Exploring Biosynthetic Pathways for Aromatic Ester Production

by

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ABSTRACT

Synthetic biology and metabolic engineering has aided the production of chemicals using renewable resources, thus offering a solution to our dependence on the dwindling petroleum resources. While a major portion of petroleum resources go towards production of fuels, a significant fraction also goes towards production of specialty chemicals. There has been a growing interest in recent years in commercializing bio-based production of such high value compounds. In this thesis the biosynthesis of aromatic esters has been explored, which have typical application as flavor and fragrance additive to food, drinks and cosmetics. Recent progress in pathway engineering has led to the construction of several aromatic alcohol producing pathways, the likes of which can be utilized to engineer aromatic ester biosynthesis by addition of a suitable enzyme from the acyltransferase class. Enzyme selection and screening done in this work has identified chloramphenicol O-acetyltransferase enzyme(*CAT)* as a potential candidate to complete the biosynthetic pathways for each of 2-phenethyl acetate, benzyl acetate, phenyl acetate and acetyl salicylate. In the end, *E. coli* strains capable of producing up to 60 mg/L 2-phenethyl acetate directly from glucose were successfully constructed by co-expressing *CAT* in a previously engineered 2-phenylethanol producing host.

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INTRODUCTION

1.1 Motivation

Chemicals and fuels are primarily derived from petroleum sources, which are non-renewable. The increasing demand and fast depleting petroleum resources pushes the need to find sustainable and renewable platforms for their production. To address this problem, scientists have been looking at biological routes as an alternative to chemical synthesis. Biological pathways offer a cleaner and greener method for synthesizing the fine chemicals and fuels to satisfy the growing demands. Biological pathways also tend to be less energy intensive as compared to chemical synthesis pathways.

Esters are industrially valuable chemicals that have been the target of biosynthesis projects. They have a wide range of applications depending on their size and structure: Ethyl and methyl esters of C14 to C20 fatty acids are used as biodiesel [1]. Fatty acid methyl and ethyl esters have properties similar to petroleum derived diesel which makes them an alternative to the conventionally used fuel [2]. Wax esters of long chain acids and alcohols are also excellent lubricants[1]. Long chain alkyl acetate esters are use in cosmetics. Aromatic esters are naturally produced in plants and fruits which gives them their peculiar aroma. They are artificially synthesized to add flavor and fragrance to food, drinks, cosmetics etc[3].

Conventional chemical synthesis of esters requires petrochemicals like benzene and toluene to start with. Chemical synthesis also requires acidic conditions and high temperature which adds to the cost of production. Microbial biocatalysts are generally

constructed using *Escherichia Coli* or *Saccharomyces Cerevisiae* as host strains which allow the production to take place in ambient temperature and neutral pH.

By applying strategies of synthetic biology and metabolic engineering, microorganisms can be engineered to bring about a shift from petrochemicals and petroleum based fuels to biochemicals and biofuels. Continued efforts have been made in this regard to expand the potential of microbial cell factories to produce a wide range of renewable chemicals. The aim of this research is to demonstrate the production of aromatic acetate esters using engineered *E.coli*.

1.2 Role of Synthetic Biology

The main goal of synthetic biology is to design biological systems that can produce useful chemicals from renewable starting materials. By applying engineering strategies microbial systems can be redesigned to direct carbon and electron flux from the substrate towards the production of target compounds. Each reaction in a biological pathway is catalyzed by a specific enzyme. As each enzyme generally catalyze only a single reaction, a series of enzymes are required for catalyzing multiple reactions required for the bio-production of a target compound. In this way, cells can function as 'microbial-factories' which takes in the substrates and excretes our target bio-product.

Thus synthetic biology provides a platform for the production of biochemical and biofuels which can act as alternatives to petrochemicals and petroleum based fuels.

1.3 Acyltransferases

Esters are naturally produced in various microbes and in higher organism like plants and animals. The final step for ester production in all these organisms is catalyzed by enzymes that belongs to the acyltransferases class (EC 2.3.1) (Fig 1). Literature survey shows that the acyltransferase enzymes in these ester producing organisms mainly belong to these two subcategories within the EC 2.3.1 group: Long-chain-alcohol O-fatty acyltransferase (EC 2.3.1.75) and alcohol O-acetyltransferase (EC 2.3.1.84). These enzymes are coenzymeA (CoA) dependent, they catalyze the transfer of acyl group from an acyl-CoA molecule to a hydroxyl, thiol or amino group of an acceptor molecule[4]. The release of CoA molecule releases the stored energy in the thioester bond which provides the free energy required for the esterification reaction to occur. Acyltransferases are known for their general ability to accept a wide range of substrates. They are very diverse in their structure, molar mass and amino acid sequence[4]. Characterization of enzymes belonging to this class from different sources, as seen in previous literature, has shown that these enzymes are capable of producing a range of esters apart from the ones naturally produced in their native sources[5-7]. The naturally produced esters in an organism depends on the precursor substrates available. Hence it is important to isolate and characterize the acyltransferase enzymes to understand their full range of functionality so they can be used to construct synthetic pathways to enable the bio-production of a variety of esters.

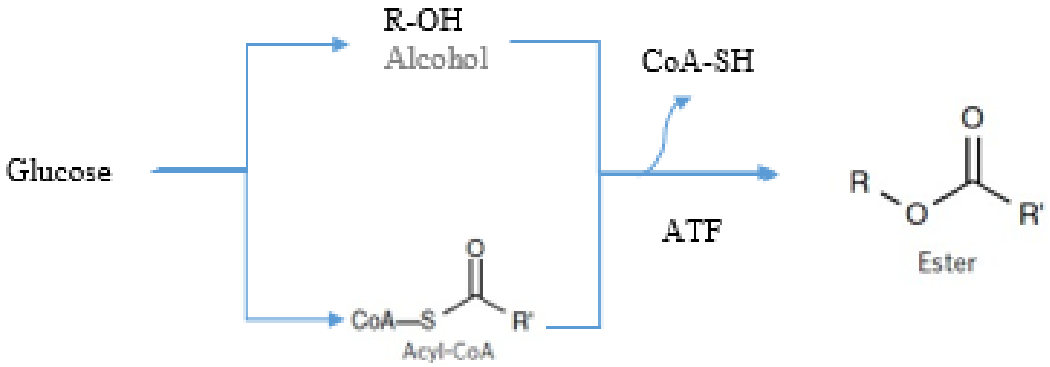

Figure 1: Ester Production Pathway from Glucose

The wide range of structure and functionality of the acyltransferases can be attributed to both converging and diverging evolution of enzymes. Several classes of acyltransferase enzyme that do not show any sequence similarity have been identified from among prokaryotes and mammals[4]. This shows the diverging evolution of genes, which enables the diversified functionality of acyltransferase class of enzyme. On the other hand, enzymes within a particular subcategory, which have similar functionality may have different evolutionary origins, which could be the result of converging evolution. Thus selecting the right candidate for catalyzing the ester biosynthesis reaction required studying acyltransferase enzymes from varied sources. Extensive studies have been done in this regard were genes form different sources are isolated and heterologously expressed in yeast or *E. coli* host cells. These studies have led to the identification of enzymes that can be used for the biosynthesis of fatty acid esters which have potential use as biodiesel. Studies have also shown that microbial biosynthesis of esters can be expanded to produce short branched or aromatic esters[3,6,8], although not much characterization studies have been done in this respect. This thesis aims at identifying

candidates which can be used as biocatalysts for the production of aromatic acetate esters which have potential use as flavors, fragrances, pharmaceutical drugs etc.

1.3.1 Wax Ester Synthase/Acyl-CoA: Diacylglycerol (WS/DGAT)

The bacterial Long-chain-alcohol O-fatty acyltransferase (EC 2.3.1.75) are also known as wax ester synthase/acyl-CoA: diacylglycerol (WS/DGAT) acyltransferase. These enzymes are responsible for the production of wax esters in various organisms. Wax esters are more common in plants and animals and is produced only in a small bracket of bacteria[9]. The WS/DGAT are bifunctional enzymes that catalyze the final steps of both triacylglyceride (TAG) and wax ester biosynthesis by condensation of Acyl-CoA and diglyceride or alcohol. Homologs of this gene have been identified in various bacterial and mammalian species.

Barney et al. studied the substrate specificity of WS/DGAT from four bacterial sources: *Marinobacter aquaeolei* VT8, *Acinetobacter baylyi, Rhodococcus jostii* RHA1, and *Psychrobacter cryohalolentis* K5. They showed that the wax esters and TAGs accumulated naturally in these species differed significantly. Also, each of these enzymes showed a conserved motif HHXXXDG, but a very low similarity in their amino acid primary sequence[5]. When these enzymes were characterized in-vitro, they showed highly conserved behavior for a range of fatty alcohols and fatty acyl-CoAs. All the enzymes they studied showed the highest activity for C-14 acyl-CoA with dodecanol used as co-substrate and the activity reduced with reducing chain length of the acyl-CoA molecule. Enzymes where also characterized for activity towards straight chain alcohols from C-8 to C-18 chain length with palmitoyl CoA (C-16 CoA) used as co-substrate. They showed highest activity towards C-11 and C-12 alcohols and the activity reduced with increase or

decrease in chain length. The enzymes also showed similar behavior when compared for their activity towards a branched (isoamyl alcohol), straight chain (hexanol) and aromatic alcohol (phenethanol) with palmitoyl CoA as co-substrate. This study gives us a comparative understanding of the behavior of alcohol acyltransferase(*AAT*) enzymes from different species of bacteria.

Based on the study we can say that the WS/DGAT enzymes derived from bacterial species show similar substrate preference and also show a conserved motif(HHXXXDG) which is believed to be the active site. We can also conclude that although the natural gene products do not tell us all the possible reactions that can be catalyzed by the enzymes, it does give us a basis to predict the possible range of substrates that the enzyme works best with. As in this study it showed that the bacterial WS/DGAT shows much lower preference for short alcohols and acyl-CoA molecules as compared to medium chain length alcohol molecules as the former is not naturally produced in the native species[3].

1.3.2 Alcohol O-acetyltransferase

The EC 2.3.1.84 subclass of enzymes are generally found in species of yeasts, flowers and fruits. These are responsible for the fruity aroma and the fragrance in flowers[6]. The acyltransferase enzymes from higher plants and fungi are broadly classified into the BAHD superfamily[3,10], including Benzyl alcohol *O*-acetyltransferase (BEAT) from *Clarkia breweri,* Anthocyanin *O*-hydroxycinnamoyltransferase (AHCT, a benzoyl/hydroxycinnamoyl-CoA acyltransferases) from *Gentiana triflora*, Hydroxycinnamoyl/benzoyltransferase (HCBT, a benzoyl/hydroxycinnamoyl-CoA acyltransferases) from *Dianthus caryophyllus* and Deacetylvindoline 4-*O*-

acetyltransferase (DAT) from *Catharanthus roseus* [11]. Most members of BAHD superfamily are found to possess two conserved motifs: HXXXD located in the middle of the protein sequence, which is responsible for catalytic activity and DFGWG located near the carboxylic end which is responsible for structural integrity[10,12]. The HXXXD motif is also found in some other acyl-CoA utilizing acyltransferase enzymes but are absolutely conserved within the BAHD family[12].

The alcohol O-acetyltransferase (EC 2.3.1.84) enzymes from numerous plants and fruits including melon, apple, banana, strawberry etc. have been isolated and characterized in numerous studies in the past two decades[6,8,13,14]. Studies have been done across species of fruits like apple, melon, strawberry, papaya, tomato etc. as well as flowers like rose, petunia etc. It has been demonstrated that the alcohol-o-acetyl transferase is responsible for the production of volatile esters which gives the peculiar flavor and fragrance to these fruits and flowers. Souleyre et.al studied the activity of *MpAAT1* enzyme from an apple species (Royal Gala) [6]. The enzyme showed activity towards medium chain length and aromatic acetate esters. The Km values calculated with alcohol as the limiting substrate were in millimolar range and the Km values calculated with acetyl-CoA as limiting substrate is in micromolar range. Hence alcohol binding with the enzyme is the rate limiting step of the reaction. Beekwilder et.al studied the functional similarity between acyltransferase enzymes of cultivated strawberry(SAAT), wild strawberry(*VAAT*) and a species of banana(*BanAAT*)[13]. The study did not show much similarity between the activity of enzymes between these species. All three enzymes showed some activity towards the tested short and medium chain alcohol, branched chain alcohol and aromatic alcohols to varying degrees. *BanAAT* showed some similar behavior towards *SAAT* in

terms of showing higher preference towards octanaol and geraniol as substrate and low preference towards short alcohols (methanol and ethanol). On the other hand, *VAAT* which is more closely related to *SAAT* did not show any similarity. This study shows that we cannot predict the functionality of the enzymes based on the genetic closeness of the native organism, different species of the same organism might show distinct behavior. Apart from fruits and flowers, volatile esters are also known to be produced by yeasts during fermentation[3]. Two of the most characterized enzymes in yeast responsible for ester formation are the *ATF1* and *ATF2* enzymes from *S. cerevisiae* which also belong to the alcohol o-acetyltransferase (EC 2.3.1.84) subclass. These studies have revealed that *ATF1* and *ATF2* are responsible for the production of volatile esters like ethyl and isoamyl acetate as well as less volatile esters like C5-C8 alcohol acetate esters and 2-phenethyl acetate during fermentation in *S. cerevisiae*.

1.3.3 Chloramphenicol O-acetyltransferase

The chloramphenicol O-acetyltransferase enzyme belongs to the acyltransferases class EC 2.3.1.28. It is commonly used for antibiotic resistance in *E. coli*. *CAT* hasn't been previously screened for activity towards different alcohol substrates, but it is reported to have similarity in structure and functionality to the BAHD family of enzymes, thus making it an interesting candidate for this research. The *CAT* type I,II and III has the conserved motif HXXXD as the BAHD family[4] . *CAT* also follows the reaction mechanism which is followed by acyltransferases that have a conserved histidine active site domain[4]. The free electron pair of nitrogen on the active histidine (His-195) site attacks the oxygen on the hydroxyl group of chloramphenicol. The nucleophilic oxygen then attacks the carbonyl carbon of the acetyl-CoA molecule to form an intermediate.

This intermediate then breaks down to give chloramphenicol 3-acetate and the free CoA molecule[4]. Thus the HXXXD motif is believed to be the active site in *CAT* which is responsible for acyltransferase reaction. *CAT* is also known to have conserved functional residues and conserved secondary structure at the HXXXD residue region, with the BAHD family.

On scanning through the amino acid sequences of enzymes grouped in the *CAT* subclass(source Uniprot database) it was observed that they have a highly conserved motif HHXXXDG towards the end of the sequence. HHXXXDG is also the conserved motif observed in long chain alcohol O-fatty acyl transferase of bacterial species which are capable of catalyzing reactions to produce wax esters. The presence of this motif could suggest a similar functionality in *CAT*. Two previous studies have shown that *CAT* shows functionality towards some ester producing reactions. In an isobutyl acetate production study using *ATF1* from *S. cerevisiae*, ester production was observed during a negative control were *ATF1* was not added. This was later discovered to be due to the activity of Chloramphenicol acetyltransferases(*CAT*) which was present in the plasmid[3]. Another work demonstrated that the chloramphenicol acetyltransferase is responsible for the acetylation of the hydroxyl group in perillyl alcohol(POH) which is an aromatic alcohol with structure as shown in figure 2[15]. These studies have shown that chloramphenicol acyltransferase is a potential candidate catalyzing a variety of ester biosynthesis reactions.

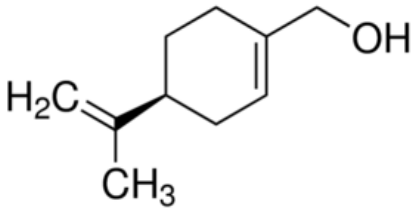

Figure 2: Perillyl Alcohol Structure

1.4 Engineering Ester Producing Pathways

Screening and characterizing of enzymes is the first step towards creating a microbial cell factory which can produce the desired product from a carbon source like glucose with no other added substrate. Constructing ester producing microbial cell factories involves combining acyl-CoA and alcohol producing pathways, using acyltransferase enzyme to catalyze the final step. Most of the research work done so far in this respect has concentrated on the production of fatty acid methyl and ethyl esters (FAMEs and FAEEs respectively) which have potential use as biodiesel[16-22]. This is achieved by overexpressing fatty acyl-CoA producing pathway through acetyl-CoA and malonyl-CoA as precursors and expressing suitable wax synthase enzyme. Rodriguez et.al showed that *ATF1* from *S. cerevisiae* can be used to catalyze the final step for synthesis of a range of esters[3]. These included acetate esters of short chain alcohols (ethanol, propanol, butanol), branched alcohol (isobutanol, 2- methyl butanol, 3-methyl butanol) as well an aromatic alcohol (2-phenylethanol) [3]. Their work demonstrated the potential of expanding the microbial cell factories towards production specialty chemicals. Recent works have demonstrated the potential of pathway engineering to produce aromatic alcohols[11]. This

progress in pathway engineering opens the door to create microbial cell factories that can produce aromatic esters.

1.5 Target Aromatic Esters of Interest

2-Phenethyl acetate is a highly valued compound in the flavor and fragrance industry due to its rose like aroma and raspberry like taste. It is used as an additive to cosmetics, bathing products, food and drinks. It is also used as a precursor for the production of styrene. It is naturally produced in many flowers and fruits during ripening season. It is ester formed by the condensation of 2-phenylethanol and acetic acid. 2-Phenylethanol is commercially produced using benzene or styrene which are derived from non-renewable sources and are environmentally unfriendly[23]. The chemical synthesis methods result in the production of bi-products which have to be separated out using expensive methods.

Benzyl acetate is also a very important chemical for the perfume and fragrance industry. Due to its sweet aroma it is used in a variety of perfume and cosmetic products. It also has application as a solvent to oils, polishes, ink etc. It is synthesized by the esterification of benzyl alcohol and acetic acid. Chlorination of toluene gives benzyl chloride which is hydrolyzed by alkali to benzyl alcohol. These reactions are very complex and involves extensive purification steps to remove by-products formed.

Phenyl acetate is a colorless liquid with sweet odor. Fries rearrangement of phenyl acetate yields valuable intermediates for the pharmaceutical industry[24]. It is produced by

the reaction of phenol and acetic anhydride. Phenol is industrially produced by cumene process which involved reaction of benzene and propene to yield phenol and acetone.

Acetyl salicylate, commonly known as aspirin is a widely used drug with a large number of medical applications. It has been used as an analgesic, to reduce fever, to reduce likelihood of heart attack or stroke etc. It is produced by the treatment of salicylic acid with acetic anhydride.

1.6 Objective

The goal of this research is to construct microbial cell factories capable of producing aromatic esters from glucose as substrate. The first step towards this goal was to identify suitable enzymes for catalyzing the terminal step in the metabolic pathways for the target products. For this purpose, enzymes from seven different sources were screened and characterized using exogenous precursors and substrates. Following identification of suitable acyltransferases, the direct biosynthesis of aromatic esters was explored using engineered pathways in *E. coli* whole cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Strains and Plasmids Used

2.1.1 Strains Used for Enzyme Assays

Table1 lists the plasmids and the respective enzymes expressed by each plasmid. These plasmids were transformed into BW25113 strain, which is the wild type *E. coli* strain. These strains expressing each of the single enzymes were used carry to out the enzyme screening tests.

Table 1: Plasmids Used for Enzyme Screening

Sr. No	Description
1	*ATF 1* from *S. cerevisiae* inserted into pET28
2	*ATF 2* from *S. cerevisiae* inserted into pET28
3	*ATF* from Strawberry plant inserted into pET28
4	*WS 1* from *M. aquaeolei* inserted into pETDuet
5	*WS 2* from *M. aquaeolei* inserted into pETDuet
6	*AtfA* from *A. baylyi* inserted into pETDuet
7	*CAT* inserted into pACYC Duet
8	Histidine-tagged *CAT* inserted into pY3

Table 2 lists the plasmids that were used to construct the 2-phenethyl acetate producing strains.

Table 2: Plasmids Used for Constructing 2-Phenethyl Acetate Producing Strains

Plasmid	**Description**
pTrc99a	*Ptrc*, pBR322 ori, *lacIq*, *Amp*
pTrcColaK	*Ptrc*, ColA ori, *lacIq*, *Kan*
pSTV28	*Plac*, p15A ori, *lacIq*, *Cmp*
pdCas9-bacteria	*pLtet-O1*, p15A ori, *Cmp*
pS3	*PlacUV5*, pBBR1 ori, *lac1*, *Cmp*
pACYC Duet	*PT7*, RK2 ori, *lacI*, *Cmp*
pTpal-fdc	*PAL2* from *A. thaliana* and *FDC1* from *S. cerevisiae* inserted into pTrc99a
pTK-styABC	*StyABC* from *P. putida* inserted into pTrcColaK
p28	*PAL2* from *A. thaliana*, *FDC1* from *S. cerevisiae* and *CAT* inserted into pSTV28
pd9	*PAL2* from *A. thaliana*, *FDC1* from *S. cerevisiae* and *CAT* inserted into pdCas9-bacteria
ps21	*PAL2* from *A. thaliana*, *FDC1* from *S. cerevisiae* and *CAT* inserted into pS3
pACYC-*CAT*	*CAT* inserted into pACYC Duet

2.1.2 Constructing 2-Phenethyl Acetate Producing Strains

2-Phenethyl acetate production was engineered using *E. coli* NST74 *ΔfeaB* as the background strain. *E. coli* NST74 is a phenylalanine overproducing strain. The *feaB* gene encodes for phenyl acetaldehyde dehydrogenase which catalyzes the conversion of phenyl acetaldehyde to phenyl acetate, thus a *ΔfeaB* strain was used to direct more flux

towards 2-phenylethanol production. In this work the styrene-dependent pathway has been expressed along with the *CAT* to complete the 2-phenethyl acetate pathway.

To construct 2-phenethyl acetate pathway, *CAT* gene was first cloned into pACYCDuet vector. This plasmid was transformed into NST74 *Δ*feaB background strain along with plasmids pTpal-fdc -*FDC1-PAL2* and pTK-styABC -*styABC*, resulting in Strain 2. Strain 1 was constructed same as strain 2, except the pACYCDuet-*CAT* plasmid was not co-transformed. This was used as control strain to confirm the functionality of *CAT*.

Strain 3, 4 and 5 were 2-phenylethanol producing strains which had been previously constructed in the lab. All the three strains contained pTK-styABC plasmid and a second plasmid expressing genes *FDC1*, *PAL2* and also containing the chloramphenicol resistance gene. The second plasmid in strain 3, 4 and 5 contains Lac, pLtet-O1and LacUV5 promoter respectively. These strains were also tested for 2-phenethyl acetate production, which is expected due to the presence of chloramphenicol resistance gene.

2.2 Cell Culturing

2.2.1 Chemically Competent Cell Transformation

An aliquot of chemically competent bacterial cells was taken from -80 freezer and thawed at 4 ^{0}C for 15 minutes. 1µl of plasmid containing the pathway genes were added for single plasmid transformation (2µl and 3 µl of each plasmid for transforming two and three plasmids respectively). This was allowed to sit on ice for 30 minutes and then heat shocked for 30 seconds in a 37 ^{0}C water bath. 0.5 ml of SOC was added to the tubes and it was allowed to incubate for 30 minutes at 37 ^{0}C with shaking. The cells were plated on LB plates with the required antibiotic and incubated overnight.

2.2.2 Culturing *E. coli* Strains in Flasks

First seeds are prepared by transferring one colony from the plate to a test tube containing 3ml of LB media and the required antibiotics. Seeds are grown overnight, then 0.5 ml of the seed culture is inoculated into 250 ml flask containing MM1/M9 media salts, 20% glucose and required antibiotics, making a total volume of 50ml. Flasks are induced when an OD_{600} of 0.5 is reached with 10 μL of 1M IPTG. pH is monitored and maintained between 6.5-7 by adding required amount of 0.4 g/l of K_2HPO_4. As 2-phenethyl acetate has very low solubility in water, in order to collect samples and analyze its production, a 5ml dodecane layer was added on top of the media in the flask. The dodecane forms a thin layer of organic phase on top of the culture thus acting as an extractant for the ester produced.

2.3 Enzyme Assays

2.3.1 Spectrophotometric Assays

Assays were carried out using a DU800 Beckman Coulter Spectrophotometer. The reactions were carried out in 3ml cuvettes. The following reagents were used to carry out the assay:

A- 100mM Trizma Base

B- 2.5 mM 5,5'-Dithio-bis (2-Nitrobenzoic Acid) (DTNB)

C- 5mM Acetyl Coenzyme A

D- 3mg/ml chloramphenicol, 3mg/ml salicylic acid, 2-Phenylethanol, Phenol, Benzyl Alcohol

E- 10mM Trizma Base

F- Cell Lysates containing the enzymes(*ATF1,ATF2,ATF,WS1,WS2,AtfA,CAT*) to be tested

Reaction mixture of 0.4 ml total volume was prepared pipetting the above reagents and adding them to a cuvette. Table 3 shows the volumes (in µL) of the different reagents used. Each of the substrates listed under reagent D (except chloramphenicol) were tested with each of the enzymes listed under reagent F. A reaction mixture with chloramphenicol and *CAT* was run as control as *CAT* is already known to have activity towards chloramphenicol.

Table 3: Volume (µl) of Each Component in Reaction Mixture and Blank for Spectrophotometric Assay

Reagent	**Test (µL)**	**Blank (µL)**
A. 100mM Trizma Base	274	274
B. 2.5mM DTNB	10	10
C. 5mM Acetyl-CoA	10	10
D. Alcohol Substrate	1	1
E. 10mM Trizma Base	-	105
F. Cell Lysates(Enzymes)	105	-

The reaction in the Test mixture was initiated by addition of the cell lysates containing the enzymes. On adding the enzymes, the following reactions takes place:

Acetyl-CoA + Alcohol $\xrightarrow{\text{Enzyme}}$ CoA + Ester ---(1)

CoA + DTNB $\longrightarrow$ TNB + CoA derivative ---(2)

The enzyme catalyzes the reaction between the acetyl-CoA and the alcohol group and releases the CoA molecule along with the production of ester. The DTNB reacts with the –SH group of the CoA molecule and results in the production of 5-Thio-2-Nitrobenzoic Acid (TNB) which has an absorbance at 412nm. As the reaction 1 proceeds, concentration of TNB in the cuvette increases due to reaction 2 which results in an increased absorbance at 412nm.

As soon as the cell lysates was added, the cuvette was placed in the sample holder of the spectrophotometer and the run was started. Absorbance at 412nm versus time reading was recorded using the kinetics mode of the spectrophotometer.

2.3.2 Whole Cell Assays

Cells expressing each of the seven enzymes to be tested were grown in M9 media. The flasks were induced with IPTG on reaching OD_{600} of 0.5. At the time of induction, the flasks were also supplied with 2-phenylethanol at an initial concentration of 0.5g/L. Alcohol and ester concentration were monitored over time.

2.4 Sampling & Analysis

Samples collected from the aqueous phase were tested in the HPLC and the samples collected in the organic phase were tested using GC-FID. Sample of 300 μL volume were collected from aqueous phase and centrifuged to separate the cell mass. The supernatant was collected in HPLC glass vials. From the organic phase 300 μL was collected in tubes

and centrifuged to separate out any aqueous phase that got transferred into the tubes. The separated out organic phase was collected in glass vials for GC detection.

2.4.1 HPLC Detection of Aromatic Alcohol Substrates

A hypersil gold C-18 column (100 X 4.6mm) was used in a Series 1100 Hewlett Packard high performance liquid chromatograph. The column was eluted at a total constant flow rate of 0.8 mL/min using as a mixture of 85% 5mM H_2SO_4 and 15% Acetonitrile for 14 minutes. The eluent was monitored using a diode array detector set at 215 nm. The peak for 2-phenylethanol was observed at 9.86 minutes, Salicylic acid peak was observed at 12 minutes, phenol peak at 6.37 minutes and benzyl alcohol peak at 5.4 minute.

2.4.2 HPLC Detection of Glucose

Aminex HPX-87X –Ion exclusion column (300X7.8mm) was used in a Series 1100 Hewlett Packard high performance liquid chromatograph. The column was eluted at a total constant flow rate of 0.55 mL/min using 5mM H_2SO_4 for 12 minutes. The eluent was monitored using a refractive index detector. The peak for glucose was observed at 9.64 minutes.

2.4.3 GC Detection of 2-Phenethyl Acetate

5890 series II Helwett Packard Gas Chromatograph and a Flame Ionization detector was used for GC analysis. An HP-5 column (30mm length x 0.32mm diameter) was used with helium as carrier gas. The temperature profile used was 40 ^{0}C for 3 minutes, ramp at 40 ^{0}C /min to 250 ^{0}C and hold at 250 ^{0}C for 5 minutes. The flowrate of helium used was 5ml/min. The peak for 2-phenethyl acetate was observed at 6.5 minutes.

CHAPTER 3

ENZYME SELECTION AND SCREENING

Seven acyltransferase enzymes were selected for activity screening for producing aromatic esters. Enzyme selection was done based on their functionality as reported in previous works. Three of the enzymes were chosen from the alcohol O-acetyltransferase family (EC 2.3.1.84). Two of these enzymes are *ATF1* and *ATF2* from *S. cerevisiae* and third one is an *ATF* from strawberry plant. *ATF1* and *ATF2* are responsible for the production of various branched, medium chain length and aromatic acetate esters in *S. cerevisiae* during fermentation. Acyltransferases from strawberry plant has shown activity towards a range straight chain length alcohols as well aromatic alcohols like 2-phenylethanol, benzyl alcohol and cinnamyl alcohol[11]. Three of the enzymes selected are from long chain alcohol O-acyltransferase (EC 2.3.1.75) family known for their ability to catalyze wax ester production. Due to the structural similarity of *CAT* with other acyltransferase groups which are known to catalyze ester biosynthesis and due to its observed functionality as seen in published literature[3,15], *CAT* (EC2.3.1.28) was also chosen as one of the candidate enzymes for this thesis.

3.1 Enzyme Screening

The aim of the section is to test which of these enzymes would show activity towards aromatic alcohols and acetyl-CoA as substrate. Two methods were used to do enzyme screening: spectrophotometric assays and whole cell assays.

3.1.1 Spectrophotometric Assays

Spectrophotometric assays were done using lysates of cells expressing the enzyme of interest to study its activity in-vitro. Cell lysates were next prepared from strains expressing different enzymes and used to test for acyltransferase activity with respect to the series of alcohol substrates of interest.

The assay used was modified from an enzymatic assay protocol for chloramphenicol O-acetyltransferase given by Sigma Aldrich. The control assay with *CAT* and chloramphenicol as shown in Fig 3 indicates that this method works as expected. The absorbance vs time reading obtained shows that the spectrophotometer is sensitive for the concentration of substrates used for this control assay.

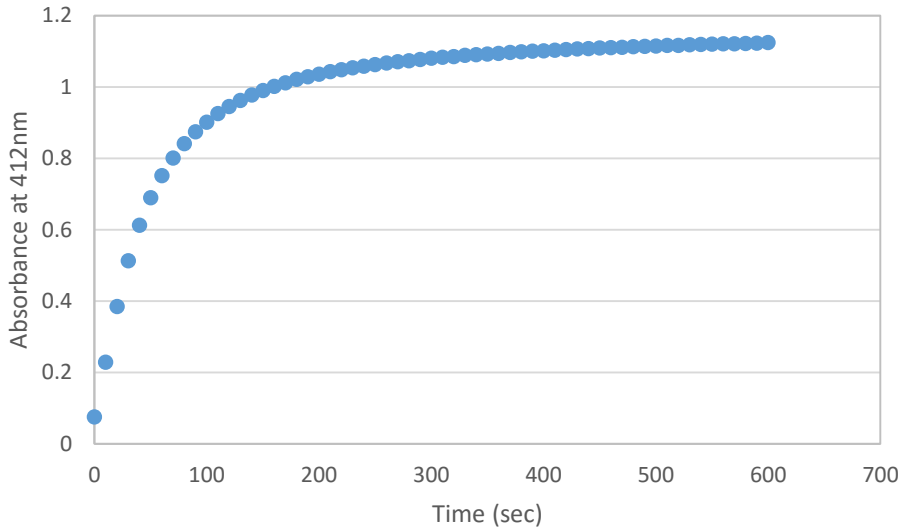

Figure 3: Absorbance vs Time for *CAT* with Chloramphenicol(0.02mM) and Acetyl-CoA(0.12mM) Substrates.

In-vitro assays were then carried out for different aromatic alcohols and acetyl-CoA as substrates. Fig 4 to 7 shows the absorbance vs time for each of the enzymes which

showed activity towards salicylic acid, 2-phenylethanol, benzyl alcohol and phenol as the aromatic alcohol substrate respectively. The absorbance reading corresponds to the production of respective esters. The initial linear slope corresponds to the initial rate of reaction, or the initial rate at which ester is formed. As the active sites on the enzyme gets occupied, the absorbance approaches a constant value, suggesting no more reaction is taking place.

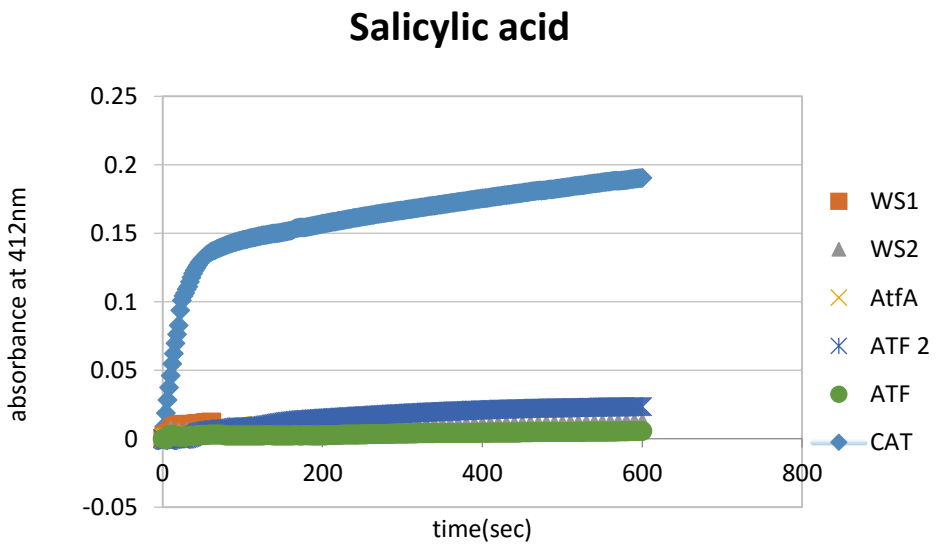

Figure 4: Absorbance vs Time with Salicylic Acid (0.05mM) and Acetyl-CoA(0.12mM) Substrates

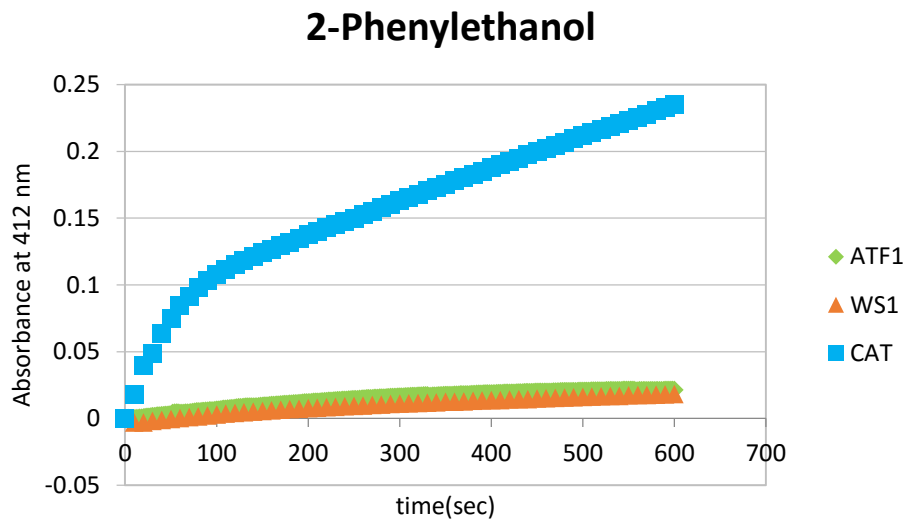

Figure 5: Absorbance vs Time with 2-Phenylethanol (20.87 mM) and Acetyl-CoA(0.12mM) Substrates.

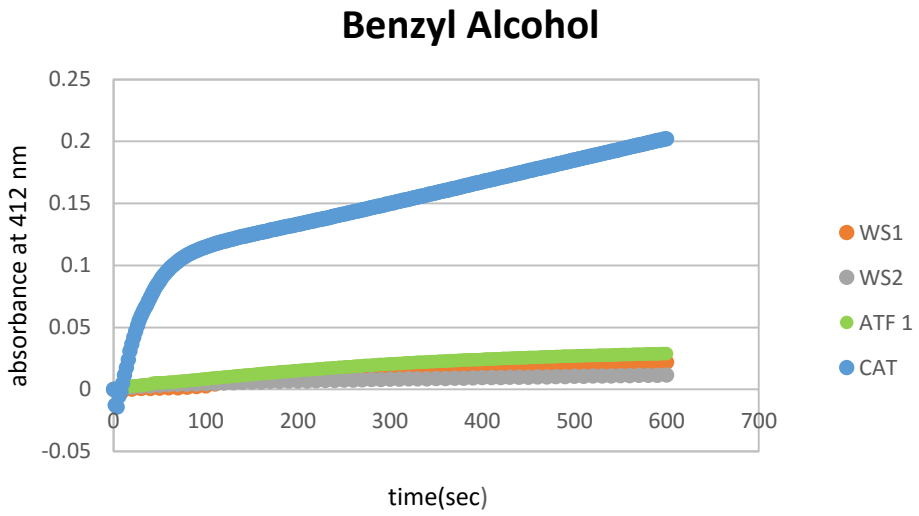

Figure 6: Absorbance vs Time with Benzyl Alcohol (24.04 mM) and Acetyl-CoA(0.12mM) Substrates.

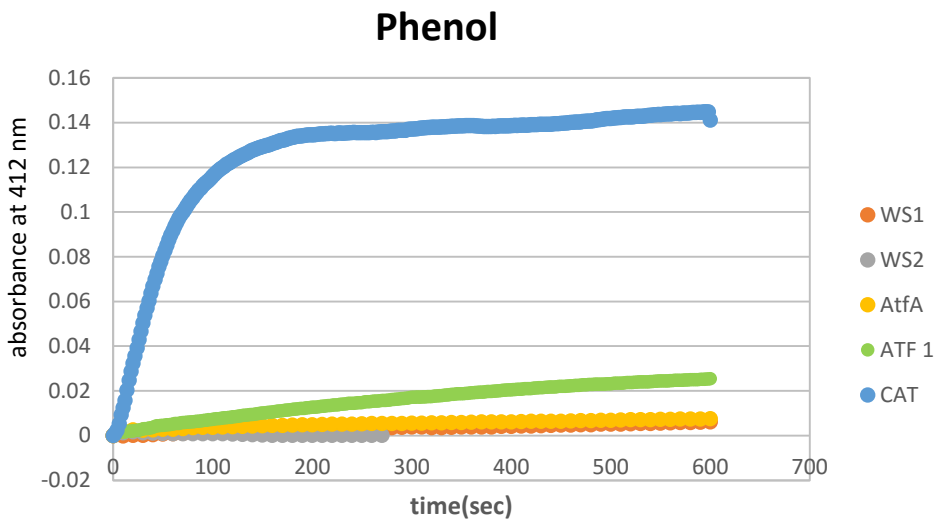

Figure 7: Absorbance vs Time with Phenol (28.42 mM) and Acetyl-CoA(0.12mM) Substrates.

The enzyme screening results are summarized below.

Table 4: Summary of Enzymes and Alcohol Substrates Screened. The Enzymes That Showed Activity with the Respective Alcohol Substrates are Marked with +.

Alcohol	Salicylic Acid	2-Phenylethanol	Benzyl Alcohol	Phenol
Enzymes				
ATF1	+	+	+	+
ATF2	+			
ATF	+			
WS1	+	+	+	+
WS2	+		+	+
AtfA	+			+
CAT	+	+	+	+

The absorbance vs time assays showed promising activity of *CAT* towards the substrates tested. Reaction mixtures which used cell lysates with *CAT* enzyme showed significantly

higher absorbance as compared with other enzymes, as can be seen from Fig 5,6,7 and 8. Thus *CAT* could potentially act as a biocatalyst for the esterification of aromatic alcohols and acetyl-CoA. The spectrophotometric assays with *CAT* for equimolar concentration of the four alcohol substrates and acetyl-CoA is shown below. Similar behavior was shown for each alcohol as substrate, though the highest conversion was observed for 2-phenylethanol. Also from Fig 8, comparing assays for phenol, benzyl alcohol and 2-phenylethanol, the reaction rate and conversion increased with increase in length of the hydroxyl group attached to aromatic ring.

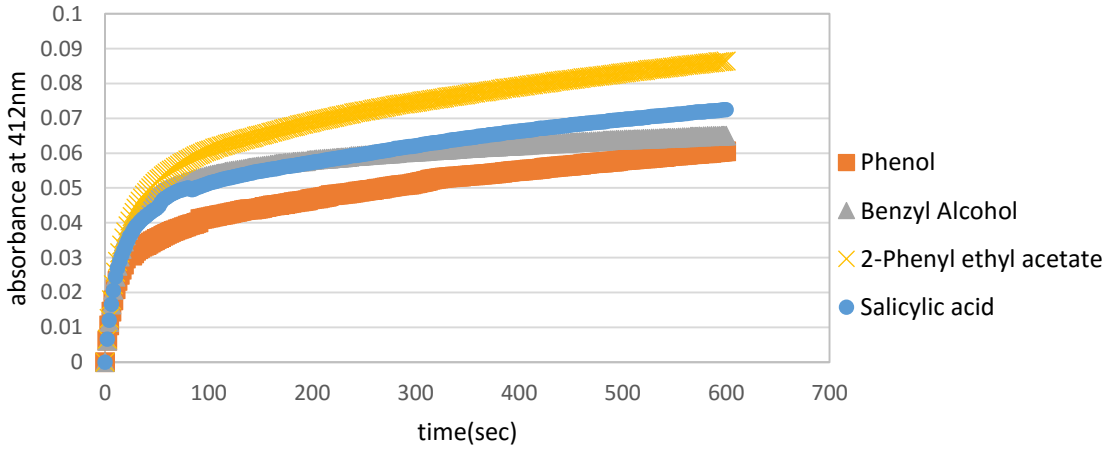

Figure 8: Absorbance vs Time with Alcohols (0.1 mM) and Acetyl-CoA(0.1mM) Substrates.

The amount of substrate consumed and the percentage conversion of each substrate in the assay shown above was calculated using the molar absorption coefficient of TNB which is 0.013.6 $\mu M^{-1}cm^{-1}$. The calculated values are shown in the table below.

Table 5: Substrate Consumption and %Conversion for each Alcohol Substrate when treated with *CAT*

Alcohol Substrate	Absorbance reading after 10minutes	Mmoles of substrate consumed	% Conversion
Chloramphenicol	1.1	3.2-06	0.16
2-Penylethanol	0.0865	2.5E-06	0.0025
Benzyl Alcohol	0.065	1.9E-06	0.0019
Phenol	0.0649	1.9E-06	0.0019
Salicylic Acid	0.0725	2.1E-06	0.0021

Although, *CAT* showed positive activity with the different alcohols, a very low conversion was observed for each alcohol. Most of the alcohol substrate is still left in cuvette after 10 minutes. The low conversion could be due to very low amount of enzymes present in the lysates. However, the lower conversion of other alcohols as compared to chloramphenicol indicates a lower activity of *CAT* towards the alcohol substrates or the presence of some inhibition in the assays with the four alcohol substrates. Further experiments need to be done with purified enzymes and different substrate concentrations in order to determine the affinity and activity of *CAT* towards the different substrates tested.

The absorbance vs time readings presented in this section indicates the activity of the enzymes present in the lysates. However, for a definitive proof that the results of the in-vitro assays are from the acyltransferase enzymes expressed in the vector, a negative control needs to be done with cell lysates of wild type *E. coli* which do not contain the vector. Also, to ensure that DTNB does not interfere with the reaction or effect the enzyme activity, future experiments can be performed with a different protocol. The first reaction can be allowed to reach completion before adding the DTNB to the cuvette. In

this way the enzyme catalyzation reaction would occur without the interference of DTNB and then DTNB can be added to react with the released CoA to produce TNB.

3.1.2 Whole Cell Assays

In-vivo assays were carried out with the recombinant cells to study enzyme activity towards 2-phenethyl acetate production. 2-phenylethanol concentrations were monitored over time, however, only the flasks containing the *CAT* expressing strain showed considerable reduction in concentration. Samples from this flask was further tested specifically for the presence and accumulation of 2-phenethyl acetate, as shown in Fig 9.

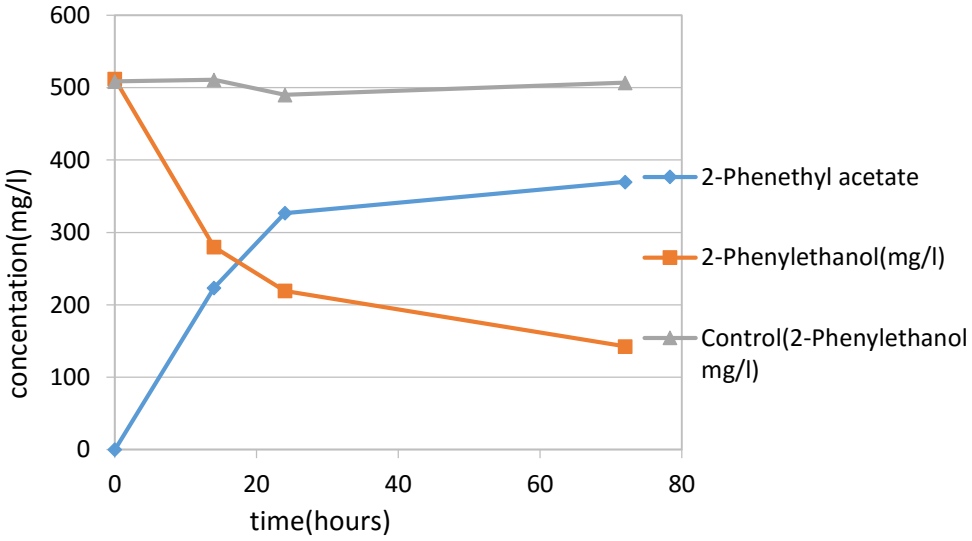

Figure 9: Whole Cell Bio Assay for Cells Expressing Chloramphenicol Acyltransferase and 0.5g/l of 2-Phenylethanol as Added Substrate.

CHAPTER 4

BIOSYNTHESIS OF 2- PHENETHYL ACETATE FROM GLUCOSE

To produce esters directly from glucose, in addition to expressing an appropriate acyltransferase, cells must also be engineered with functional alcohol and acyl- CoA producing pathways. As acetyl-CoA is naturally produced by and abundant in *E. coli*, the use of this specific acyl-CoA requires minimal additional modifications. Thus the focus here was to investigate different aromatic alcohol pathways in *E. coli*, before introducing the desired acyltransferase activity.

4.1 Styrene-derived Pathway for 2-Phenylethanol Production

The shikimate pathway is an endogenous pathway found in plants and bacteria for the production of aromatic amino acids and other aromatic compounds. Fig 10 shows the shikimate pathway in *E. coli* from 3-dehydroshikimate to the terminal amino acids phenylalanine and tyrosine. Intermediates in this pathway provide the precursors that can be used to produce a range of aromatic products through further pathway engineering as demonstrated in previous works[7,23-26].

For example, 2-phenylethanol can be produced using distinct pathways constructed from either phenyl-pyruvate or phenylalanine as precursors. Bioconversion of phenylalanine to 2-phenthanol takes place in yeast by its endogenous Ehrlich pathway in which phenylalanine is converted to phenylacetaldehyde which is then converted to 2-phenylethanol. The pathway used in this research is the styrene-derived pathway (unpublished work), which was constructed by extending the styrene oxide pathway in *E. coli* as described below.

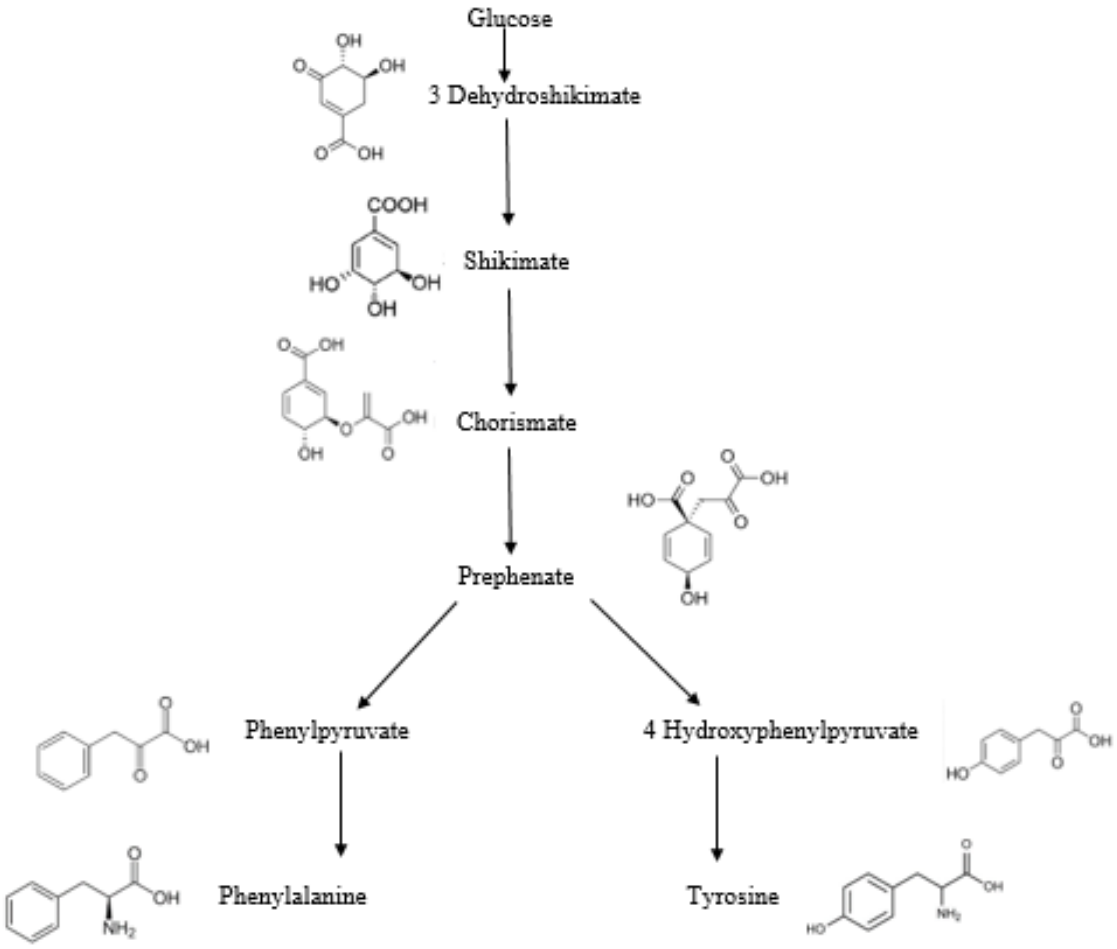

Figure 10: Shikimic Acid Pathway in *E. coli*

McKenna et.al constructed styrene producing *E. Coli* strains using phenylalanine from shikimate pathway as precursor[23]. *E. coli* NST74 strains which produced 700-1000mg/l of phenylalanine from glucose was used as background strain. Styrene producing pathway was constructed by overexpressing phenylalanine ammonia lyase, *PAL2* from *A. thaliana* and trans-cinnamate decarboxylase, *FDC1* from *S. cerevisiae*. This pathway was further extended to create a styrene oxide producing strain by expressing styrene monooxygenase, *styAB* from *P. putida*[24]. *StyC* from *P. putida* was expressed for conversion of styrene oxide to phenylacetaldehyde. Conversion of phenylacetaldehyde to

2-phenylethanol is catalyzed by aldehyde reductase in *E. coli* expressed by genes *yqhD, yigB, yahK*. The complete pathway for 2-phenylethanol production using styrene as intermediate precursor is shown in Fig 11.

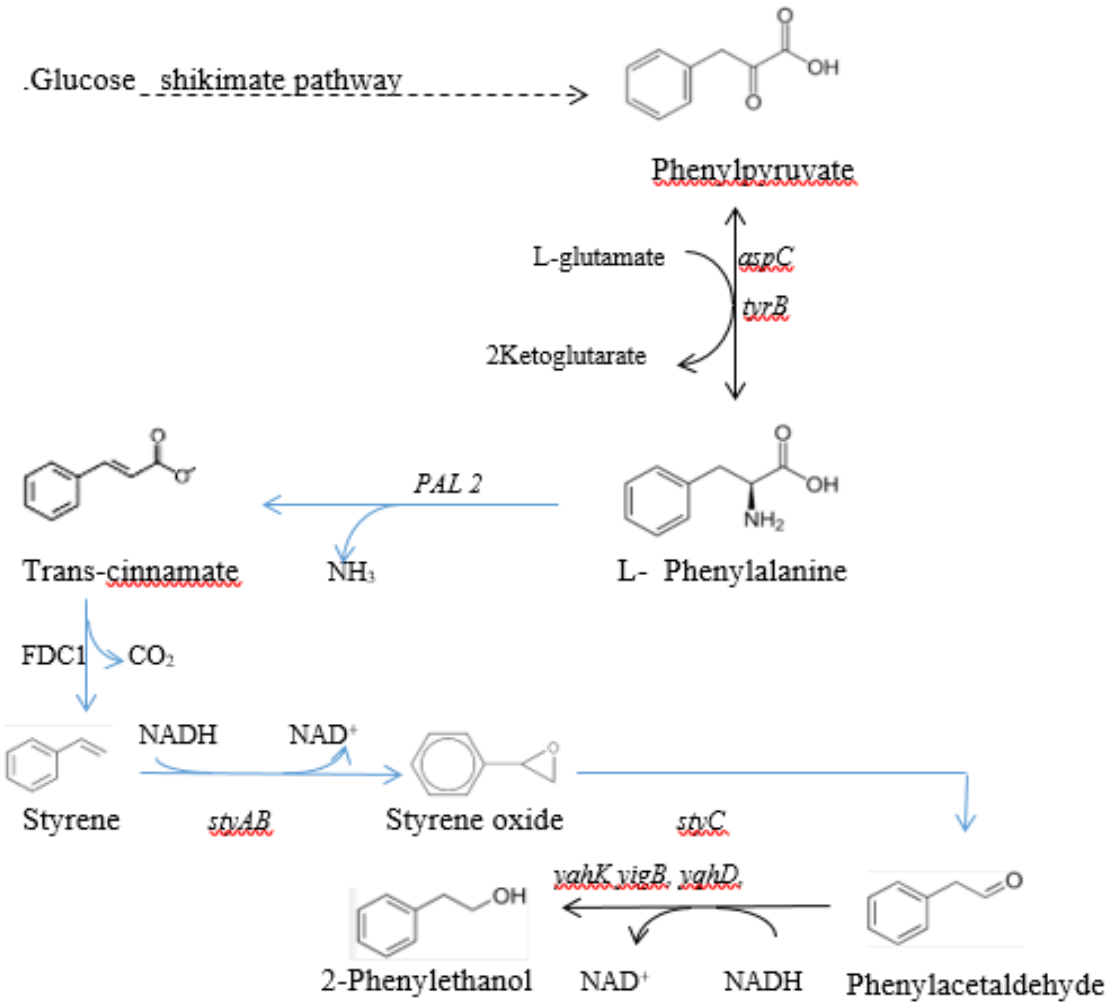

Figure 11: Styrene-derived Pathway for 2-Phenylethanol Production (Unpublished Work)

4.2 2-Phenethyl Acetate Production

4.2.1 Results and Discussions

Flasks grown with and without the dodecane layer showed similar OD_{600} after 120hours, thus addition of dodecane did not affect the growth of *E. coli*.

Fig 12 shows the concentration of 2-phenethyl acetate in the aqueous phase corresponding to the measured concentration of 2-phenethyl acetate in the dodecane phase. The calculated partition coefficient is 100 (g/l in dodecane phase / g/l in aqueous phase).

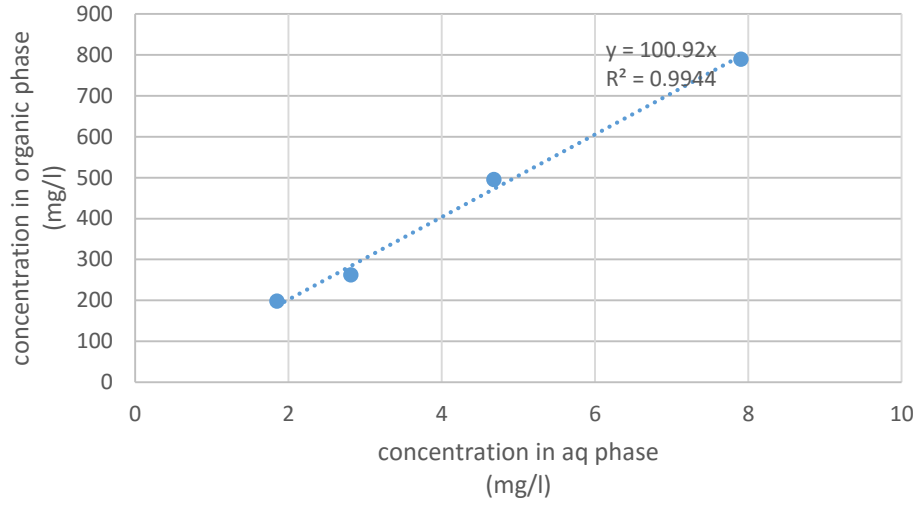

Figure 12: Total Concentration vs Concentration in Dodecane Phase after Extraction

Four different strains expressing the styrene dependent pathway along with *CAT* were tested for 2-phenethyl acetate production. One control strain was constructed without *CAT*. The strains were named strains 1,2,3,4 and 5 and have been summarized in the table below.

Table 6: Strains Constructed for 2-Phenethyl Acetate Production

Strains	**Plasmids**
strain 1(control strain)	pTpal-fdc-*FDC1,PAL2;* pTK-styABC-*StyABC*
strain 2	pTpal-fdc-*FDC1,PAL* ; pTK-styABC-*StyABC* ; pACYC Duet- *CAT*
strain 3	p28-*FDC1,PAL2,CAT*; pTK-styABC-*StyABC*
strain 4	pd9-*FDC1,PAL2,CAT*; pTK-styABC-*StyABC*
strain 5	ps21-*FDC1,PAL2,CAT*; pTK-styABC-*StyABC*

Strain 3,4 and 5 were previously constructed for 2-phenylethanol production, but they were tested for 2-phenethyl acetate production as one of the plasmids in these strains contains the chloramphenicol resistance gene. Strain 1 and 2 were constructed in this work. Strain 2 contains two plasmids expressing the pathway genes for 2-phenylethanol production and an additional plasmid was added expressing *CAT.* Strain 1 is a control strain that is constructed same as strain 1 but without the plasmid carrying *CAT.*

The four ester producing strains were grown in flasks and the 2-phenethyl acetate and 2-phenylethanol concentration were monitored for each strain as shown in figures 13 and 14. *CAT* showed recombinant activity in each of the strains as ester is produced in each strain. Although, none of the strains could completely convert the available alcohol precursor. The difference in accumulation of alcohol and ester in each strain suggests different levels of protein expression of the pathways enzymes for each strain. Strain 5 showed the maximum ester titer of 60mg/l.

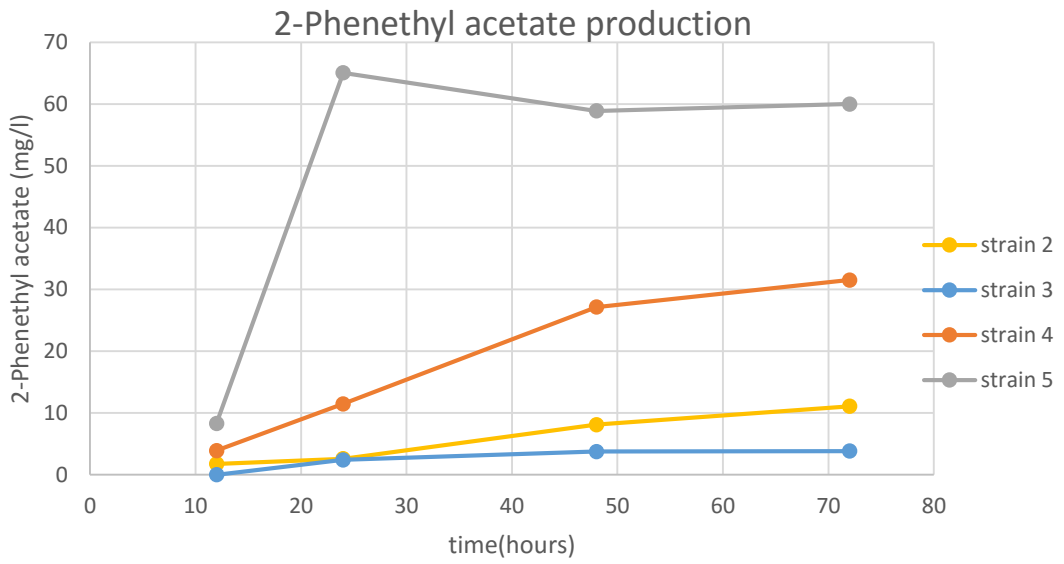

Figure 13: 2-phenethyl acetate production with time

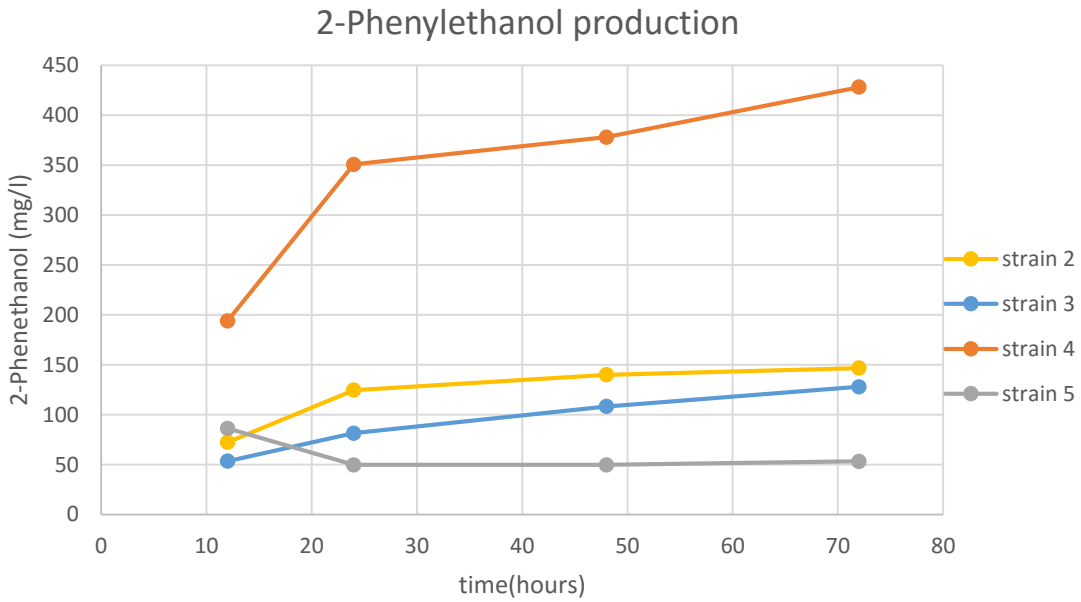

Figure 14: 2-Phenenthanol Concentration with Time for Each Strain

As seen in the figures above, strain 2 and 3 produced similar amount of 2-phenylethaol and the reduction in the amount of 2-phenylethanol produced by strain 5 compared to strains 2 and 3 corresponds to the higher amount of ester produced by the strain. Also strain 4 shows much higher amount of 2-phenylethanol as compared to strains 2,3 and 5

but isn't showing a corresponding amount of ester production. This difference shows that the pathway enzymes are expressed at different levels in these strains due to the different plasmids used for their construction.

The control strain which does not contain *CAT* was also grown and monitored for alcohol and ester production. Figure 15 shows the alcohol and ester concentrations in the control strain and strain 2, which illustrates the effect the of adding *CAT* to the pathway. The control showed no 2-phenethyl acetate production and also higher accumulation of alcohol, thus confirming the role of *CAT* in the ester production.

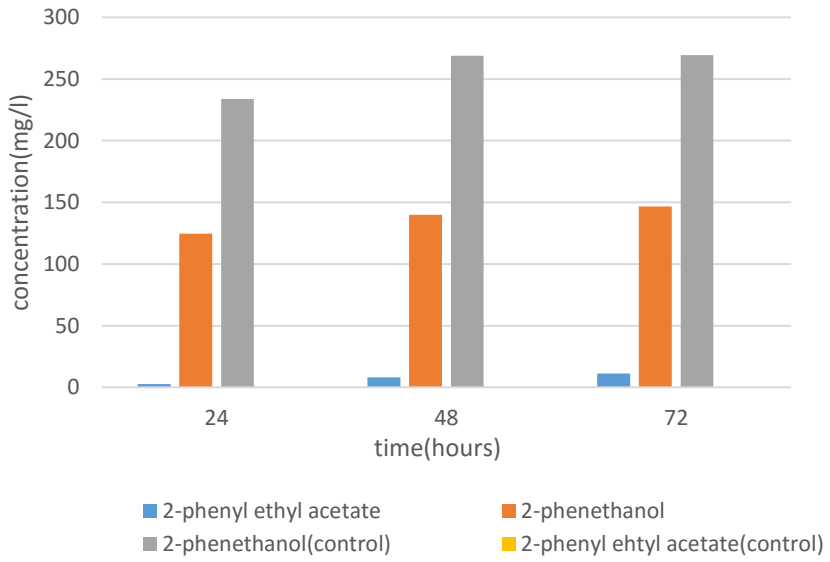

Figure 15: 2-Phenethyl Acetate and 2-Phenylethanol Concentration in Flasks with Strain1

As seen in Fig 17, a very small fraction of the available 2-phenylethanol produced by strain 2 has been converted to ester. The low conversion of alcohol to ester could be due to limited availability of acetyl-CoA or due to limitation in the functionality of the pathway enzyme.

Also, the reduction in 2-phenylethanol production in strain 1 as compared to strain 2 does not correspond to the 2-phenethyl acetate production in strain 1 as seen in Fig 15. If the reduction was entirely due to conversion to 2-phenethyl acetate, a much higher amount of 2-phenethyl acetate production should have been observed. This suggests that the initial production of 2-phenylethanol is itself lower in strain 1 as compared to strain 2, probably due to the addition of an extra plasmid.

Triplicate experiments were run with strain 5 which showed the highest titer for ester. The ester and alcohol accumulated is shown in the figure below.

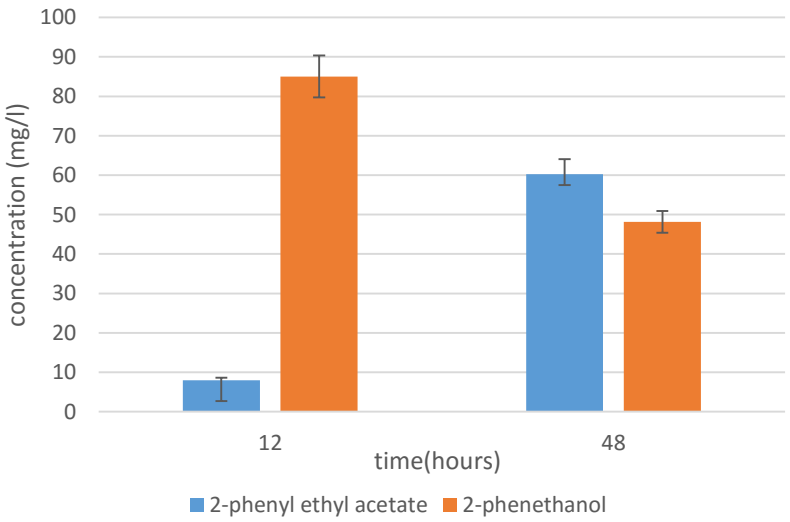

Figure 16: 2-Phenethyl Acetate and 2-Phenathanol Concentration in Flasks with Strain 5.

As seen in the above figure, after 48 hours there is still 48mg/l of 2-phenylethanol left in the flask. The alcohol and ester titers remained constant after 48 hours.

Future experiments need to be directed towards troubleshooting both the alcohol and acetyl-CoA pathway. The intermediate metabolites could be getting accumulated due to low conversion at different points in the pathway. The bottleneck in the alcohol pathway

could be determined by analyzing the samples from the flasks for all the intermediates along the constructed pathway: phenylalanine, trans-cinnamate, styrene, styrene oxide and phenylacetaldehyde. These bottlenecks can then be eliminated by better expression of the corresponding pathway enzyme to achieve higher conversion of the intermediate metabolites. Also, it needs to be tested if the availability of acetyl-CoA is limiting the final step of the pathway which produces the ester. This could be done by expressing pyruvate formase lyase in the strain which will increase acetyl-CoA availability. These strains can then be tested to see if increase in acetyl -CoA availability increases the amount of ester produced.

4.2.2 Conclusions

2-Phenethyl acetate producing strains were created by extending the styrene dependent 2-phenylethanol producing pathway by addition of *CAT* gene. Four different strains with the 2-phenethyl acetate producing pathway were screened. Ester production was observed in all the four strains. Three of these strains were previously constructed for 2-phenylethanol production (3,4 and5), but contained the *CAT*. Thus while constructing, alcohol producing strains, it should be kept in mind that *CAT* should not be used as antibiotic resistant as it will direct flux towards ester production. All the four strains that produced 2-phenethyl acetate showed different productivities and titers with a maximum titer of 60mg/l observed in one of the strains.

CHAPTER 5

CONCLUSIONS

5.1 Summary

This thesis explored the potential of microbial cell factories to produce aromatic esters. To do so, first literature survey was conducted to study classes of acyltransferases which have been proven to catalyze ester producing reactions. The acyltransferases are shown to have very diverse functional and structural backgrounds. Enzymes from three classes of acyltransferases were selected and characterized for their activity towards different aromatic alcohols and acetyl-CoA as substrate. The in-vitro assays identified acyltransferases from each of these classes that showed some activity towards one or more of the aromatic alcohols, though *CAT* stood out from the other enzymes, showing promising activity. Thus *CAT* has been identified as a potential enzyme for expanding microbial synthesis of aromatic esters. Production of aromatic ester in *E. coli* directly from glucose, was demonstrated by constructing, 2-phenethyl acetate producing strains.

5.2 Future Works

The applicability of *CAT* for making aromatic ester producing microbial cell factories has been demonstrated in this work. The activity assays with *CAT* showed its potential to enable construction of phenyl acetate, benzyl acetate and acetyl salicylate producing pathways. Thus this knowledge can be used to expand the aromatic ester products that can be microbially synthesized. It could be tested for its ability to catalyze similar chemistries to biosynthesize other target compounds as well.

Knowing the kinetic parameters for an enzyme gives a good comparison of the enzyme against other candidate enzyme. In this thesis, the purification of *CAT* for further kinetic studies were attempted, but did not work out successfully. Thus kinetic studies with purified *CAT* is an important next step that needs to be done.

In this work, the functionality of *CAT* towards aromatic alcohols with different alkyl chain lengths was studied. *CAT* also showed activity towards, salicylic acid which is an aromatic alcohol with an attached carboxylic group. Further characterization studies towards substrates such as straight chain alcohols, branched chain alcohols and diols could give better insight into the functionality of this enzyme.

The acyltransferase class has enzymes categorized over two hundred subcategories. Many of these enzymes, like *CAT,* are known for their ability to catalyze very specific reactions. A broader enzyme screening might reveal potential candidates from other subclasses as well for producing different target esters.

Titer, productivity and yield are important factors for a biosynthesis process to make it economically viable. Thus future work also needs to be directed towards troubleshooting the pathway to achieve desirable productivity and yield of 2-phenethyl acetate. The different production results from different strains have shown that the same pathway can give different yields depending upon the plasmid construction and the promoters used. Thus experiments directed towards strain engineering strategies could help in further increasing the yield of the target compound.

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APPENDIX A

PURIFICATION OF *CAT*

The *CAT* gene of interest was amplified by conducting polymerase chain reaction (PCR). pACYC Duet plasmid containing the gene and primers containing polyhistidine-tag were used for the PCR reaction. The forward primer contained the histidine codons, so the his-tag was added to the N-terminus of the protein. After the reaction, the PCR samples were purified using DNA wash buffer. The enzymes were then digested using BamH1 and Xhol restriction enzymes. The digested his-tagged proteins were then ligated into pY3 plasmid.

The pY3 plasmid was transformed into a BW25113(DE3) background strain. The cells were grown in flasks and induced with 10µl of 1mM IPTG. The cells were allowed to grow overnight after induction. 10 ml of culture was pipetted into a test tube and spun down in a centrifuge. The supernatant was discarded and the cells were re-suspended in 1ml of His wash binding buffer. The re-suspended cells were lysed using Fast Break cell lysis reagent. The lysed cells were then purified using His-Spin Protein Miniprep kit from Zemo Research Corp.

APPENDIX B

BICINCHONINIC ACID ASSAY (BCA ASSAY)

The purified proteins were quantified using Thermo Scientific Pierce BCA protein assay kit. The method utilizes the reduction of Cu^{+2} to Cu^{+1} by protein in alkaline medium. The Cu^{+1} cations combine with bicinchoninic acid (BCA) to give a purple colored product which shows absorbance at 562 nm.

Absorbance measurements for the assay was found using DU800 Beckman Coulter Spectrophotometer. Fig 17 shows the calibration curve for BCA assay for known quantities of Bovine Serum Albumin(BSA). Ten times diluted samples of purified proteins gave an absorbance of 0.0363 at 562 nm. Using the calibration curve the concentration of purified protein was found to be 1903.8 µg/ml.

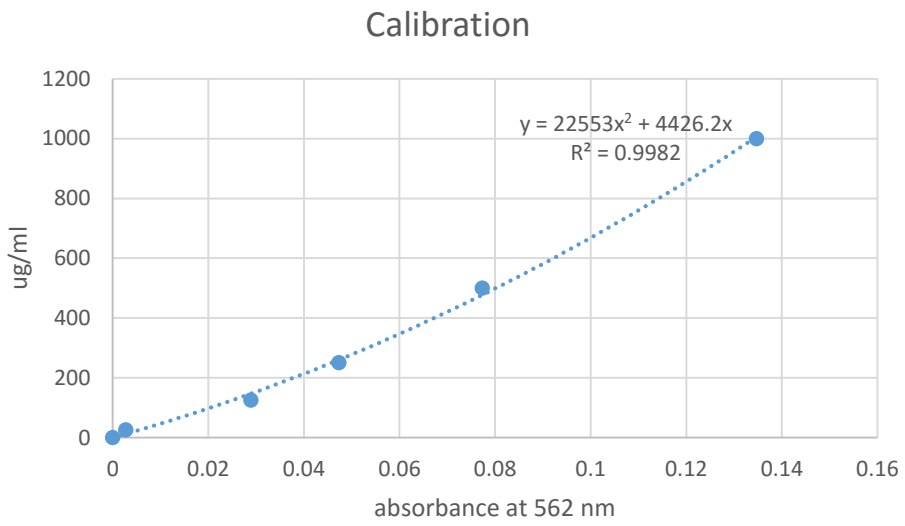

Figure 17: Concentration vs Absorbance Curve for BSA Protein

APPENDIX C

SDS-PAGE

Protein gel was run using a Bio-Rad precast polyacrylamide protein gel. Gels were run in a Bio-Rad Mini-PROTEAN tetra cell at 200 volts for 30 minutes. Samples were prepared by mixing 15µl of purified *CAT* protein solutions with 15 µl of protein loading dye. Three 15µl protein sample solutions were made containing 28µg,14 µg and 6 µg of protein (based on BCA assay). Other gel wells were added with control samples. Three wells were added with different concentrations (100%, 50%, 20% by volume) of supernatants from lysed cell solutions expressing *CAT*, two wells with supernatants from lysed cell solutions of BW25113 strain and one with lysed cells which contained pTrcColaK plasmid. Standard protocol for running protein gel using precast polyacrylamide protein gel was followed.

APPENDIX D

DISCUSSIONS ON *CAT* PURIFICATION AND ANALYSIS

Assays were conducted using purified protein to calculate the activity of the enzyme towards 2-phenylethanol and acetyl-CoA. On using equimolar amount of 2-phenethyl acetate, the spectrophotometric assay showed almost no detectable activity, suggesting that the aromatic alcohol is limiting in the reaction, likely due to a low affinity of *CAT* for 2-phenethyl acetate (relative to acetyl-CoA). Fig 18 shows the assay for *CAT* with excess of 2-phenylethanol. Detectable activity was observed only on adding about a thousand molar excess of 2-phenylethanol.

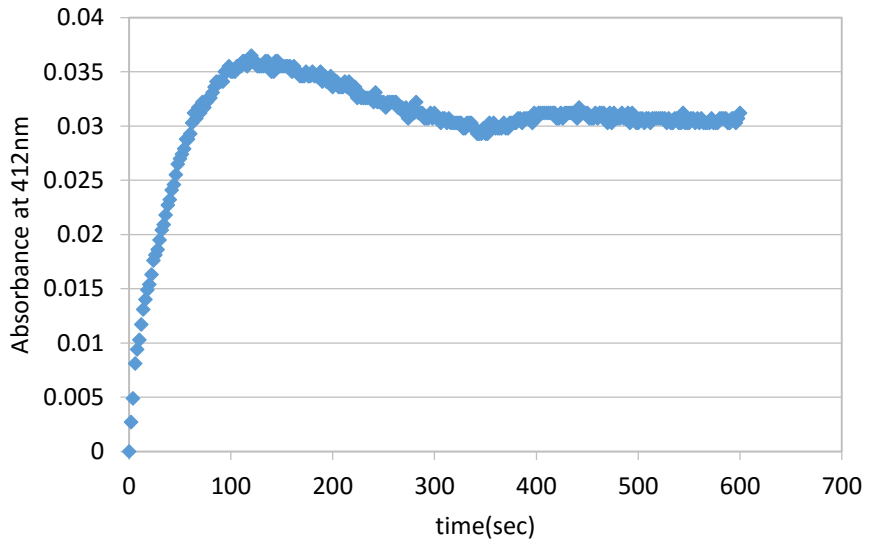

Figure 18: Absorbance vs Time with 2-Phenylethanol (139.16 mM) and Acetyl-CoA(0.1mM) as Substrates.

The very low activity shown by *CAT* as seen in the figure above indicated that the His-Tagged enzyme was not being expressed in the strain or wasn't concentrated in the purification step. The results of the protein gel did not show any band in the wells run with purified *CAT* thus confirming the absence or very low amount of *CAT* in the purified enzyme solution. Thus the cloning and purification steps need to be repeated to eliminate possible errors. The His-tag could be inserted on the C-terminus of *CAT* instead of

N-terminus. This would help if the His-tag is inaccessible to the column at the N-terminus. The purification step could also be improved by allowing the sample to interact with the column for a longer period of time and resuspending the gel more frequently during the incubation period.