SCREENING AND CHARACTERIZATION OF BIOCATALYSTS FOR SYNTHESIS OF WIELAND MIESCHER KETONE: A VERSATILE SYNTHETIC INTERMEDIATE

by

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Major Department: Chemistry

Biocatalysts derived from biological molecules such as proteins, RNA, and DNA have long been exploited for applications in synthetic chemistry. Lipases, a versatile class of biocatalysts, are known to exhibit significant promiscuity for non-native substrates in non-aqueous and mixed organic/aqueous solvents. While lipases are known to possess catalytic activity for a wide range of organic transformations, predicting which lipases will catalyze specific carbon-carbon bond forming reactions remains a significant challenge. In this study, the catalytic potential of a library of commercially available lipases was investigated by screening them for catalysis of the Robinson Annulation, a synthetically important carbon-carbon bond forming reaction. Specifically, the lipase library was screened for synthesis of the Wieland-Miescher Ketone, an important intermediate in the synthesis of biologically active compounds such as steroids and terpenoids, from methyl vinyl ketone and 2-methyl-1,3-cyclohexanedione. An optimized procedure is presented for Wieland-Miescher Ketone synthesis using a crude preparation of porcine pancreatic lipase (PPL), reporting on both yield and enantiomeric excess. Finally, commercially available lipases are often sold as crude preparations, containing many different proteins, and are often used by many researchers without further purification,

potentially obscuring the identity of the catalytic species. Therefore, a methodology to isolate and characterize the active component(s) of these lipase formulations is described in this study.

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LIST OF ABBREVIATIONS

RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
PPL	Porcine Pancreatic Lipase
EPA	Environmental Protection Agency
EtOH	Ethanol
H_2O	Water
KC	Knoevenagel Condensation
MBH	Morita-Baylis-Hillman
WMK	Wieland Miescher Ketone
His	Histidine
Asp	Aspartic Acid
C-C	Carbon-Carbon
WGL	Wheat Germ Lipase
TLC	Thin Layer Chromatography
LC	Liquid Chromatography
MS	Mass Spectrometry
HPLC	High Pressure Liquid Chromatography

FPLC	Fast Protein Liquid Chromatography
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
MW	Molecular weight
MRM	Multiple Reaction Monitoring
MALDI	Matrix-Assisted Laser Desorption Ionization
TOF	Time of Flight
DMSO	Dimethyl Sulphoxide
BSA	Bovine Serum Albumin
Hi-Res	High Resolution
NMR	Nuclear Magnetic Resonance
DMAP	Dimethylaminopyridine
µL	microliter
^{0}C	Degrees centigrade
rpm	Revolutions per minute
ESI	Electrospray Ionization
mmol	millimole
mL	milliliter
h	Hour

α	Alpha
β	Beta
HCl	Hydrochloric Acid
$CaCl_2$	Calcium Chloride
NaCl	Sodium Chloride

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Biocatalysis in Organic Synthesis

The world of synthetic chemistry has been revolutionized by transition-metal based catalysts, which allow scientists to control stereochemistry and perform complex chemical reactions.[1,2] However, these have several drawbacks including high cost, toxicity, and adverse environmental effects. As a result, chemists have been exploring the potential of biocatalysts as less expensive and non-toxic alternatives to the traditional transition-metal based catalysts, while also promoting green chemistry at the same time.[1-4] Biocatalysts are a group of molecules derived from biological molecules such as proteins, RNA, and DNA. They can be generated in large quantities, and obtained from a wide variety of sources including plant, mammalian, fungal, and bacterial species using traditional molecular biological methods.[1,2] These enzymes (biocatalysts) are already being utilized in the industrial settings, particularly in the pharmaceutical industry.[3] Recently, a U.S. EPA award-winning study conducted by protein engineering company Codexis employed an enzyme (halohydrin dehydrogenase) to synthesize an atorvastatin (cholesterol lowering drug) intermediate on an industrial scale with high yields and enantiomeric excess.[4]

Within this group of biocatalysts is a versatile class of enzymes called lipases, which have been studied extensively in the past two decades.[5] Lipases have been reported to function in non-aqueous media/organic solvents, possess the catalytic ability for a wide range of organic reactions, and provide recyclability via immobilization.[1,5,6,7] Specifically, lipases are a subclass of esterase that catalyze the hydrolysis of esters from triacylglycerides under *in vivo* conditions (high abundance of water) (**Figure 1A**). When lipases are under very low or no-water conditions, they can function alongside non-aqueous nucleophiles to catalyze various types of

organic transformations such as Michael addition and Aldol addition (**Figure 1B,C**).[6,8-11] Several different previous studies performed under *ex vivo* conditions have demonstrated that lipases catalyzed unexpected chemical reactions, therefore, deeming these catalysts as "promiscuous".[12,13] Hult and Berglund further described the promiscuity by breaking it down into three different types: condition, substrate, and catalytic.[12]

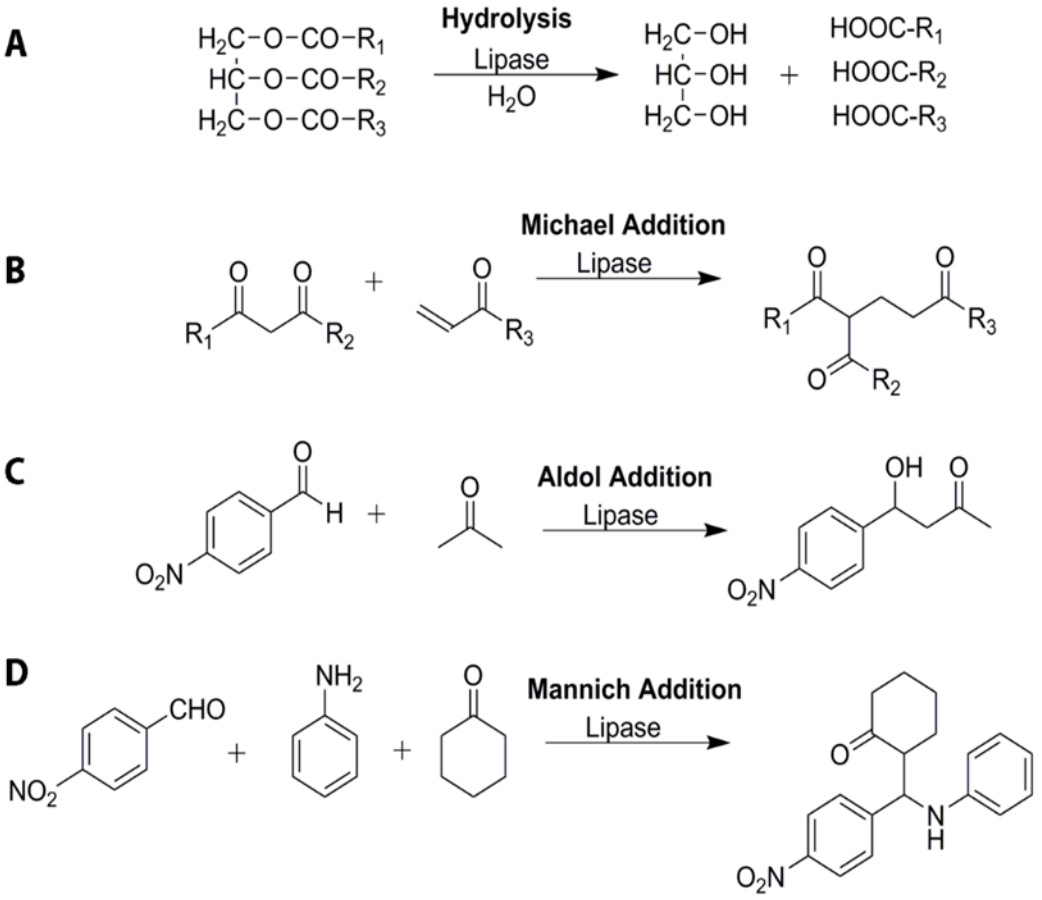


Figure 1. Native and "promiscuous" transformations catalyzed by lipases. **A**. Native lipase transformation catalyzed in the aqueous environment. **B**. Michael addition catalyzed by lipase in non-aqueous environment (solvent free).[8] **C**. Aldol addition catalyzed by lipase (acetone/0-55% H_2O).[9] **D**. Three-component Mannich addition catalyzed by lipase (EtOH/10% H_20).[10]

The “condition promiscuity” pertains to enzymes with catalytic activity in reaction conditions different from their natural ones. There have been thousands of studies that demonstrated enzymes which possess condition promiscuity can catalyze reactions conducted in organic solvents, in the absence of solvents, and at extreme temperatures (< 15 °C and >80 °C) or pH (< 2 and > 10).[14-17] When an enzyme shows broad substrate specificity, it is deemed to possess “substrate promiscuity”.[18-21] The “catalytic promiscuity” refers to the ability of an enzyme’s active site to catalyze different chemical transformations other than its native reaction. The “catalytic promiscuity” of lipases has been the foundation over which various studies have been conducted to demonstrate a broad spectrum of lipase catalyzed organic transformations.[22-27] Additionally, in organic solvents the lipases are known to catalyze a wide variety of base-activated carbon-carbon bond forming reactions, where histidine on the lipase acts as the base. These chemical interactions have been extended and applied to various carbon-carbon bond forming reactions including but not limited to the Robinson annulation, the Knoevenagel condensation, and the Morita-Baylis-Hillman reaction (**Figure 2**).[7,28,29]

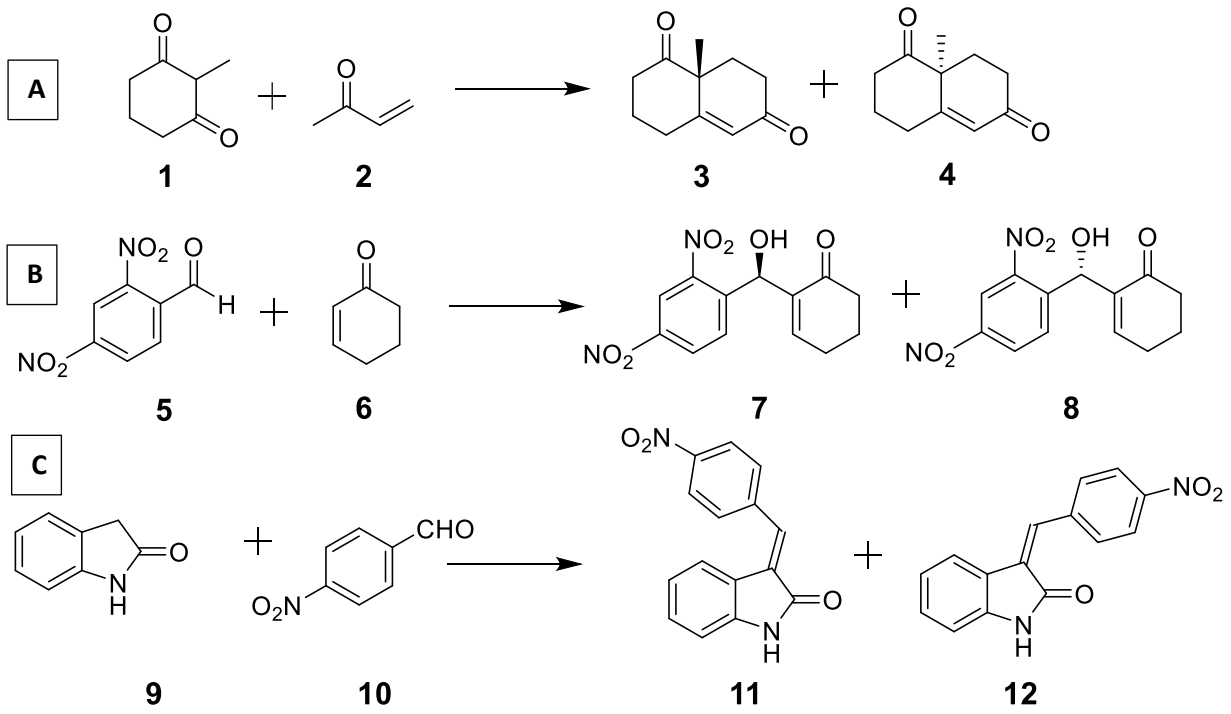


Figure 2. Examples of previously reported carbon-carbon bond forming reactions catalyzed by lipases. **A**. Robinson Annulation: reaction between 2-methyl-1,3-cyclohexanedione **1** and methyl vinyl ketone **2** results in bicyclic products **3** and **4** also known as Wieland Miescher Ketone. **B**. Morita-Baylis-Hillman: reaction between 2,4-dinitrobenzaldehyde **5** and cyclohexenone **6** results in MBH products **7** and **8**. **C**. Knoevenagel Condensation: reaction between 2-oxindole **9** and 4-nitrobenzaldehyde **10** results in KC products **11** and **12**.

1.2 Synthesis of Wieland Miescher Ketone via Robinson Annulation

The Robinson Annulation reaction, named after the chemist (Robert Robinson) who first reported the transformation in 1935, has been heavily utilized throughout the years as a synthetic tool to produce six-membered rings in polycyclic compounds.[30,31] The term "annulation" means "building a ring". Generally in this reaction, α, β-unsaturated cyclic ketones are produced from a

reaction between α, β-unsaturated ketones and aldehydes or ketones. Since its initial report, the Robinson Annulation reaction has been studied and applied extensively to produce various polycyclic compounds which act as either starting materials or intermediates for synthesis of many natural products. One example of those polycyclic compounds is the Wieland Miescher Ketone as previously mentioned (**Figure 2A**).

Synthesis of WMK via Robinson Annulation has had a tremendous impact on the field of organic synthesis, as it has provided chemists with a great synthetic tool to explore different reaction paths for various organic compounds. Interestingly, the Robinson Annulation is a simple yet very important reaction which involves combination of two commonly used organic transformations: Michael Addition and Aldol Condensation (**Figure 3**). Specifically, for the synthesis of Wieland Miescher Ketone, the reaction begins with the Michael Addition step, where the α,β-unsaturated ketone(methyl vinyl ketone **2**) goes under a nucleophilic attack by the activated 2-methyl-1,3-cyclohexanedione **1** to produce the triketone **13**. This Michael product then acts as the starting material for the subsequent step, Aldol Condensation, in the Robinson Annulation reaction cascade. During the second segment of Robinson Annulation reaction, the triketone intermediate goes through an intramolecular aldol condensation producing an enolate **14**. The hydroxide ion is expelled through dehydration step producing both enantiomers of the desired product Wieland Miescher Ketone **3** and **4**.

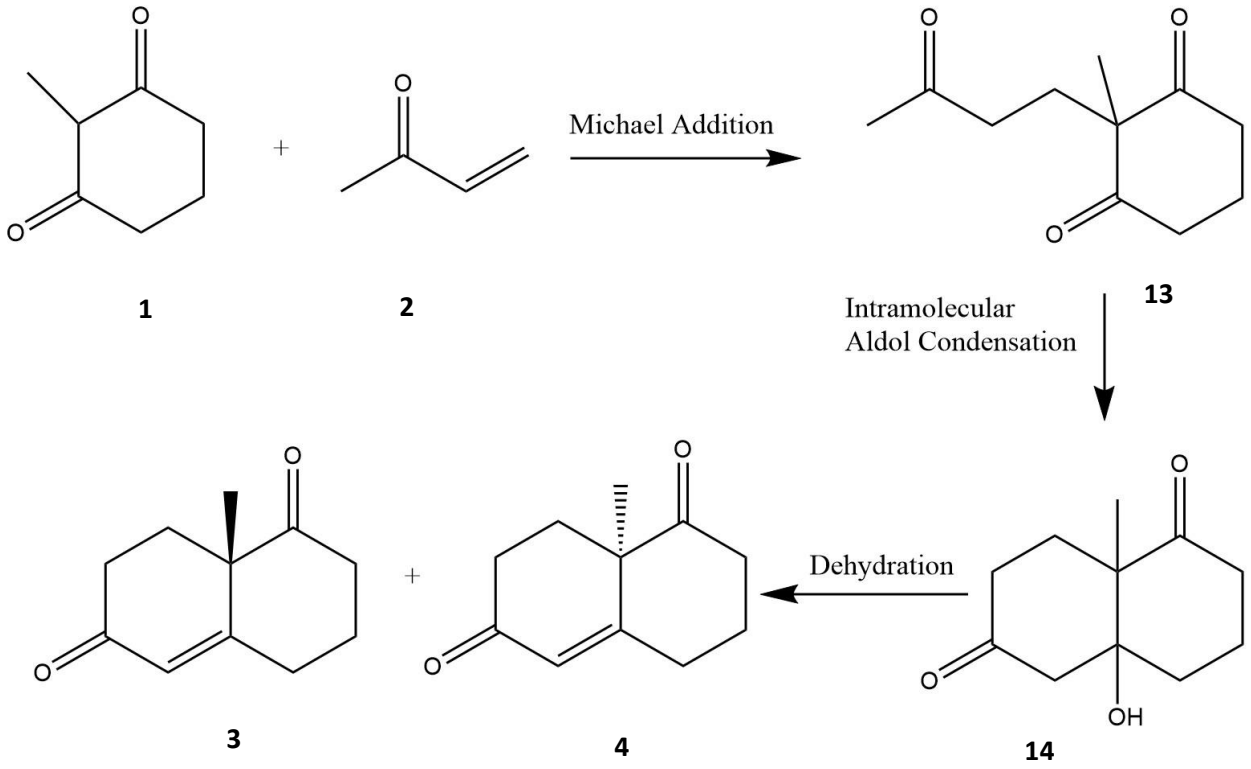


Figure 3. Wieland Miescher Ketone synthesis scheme.

The Wieland Miescher Ketone, named after the two chemists who first reported its synthesis in 1950, is a bicyclic α, β-unsaturated cyclic ketone.[32] Throughout the years, the synthetic potential of WMK has been explored by many scientists. The presence of two rings (AB ring structure) makes it a very versatile building block for synthesis of many different compounds such as steroids, terpenoids, Taxol, and other natural products. WMK has many applications in synthesis of complex organic compounds, especially in the pharmaceutical industry. The importance of WMK could not be emphasized enough, as many studies have been reported where biologically important steroids, anticancer compounds, and other natural products were synthesized starting from this versatile bicyclic compound and its analogues.[33-39]

1.3 Research Relevance and Objective

Recent advancements in molecular biotechnology and protein engineering[40,41], along with increasing interest for "green chemistry" has led to an emergence of biocatalysts being adopted as an alternative catalyst for organic transformations.[42] Specifically, lipases have been extensively researched for synthesis of different organic compounds. A generally accepted mechanism of lipase catalysis, as described by Hult and Berglund, is that the active site responsible for native function of hydrolysis also plays a major role in the promiscuous catalysis of organic reactions.[12] The catalytic triad present in the active sites are believed to be the driving force of catalysis in enzymes, where they create electronic environment conducive to the activation of a specific substrate initiating the reaction. The term catalytic triad refers to a set of three amino acids commonly found in active sites of enzymes: histidine, aspartic acid, and serine.[43] The active sites provide an oxyanion hole which are very crucial for stabilization of the high energy oxyanion intermediates or transition states during a reaction. The oxyanion hole is basically a pocket that consists of backbone amides or positively charged residues which would stabilize the negative charge on a deprotonated oxygen or alkoxide in the transition state. This is where the presence of serine in the catalytic triad becomes important, as it contains a neutral polar side chain. The hydroxy side chain in serine creates a positively charged microenvironment to stabilize the high energy negatively charged transition states, and also act as an anchor through catalysis.[44]

The mechanism for Porcine Pancreatic Lipase-catalyzed Robinson annulation reaction, proposed by Lai and Zhang, is shown in **Figure 4**. The proposed mechanism starts with the first step of Robinson Annulation, which is Michael reaction, in the active site consisting of the catalytic triad. The 2-methyl-1,3-cyclohexanedione is activated via deprotonation by His264-

Asp177 amino acid pair to form an enolate nucleophile. The carbonyl on methyl vinyl ketone interacts with the oxyanion hole in the active site increasing its electrophilic ability. The activated enolate performs a nucleophilic attack on the methyl vinyl ketone followed by proton transfer producing the intermediate **b**. The next step involves deprotonation by the His264-Asp177 pair to form an enolate anion, influenced by the stabilizing effects of the oxyanion hole. This enolate anion goes under intramolecular Aldol Condensation to produce the aldol adduct **c**. Finally the aldol adduct underwent dehydration resulting in the desired product **d**, Wieland Miescher Ketone(WMK).

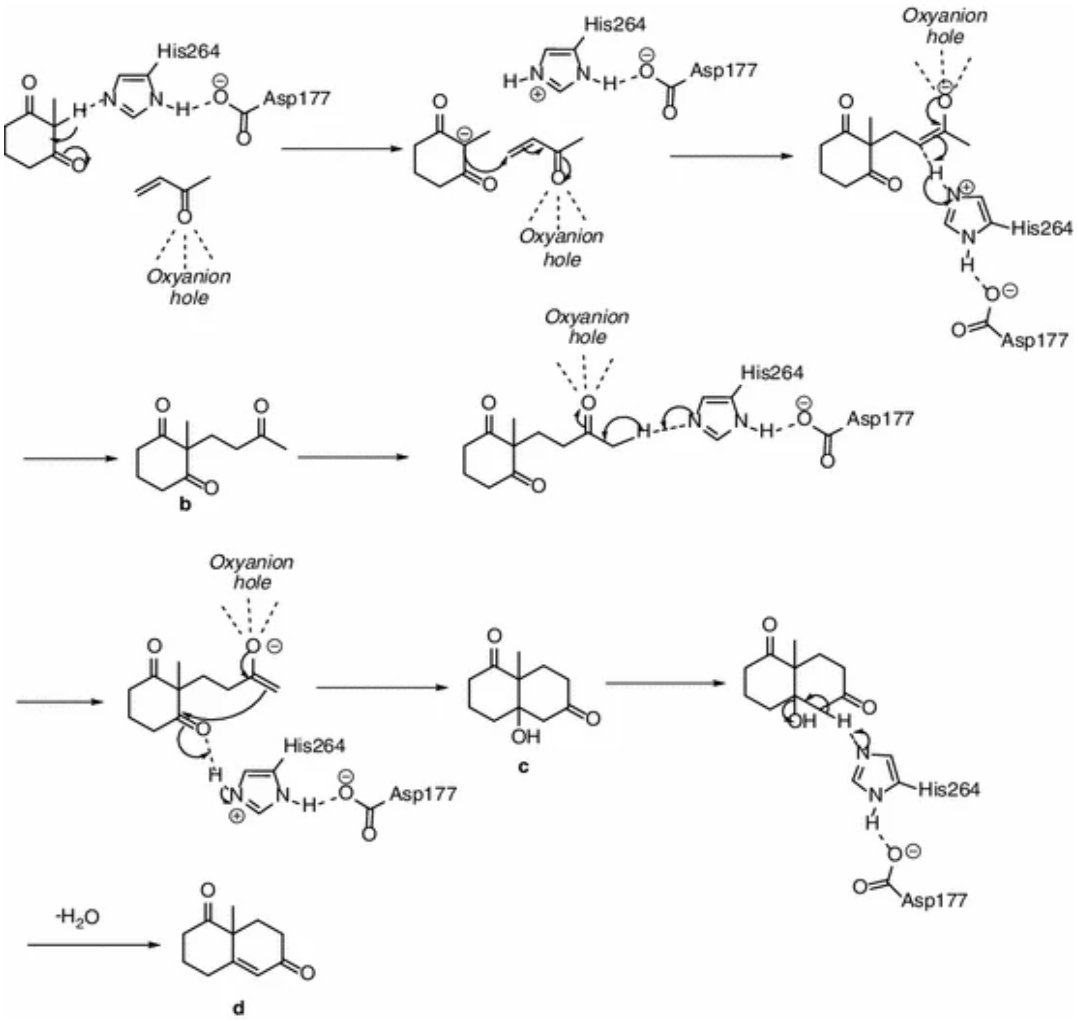


Figure 4. Proposed mechanism for PPL-catalyzed Robinson annulation for WMK synthesis.[29]

Based on previously reported results it is hypothesized that lipases proceed to catalyze the Robinson Annulation reaction through their active site. The objective of this research is to screen a library of lipases, consisting of some lipases with previously reported promiscuity while others with unknown catalytic activity for synthesis of WMK. Also, it is the hope of this research to characterize the lipases that show promising catalytic promiscuity towards synthesis of WMK, for future application of directed evolution or enzyme engineering to maximize the lipases activity for possible industrial application[20,40,41]. Finally the last objective of this research is to better understand the role of active site in lipase promiscuity towards organic transformation such as the Robinson Annulation.

Different biocatalysts had been previously reported to catalyze the Robinson Annulation reaction. In the original biocatalytic preparation of WMK, the triketone Michael product from the first step of the Robinson Annulation was synthesized using traditional organic catalysts, and then isolated. The isolated Michael adduct was then reacted in organic solvent along with organocatalysts such as L-Proline and its analogues to produce Wieland Miescher Ketone via intramolecular aldol condensation as a mixture of enantiomers. Isolating one of the enantiomers is a very challenging task, which generally requires additional processing after the initial synthesis of a racemic mixture. One of the many reasons for producing one or the other enantiomers is pharmaceutical application and natural product synthesis. Although they might be the same molecule with the only difference being in their stereochemistry, this difference is very critical in the efficacy of drugs where one enantiomer could help alleviate a health problem while the other could have harmful side effect. Since WMK is a very important and versatile starting material for synthesis of many natural and pharmaceutical products as mentioned previously, it is imperative that efficient methods to synthesize it be explored. Interestingly in

the past couple of decades, biocatalysts such as lipases have caught the attention of organic chemists for synthesis due their ability to provide great control over stereoselectivity.

CHAPTER 2: SCREENING OF LIPASE LIBRARY FOR SYNTHESIS OF WIELAND MIESCHER KETONE

2.1 Assembly of Lipase Library

The study began by assembling a 14-member library of commercially obtained lipases. The library consisted of lipases that had been previously reported to catalyze C-C bond forming reactions such as porcine pancreatic lipase (PPL) and *Candida Antarctica lipase* B[8,28,29], in addition to lipases with unknown biocatalytic properties for promiscuous biocatalysis such as wheat germ lipase (WGL). The library consisted of lipases originating and extracted from different species such as bacterial, fungal, plant, and mammals (**Table 1)**.

Table 1. Commercially obtained lipase library

Lipase Code	*Lipase*	*Origin*	*Sigma Aldrich Catalog Number*
L1	Porcine Pancreatic Lipase	Mammalian	L3126
L2	Rhizopus Oryzae	Fungal	62305
L3	Wheat Germ Lipase	Plant	L3001
L4	Candida Rugosa	Fungal	L1754
L5	Aspergillus Niger	Fungal	62301
L6	Aspergillus Oryzae	Fungal	L4277
L7	Pseudomonas Cepacia	Bacterial	62309
L8	Candida Sp.	Fungal	L3170
L9	Rhizopus Niveus	Fungal	62310
L10	Mucor Miehei	Fungal	62298
L11	Mucor Javanicus	Fungal	L8906
L12	Candida Antarctica B	Fungal	62288
L13	Candida Antarctica A	Fungal	62287
L14	Pseudomonas Fluorescens	Bacterial	534730

2.2 Screening of Lipase Library

The biocatalytic potential of our lipase library was assessed by screening the lipases for a one-pot synthesis of WMK from methyl vinyl ketone and 2-methyl-1,3-cyclohexanedione using previously reported literature conditions.[29] Interestingly, after 48 hours of reaction it was noted that each of the reaction mixtures had varying degree of brownish-orange color, possibly indicating that product had formed. Reaction progress was monitored using TLC upon completion of reaction after 48 hours. TLC analysis showed spots for product along with one of the starting materials (methyl vinyl ketone), indicating that reaction had consumed all the limiting starting material (2-methyl-1,3-cyclohexanedione). LC/MS method was adopted to quickly assess the yields of biocatalyzed reaction. Using WMK standard, an LC/MS method was developed (Experimental Section) to detect only the main fragmentation pattern for the WMK product in reaction samples (**Figure 5**).

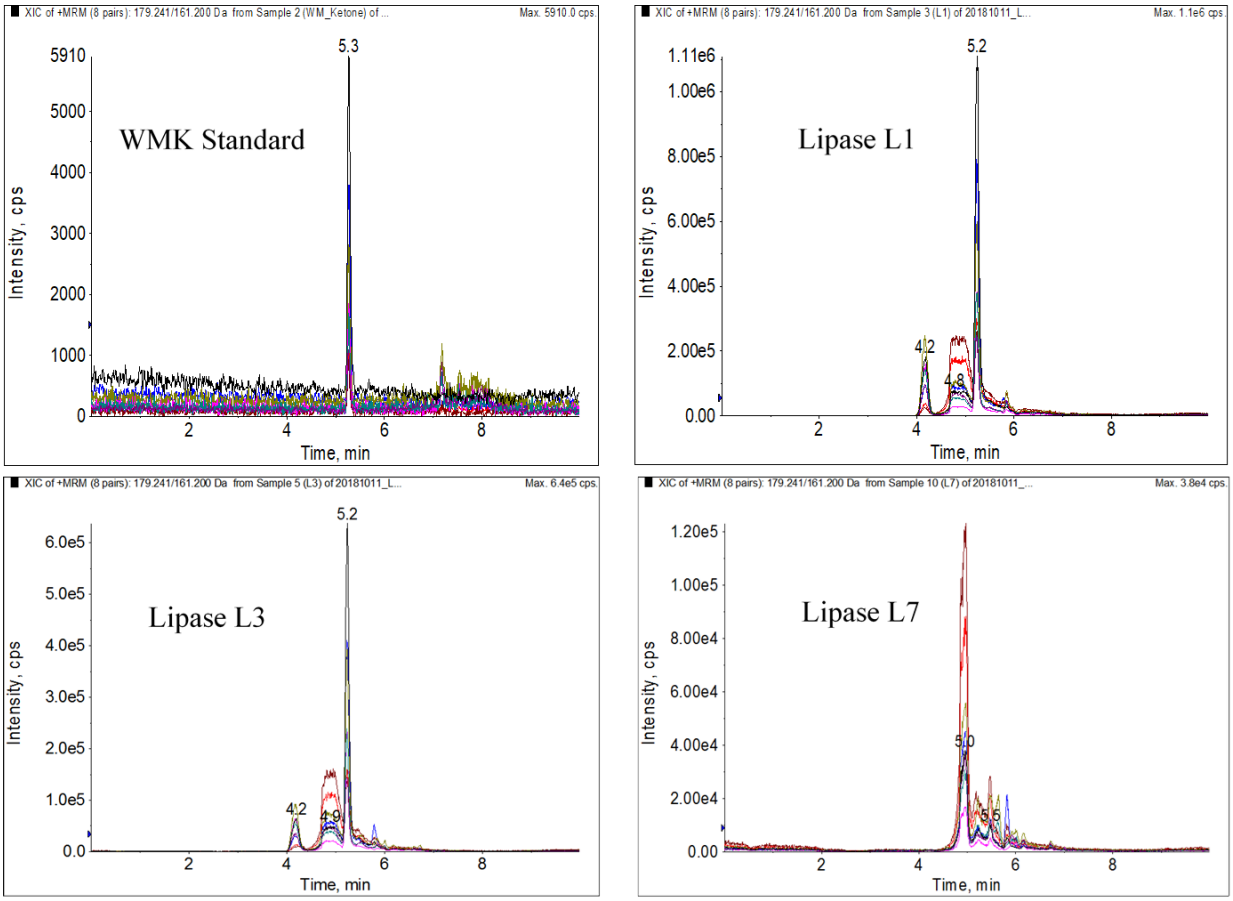


Figure 5. HPLC chromatograms from LC/MS analysis. **Top left:** WMK standard. **Top right:** Lipase L1 (Porcine Pancreatic Lipase, PPL). **Bottom left:** Lipase L3 (Wheat Germ Lipase, WGL). **Bottom right:** Lipase L7 (Pseudomonas Cepacia). The overlaying peaks with different color in each chromatogram represent the eight most abundant ion pairs from MS fragmentation. Target mass (*m*/*z*) = 178.10

A series of standard solutions were used to generate a standard curve (**Figure 6**) on the LC/MS for quantification of WMK yield for reaction samples.

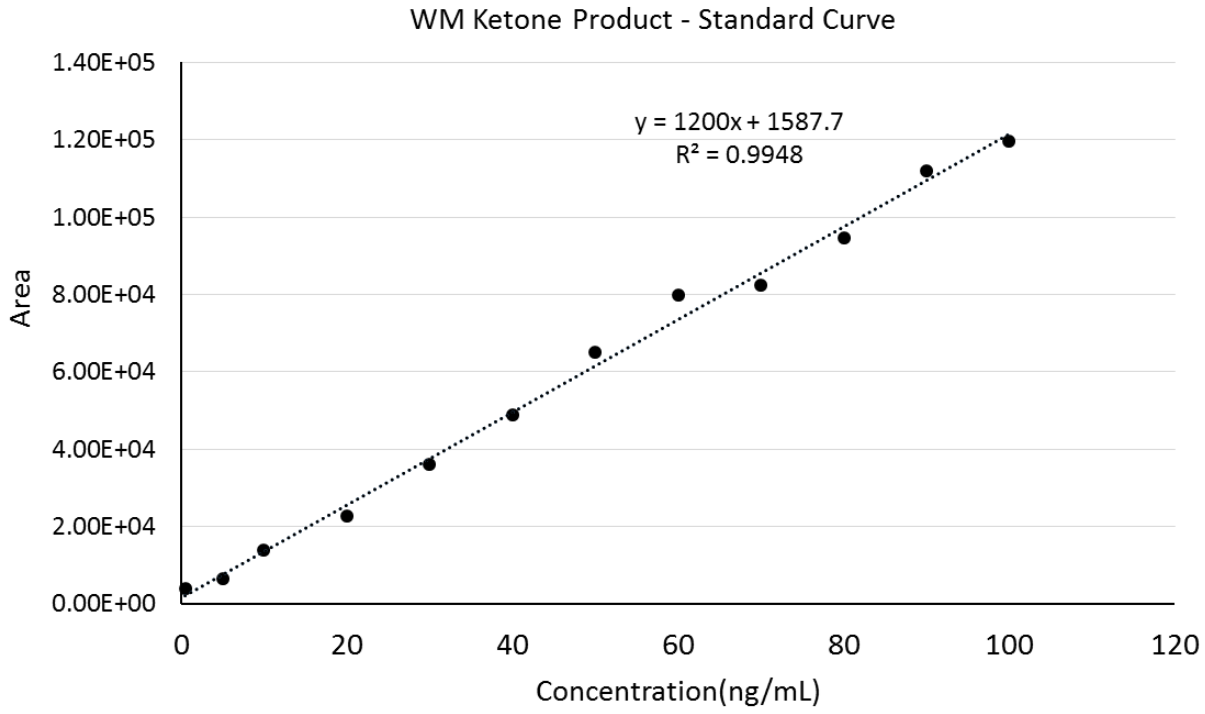


Figure 6. Wieland Miescher Ketone standard curve used for quantification of yields

The LC/MS data for reaction samples was quantified and their reaction yields can be seen below in **Table 2** and **Figure 7**.

Table 2. Screening of lipase library for synthesis of Wieland Miescher Ketone

Entry	Lipase Code	Lipase Name	Yield(ng/μL)[a]	Yield(%)[a]
1	L1	Porcine Pancreatic Lipase(PPL)	6140	<1
2	L2	Rhizopus Olyzae	92	<1
3	L3	Wheat Germ Lipase(WGL)	2880	<1
4	L4	Candida Rugosa	269	<1
5	L5	Aspergillus Niger	591	<1
6	L6	Aspergillus Oryzae	242	<1
7	L7	Pseudomonas Cepacia	59	<1
8	L8	Candida Sp.	148	<1
9	L9	Rhizopus Niveus	457	<1
10	L10	Mucor Miehei	211	<1
11	L11	Mucor Javanicus	331	<1
12	L12	Candida Antarctica “B”	15	<1
13	L13	Candida Antarctica “A”	77	<1
14	L14	Pseudomonas Fluorescens	648	<1

[a] The yields were determined by LC/MS

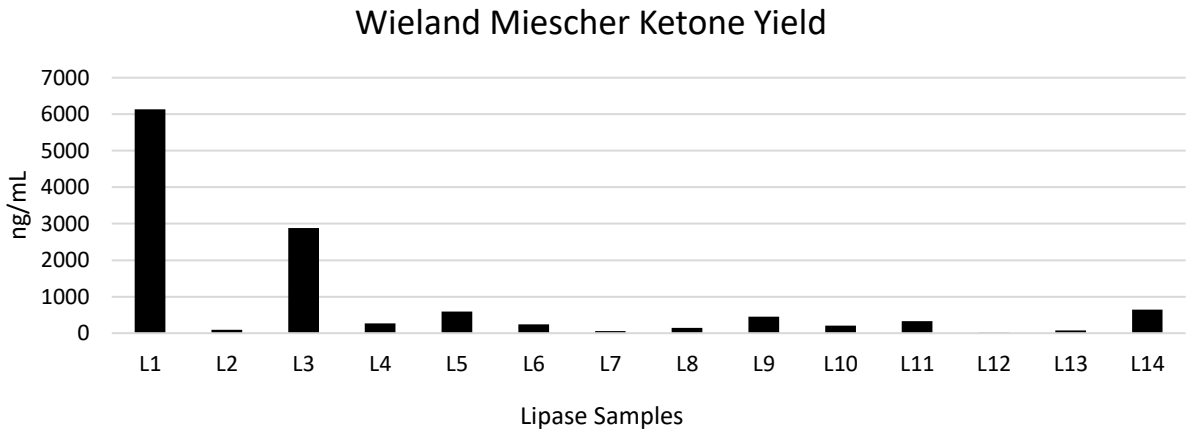


Figure 7. Quantification of data for lipase library using the standard curve shown in **Figure 6**. Reaction conditions: The following reagents were mixed in a centrifuge tube for one-pot synthesis of Wieland Miescher Ketone: methyl vinyl ketone (81 μL,), 2-methyl-1,3-cyclohexanedione (23 mg), water (100 μL), and lipase (5 mg). After all the reactants were assembled, the centrifuge tubes were placed in an incubator shaker (30 °C, 250 rpm) for 48 hours.

The results from screening study showed some lipases (Entries 1, 3, 5, and 14 in **Table 2**) possessed biocatalytic activity, but none of the yields were above 1%. Interestingly, the literature report from which the reaction conditions were obtained had reported yields of up to 95% using Porcine Pancreatic Lipase as the biocatalyst.[29] Although reaction yields from the screening study were all below 1%, it is important to note that out of all the lipases screened the minimal yet best yield was achieved using PPL; the biocatalyst which resulted in 95% yield in a literature report using the same conditions. Besides PPL (L1), there were other lipases such as L3, L5, L9 and L14 (**Figure** 7 and entries 3, 5, 9, and 14 in **Table 2**) which also produced WMK **3** and **4**.

2.3 Identification of the Primary Components of Lipase Preparations

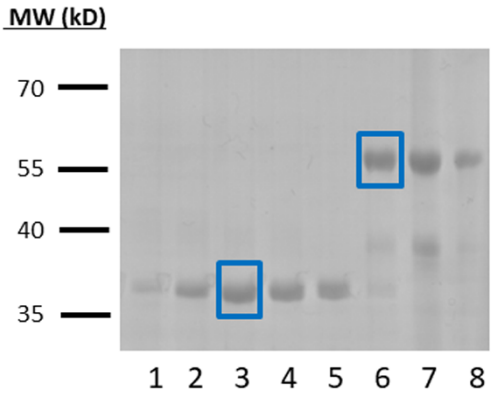
Lipases are the most commonly used biocatalysts in organic synthesis because of their broad specificity for various molecules, and the high degree of regio- and enantioselectivity they provide.[1-4] However, most of the commercially available lipases are reported to be crude preparations, meaning that they contain contaminants such as other proteins.[45,46] This could create a huge problem since there is no control over which proteins are present in the commercially obtained lipase. The presence of other contaminant proteins could possess some form of catalytic activity, which could potentially have opposite activities that would make the interpretation of the results very difficult. These contaminants could even hinder the efficiency of these commercially available lipases, specifically when being used for organic reactions, by causing competing/reverse reactions. For example, a widely used lipase for biocatalysis, PPL (porcine pancreatic lipase), was previously reported to contain other proteins.[47] Another study

reported that commercially available crude preparation of PPL contained between 10 and 50 different enzymes.[48] The recent advancements in molecular biotechnology and enzyme engineering have tremendous application to optimize the protein to its full potential as previously demonstrated in an EPA award winning study.[4] The study conducted in collaboration with Codexis, a protein engineering company, demonstrated the potential of directed evolution. The researchers in the study evolved a lipase to develop a more efficient catalyst that gave yields for an atorvastatin intermediate above 90% on a commercial scale.[4] Directed evolution allows scientists to modify the lipases or any other enzyme to obtain higher yields and greater control over the stereoselectivity of the target product. But performing directed evolution or applying other enzyme engineering techniques requires the identity or peptide sequence of the enzyme of interest. Therefore, it is very important to identify the primary component of the crude lipase which is catalyzing the reaction.

Since PPL(L1) and WGL(L3) were the biocatalysts that had the best yields of WMK from the initial screening, it was important to isolate, identify, and characterize the biocatalytic component of those lipases. That was achieved by separating the crude lipase mixture of both the PPL and WGL using size exclusion chromatography with AKTA FPLC. The resulting fractions were analyzed using SDS-PAGE to obtain the desired bands. Two distinct bands were found in SDS-PAGE analysis of fractions from crude PPL (**Figure 8A**), while many bands were found for WGL (**Figure 9**). The bands were excised and sent to UNC Proteomics Center for mass spectrometry fingerprinting analysis for characterization. The results (**Figure 8B**) indicated that the lower molecular weight band from Lane 3 in **Figure 8A** contained truncated version of two peptides, Carboxypeptidase A1 and Carboxypeptidase B, while higher molecular weight band from Lane 6 in **Figure 8A** contained peptide Alpha-amylase. Surprisingly, neither

one of the primary components found in the PPL crude preparation was namesake Porcine Pancreatic Lipase. This is very significant as many different studies have reported promising results using this commercially available crude preparation of PPL (Type II) and other enzymes without any further purification or characterization.[29,49] The same method was applied to characterize the peptides present in WGL crude preparation, which were identified to be HSP70 and Phosphoglycerate Kinase (**Figure 8B**). Interestingly, none of the identified proteins were the namesake Wheat Germ Lipase.

A

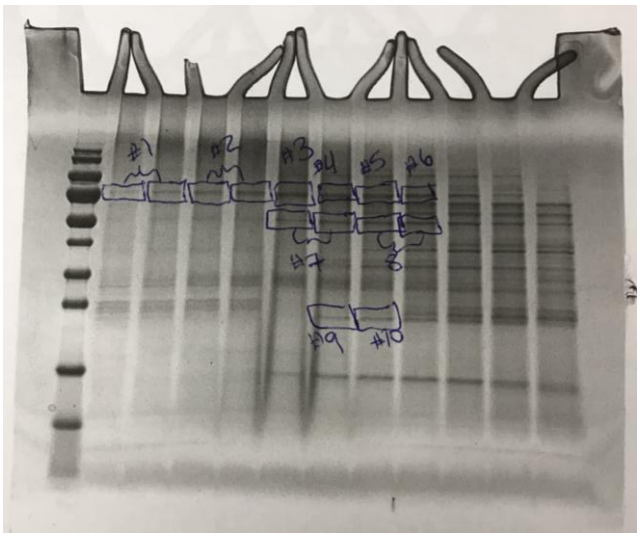


B

	Sample(Band/Spot)	Protein Name	Species	MW(Da)
Lane 6	PPL - High MW	Alpha-amylase	Sus scrofa	57094
Lane 3	PPL - Low MW	Carboxypeptidase A1	Sus scrofa	47206
Lane 3	PPL - Low MW	Carboxypeptidase B	Sus scrofa	47351
	WGL – High MW	HSP70	Triticum aestivum	70986
	WGL – Low MW	Phosphoglycerate kinase	Triticum aestivum	39331

Figure 8. Identification of primary components of PPL (Porcine Pancreatic Lipase, L1) and WGL (Wheat Germ Lipase, L3) preparations. **A**. The PPL crude preparation was purified using a size exclusion chromatography and the resulting fractions were analyzed using SDS-PAGE. Two distinct bands were found in crude preparations of PPL, which were then characterized by mass spectrometry fingerprinting analysis. **B**. Peptides identified from the fingerprint analysis. The lower molecular weight band from Lane 3 in (**A**) contained two proteins (truncated), Carboxypeptidase A1 and Carboxypeptidase B. The higher molecular weight band from Lane 6 in (**A**) contained protein Alpha-amylase. The two proteins identified in the distinct bands from WGL crude preparation were HSP70 and Phosphoglycerate kinase.

A



B

Bands 1-6
Description
Protein disulfide-isomerase [OS=Triticum aestivum]
Adenosylhomocysteinase [OS=Triticum aestivum]
Beta-amylase [OS=Triticum aestivum]
Bands 7-8
Description
Adenosylhomocysteinase [OS=Triticum aestivum]
Protein disulfide-isomerase [OS=Triticum aestivum]
Beta-amylase [OS=Triticum aestivum]
Glucose-1-phosphate adenylyltransferase large subunit, chloroplastic/amyloplastic [OS=Triticum aestivum]
Serpin-Z1B [OS=Triticum aestivum]
Serpin-Z1C [OS=Triticum aestivum]
Serpin-Z1A [OS=Triticum aestivum]
Bands 9-10
Description
2-Cys peroxiredoxin BAS1, chloroplastic [OS=Triticum aestivum]

Figure 9. Proteins identified from crude lipolytic preparation derived from Wheat Germ. **A**. Wheat germ lipase preparation was purified using size exclusion chromatography. The fractions were analyzed using SDS-PAGE and numerous proteins were found to be present in the crude preparation. **B**. Proteins from *T. aestivum* identified from the fingerprint analysis.

2.4 Experimental Section

All chemicals, substrates, and solvents were purchased from commercial sources (VWR, Millipore-Sigma, TCI America, and Fisher Scientific. Lipases were purchased from Sigma-Aldrich. Thin layer chromatography was performed with silica gel 60 F254 plates, purchased from EMD chemicals.

Biocatalysis Reaction Conditions

The following reagents were mixed in a centrifuge tube for one-pot synthesis of Wieland Miescher Ketone: methyl vinyl ketone (81 µL), 2-methyl-1,3-cyclohexanedione (23 mg), water (100 µL), and lipase (5 mg). After all the reactants were assembled, the centrifuge tubes were placed in an incubator shaker (New Brunswick Excella e24; 30 °C, 250 rpm) for 48 hours.

LC/MS Instrumental Method

Liquid chromatography (LC) was performed on a HPLC system (3200 Exion LC100; SCIEX Corporation). 5 µL of samples were injected into the reversed-phase Gemini NX-C18 column (50 × 2.0 mm, 3 µm; Phenomenex, Torrance, CA, USA) by using auto sampler. The mobile phase composition for A was Water/Acetonitrile mixture (95/5, v/v) with 0.1% formic acid, and for B was Methanol. The gradient method was as follows: 100% A for 1 min, decrease to 15% over 4 min, hold at 15% for 1 min, increase back to 100% over 1 min, and hold at 100% for 3 min. The flow rate was 0.3 mL min^{-1} and total run time was 10 min for each sample injection. The column temperature was kept at 35 °C. The LC elute was introduced into the Applied Biosystem Triple Quad API 3200, a triple-quadrupole tandem mass spectrometer equipped with a turbo spray ionization source, for quantification of compounds in positive

ionization mode. Detection of the target molecule was performed in a multiple reaction monitoring (MRM) mode, and transition of *m/z* 179.24 to 161.20 was used for detection of WMK. Mass of WMK: 178.23 g/mol.

Lipase Purification and Proteomics Method

Solutions of porcine pancreatic lipase and wheat germ lipase were fractionated on a HiPrep 26/60 Sephacryl S-200 HR column connected to an Akta FPLC (GE Healthcare Life Sciences). Protein fractions were characterized via SDS-PAGE stained with SimplyBlue SafeStain (ThermoFisher). The bands of greatest intensity were excised and digested with trypsin overnight using a standard in-gel digestion protocol. The resultant tryptic peptides were desalted using a C18 Zip-Tip and applied to a MALDI target plate with α-cyano-4-hydroxycinnamic acid as the matrix. The sample was analyzed on an AB Sciex 5800 MALDI TOF/TOF mass spectrometer (Framingham, MA, USA). MS/MS spectra were searched against Uniprot databases using Mascot version 2.3 (Matrix Science) (Boston, MA, USA) within ProteinPilot software version 3.0 (AB Sciex, Framingham, MA, USA). A significance score threshold was calculated in Mascot, with ion scores above the threshold considered positive IDs ($p < 0.05$).

2.5 Summary of Results

At the conclusion of this investigation, it was found that Porcine Pancreatic Lipase and Wheat Germ Lipase were the two biocatalysts which produced the most amount of the target molecule, Wieland Miescher Ketone, from the screening experiment. The yields from lipase library screen were minimal (<1%) in contrast to the literature report that used the same reaction conditions. Interestingly, the biocatalyst PPL which was reported to give 95% WMK yield in

that study also gave the best yield out of all the biocatalyst from the screening experiment. After identifying PPL as the top-hit biocatalyst from the screening experiment, it was important to characterize the primary component of the crude preparation of lipase. This was achieved via purification of lipase (PPL and WGL) solution using size exclusion chromatography. The resulting fractions were analyzed on SDS-PAGE and two distinct bands were found to be present in PPL. Same method was applied to WGL and the resulting bands from SDS-PAGE analysis for both biocatalysts were excised and sent to UNC Proteomics Center for protein identification via mass spectrometry fingerprinting analysis. The results were surprising, as none out of the three peptides (Carboxypeptidase A1, Carboxypeptidase B, and Alpha-amylase) found to be the principle components of PPL were the namesake Porcine Pancreatic Lipase. This was a very significant finding as there have been numerous reports using this commercially available crude preparation of PPL (Type II) for catalyzing organic transformations without any further purification or characterization. Which means that there could possibly be a group of proteins contributing towards the overall yields of these biocatalyzed organic reactions. Identifying the primary components of PPL creates many possibilities for future enzyme engineering application to optimize the enzyme specifically to meet the goals of a study or an experiment.

CHAPTER 3: REACTION OPTIMIZATION

3.1 Addition of Imidazole as Co-catalyst

After identifying PPL as the model biocatalyst for one-pot synthesis of WMK based on the lipase library screening results, optimization experiments were undertaken. To optimize this reaction, reaction conditions from a previous study were adopted where one-pot synthesis of WMK was achieved using L-proline as organocatalyst in DMSO.[50] There were several changes to the reaction conditions from the initial screening experiment. The reaction time was increased from 48 hours to 89 hours, reaction temperature was increased to 35 °C, and the solvent was switched from methanol to DMSO. The L-proline reaction sample was included as a positive control. Also, a HPLC method was developed to analyze the samples for optimization experiments. The first set of reactions involved a positive control of L-proline along with the top-hit biocatalyst PPL from the screening experiment, and Bovine Serum Albumin.

Table 3. Reaction yields for L-Proline catalyzed Robinson Annulation

Entry	Biocatalyst	Yield(%)[a]
1	-	n.r.
2	L-Proline	68.8
3	PPL	2.6
4	BSA	n.r.

[a] Yields were determined by HPLC; n.r.= no reaction

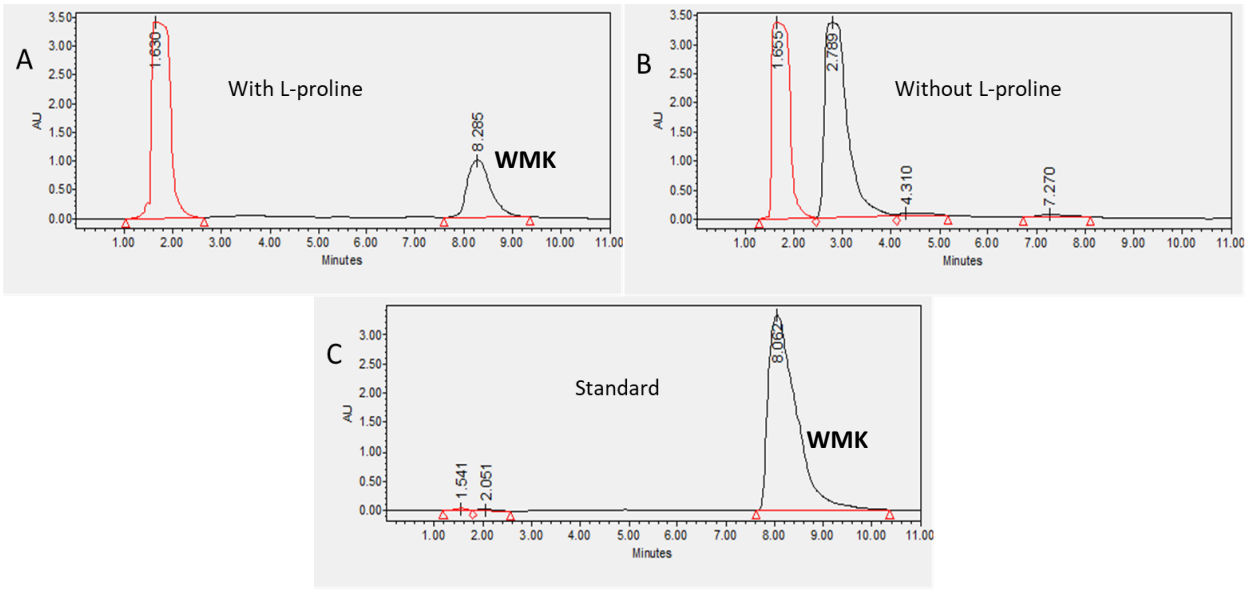


Figure 10. HPLC chromatograms of L-proline catalyzed Robinson Annulation reaction. Reaction conditions: The following reagents were mixed in a centrifuge tube for one-pot synthesis of Wieland Miescher Ketone: DMSO (1000 µL), methyl vinyl ketone (19.8 µL, 0.24 mmol), 2-methyl-1,3-cyclohexanedione (20 mg, 0.16 mmol), L-Proline (6.4 mg), PPL/BSA (5 mg). After reactant assembly, centrifuge tubes were placed in a shaker (35 °C, 250 rpm) for 89 hours.

The results from the positive control study (**Table 3** and **Figure 10**) clearly indicate that L-proline was catalyzing the synthesis of WMK with good yield (68.8%), while PPL under the same reaction conditions was only able to achieve 2.6% yield. Interestingly we found that the PPL catalyzed reaction in DMSO was producing the triketone Michael product **13**, while the L-proline reaction was producing the expected WMK products **3** and **4**. This was confirmed by Hi-Res mass spectrometry and proton NMR. Therefore, we hypothesized that L-proline plays a dual role as a biocatalyst where the amino acid stabilized the necessary transition state for conversion of **13** to **14** through intramolecular aldol condensation, and the side chain functions as a base to

produce **3** and **4** through dehydration step. This indicated that an addition of an acidic or basic co-catalyst might be necessary to help with the dehydration step to push the reaction forward. A previously reported study had utilized acid to promote the dehydration and push reaction forward after the intramolecular aldol condensation step had completed to produce high yields of WMK.[49] Interestingly, imidazole is the side chain of amino acid histidine, which is a part of the catalytic triad present in the active sites of enzymes. Therefore, it was reasoned that basic co-catalyst such as imidazole would possibly play a bi-functional role by helping with the activation of ketone for the Michael step and also by allowing the reaction to progress forward post-intramolecular aldol condensation by promoting dehydration and potentially increasing the yield of WMK.

The next step in reaction optimization was to test the addition of imidazole as co-catalyst along with the PPL while keeping the other reaction conditions same. Reaction samples were setup with and without imidazole to test our hypothesis and better understand the role of co-catalyst in reaction.

Table 4. Addition of Imidazole as Cocatalyst

Entry	Biocatalyst	Cocatalyst(Imidazole)	Yield(%)[a]
1	-	-	n.r.
2	-	+	n.r.
3	PPL	-	2.6
4	PPL	+	14.4
5	BSA	-	n.r.
6	BSA	+	13.4

[a] Yields were determined by HPLC; n.r. = no reaction

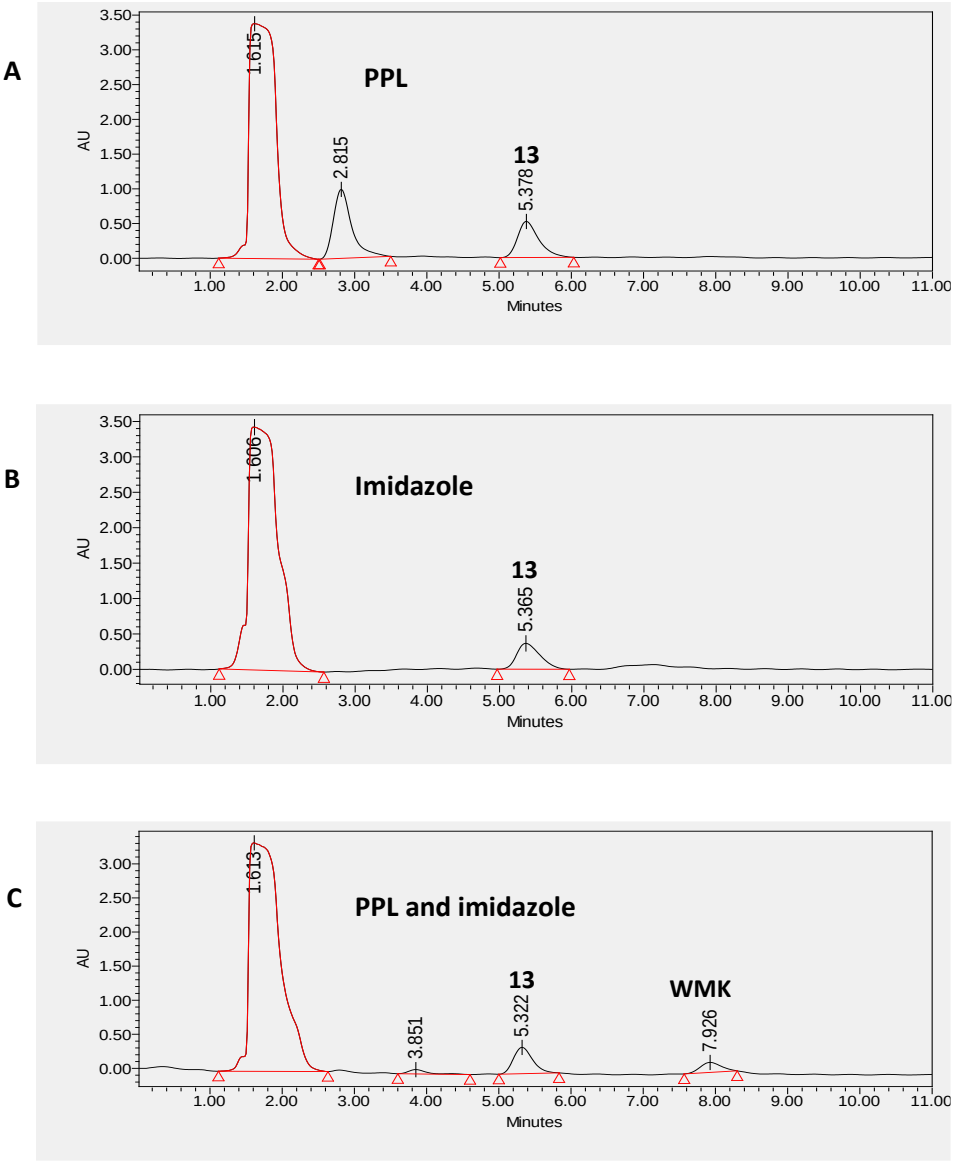


Figure 11. HPLC chromatograms corresponding to reaction samples in **Table 4**. **A.** HPLC chromatogram of sample with only PPL. **B**. HPLC chromatogram of sample with only imidazole. **C**. HPLC chromatogram of sample with both PPL and imidazole. Reaction conditions: The following reagents/reactants were mixed in each centrifuge tube for synthesis of Wieland Miescher Ketone: DMSO (900 µL), methyl vinyl ketone (19.8 µL, 0.24 mmol), 2-methyl-1,3-cyclohexanedione (20 mg, 0.16 mmol), Deionized water (100 µL), lipase (L1, 5 mg),

imidazole (2.7 mg, 0.04 mmol). After reactant assembly, centrifuge tubes were placed in an incubator shaker (35 °C, 250 rpm) for 89 hours.

The results clearly indicated the impact of imidazole on the yield after its addition as a co-catalyst. The reaction sample with just the biocatalyst PPL had no product formation as clearly indicated by the absence of a peak around 8 minutes in HPLC chromatogram (**Figure 11A**). The reaction sample with just the co-catalyst imidazole also had no product formation as indicated by the absence of product peak in the chromatogram (**Figure 11B**). Although no product was being formed, one of the starting materials had been completed consumed during the reaction in the presence of just the imidazole, which was not the case when only PPL was present in the reaction mixture. This is possibly due to imidazole acting as base during the first segment of Robinson Annulation reaction and promoting the Michael addition. That would generally be catalyzed by the Histidine amino acid present in the active site as a part of the catalytic triad mentioned earlier. Since imidazole is the side chain of Histidine which plays a role in its functionality, the presence of free imidazole in the reaction allowed for the Michael reaction segment to go to completion while consuming all of the limiting reagent (2-methyl-1,3-cyclohexanedione). Interestingly, when both the PPL and imidazole were present together in the reaction mixture, minimal product formation was detected (**Figure 11C** and entry 4 in **Table 4**). These results indicate that addition of imidazole as co-catalyst could be playing a role in the dehydration step of the reaction after the intramolecular aldol condensation step pushing the reaction equilibrium towards the product. Also it is important to note that BSA(bovine serum albumin) also produced the WMK in the presence of imidazole.

3.2 Catalyst Loading Study

The co-catalyst study showed that the presence of imidazole was important along with the biocatalyst for product formation. The next step in reaction optimization was to determine the optimal catalyst loading. Reaction samples with the biocatalyst PPL ranging from 5 to 40 mg/mL were assembled in DMSO along with the co-catalyst imidazole. No other reaction conditions were changed except the amount of biocatalyst present in the reaction mixture.

Table 5. Reaction yields from PPL loading study

Entry	Biocatalyst	Catalyst Load (mg)	Yield(%)[a]
1	PPL	5	14.4
2	PPL	10	23.9
3	PPL	15	47.9
4	PPL	20	51.4
5	PPL	25	60.4
6	PPL	30	62.2
7	PPL	35	51.9
8	PPL	40	11.2

[a] Yields were determined by HPLC

The results in **Table 5** show the yields obtained from the catalyst loading study, where the amount of catalyst in each sample was varied from 5 mg/mL to 40 mg/mL. The results were intriguing, as the yields increased in every reaction sample with catalyst loading all the way up to 30 mg/mL, but started decreasing with higher catalyst loading. This observed decrease in the yield with higher catalyst loading could be contributed mostly to an increased amount of insoluble material from the crude lipase preparation in the reaction vials, preventing an efficient mixing between the catalyst and the substrates. This problem could be addressed by applying mechanical stirring instead of the shaking during the incubation/mixing period, which might result in better yields for the higher catalyst loading samples.

3.3 Solvent Effects and Enantiomeric Excess Determination

The WMK is a chiral compound, meaning that there are two enantiomers of the molecule. Since WMK is a versatile starting material for steroids, terpenoids, and other natural products, the stereochemistry of the product being synthesized is very important. Therefore, a chiral HPLC method was developed to determine enantiomeric excess of the WMK being produced by lipase catalyzed Robinson Annulation reaction. Chiral HPLC is a common technique used for finding the enantiomeric excess of a sample mixtures. The peak area for each enantiomer in a sample mixture are used to calculate the enantiomeric excess value. The chiral HPLC chromatograms can be seen below in **Figure 12** for the standards, L-Proline catalyzed reaction sample, and PPL-imidazole in DMSO sample from co-catalyst study. The product from samples was isolated and purified via column chromatography on silica gel with hexanes-ethyl acetate (2:1) as the eluting solvent. The collected fractions were monitored for presence of WMK using TLC. The fractions with the product were combined and excess solvent was evaporated in a rotary evaporator, which resulted in a orangish brown oil which was resuspended in methanol for chiral HPLC analysis.

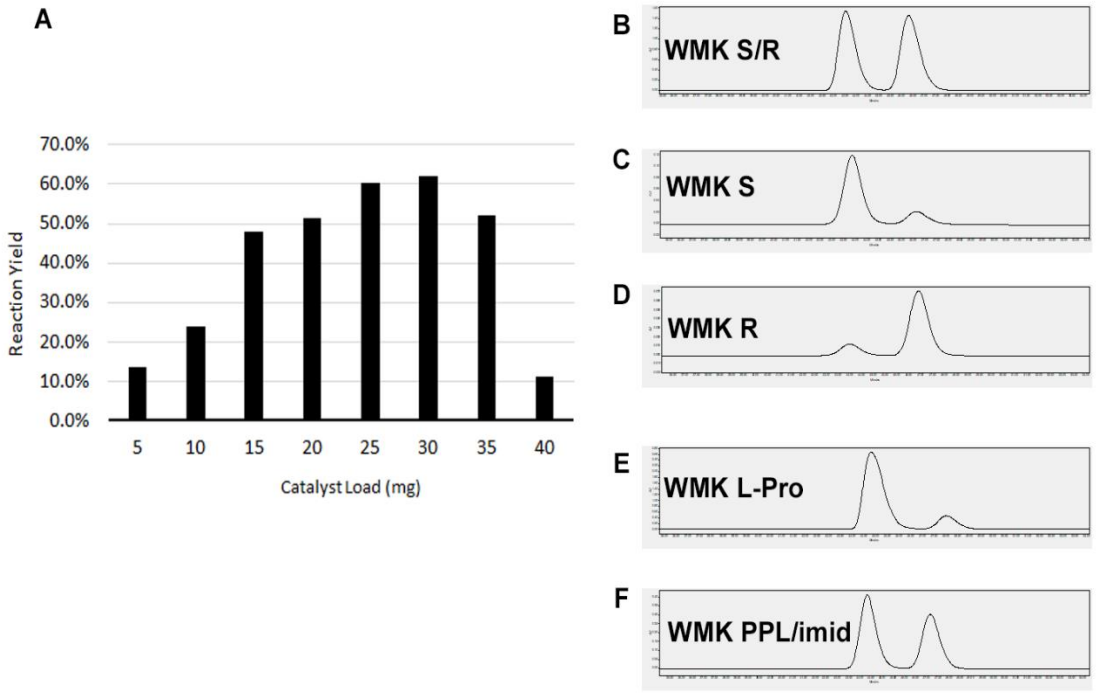


Figure 12. Development of Chiral HPLC Method and Determination of Enantiomeric Excess in Reaction Samples. **A**. Reaction yields from catalyst loading study. Chiral HPLC chromatograms (Chiralcel OD-H column; mobile phase: isopropanol/heptane mixture (20/80, *v*/*v*) containing 0.1% formic acid) used for enantiomeric excess determination are shown for: **B**. Racemic WMK standard; **C**. S-WMK enriched standard; **D**. R-WMK enriched standard; **E**. Purified L-proline catalyzed WMK product; and **F**. Purified PPL/imidazole in DMSO catalyzed WMK product.

Having optimized the catalyst loading, the next step in the optimization was to determine the optimal solvent that resulted in good yield and enantiomeric excess. Previously reported studies had shown that the polar solvents were generally favored and showed promising results for biocatalyzed Robinson Annulation reaction. Therefore, different solvents were tested while

using the conditions optimized so far such as optimal catalyst load (30 mg) and addition of imidazole as co-catalyst. The resulting yields along with enantiomeric excess of WMK products can be seen below in **Table 6**.

Table 6. Determination of optimal solvent for reactions with PPL and imidazole

Entry	Biocatalyst	Solvent	Yield(%)[a]	Ee(%)[b]
1	L-Proline	DMSO	68.8	70.1
2	PPL-5 mg	DMSO	14.4	9.8
3	PPL-35 mg	DMSO	51.8	17.7
4	PPL-5 mg	Methanol	7.3	24.1
5	PPL-30 mg	Methanol	55.1	13.7
6	PPL-30 mg	Ethanol	48.3	10.3
7	PPL-30 mg	Propanol	35.3	8.3
8	PPL-30 mg	Butanol	28.7	6.1
9	PPL-30 mg	Pentanol	29.6	3.6

[a] Yields were determined by HPLC

[b] Enantiomeric excess determined by chiral HPLC (Chiralcel OD-H column); *S*-configuration

The reaction yields in solvents other than DMSO were investigated. The positive control reaction with L-Proline produced good yield and enantiomeric excess of WMK products, similar to the literature report.[50] In contrast, the yields and enantiomeric excess of WMK products produced by the PPL/imidazole in DMSO was substantially lower, but increased after the catalyst loading was increased (Entries 1-3 in **Table 6**). The reaction with lower catalyst load in methanol produced the best enantiomeric excess of WMK products but had a very minimal yield of 7.3% (Entry 4 in **Table 6**). The reactions in methanol produced yields similar to DMSO, but reactions with increasingly longer chain alcohols (ethanol, propanol, butanol, and pentanol) showed a steady decrease in the reaction yield (Entries 6-9 in **Table 6**). The same trend was observed for the enantiomeric excess of WMK products produced in reactions with those solvents. Until this point, it has been determined that the reaction required co-catalyst

imidazole to push the reaction forward and promote product formation. Also the optimal catalyst loading (30 mg) and solvent (methanol) were identified for the reaction to produce moderate yield and enantiomeric excess of WMK products.

3.4 Exploration of Co-catalysts

Although it had been established that imidazole was improving the reaction yield, it was important to explore other co-catalysts. The optimal PPL loading and solvent were screened with different co-catalysts, with each one varying in basicity. The dimethylaminopyridine (DMAP) gave substantially lower yield compared to imidazole, while the ionic base co-catalysts sodium hydroxide and sodium bicarbonate gave lower yields than imidazole but not as significantly reduced as DMAP (**Table 7**). It is important to note that DMAP which is more nucleophilic but less basic than imidazole gave a lower yield. The results indicate that basicity of co-catalyst could play an important role especially during the dehydration step after the intramolecular aldol condensation. The co-catalyst's ability to abstract proton from aldol product **14** depends on its basicity. That correlation can be seen from the results of testing of different co-catalysts.

Table 7. Screening optimal PPL loading with different cocatalyst

Entry	Cocatalyst	Solvent	Yield(%)[a]
1	Sodium Hydroxide	Methanol	43.0
2	Sodium Bicarbonate	Methanol	46.5
3	Imidazole	Methanol	55.1
4	DMAP	Methanol	24.5

[a] Yields were determined by HPLC

The reaction conditions were optimized through various experiments to increase WMK products yield. Different solvents were tested to explore any effects on the yield as well as the

enantiomeric excess of WMK products. The optimal catalyst loading was determined to be 30 mg, and higher catalyst loading than that resulting in a decrease in the product yield. As the carbon chain on alcohol solvents increased, the yields and enantiomeric excess decreased, with methanol giving the best results.

3.5 Rescreening of Biocatalysts using Optimized Reaction Conditions

After identifying the optimal catalyst loading, solvent, and basic co-catalyst, the lipase library was rescreened to determine the impact of optimized conditions on their biocatalytic activity. In addition to the lipases from the initial screening, commercially available proteins that were identified from the proteomics as principal components of crude PPL (carboxypeptidase A, carboxypeptidase B, alpha amylase) were also included in the rescreen. The immobilized forms of *Candida Antarctica* lipase B (Lipase codes: L12-acr and L12-imo) were also added to the lipase library. The results from the rescreening of updated lipase library using optimized conditions can be seen below in **Table 8**. The samples with the highest yields were further purified via column chromatography and the isolated product was used for enantiomeric excess determination (Entries 1, 3, 5, 9, 13, 17 in **Table 8**).

Table 8. Rescreening of updated lipase library using optimized reaction conditions

Entry	Biocatalyst	Yield(%)[a]	Ee (%)[b]
1	L1	51.9	21.7
2	L2	0.7	-
3	L3	17.1	1.2
4	L4	7.2	-
5	L5	13.5	19.9
6	L6	0.8	-
7	L7	1.3	-
8	L8	n.r.	-
9	L9	16.0	14.4
10	L11	4.5	-
11	L12-Acrylic Resin	1.7	-
12	L12-Immobeads	2.3	-
13	L14	10.9	(-)1.7
14	α-Amylase (liquid)	5.3	-
15	Carboxypeptidase A	0.4	-
16	Carboxypeptidase B	4.2	-
17	α-Amylase(solid)	26.4	19.3

[a] Yields were determined by HPLC

[b] Enantiomeric excess determined by chiral HPLC (Chiralcel OD-H column); *S*-configuration

In addition to the HPLC and mass spectrometry screening method, absorbance was discovered to be an effective method for a quick screening of biocatalytic potential towards the WMK synthesis. The absorbance (350 nm) of crude reaction mixture was correlated with the reaction yield, demonstrating a correlation between biocatalytic potential and yield of WMK from HPLC (**Figure 13A**). Additionally, plotting absorbance (350 nm) versus percent yield revealed that if a colorimetric cutoff of OD_{350} = 1.0 was applied, all the reactions with yields below 10% would be eliminated (**Figure 13C**). This demonstrates that colorimetric analysis could be very convenient and quick approach towards a large-scale screen of biocatalysts for synthesis of WMK.

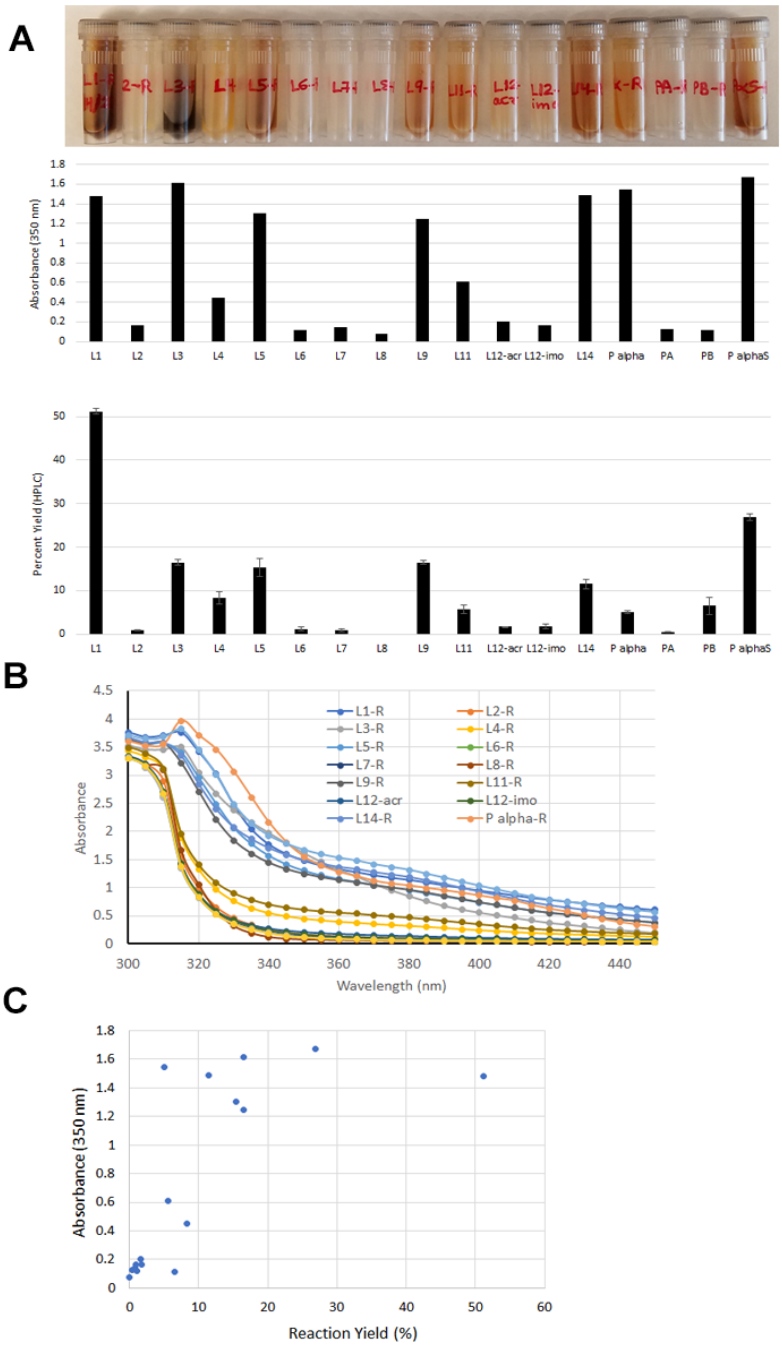


Figure 13. Rescreening of Lipase Library using Optimized Conditions. **A**. Image of reaction samples after 89 h (top); absorbance of crude reaction samples at 350 nm (middle); and reaction yields (**Table 8**) from rescreening of updated lipase library (bottom). Error bars were

determined from replicate HPLC injections. **B**. Wavelength scan of crude reaction sample absorbance. **C**. Plot of absorbance at 350 nm versus reaction yield.

Comparing the reaction yields from the rescreen (**Table 8**) with the initial screening (**Figure 7**) revealed that the biocatalysts which were identified as top hits from the initial LC/MS screen (L1, L3, L5, L9, and L14) were also the top biocatalysts in the colorimetric screening method (**Figure 13**). Out of the three principal components identified in the crude PPL preparation, only alpha amylase produced a significant yield of WMK while the other two proteins (carboxypeptidase A and carboxypeptidase B) have very minimal yield. Interestingly the yield was not similar to that of crude PPL preparation, indicating that multiple proteins maybe involved in the catalysis of Robinson Annulation reaction. It is important to note that a non-lipase protein (BSA) had shown product formation (Entry 6 in **Table 4**) in the presence of imidazole as co-catalysts. Based on these observations, we hypothesized that the endogenous catalytic activity of proteins present in lipase preparations was not contributing to product formation as previously reported.[29]

3.6 Experimental Section

All chemicals, substrates, and solvents were purchased from commercial sources (VWR, Millipore-Sigma, TCI America, and Fisher Scientific. Lipases were purchased from Sigma-Aldrich. Thin layer chromatography was performed with silica gel 60 F254 plates, purchased from EMD chemicals.

Table 9. Updated Lipase Library

Lipase	***Lipase***	***Catalog Number***
L1	Porcine Pancreatic Lipase	L3126
L2	Rhizopus Olyzae	62305
L3	Wheat Germ Lipase	L3001
L4	Candida Rugosa	L1754
L5	Aspergillus Niger	62301
L6	Aspergillus Oryzae	L4277
L7	Pseudomonas Cepacia	62309
L8	Candida Sp.	L3170
L9	Rhizopus Niveus	62310
L10	Mucor Miehei	62298
L11	Mucor Javanicus	L8906
L12	Candida Antarctica B	62288
L13	Candida Antarctica A	62287
L14	Pseudomonas Fluorescens	534730
L12-acr	Candida Antarctica B	L4777
L12-imo	Candida Antarctica B	52583
PA	Carboxypeptidase A	C9268
PB	Carboxypeptidase B	C9584
P alpha	Alpha amylase (suspension)	10102814001
P alpha S	Alpha amylase (powder)	A3176

Biocatalysis Reaction Condition

The following reagents/reactants were mixed in a microcentrifuge tube for synthesis of Wieland–Miescher ketone: solvent (900 µL), methyl vinyl ketone (19.8 µL, 0.24 mmol), 2-methyl-1,3-cyclohexanedione (20 mg, 0.16 mmol), deionized water (100 µL), and lipase (0–40 mg) or L-proline (6.4 mg), with or without imidazole (2.7 mg, 0.04 mmol). After all the reactants were assembled, the centrifuge tubes were placed in an incubator shaker (New Brunswick Excella e24; 35 °C, 250 rpm) for 89 hours.

Cocatalyst and Solvent Optimization Reaction Condition

The following reagents/reactants were mixed in a microcentrifuge tube for synthesis of Wieland–Miescher ketone: alcohol solvent (900 µL), methyl vinyl ketone (19.8 µL), 2-methyl-1,3-cyclohexanedione (20 mg), deionized water (100 µL), and lipase (L1, 30 mg), cocatalyst (0.04 mmol). After all the reactants were assembled, the centrifuge tubes were placed in an incubator shaker (New Brunswick Excella e24; 35 °C, 250 rpm) for 89 hours.

Hi-Res Mass Spectrometry Analysis of triketone and WMK

Samples were analyzed with a Q Exactive HF-X (ThermoFisher, Bremen, Germany) mass spectrometer. Samples were introduced via a heated electrospray source (HESI) at a flow rate of 10 µL/min. One hundred times domain transients were averaged in the mass spectrum. HESI source conditions were set as: nebulizer temperature 100 deg C, sheath gas (nitrogen) 15 arb, auxillary gas (nitrogen) 5 arb, sweep gas (nitrogen) 0 arb, capillary temperature 250 degrees C, RF voltage 100 V, spray voltage 3.5 KV. The mass range was set to 150-2000 m/z. All measurements were recorded at a resolution setting of 120,000. Solutions were analyzed at 0.1 mg/mL or less based on responsiveness to the ESI mechanism. Xcalibur (ThermoFisher,

Breman, Germany) was used to analyze the data. Molecular formula assignments were determined with Molecular Formula Calculator (v 1.2.3). All observed species were singly charged, as verified by unit *m/z* separation between mass spectral peaks corresponding to the ^{12}C and $^{13}C^{12}C_{c-1}$ isotope for each elemental composition.

Characterization of triketone (2-methyl-2-(3-oxobutyl)-1,3-cyclohexanedione):

Hi-Res mass spec: m/z = 197.11761 (mass error = 2.0 ppm) Assigned Chemical Formula: $C_{11}H_{17}O_3$ $[M+H]^+$; 1H-NMR (400 MHz, CDCl3): 2.60-2.77 (m, 2H), 2.35 (t, 2H), 2.11 (s, 3H), 2.04-2.08 (m, 2H), 1.89-1.95 (m, 2H), 1.25 (s, 3H).

Characterization of WMK ((S,R)-(+/-)-8a-methyl-3,4,8,8a-tetrahydro-1,6(2H,7H)-napthalenedione):

Hi-Res mass spec: m/z = 179.10583 (mass error = -4.6 ppm) Assigned Chemical Formula: $C_{11}H_{15}O_2$ $[M+H]^+$; 1H NMR (400 MHz): 5.85 (s, 1H), 2.67–2.76 (m, 2H), 2.38–2.52 (m, 4H), 2.09–2.19 (m, 3H), 1.65–1.78 (m, 1H), 1.45 (s, 3H).

HPLC analysis for reaction yield

Liquid chromatography was performed on a HPLC system(Waters Corporation) consisting of binary pump(1525 Binary HPLC Pump), absorbance detector(2487 Dual Wavelength Absorbance Detector), and auto sampler(717-plus Autosampler). 5µL of sample were injected into the reversed-phase Xterra MS C18 column (4.6 × 100 mm, 5µm; Waters, Milford, MA, USA) using auto sampler. The samples were analyzed using an isocratic method: 80% Solvent A(water) and 20% Solvent B(methanol). The flow rate was 1.0 mL min^{-1} and the total run time was 13 minutes for each sample injection. The detector wavelength was set to 210 nm and the retention time for WMK was 7.9 minutes.

Chiral HPLC analysis for enantiomeric excess

Liquid chromatography for enantiomeric excess was performed on a HPLC system(Waters Corporation) consisting of binary pump(1525 Binary HPLC Pump), absorbance detector(2487 Dual Wavelength Absorbance Detector), and auto sampler(717-plus Autosampler). 5μL of sample were injected into the Chiralcel OD-H column (0.46 cm × 25 cm; Daicel, Minato, Japan) using auto sampler. Isopropanol/Heptane mixture (20/80, v/v) containing 0.1% formic acid was used as a mobile phase and pumped at a flow rate of 0.1 mL min^{-1}. Total run time was 111 minutes for each sample injection. The detector wavelength was set to 250 nm and the retention times for (S)-WMK and (R)-WMK were 43.5 and 46.3 minutes respectively.

CHAPTER 4: MECHANISTIC INSIGHT

4.1 Exploring the effects of biocatalysts inhibitors on reaction yield

Based on the results from optimization studies, it was hypothesized that the native catalytic activity of the proteins was not responsible for product formation. To investigate this, an experiment was conducted using inhibitors acarbose and orlistat (amylase and lipase inhibitors respectively). The biocatalytic reactions were setup using alpha amylase and PPL in the presence of commercially obtained inhibitors acarbose and orlistat.

Table 10. Biocatalytic reaction in the presence of biocatalyst inhibitors

Entry	Biocatalyst	Inhibitor(Acarbose)	Inhibitor(Orlistat)	Yield(%)[a]
1	PPL	-	-	50.7
2	PPL	+		52.5
3	PPL	-	+	54.9
4	α-Amylase (solid)	-	-	31.0
5	α-Amylase (solid)	+	-	27.4
6	α-Amylase (solid)	-	+	27.6
7	Carboxypeptidase B + α-Amylase (solid)	-	-	31.9

[a] Yields were determined by HPLC

The results (**Table 10**) from the inhibitor experiment were very interesting, as the yield for PPL in the presence of excess lipase inhibitor had not decreased compare to the reaction without the inhibitor. Interestingly the excess amylase inhibitor did reduce the yield of reaction catalyzed by alpha amylase, but only minimal amount. The intrinsic lipase activity of crude PPL preparation was confirmed to be fully inhibited in the presence of orlistat. This was achieved by performing a simple lipase assay monitoring the hydrolytic activity with p-nitrophenylpalmitate with and without inhibitor (**Figure 14**).

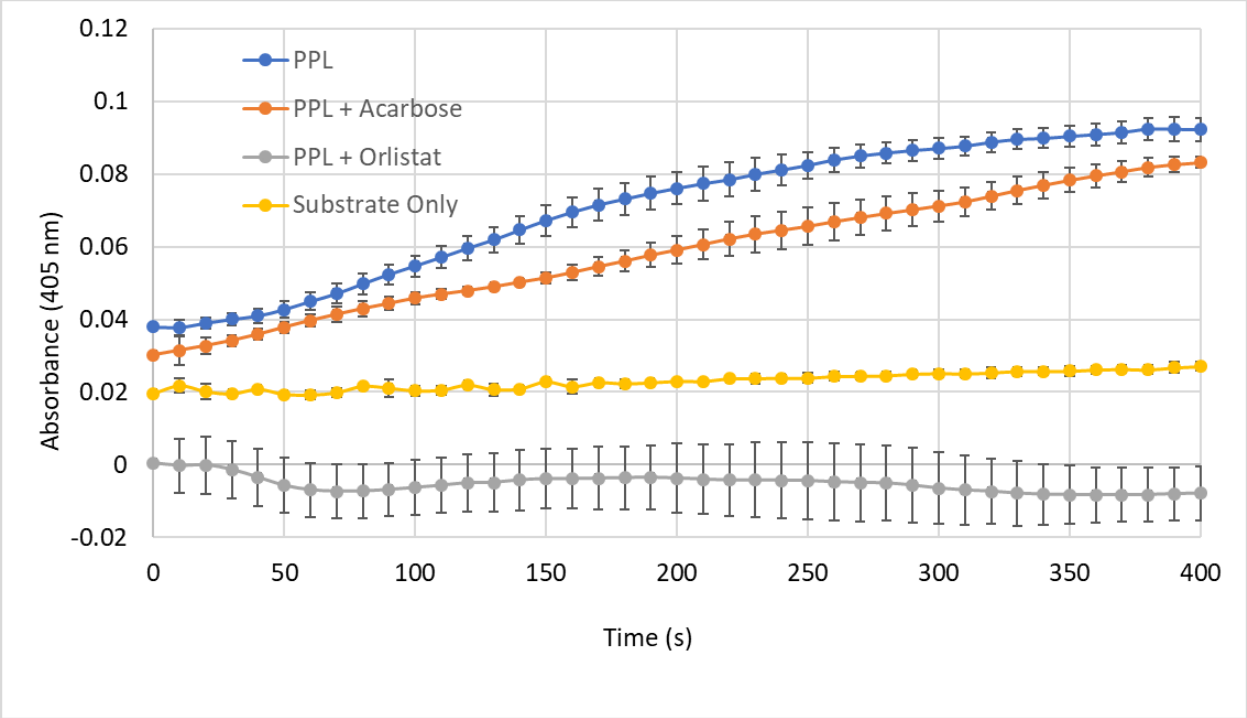


Figure 14. Effects of inhibitors on lipolytic activity of a crude preparation of PPL.

4.2 Experimental Section

Biocatalysis reaction condition

The following reagents/reactants were mixed in a microcentrifuge tube for synthesis of Wieland–Miescher ketone: solvent (900 µL), methyl vinyl ketone (19.8 µL), 2-methyl-1,3-cyclohexanedione (20 mg), deionized water (100 µL), and lipase (30 mg), imidazole (2.7 mg, 0.04 mmol). For reaction involving inhibitors, the biocatalyst was mixed with the inhibitor (5 mg) for 10 min at room temperature before adding the substrates and cocatalyst. After reaction assembly, capped microcentrifuge tubes were placed in a temperature-controlled shaker (New Brunswick Excella e24; 35 °C, 250 rpm) for 89 h.

Lipase assay for native activity

PPL (70 mg) was suspended in 3 mL of Tris-Glycerol buffer (13 mM Tris HCl, pH 8, 150 mM NaCl, 3 mM CaCl2, 5% glycerol) for 10 min, then centrifuged to remove insoluble material. The resulting supernatant (1 mL) was then combined with the inhibitor (5 mg Orlistat or Acarbose) dissolved in 200 µL of methanol. The resulting solutions were incubated for 10 min at room temperature on an end-over-end rotator, then filtered (Millipore centrifugal filter) to remove insoluble material. 100 µL of the resulting solutions were then assayed in the presence of p-nitrophenylpalmitate (5 µL of a 2.5 mM stock solution in DMSO) with a Molecular Devices M2e plate reader (405 nm, readings acquired every 10 s). Assays were performed in triplicate, error bars represent S.E.M.

4.3 Summary of Results

Based on the results above, it was concluded that lipase activity is not responsible for the promiscuous catalysis observed with crude preparation of PPL. Additionally, while the reaction was shown to be catalyzed by multiple proteins regardless of whether they possess intrinsic enzymatic activity, not all proteins were capable of catalyzing WMK product formation as seen from the screening results. While many literature reports have attributed the promiscuous biocatalytic activity of PPL directly to the lipase active site, that was not the case based on the results discovered in this study. The generally accepted mechanism for lipase promiscuous catalysis is believed to be occurring in the active site of lipase where the catalytic triad is present. Based on the results found in this study, the lipase promiscuous catalysis was still happening even when the lipase inhibitor (orlistat) was present in the reaction mixture. This promiscuous activity even in the presence of inhibitor could possibly be attributed to the surface exposed amino acids which could be catalyzing the Robinson Annulation reaction. For these biocatalysts

to be potentially used in industrial applications, it is important to characterize their mode of action. Interestingly in this study, the crude PPL preparation was found to contain alpha amylase as a principal component, which could become a candidate for future optimization. There are several different ways to potentially understand the role of active site possibly via mutagenesis of amino acids that are part of the catalytic triad. Also, alpha amylase could be optimized through directed evolution to further increase the yield and stereoselectivity for WMK. Furthermore, this study demonstrated that a quick colorimetric screening can be very useful for large-scale screening for identification of biocatalysts that possess promiscuous activity towards WMK formation. This screening methodology could be extended to other variations of Robinson Annulation reaction by screening for substrate scope to produce analogues of WMK and other important molecules.

CHAPTER 5: CONCLUSIONS

In this research different methods for screening commercially available lipases to assess their biocatalytic potential for synthesis of Wieland Miescher Ketone were demonstrated. After the initial screening of lipase library, optimization experiments were conducted using PPL as the model lipase. Through optimization experiments, the importance of a basic co-catalyst Imidazole was demonstrated for efficient production of Wieland Miescher Ketone. The yield was improved from less than 1% up to 55%. The enantioselectivity was lower than previously reported L-Proline catalyzed WMK synthesis. Interestingly after identifying the principle components of crude preparation of PPL, those proteins could become candidates for future studies. The product yield and enantioselectivity could be further improved by optimizing the identified proteins through techniques such as directed evolution or enzyme engineering. Colorimetric screening method for assessing biocatalytic potential of lipases was also demonstrated, which could be a quick method for future screening of other biocatalysts. Furthermore, the screening methods could be applied for a substrate scope study or even characterizing biocatalysts for other carbon-carbon bond forming reactions.

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