HPLC (high-performance liquid chromatography) vials in triplicates performing the HPLC analysis with 2 % acetonitrile and 98% H_2O with 0,1 % trifluoro acetic acid for 6 min, with detection at 210 nm. Using the area on the chromatogram plotted against the concentration, an equation was obtained for determining unknown concentrations of HMFA. This was repeated for 5-(hydroxymethyl)furfural (HMF).

5.8 Transamination of HMF

Three different transaminase concentrations (0.1 mg/mL, 0.05 mg/mL, and 0.02 mg/mL) were prepared in separate Eppendorf tubes. To each tube, 5 mM HMF, 0.1 mM PLP, and 5 mM Cadaverine were added in 50 mM pH 8 HEPES buffer, resulting in a final volume of 1 mL. The Eppendorf tubes were then incubated for 2 hours at 500 rpm and 37 degrees Celsius to allow the transamination reaction to occur. To stop the reaction, the reaction mixtures were subjected to a temperature of 95 degrees Celsius for 10 minutes. After denaturation, 1 mL of each sample was transferred to HPLC vials. The HPLC analysis was conducted using 2 % acetonitrile and 98 % H_2O with 0,1 % trifluoro acetic acid for 6 min with detection at 210 nm. By quantifying the HMFA concentration, it was possible to assess

the conversion of HMF to HMFA and determine its proficiency in catalysing the conversion of HMF to HMFA.

5.9 pH optimization of transaminase reaction

The assess the optimal pH value for the transaminase reaction, 0.05mg/ml enzyme was added to separate Eppendorf tube together with 2.5 mM HMF, 2.5 mM cadaverine and 0.1mM PLP in 100 mM CHES buffer at pH 8.5, 9, 9.5, and 10. The samples were incubated in a Thermomixer at 500 rpm for 2 h and analysed with HPLC.

5.10 Negative test analysis

In order to verify that the observed HPLC results were solely a result of the enzyme activity and not influenced by other components, a negative control test was performed. Six Eppendorf tubes were prepared, the first Eppendorf tube contained HMF, PLP, cadaverine, buffer but no enzyme. The second Eppendorf tube contained cadaverine, PLP, buffer but no HMF nor enzyme. The third Eppendorf tube contained HMFA, PLP, buffer and no enzyme. The fourth Eppendorf tube contained the enzyme, PLP, cadaverine, buffer but no HMF. The fifth Eppendorf tube contained enzyme, HMF, cadaverine, buffer but no PLP. Lastly, the sixth Eppendorf tube contained enzyme, PLP, HMF, buffer but no cadaverine. By including these negative control samples, we ensured that the observed changes in the chromatogram were specifically a result of the enzymatic activity and not influenced by other components.

5.11 Cadaverine derivatization

To obtain cadaverine derivatives for cadaverine detection through HPLC analysis, duplicate Eppendorf tubes were prepared, to each tube a reaction mixture was added consisting of 300 µL 100 mM pH9 CHES buffer, 100 µL of 100% methanol, 47 µL of distilled water, 50 µL of a 5 mM cadaverine solution in 100 mM pH9 CHES buffer, and 3 µL of diethylethoxymethylenemalonate (DEEMM). These tubes were then incubated for a duration of 2h at 70 degrees Celsius. HPLC analysis was performed using a mobile phase comprising 100% acetonitrile and 25 mM pH 4.8 acetate buffer. The HPLC program was set as follows: from 0 to 2 minutes, a gradient of 20% to 25% acetonitrile was applied; from 2 to 32 minutes, the gradient increased from 25% to 60% acetonitrile; and finally, from 32 to 40 minutes, the gradient decreased from 60% to 20% acetonitrile. The product was detected at 284 nm.

5.12 Cadaverine derivatisation with varying concentrations

The same cadaverine derivatisation method was conducted except the cadaverine concentration was tested for 10 mM, 20 mM and 30 mM with the same reaction mixture. Similarly, the same reaction was conducted using 300 µL 50 mM pH9 Borate buffer with 5 mM and 10 mM cadaverine.

5.13 Enzymatic approach to cadaverine detection

To assess the derivatisation of the cadaverine, the transaminase reaction was conducted in duplicates in which one of the samples didn't contain any enzyme. Both reactions were incubated for 2h at 37 degrees Celsius. After 2 h, 6 µL of diethylethoxymethylenemalonate was added together with 200 µL of 100 % methanol and the reaction was incubated for 2h in 70 degrees Celsius, followed by HPLC analysis in 2 % acetonitrile and 98 % H_2O with 0,1 % trifluoro acetic acid for 6 min.

5.14 Enzyme Immobilisation

The immobilization of the enzyme was carried out using EziG carriers of three different types, namely Opal, Amber, and Coral. A total of 20 mg of each respective carrier was placed in separate Eppendorf tubes. To achieve a final volume of 1 ml, 1 mM PLP and 10 mg of enzymes were added along with 50 mM HEPES buffer, pH 8. The tubes were placed on an end-to-end rotator at 25 rpm for 3h at room temperature. Following immobilization, the supernatant was carefully separated from the carriers through spin columns, and the enzyme concentration in the flow-through was determined using the Bradford assay, as described previously, to calculate the immobilization yield. The carriers were then stored in 50 mM pH 8 HEPES buffer for further use.

5.15 Activity assay of immobilised enzyme

An adapted acetophenone assay was performed to determine the activity of the immobilized enzyme. 1 mg of each carrier was added to a separate Eppendorf tube. To this tube, 2.5 mM *S*-(α)-methylbenzylamine, 2.5 mM sodium pyruvate, 0,1 mM PLP, in 100 mM CHES buffer at pH 9 were added to a final reaction volume of 1 mL. The Eppendorf tubes were placed in a Thermomixer at 1000 rpm and incubated at 37 degrees Celsius. Immediately, a 5 µL sample was taken and added to UV cuvettes and diluted with 995 µL of distilled water. Samples were

taken every 2 min for 10 min. The absorbance of the samples was measured at 245 nm using a spectrophotometer.

5.16 Assessment of optimal EziG carrier type

To determine which EziG carrier was most suitable, the transamination reaction was carried out by the addition of 1 mg of immobilised enzyme from each carrier type, 5 mM cadaverine, 5 mM HMF, and 0.1 mM PLP and 100 mM pH 9 CHES buffer to separate Eppendorf tubes. The samples were then incubated on a Thermomixer for 2h at 37 degrees Celsius at 1000 rpm. Followed by HPLC analysis. A leaching assessment was performed using a Bradford assay on a sample of the completed transamination reaction as described.

5.17 Enzyme immobilisation yield with varying loading

In order to determine the optimal enzyme loading on the EziG carrier, a series of varying concentrations of enzyme loading was immobilised. Specifically, enzyme loadings of 10%, 20%, 30%, and 40% (*w/w*) were tested. Each tube contained 0.1 mM PLP, 10 mg EziG beads of carrier type "Coral", varying amounts of enzyme and 100 mM pH 9 CHES buffer, resulting in a final volume of 1 ml. The carriers with the enzymes were then incubated for 3h in an end-to-end rotator. Following the incubation, the samples were transferred to spin columns, centrifuged, and stored in 100 mM pH9 CHES buffer. The supernatant was analysed using a NanoDrop spectrophotometer to determine the enzyme concentration in the supernatant. This information, combined with the initial enzyme concentration, was then utilized to calculate the overall immobilization yield.

5.18 Testing the reusability of immobilised enzyme

To evaluate the reusability of the immobilized enzymes, 1 mg/ml immobilised enzyme from each EziG carrier type was added to 3 separate Eppendorf tubes together with 5 mM HMF, 5 mM cadaverine, 0,1 mM PLP and 100 mM CHES buffer at pH 9. The samples were incubated for 2h at 37 degrees Celsius on shaker table at 1000 rpm. Followed by centrifugation for separation of the enzyme coated beads enabling extraction of the reaction mixture into HPLC vials. The vials were then used for HPLC analysis as described. The immobilised enzyme was washed using H_2O twice and new reaction mixture was added to repeat the reaction 4x to determine the HMFA concentration at each round.

6. Results and Discussion

6.1 Identification of transaminase

SpTA was obtained through growing the cell cultures, lysing, and lastly purifying it using the polyhistidine-tag of the enzyme for affinity chromatography. For the identification, SDS PAGE was conducted as explained in section 5.3. The obtained results from the SDS PAGE were compared with the theoretical value of 51.7 kDa for SpTA. A distinct band corresponding to a molecular weight of approx. 47 kDa was successfully identified, see Figure 3. [24]

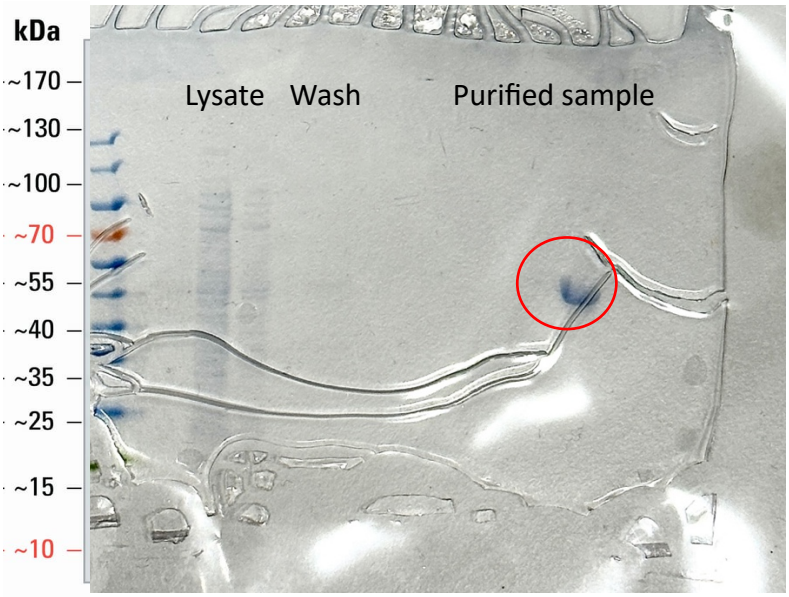

Figure 3. SDS gel of cell lysate, wash steps and purified SpTA, showing a thick band around 47 kDa circled in red with a protein ladder to the left.

6.2 Determination of transaminase concentration

The concentration of SpTA was determined using both Bradford assay explained in section 5.4, and a Nanodrop spectrophotometer as explained in section 5.5. The Bradford assay standard curve shown in Figure 4 was used to calculate the concentration of SpTA to be 8,61 mg/ml. The Bradford assay was used in the beginning to determine the enzyme concentration, however when the Nanodrop spectrophotometer was later available, it was used instead.

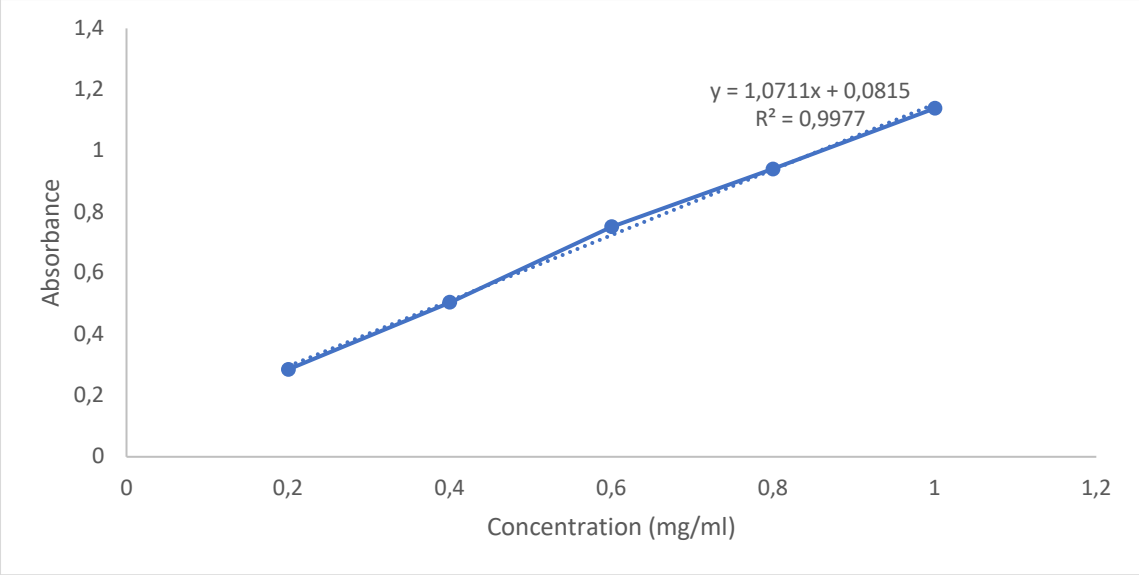

Figure 4. Calibration Curve for Protein Concentration Determination.

The calibration curve illustrates the relationship between the absorbance at 595 nm and the concentrations of Bovine Serum Albumin (BSA) used as standards in the Bradford assay. Each data point represents the average absorbance value obtained from duplicate measurements with a 5 % error. The BSA concentrations ranged from 0.2 mg/mL to 1.0 mg/mL.

6.3 Determination of transaminase activity

The SpTA activity was determined using an acetophenone assay, described in section 5.6. Where the S-methylbenzylamine was used as the amine donor for pyruvate providing the final product, acetophenone together with by product alanine. The activity was then quantified using Beer-Lambert's law, resulting in a value of 2.5 U/mg.

In other studies, values of 4.6 U/mg [19] have been obtained indicating a decrease. The observed activity may be low due to unfavourable conditions. Such as the repeated freezing and thawing cycles to which the SpTA enzyme was subjected during various stages of the experiment. Additionally, carrying out the experiments at room temperature, may have affected the enzyme's stability and activity. [19] The enzyme activity could also vary between batch to batch making it highly specific to the enzyme.

With the known activity of the SpTA it was possible to continue with the transamination reaction using the purified SpTA. For this the SpTA concentration was determined again but instead of a Bradford assay, it was measured using the Nanodrop spectrophotometer method

specified in section 4.5 which gave concentration of 601,2 µg/ml. This can also be due to variation in batches causing the observed difference.

6.4 Determining the optimum transaminase concentration

The transaminase reaction involves the transamination of HMF using cadaverine as the amine donor providing the final product HMFA and by product 1-piperdiene. To identify the most optimum concentration to use in the further experiments, the transamination reaction was carried out using three different enzyme concentrations to compare the concentration of HMFA produced. Table 1 shows that the results were negligible since more than 5 mM HMFA was detected. 5mM HMF was added in the reaction mixture, meaning the conversion cannot surpass 5 mM HMFA. This could be due to incorrect integration of peaks or faulty standard curve for the HMFA. The equation used for the calculation of the HMFA was y = 239704x obtained from a previous study.

Table 1. The three different SpTA concentration is shown in the left column. The middle column shows the peak area of the measured HMFA and the corresponding HMFA concentration is shown in the right column.

Enzyme concentration (mg/ml)	Area	HMFA (mM)
0.1	1062218	4.43
0.05	1483138	6.19
0.02	1551016	6.47

To make sure the HPLC method works correctly, the reaction mixture was added omitting the enzyme and the HMF concentration was measured as shown in Table 2. The concentration was calculated using the following equation, y = 577626x. Table 2 shows a HMF concentration of lower than 5 mM which should not be the case since the enzyme did not participate in the reaction for the conversion of HMF to HMFA to happen. This can be due to dilution errors since buffer was added to replace the volume of the enzyme.

Table 2. The left column shows the peak area of the measured HMF and the corresponding HMF concentration is shown in the right column.

Area	HMF (mM)
1955541	3.39
1994637	3.45
1793402	3.10

A new HMFA standard curve as described in section 5.7 was made seen in Figure 5 to quantify the HMFA product in the transamination reaction for the future experiments instead of using the old equation from another study.

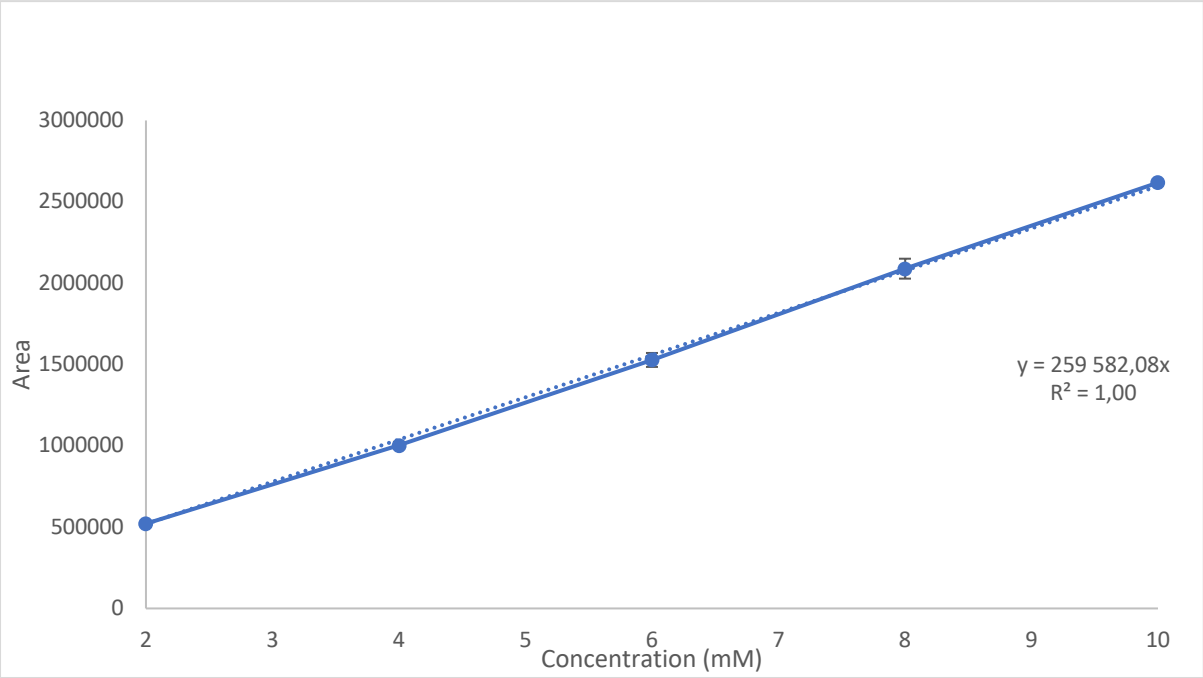

Figure 5. HMFA calibration curve measured with HPLC as described in section 5.7 in triplicates.

6.5 pH test for transaminase activity

To optimise the SpTA activity in the transamination reaction, four different pH conditions were tested in CHES buffer to see how much HMFA was produced as described in section 5.9. The transamination reaction was conducted as explained in section 5.8. The HPLC analysis showed no difference in the HMFA peaks in all of the different pH solutions meaning there could have been a too high enzyme concentration. This could mean that there was so much enzyme present that all of the HMF was converted to HMFA in a short period of time before its activity got negatively impacted by a possibly unsuitable pH.

Since it was not possible to observe any difference in enzyme activity based on its conversion of HMF, the SpTA concentration was diluted 10x and the reaction was carried out. This

resulted in close to no conversion for pH higher than 9 as seen in Figure 6. The HMF peak (RT 4.6 min) is visible in samples with higher pH such as pH 9.5 and 10 leading to the anticipation that the SpTA has lower activity when exposed to pH above 9 inhibiting it from converting HMF into HMFA. The HMFA peak (RT 2.7 min) appears only at pH 9 and 8.5 as well as a smaller peak at pH 9.5. Observing a decrease in HMF concentration and an increase in HMFA concentration. Suggesting that there is an increase in the HMFA concentration in pH 9 and hence allowing for the transaminase to convert HMF to HMFA in those conditions.

Based on these peaks it's clear to see that the HMFA concentration is very low for higher pH while pH around 8.5 and 9 seemed to provide the most conversions. However, transaminases do show a slightly higher activity for pH conditions that are more towards basic conditions [20]. The future experiments were hence decided to be carried out in pH 9 CHES buffer.

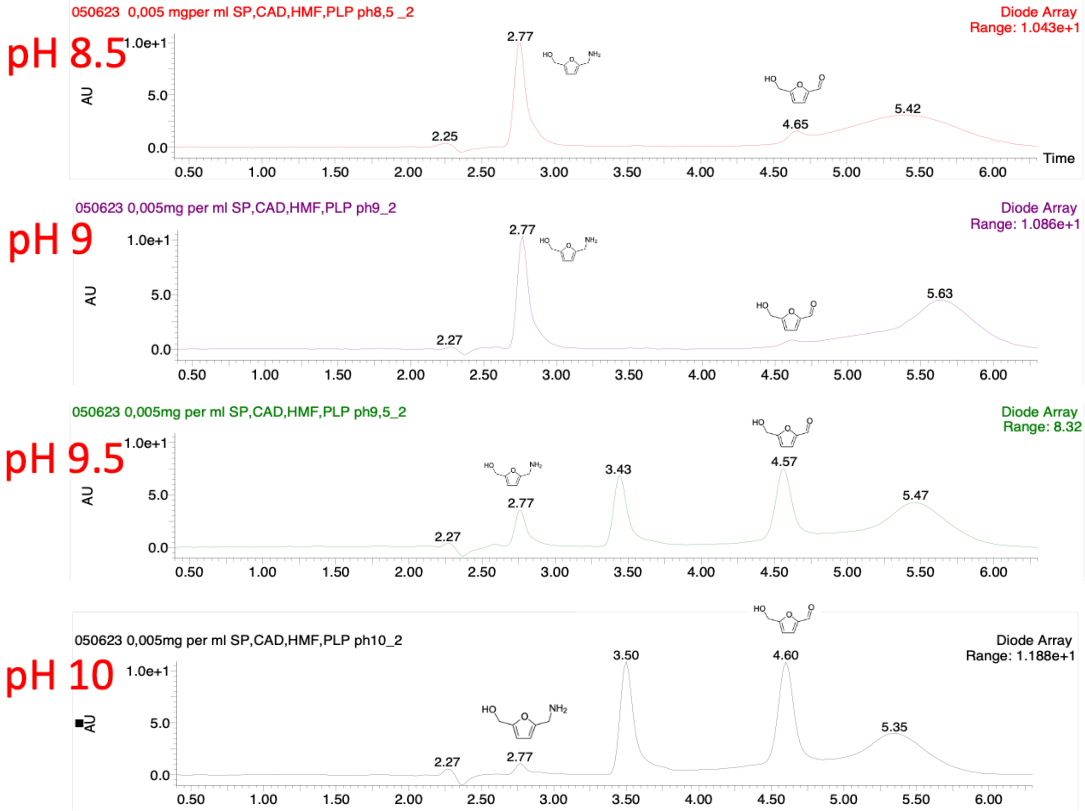

Figure 6. HPLC chromatogram of the transaminase reaction of HMF to HMFA at different pH ranging from pH 8.5, 9.5 and 10. HMF has a retention time of 4.6 min and HMFA has a retention time of 2.7 min.

6.6 Negative control of transaminase reaction

A Negative control were carried out at pH 9 to verify that the findings were not a product of any other unknown side reactions occurring and to ensure validity of the results obtained. The negative test included six different reaction mixture shown in Table 3. As seen from the results, it was possible to verify that the finding solely was due to the transamination reaction and no other side reactions were occurring producing the same results. For the reactions without any SpTA, it can be concluded that the product HMFA was not formed which is desirable. It potentially also verified an unknown peak that would occur around the time point 3.5 minutes that can also be seen in Figure 6 which was hypothesised to be the imine product created as a by-product when cadaverine donates its amine group. The last three reactions had SpTA incorporated however, by removing the substrate HMF it was shown that no product was formed. This was also tested by not adding the amine donor cadaverine. Lastly, by not incorporating the cofactor PLP, it could also be seen that the enzyme was unable to perform its function as it should to form HMFA.

Table 3. Six reactions containing different components shown in the left column and the results of those reactions based on HPLC analysis shown in the right column.

Reaction mixture	Result
HMF, PLP, Cadaverine, CHES buffer	No HMFA formation
PLP, Cadaverine, CHES buffer	No imine formation
PLP, HMFA, CHES buffer	No imine formation
SP, PLP, Cadaverine, CHES buffer	No HMFA
SP, PLP, HMF, CHES buffer	No HMFA
SP, HMF, Cadaverine, CHES buffer	No HMFA

6.7 Cadaverine detection

Once the Negative control were successful, the second reaction of the cascade was ready to be tested. Where the substrate lysine gets decarboxylated by lysine decarboxylase producing cadaverine and carbon dioxide. However, a method for detecting cadaverine is needed. Previous methods used the derivatization agent DEEMM to make cadaverine detectable and providing a peak with detection at 284 nm (RT 30 min) through HPLC analysis. [21] When the method was carried out small peak at 15 minutes was observed instead, whereas a cadaverine peak at 30 min us expected in literature. This could be due to the difference in HPLC analysis column used.

To confirm that the peak was associated with the cadaverine detection, another analysis using three different concentrations of cadaverine was conducted as explained in section 5.12. The rationale behind this approach was that a larger peak would be correlated to the higher concentration of cadaverine. Through this method it could be concluded the peak observed was cadaverine. The results from these reactions as shown in Figure 7 are not consistent. At 5 mM cadaverine, a small peak was observed, and for 10 mM the peak was larger however for the 30 mM the peak was smaller again. The detection of cadaverine with this method was unsuccessful. A hypothesis for this behaviour could be that not enough DEEMM has been used and it would require further calculations to optimise the amount of DEEMM needed for a certain concentration of cadaverine to undergo complete derivatisation.

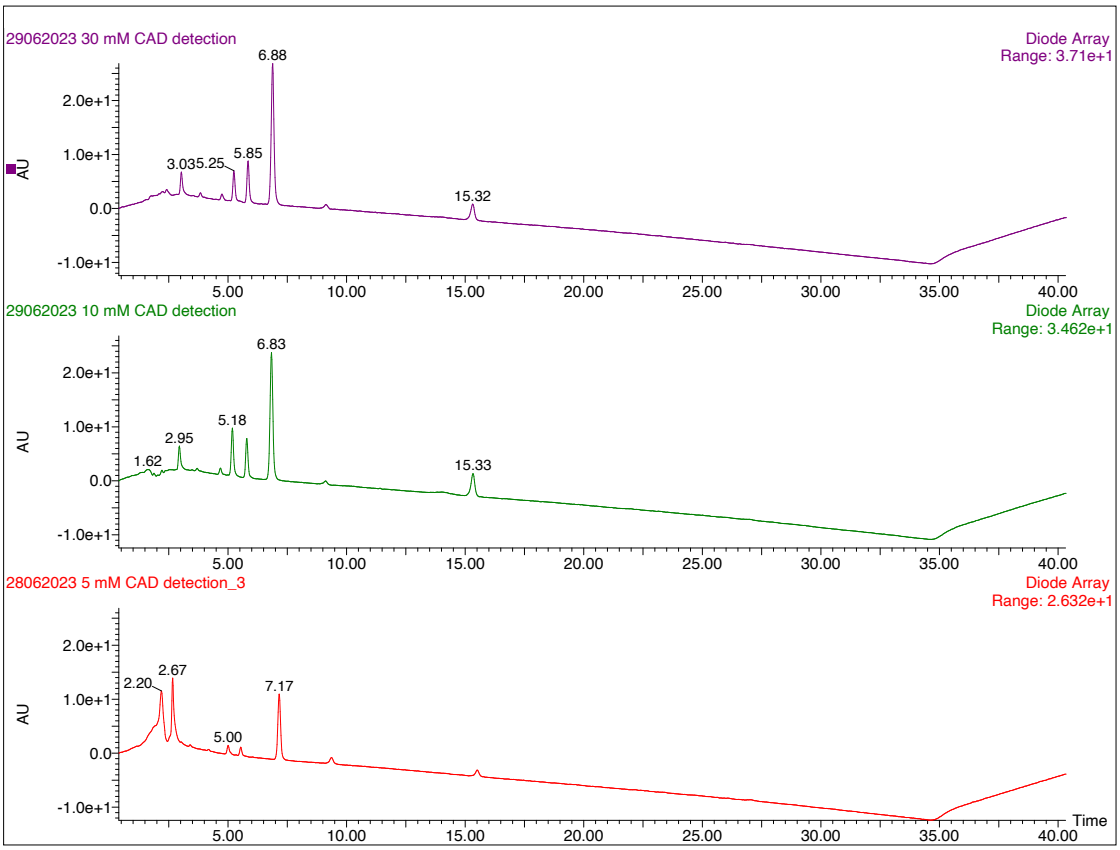

Figure 7. The HPLC chromatogram of derivatized cadaverine with 30 mM, 10 mM and 5 mM of cadaverine. The peak of interest (RT 15 min) is presented.

An alternative method to try and detect the cadaverine was through an enzymatic approach as explained in section 5.13. The transaminase reaction was conducted in duplicates with one of the reactions was lacking SpTA. This would allow for potentially observing a difference between the sample with SpTA and without. For the sample with SpTA, the hypothesis is that the transaminase would convert the cadaverine leading to the absence of its peak on the HPLC analysis while the sample without SpTA would still have the cadaverine and would hence provide a peak. The samples could not be analysed, the HPLC machine was experiencing technical malfunctions and was temporarily inoperable. The problem could not be resolved within the timeframe; hence this part of the study could not be fulfilled.

6.8 Immobilization of transaminase

A key part of this study was to conduct the conversion of HMF to HMFA using immobilized enzymes. The immobilization was conducted as explained in section 5.14 in which three different carrier types of Ezig carriers were used. To analyse immobilization yield, the flow through of the samples after immobilization were analysed to measure the concentration of unbound SpTA. The flow through concentration is presented in Table 4 for each carrier type showing the immobilization yield. Depending on the carrier, a 50 -60 % immobilization yield from the initial 10 mg/ml SpTA was achieved. The expected immobilization yield was around 60 %. [22]

Table 4. Each carrier type is shown in the left column with their corresponding flow through concentration observed in the right column.

Carrier type	SpTA concentration in flow through concentration (mg/ml)	Immobilization yield (%)
Opal	4.44	55.6
Amber	4.76	52.4
Coral	3.95	60.4

6.9 Activity assay of immobilized transaminase

Once the enzyme was immobilized an activity assay was performed on the immobilised enzyme as explained in section 5.15, Where the S-methylbenzylamine was used as the amine donor for pyruvate providing the final product acetophenone together with by product alanine. This was done to understand how the SpTA activity was affected by the immobilization in comparison to when it was purified. The assay was also performed to determine which of the carriers allows for the highest activity of the immobilized enzyme.

The results are shown in Figure 8 for Amber, Figure 9 for Coral, and Figure 10 for Opal. The absorbance of acetophenone showed an increase as time progressed however for Opal and Amber the absorbance decreased after eight minutes. Similarly for Coral it started fluctuating after six minutes.

The preferred outcome would have been a linear increase in absorbance as the reaction progressed. The alternative expectation would be for the absorbance to increase until it reaches a steady state. Meaning more acetophenone isn't produced after a certain time. However, the obtained results show a steady increase for the first 6-8 minutes and then a lower absorbance is observed meaning less acetophenone is measured. A hypothesis for this could be that the acetophenone is experiencing degradation over time from being unstable in the reaction environment. Another hypothesis is that acetophenone has a higher density than water, hence could be sinking in the Eppendorf tube. [23] Meaning when new samples were taken every 2 minutes, acetophenone might now have been dispersed properly in the tubes leading to lower amounts being pipetted out for analysis, which makes the results faulty and unreliable. Hence to determine which carrier type would be a better fit for the enzyme based on enzyme activity could not be concluded.

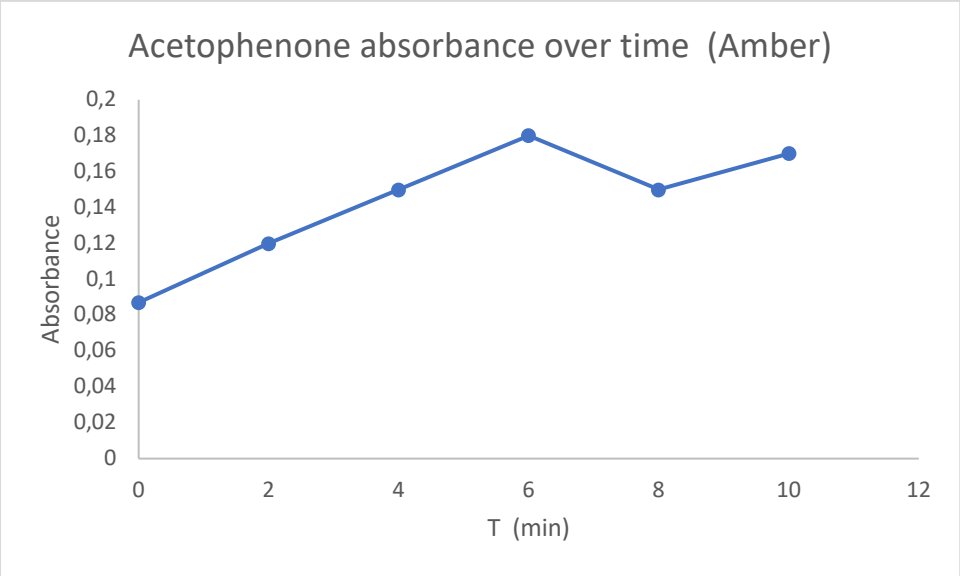

Figure 8. Absorbance of acetophenone of several points in time produced by transaminase reaction of S-MBA and pyruvate using SpTA immobilized on Amber.

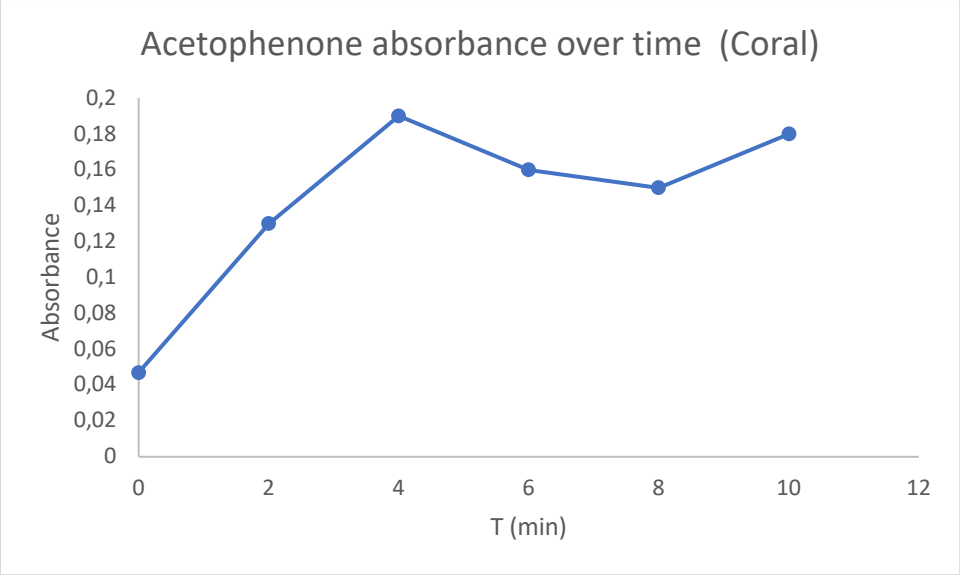

Figure 9. Absorbance of acetophenone of several points in time produced by transaminase reaction of S-MBA and pyruvate using SpTA immobilized on Coral.

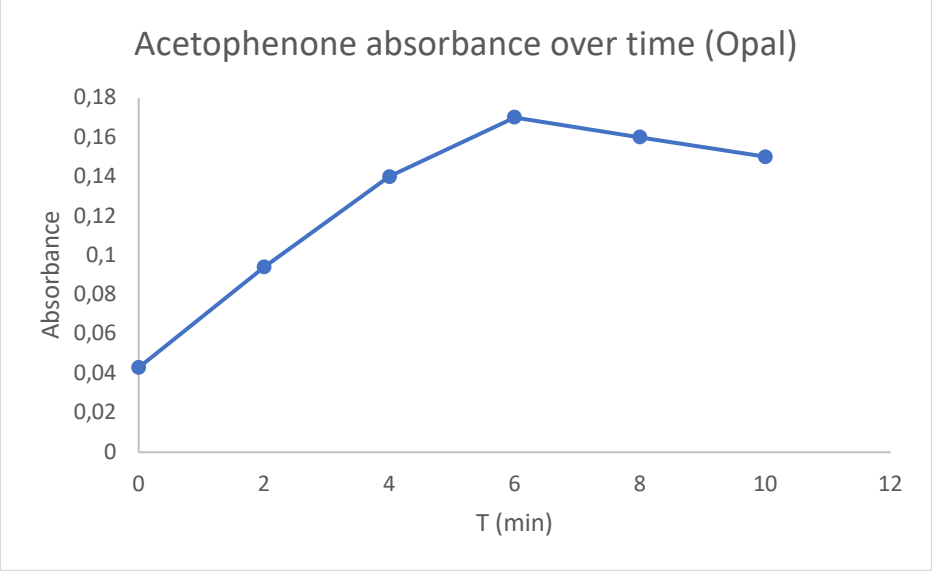

Figure 10. Absorbance of acetophenone of several points in time produced by transaminase reaction of S-MBA and pyruvate using SpTA immobilized on Opal.

To determine the ideal carrier type, another approach was also conducted as explained in section 5.16. The transamination of HMF with cadaverine was performed using the immobilized enzymes and the HMFA produced was quantified. By comparing the concentration of HMFA formed between the three types of carriers, the most effective carrier type out of Amber, Coral and Opal could be picked.

As seen in Table 5, the carrier type Coral has the highest conversion of HMFA. Additionally, the leaching was also assessed using Bradford assay. The results in Table 6 show that the carrier type Coral had the most leaching. Since Coral showed the highest conversion with Opal coming second place, as well as Opal coming second place for leaching, it was decided to continue with Coral and Opal for the following planned experiments.

Table 5. HMFA concentration after conversion of HMF with SpTA immobilized of Opal, Amber and Coral.

Carrier type	HMFA concentration (mM)
Opal	3.10
Amber	2.79
Coral	3.63

Table 6. SpTA concentration in supernatant after transaminase reaction using Nanodrop spectrophotometer.

Carrier type	SpTA concentration (mg/ml)
Opal	0.0559
Amber	0.0375
Coral	0.0679

6.10 Determination of ideal transaminase loading for immobilization

To determine the ideal enzyme loading for the immobilization, different enzyme loadings were tested as explained in section 5.17. The test was only conducted for carrier type Coral and the results are shown in Table 7. The immobilization yield has been calculated based on the enzyme concentration at start, subtracted by flow through concentration divided by the enzyme starting concentration. Table 7 shows that the lowest enzyme loading of 10 percent gave the highest yield of 94 %. The immobilization yield also appears to be decreasing as the enzyme loading increases. This could be because the carrier may have a maximum capacity for enzyme binding which only allows for the lower loading to be efficient.

Table 7. immobilization yield (%) of different amount of enzyme loading on carrier Coral.

Enzyme loading (%) (w/w)	Immobilization yield (%)
10	94
20	82
30	74
40	62

6.11 Reusability assessment of immobilized SpTA on Coral and Opal

The reusability of SpTA immobilized on carrier type Opal and Coral was tested. This was done through the transaminase reaction involving SpTA using cadaverine to convert HMF to HMFA. This was conducted four times reusing the SpTA immobilized on Coral and Opal. The HMFA concentration from each reaction cycle for both carrier types, Coral and Opal, is shown in Table 8. The carrier Coral has the higher conversion of HMFA compared to Opal. In the last round, both carriers show zero amount of HMFA. With these results it was able to conclude that the carrier Coral was able to continue to produce HMFA when being reused which was surprising since Coral was also the one that would show the highest leaching. However, regarding the reusability, it was not expected that the SpTA would stop converting HMF to HMFA in only four rounds. Further optimization could be investigated to achieve higher concentration of HMFA in each reaction cycle allowing for the immobilized SpTA to be continuously used.

Table 8. HMFA concentration after reaction of each round for the SpTA immobilized on Coral (middle column) and Opal (right column).

Round	HMFA concentration(Coral)	HMFA concentration(Opal)
1st	2.74 mM	2.37 mM
2nd	0.068 mM	0.044 mM
3rd	0.021 mM	0 mM
4th	0 mM	0 mM

7. Conclusion

In this study, the aim was to explore the feasibility of utilizing an enzymatic cascade for the production of 5-hydroxymethylfurfurylamine (HMFA). This involved two main reactions: the decarboxylation of lysine using lysine decarboxylase to produce cadaverine as amine donor, followed by the transamination of cadaverine with 5-hydroxymethylfurfural (HMF) using a transaminase from *Silicibacter pomeroyi* (SpTA). While the cascade could not be completed due to multiple challenges, some insights were gained. The work and challenges provided a deeper understanding on the intricacies of the individual reactions and the immobilization processes and conditions applied. Challenges such as delayed arrival of the lysine decarboxylase hindered the cascade reaction to be performed as well as the time frame limit regarding the optimization of cadaverine detection which required more time than anticipated. Other challenges such as inconsistent results seen with the immobilization carriers also contributed to the excessive amount of time used and simultaneously underscored the complexity of enzyme immobilization and the quality of the carriers. Nevertheless, the transamination reaction itself yielded positive results and with extended time could be used to complete the cascade. In conclusion, while the study encountered challenges preventing full disclosure of the intended cascade, invaluable insight was gained. Sustainable and efficient biocatalytic pathways are still essential from an environmental perspective and hence should be continuously explored and refined.

8. Future Perspectives

With regards to future improvement many experiments in this study could be revaluated in terms of the duration that was expected for them to be done. Since many crucial milestones required more time and effort than planned, such as determining enzyme concentration required a calibration curve which needed to be close to accurate in order to be used and hence redone multiple times. This in turn delayed the process of verifying the concentration and being able to continue with the other experiments. Similarly, the use of common equipment such as HPLC machines and spectrophotometers contributed to delays as well since booking of these equipment were not considered when planning this project.

Certain methods used in this study required to be refined in order to carry out the experiments more accurately and effectively. As the enzyme was immobilised, the activity assay performed on them needed different concentrations of enzyme to be tested, hence the carriers had to be manually removed from Eppendorf tubes and weighed. This led to the carriers to be spilled and wasted but also due to the wet weight, not accurately weighed either. This in turn leads to many errors in the final results and can't necessarily be reliable unless the loss is negligible. An alternative method was used towards the end of this study, which was creating a slurry and pipetting from that, however the accuracy can not be certain. Therefore, further investigation needs to be done for optimising the extraction if immobilised enzyme without too much loss or waste.

Another aspect to consider in the future is how well functioning the equipment used is, in this case for the HPLC analysis. In regards to detecting cadaverine, different methods were carried out to find correlation between cadaverine and the observed peak. The last attempt was using two reactions in which only one of them contained the SpTA. The reaction mixture was prepared, and the samples had been incubated for the reaction to occur, however the HPLC machine had malfunctions preventing the samples from being analysed. Causing waste of reaction mixture, time used to prepare but also incomplete results. In general, many tries had to be performed for analysing samples due to sudden errors or samples not being processed for hours. At certain times, the column had experienced difficulties with washing as well which leads to wrong or inconsistent results. This in turn led to the samples to be re-run and causing the time needed to be doubled and delaying the next steps even further.

The main hurdle this study faced was the delayed arrival of the lysine decarboxylase which was involved in the first reaction. There were also problems identifying it, once purified, since no protein sequence or molecular wight was known. Hence the main idea of this study, the creation of an enzymatic cascade, was never fulfilled and little data for future steps could be concluded.

9. Acknowledgements

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