Title
Towards the Development of Biosynthetic Pathways for the Short Chain Acetate Ester Biosynthesis

Permalink
https://escholarship.org/uc/item/95q3g4vt

Author
Palomec, Leidy Iracema

Publication Date
2014

Peer reviewed|Thesis/dissertation
Towards the Development of Biosynthetic Pathways for the Short Chain Acetate Ester Biosynthesis

A Thesis submitted in partial satisfaction of the requirements for the degree of

Master of Science

in

Chemical and Environmental Engineering

by

Leidy Iracema Palomec

June 2014

Thesis Committee:
Dr. Ian Wheeldon, Chairperson
Dr. Xin Ge
Dr. Charles Wyman
The Thesis of Leidy Iracema Palomec is approved:


Committee Chairperson

University of California, Riverside
ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to the individuals that made this thesis possible. First and foremost, I would like to thank my research group. At the head of the research group is my graduate advisor, Dr. Ian Wheeldon, who gave me support, training, advice, encouragement and knowledge throughout my studies and research experience. I want to thank him for fostering my research skills, sharing his incredible ideas, and for his continued mentorship. I would like to thank Jyun-Liang (Aaron) Lin for his contribution in this work and for his continued guidance, training and friendship. I would also like to thank my fellow present and past lab members, Jie Zhu, Aaron Toop, Yingning Gao, Ann-Katherin Loebs, Samson Or, Andrew Flores, Stephanie Eatinger, Scott Hernandez, Rene Henderson, Patrick Ho, Micheal Amendola and Gina Flores for their collaborations, friendship, camaraderie and for special memorable times in the lab.

Furthermore, I would like to recognize the contributions of several individuals in the University to this work. I would like to thank Dr. Xin Ge and Dr. Ashok Mulchandani for allowing my lab and I access into their laboratory and for sharing their experimental equipment. I would also, like to demonstrate my gratitude towards their respective laboratory students who were considerate and accommodating. I would also like to thank Kathy Cocker for her patience, support and training with experimental equipment. In addition, I would like to thank the members of my committee Dr. Xin Ge, Dr. Charles Wyman and Dr. Ian Wheeldon for their collaboration, time and guidance.
Also, I would like to thank the University of California, Riverside, the Chemical and Environmental Engineering Department, the staff and the faculty for giving me the opportunity to enhance my education, for their constant support, and for the continued support.

Finally, I would like to thank my family and friends. I would like to thank my mom, dad and my two younger brothers for always believing in me, for allowing me the time necessary to continue my studies in graduate school, for the long distance family visits, their constant love, their constant generosity, and for their unwavering support throughout my education program. I would also like to thank my extended family for their motivations and encouraging me to push forward. I would like to thank my roommates for their friendship, their support, for keeping me company during late study night study sessions, for listening and sharing lab stories, and for their generous companionship. I would like to thank my undergraduate students in the ChE lab class for allowing me to practice my mentorship skills as a teaching assistant. I would also like to thank my UCR, CSULB, SHPE, Church, work friends who have shared their wisdom, time and experiences with me and have made my experience at UCR an amazing one.
DEDICATION

ABSTRACT OF THE THESIS

Towards the Development of Biosynthetic Pathways for the Short Chain Acetate Ester Biosynthesis

by

Leidy Iracema Palomec

Master of Science, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, June 2014
Dr. Ian Wheeldon, Chairperson

Acetate esters are ubiquitous in nature and broadly used for a range of applications including as solvents, aromas, and flavours in the polymer cosmetics, pharmaceutical and beverages industries. In yeast and fruit ripening, alcohol-o-acetyltransferase (AATase, E.C. 2.3.1.84.) is responsible for the biosynthesis of a range short and medium chain esters from an alcohol and an acetyl-CoA. Microbial production of acetate esters has been the focus of a number of metabolic engineering efforts; however, a poor understanding of kinetic characteristics of the AATase family has limited the success of acetate ester biosynthesis via metabolic engineering. The overall goal of the study is to work towards the development of a biosynthesis pathway for the production of acetate esters from the metabolic engineering of AATase enzymes. These studies include 1) activity screening of AATase orthologs from Saccharomyces and non-Saccharomyces yeast and various fruit species; 2) observation of the acetate ester effect on cell cultures; and 3) substrate channeling simulations of coupled enzyme complexes. These studies of the AATase family can lead to a better understanding of these enzymes.
and provide insight into the selection of the most suitable candidate to develop a biosynthetic pathway for acetate ester production.
# TABLE OF CONTENTS

## CHAPTER 1: INTRODUCTION

1.1 Motivation

1.2 Common acetate ester production

1.3 Microbial biosynthesis of acetate ester

1.4 Objectives

## CHAPTER 2: STUDIES OF ACETATE ESTER FORMATIONS BY AATASE ORTHOLOGS FROM SACCHAROMYCES AND NON-SACCHAROMYCE YEAST AND VARIOUS FRUIT SPECIES

2.1 Introduction

2.2 Materials and Methods

2.2.1 Chemicals

2.2.2 Strain, media and culturing conditions

2.2.3 Overexpression plasmids

2.2.4 Alcohol acetyltransferase activity

2.2.5 Headspace gas chromatography

2.2.6 Acetate ester toxicity assays

2.3 Results

2.3.1 Multiple alignment of amino acid sequence for AATase orthologs

2.3.2 AATase activity screening

2.3.3 Acetate ester toxicity
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3. Discussion</td>
<td>25</td>
</tr>
<tr>
<td>CHAPTER 3: ANALYSIS OF COUPLED-ENZYME REACTION FOR PATHWAY ENGINEERING</td>
<td>30</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>30</td>
</tr>
<tr>
<td>3.2 Engineering substrate channeling in multi-enzyme structures</td>
<td>31</td>
</tr>
<tr>
<td>3.3 Substrate channeling in nature and transfer mechanisms</td>
<td>32</td>
</tr>
<tr>
<td>3.4 Kinetic analysis of substrate channeling</td>
<td>38</td>
</tr>
<tr>
<td>3.5 Numerical solutions of kinetics equations</td>
<td>42</td>
</tr>
<tr>
<td>3.6 GOx-HRP: Effect of adding a competitive enzyme</td>
<td>46</td>
</tr>
<tr>
<td>3.7 TS-DHFR: Effects of adding a competitive inhibitor</td>
<td>48</td>
</tr>
<tr>
<td>3.8 AATase coupled enzyme channeling application</td>
<td>50</td>
</tr>
<tr>
<td>3.9 Discussion</td>
<td>52</td>
</tr>
<tr>
<td>CHAPTER 4: CONCLUSION &amp; RECOMMENDATIONS</td>
<td>54</td>
</tr>
<tr>
<td>4.1 Recommendations</td>
<td>55</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>57</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>61</td>
</tr>
</tbody>
</table>
LIST OF TABLES

TABLE 2-1: Microbial strains and plasmids used---------------------------------------------12

TABLE 2-2: Protein sequence similarity and identity of S. cerevisiae Atf1 and all orthologs protein sequence found---------------------------------------------19

TABLE 2-3: Whole cell lysate Atf activity with acetyl-CoA and varied short chain alcohol substrates---------------------------------------------23

TABLE 2-4: ATF from S. cerevisiae activity with acetyl Co-A and isopentanol substrate ---------------------------------------------28
LIST OF FIGURES

FIGURE 1-1: Industrial ester preparation types---------------------------------3

FIGURE 1-2: Microbial Ester production. Three enzymatic routes are shown a) alcohol acyltransferase b) alcohol dehydrogenase, and c) Baeyer-Villiger monooxygenase------5

FIGURE 2-1: Biosynthesis of short and medium chain length acetate esters from an alcohol and acetyl-CoA by AATase---------------------------------10

FIGURE 2-2: pR426-PGK1p-ATF-myc-PGK1t plasmid used for this study. The promoter and terminator were amplified from genomic DNA of BY4742 and cloned into pR426 (vector). PGK1p was inserted using SacI and SacII sites. PGK1t was inserted using SpeI and KpnI sites while ATF-myc was inserted at SacII and NotI-----------------13

FIGURE 2-3: Testing methods. (A) Transformant cell cultures in 50 ml SD/-U medium in a 125 ml Erlenmeyer flasks, grown at 30 °C for 16h in an orbital shaker at 220 rpm. (B) Sample cuvettes with Bradford reagent and crude extract used to determine the protein concentration present. (C) Reaction mixtures of crude extract, corresponding alcohol, acetyl-CoA and buffer in a 10 ml capped GC vials. (D) Agilent 7890 gas chromatography equipped with a CombiPAL autosampler used for enzyme assay sample-----------------15

FIGURE 2-4: Multiple alignment of the amino acid sequence of Atf1 and Atf2 from S. cerevisiae (sc) and their corresponding orthologs from S. lycopersicum (sl), S. pastorianus (sp), K. lactis (kl), C. melo (cm), and P. anomala. The alignments were performed using T-coffee online software. Non-conserved residues are shaded in green and yellow, similar residues are shaded in orange, and conserved and identical residues
are shaded in red. The conserved residues H-X-X-X-D, possibly essential for catalytic activity and considered the putative active site, is boxed and marked by arrows--------17

FIGURE 2-5: GC analysis of the acetate ester products formed by Atf1-S.c. enzyme with acetyl Co-A and a range of short chain alcohol substrate--------------------------21

FIGURE 2-6: Enzymatic ester synthesis of Atf1 overexpressed transformant by combining alcohols and acetyl-CoA. Substrate specificity of Atf activity in cell-free extract is demonstrated. Substrate concentration was of 500mM (methanol, ethanol, propanol and isopropanol) and 200 mM (butanol, isobutanol, pentanol and isopentanol). Ester formation was measured utilizing the kinetic assay procedure described under materials and methods-------------------------------22

FIGURE 2-7: Cell growth of S. cerevisiae in a spectrum of acetate ester toxic environments. Cells were cultured in 2% YPD medium and a corresponding acetate ester percentage (0, 0.5, 2.5 or 5%). Cultures were grown at 30°C in an incubated orbital shaker. Samples were taken at 0, 2, 4, 6, 8, 24 and 48h--------------------------------24

FIGURE 2-8: Cell growth rate of S. cerevisiae in a spectrum of acetate ester toxic environments. The cell growth rate (OD/hr) is calculated from the growth rate analysis and the corresponding log phase region. Cells were cultured in 2% YPD medium and a corresponding acetate ester percentage (0, 0.5, 2.5 or 5%). Cultures were grown at 30°C in an incubated orbital shaker. Samples were taken at 0, 2, 4, 6, 8, 24 and 48h--------25

FIGURE 3-1: Substrate channeling schematics of (A) General process for the direct delivery of a reaction intermediate (I) from the active site of one enzyme (E1) to the active site of a second enzyme (E2) without dissociation into bulk solvent. (B) The general process
with the presence of an inhibitor intermediate ($I^*$). (C) The general process with the presence of a third competing enzyme ($E_2$) (Huang et al., 2001).

**FIGURE 3-2:** Natural bifunctional enzyme that exhibit substrate channeling. Channeled intermediates are boxed. The represented examples include (A) Tryptophan Synthase, (B) Dihydrofolate Reductase-Thymidylate Synthase. Abbreviations definition: IGP, indole-3-glycerol phosphate; G3P, D-glyceraldehyde 3-phosphate; H2folate, dihydrofolate; H4folate, tetrahydrofolate; C2H4folate, methylene tetrahydrofolate; (Huang et al., 2001; Lin et al., 2014; Miles et al., 1999). Figure adapted from (Miles et al., 1999).

**FIGURE 3-3:** Natural examples of tunneling and electrostatic channeling. **Tunneling:** (A) One αβ-heterodimer of Tryptophan Synthase (TS) with a α-subunit that contains the indole-3-propanol phosphate and the β-subunit that contains the L-serine. This tunnel is ~25 Å long. The cyan/blue section and the yellow section represent the different active sites of each enzyme. The orange/yellow spheres represent the course of the tunnel from the different subunits (active sites). **Electrostatic channeling:** (B) Dihydrofolate Reductase-Thymidylate synthase (DHFR-TS) reveals an electrostatic potential distribution (negative charge shown in red, positive charge shown in blue, active sites are yellow), and connects the two active sites by a positive pathway. Figure adapted from reference (Huang et al., 2001; Miles et al., 1999).

**FIGURE 3-4:** Transient time as a function of $p_c$ values as described by equation 3-2.

**FIGURE 3-5:** Graphical calculation of the transient time, $\tau$, in MDH-CS coupled-enzyme reaction with varied channeling probability ($P_c$). A linear fit to the product concentration.
as a function of time at steady state crosses x-axis at $\tau$. Simulations values as described by equation 3-3 to 3-5.

**FIGURE 3-6:** Reaction scheme used in numerical solution of differential equations. Figure adapted from reference (Elcock et al., 1997).

**FIGURE 3-7:** First-order differential equations describing time evolution of the reaction scheme shown in Figure 3-6. Figure adapted from (Elcock et al., 1997).

**FIGURE 3-8:** Simulated substrate (A) and product (B) concentration as a function of time for bifunctional GOx-HRP, at various $k_{cat_1}/k_{cat_2}$ ratios.

**FIGURE 3-9:** Simulated product concentration behavior as a function of time for bifunctional GOx-HRP at various competitive third enzymes (catalase) concentrations including $[E_1]=1e^{-8}M$ and $[E_2]=1e^{-8}M$ with $[E_3]=1e^{-6}M$, $[E_3]=1e^{-7}M$, $[E_3]=1e^{-8}M$, $[E_3]=1e^{-9}M$ and $[E_3]=1e^{-10}M$. A mono-functional system is represented by (A) with $P_c=0.01$. A substrate channeling bi-functional system is represented by (B) with $P_c=0.95$.

**FIGURE 3-10:** Simulated production concentration behavior as a function of time for bifunctional DHFR-TS at various competitive inhibitor substrates concentrations including $[TMP]=1\mu M$, $[TMP]=5\mu M$, $[TMP]=10\mu M$, $[TMP]=30\mu M$, $[E_1]=2e^{-8}M$ and $[E_2]=2e^{-8}M$. Images (A) and (B) are validations of the model obtained from the external reference. (A) represent a mono-functional system with no channeling of $P_c=0.01$. (B) represent a bi-functional system with channeling of $P_c=0.95$. Figure adapted by (Elcock et al., 1997).
FIGURE 3-11: Natural bifunctional enzyme that exhibit substrate channeling. The channeled intermediate is boxed. The coupled enzyme complex is acetyl coenzyme-A synthase-alcohol acetyltransferase (ACS1-AAT).

FIGURE 3-12: For the coupled enzyme acetyl-coenzyme A synthase-alcohol acetyltransferase is simulated to demonstrate the product formation with E1:E2 ratios and a Pc value of 0.01 (A).
LIST OF APPENDICES

APPENDIX A: ALCOHOLS AND ESTERS--------------------------------------------61-62

APPENDIX B: KINETIC PARAMETERS OF COUPLED ENZYME SYSTEMS-----63

APPENDIX C: MATLAB CODE--------------------------------------------------------64-78

APPENDIX D: GAS CHROMATOGRAPHY STANDARDS--------------------------------------79

APPENDIX E: RESEARCH PAPER-------------------------------------------------------80-106
CHAPTER 1: INTRODUCTION

1.1 MOTIVATION

Acetate esters are prevalent in nature and broadly used in industry. Esters are responsible for the fragrances and aromas of a wide range of fruit including apples, pears, bananas and strawberries (Cumplido-Laso et al., 2012; Khanom & Ueda, 2008; Shalit et al., 2001). Some fungi, including yeast, are known to produce volatiles esters during fermentation (e.g. during wine and beer production) (Mason & Dufour, 2000; Verstrepen, Derdelinckx, et al., 2003; Verstrepen, Laere, et al., 2003). Short-chain volatiles esters are also added to foods and beverages as flavor compounds and used to produce fruity or floral aromas in the fragrance and cosmetics industry (Dhake, Thakare, & Bhanage, 2013; Park, Shaffer, & Bennett, 2009; Rodriguez, Tashiro, & Atsumi, 2014; Saerens, Delvaux, Verstrepen, & Thevelein, 2010; Stellman Mager et. al, n.d.). Some short chain acetate esters, for example ethyl acetate, are good solvents and have high volatility, which make them important for the manufacturing of many products including paints, coatings and inks (J. Stellman Mager et. al; Oxide, 2004; Rodriguez et al., 2014)

In 2012, the global market for flavor and fragrance compounds was $16.6 billion and demand worldwide is expected to continue to grow (Dhake et al., 2013; Mason & Dufour, 2000; Rodriguez et al., 2014). The vast majority of this demand is met by acid catalyzed esterification; however, this process has undesirable characteristic including the need to conduct reactions at high temperatures (150-280 °C), the use of strong acid catalysts, poor selectivity, and undesired side reactions that can reduce yields (Gross, Ganesh, & Lu, 2010). New versatile catalysts that catalyze reactions at mild
temperatures, limit the production of undesired by-products, and have high selectivity would be beneficial to the industrial production of acetate esters (Gross et al., 2010). The synthesis of volatile esters by biocatalytic processes represents an important opportunity to develop such catalysts. The overall goal of this thesis is to study acetate ester synthesizing enzymes and work towards the development of biosynthetic pathways for the production of short chain acetate esters.

1.2 COMMON ACETATE ESTER PRODUCTION

In industry, esters are commonly produced by esterification of an alcohol and an organic acid. This reaction is highly reversible but high yields can be attained by the application of Le Chatelier’s Principle to drive the reaction towards ester synthesis. For example, (1) excess reactant (i.e. alcohol or organic acid); (2) using dehydration agents (e.g. sulfuric acid), (3) removing water by physical means (e.g. distillation) and (4) applying high temperatures (B. Neises and W. Steglich & Steglich; Dhake et al., 2013; Ignatyev, Doorslaer, Mertens, Binnemans, & Vos, 2012; J. Stellman Mager et. al; W. Reusch & Reusch). A typical example of esterification is the synthesis of esters by Fischer esterification, which involves treating a carboxylic acid with an alcohol in the presence of a dehydrating agent (Figure 1-1A). This reaction is slow, thus typically a catalyst (e.g. sulfuric acid) is used to improve reaction rate (B. Neises and W. Steglich & Steglich; Ignatyev et al., 2012).
Another example of esterification is the alcoholysis of acyl chlorides and acid anhydrides to produce esters (Figure 1-1B). These reactions are irreversible, prefer anhydrous conditions, and are expensive due to the high costs of components (B. Neises and W. Steglich & Steglich; Ignatyev et al., 2012). Alkylation of carboxylate salts can also produce esters (Figure 1-1C), but this reaction is not a widely used form for esterification. Another synthesis method is transesterification, which involves changing one ester into another. This reaction is catalyzed by an acid or a base and is typically used to degrade triglycerides (e.g. production of fatty acid esters and alcohols). Carbonylation of alkenes by metal carbonyl catalysts can also forms esters. An example is the production of methyl formate by carbonylation of methanol with a sodium methoxide catalyst (Figure 1-1-D) (B. Neises and W. Steglich & Steglich; Ignatyev et al., 2012).

Despite the prevalence of esterification via chemical catalysis, these procedures often lead to the formation of undesired side products and impurities (e.g. unreacted excess alcohol), are reversible, require extra manipulations (e.g. high temperatures) and, can be costly (e.g. Alcoholysis of acyl chlorides) (B. Neises and W. Steglich & Steglich; Dhake et al., 2013; Ignatyev et al., 2012; J. Stellman Mager et al.). Environmental
legislation, industrial competitiveness, and environmental responsibility along with vast advancements in biotechnology and metabolic engineering point industries towards the development of more environmentally friendly and economical ester production processes. Enzymatic catalysis is a potentially useful alternative for the formation of acetate esters (Dhake et al., 2013; Rodriguez et al., 2014).

1.3 MICROBIAL BIOSYNTHESIS OF ACETATE ESTERS

Microbial fermentation offers a sustainable, green chemistry alternative to the current chemical-based synthesis methods for acetate ester production. The microbial production of esters has been the focus of a number of works including whole cell catalysis development and metabolic engineering of microbial cell factories (Barney, 2014; Bornscheuer et al., 2012; Brault, Shareck, Hurtubise, Lépine, & Doucet, 2014; Inoue et al., 1997; Leo, Fernandes, Pinheiro, & Cabral, 1998; Rodriguez et al., 2014). The biosynthesis pathways that can produce esters via an enzymatic routes include: 1) Alcohol acyltransferases (AATase; EC 2.3.1.84) that convert acetyl- or acyl-CoA and an alcohol to the corresponding esters (Figure 1-2a); 2) Ester formation from hemiacetals via reactions with alcohol dehydrogenase (EC 1.1.1.1.) (Figure 1-2b); and, 3) The reaction of ketones with molecular oxygen by a Baeyer-Villiger monooxygenases (BVMO; EC 1.14.13.92) to form esters (Figure 1-2c) (Park et al., 2009; Saerens et al., 2010). In fruit and yeast it has been demonstrated that the primary synthesis route is by AATase and as such this work focuses on engineering and understanding AATase and their ability to synthesize short chain acetate esters. Yields can approach 100 percent product formation
from methods such as enzyme processivity where the enzyme is able to catalyze consecutive reactions without releasing the substrate (Wyman, Decker, Himmel, Brady, & Skopec, 2005).

**FIGURE 1-2:** Microbial Ester production. Three enzymatic routes are shown a) alcohol acyltransferase b) alcohol dehydrogenase, and c) Baeyer-Villiger monooxygenase.

The first identification of AATase activity in yeast was of Atf1 and Atf2 by Fujji et al. and Nogasawa et al. (Park et al., 2009; Verstrepen, Derdelinckx, et al., 2003). In these early works, Atf1 was overexpressed in a sake strain of *S. cerevisiae* leading to a 9-fold increase in ethyl acetate production and 27-fold increase in isoamyl acetate formation. An AATase of *S. pastorianus* was also overexpressed in *S. cerevisiae* and ester acetate synthesis was shown to increase by 17-fold for isoamyl and 2-fold for ethyl acetate. The AATase activity levels in cell-free extracts were also measured for the overexpression of Atf1 and Atf2 in *S. cerevisiae* and resulted in a 4.7-fold and 2.4-fold
increase in isoamyl acetate, respectively, versus vector control strains (Park et al., 2009; Verstrepen, Derdelinckx, et al., 2003). Similarly, Vestrepen et al. (Verstrepen, Derdelinckx, et al., 2003) overexpressed Atf1 and Atf2 in a commercial brewer’s strain of *S. cerevisiae*. The beer produced with overexpressed Atf1 provided 5-times more acetate esters than beer produced with the wild type strain. In the case of Atf2 overexpression, only a small increase of isoamyl acetate and insignificant changes of ethyl acetate were observed.

In a later study by Vestrepen et al (2008), Atf1 and Atf2 from *S. cerevisiae* were homologously overexpressed. Overexpression of Atf1 was found to have a >20-fold increase in ester formation. Atf2 overexpression resulted in a 1.5-fold and 2-fold increase in ethyl acetate and isoamyl acetate, respectively. Atf from *K. lactis* was also overexpressed in *S. cerevisiae* and demonstrated only a small effect on the ester formation. The introduction of ATF genes in other organisms such as *E. coli* or *Clostridium acetobutylicum* for the production of acetate esters was also observed (Park et al., 2009).

The substrate specificity of Atf1 and Atf2 is not limited to ethanol and isoamyl alcohol. Broad substrate specificity for both alcohol and acyl-CoA chain lengths creates the possibility to engineer yeast to produce a desire ester product, including variable chain length acetate esters and ethyl esters (Van Laere et al., 2008). In addition, expression of non-saccharomyces and fruit AATase could yield different product profiles. Understanding the mechanisms of ester formation and the enzymes ability to control levels of the end product is of major concern and interest for industry.
1.4 OBJECTIVES

Esters have a widespread application in the fragrance, flavour, pharmaceutical and chemical industries. Protein and metabolic engineering arise as potentially useful tools to meet the market needs. The overall goal in this thesis is to work toward the development of biosynthetic pathways for acetate ester synthesis.

Chapter 1, this chapter, provides an introduction to the motivation towards ester synthesis. A brief overview of current industrial ester synthesis methods are discussed and compared. In addition an overview of microbial synthesis of esters is introduced with an emphasis on alcohol acetyltransferase enzyme catalysis. Lastly, the objective of the thesis for each chapter is presented.

Chapter 2 presents the study of seven AATase genes from various yeast and fruit species and activity screening of the corresponding AATase against a series of linear and branched primary alcohols. Bioinformatics tools were used to create amino acid sequence alignments and analyze the sequences of different AATases. Headspace gas chromatography was used to screen enzyme activity and the toxicity of each studied acetate ester towards an S. cerevisiae host was evaluated.

Chapter 3 provides the analysis of coupled enzyme reactions by using MATLAB to model and simulate a series of coupled enzyme reactions. This work included the analysis of a coupled enzyme reaction with a competitive third enzyme and a competitive inhibitor was performed. Transient time simulation was determined based on the probability of channeling between the coupled enzymes. Analogous MATLAB code was
used to produce a coupled enzyme reaction simulation with alcohol acetyltransferase parameters.

Finally, the studies present in chapters 1-4 are summarized and recommendations for future research are given in chapter 4.
CHAPTER 2: STUDIES OF ACETATE ESTER FORMATIONS BY AATASE ORTHOLOGS FROM SACCHAROMYCES, NON-SACCHAROMYCES YEAST AND VARIOUS FRUIT SPECIES

2.1 INTRODUCTION

Acetate esters are widespread in nature and industry and account for the flavour and fragrance additives used by the food, beverage, pharmaceutical and personal care industries. In yeast fermentation and fruit ripening, alcohol acetyltransferases (AATase or Atf) are responsible for the biosynthesis of short and medium chain length acetate esters from an alcohol and acetyl-CoA (Barney, 2014; Park et al., 2009; Rodriguez et al., 2014; Shalit et al., 2001). Examples of C3 to C7 chain length acetate esters produced by yeast and fruit including ethyl and isoamyl acetate are shown in Figure 2-1. Despite some success of metabolic engineering for microbial ester biosynthesis (Vadali, Horton, Rudolph, Bennett, & San, 2004; Van Laere et al., 2008; Verstrepen, Derdelinckx, et al., 2003; Wang, Xing, Chin, Ho, & Martin, 2001), a poor understanding of the kinetic characteristics of AATases has limited many of these works to low yields and titers. In addition, the substrate specificity of different AATase is not well understood and the toxicity of different esters towards suitable microbial hosts has not yet been studied.

In this chapter, seven different AATases from yeast and fruit species were identified and screened for activity with a series of linear and branched primary alcohols. Eight different alcohol substrates including methanol, ethanol, propanol, isopropanol, butanol, isobutanol, pentanol, and isopentanol were screened against AATases from S. cerevisiae, S. pastorianus, K. lactis, P. anomala, S. lycopersicum, and C. melo for the production of the corresponding acetate ester. Gas chromatography was used to test for
the presence of ester products. From the experimental results, fingerprints of substrate specificity describing the selectivity towards various short chain alcohols were determined. In addition to the screening efforts, the toxicity of each ester towards the *S. cerevisiae* host was evaluated. The range of short-chain acetate esters tested includes: methyl acetate, ethyl acetate, propyl acetate, isopropyl acetate, butyl acetate, isobutyl acetate, pentyl acetate and isopentyl acetate. The overall goal for these studies is to work towards the development of acetate ester synthesis pathways by microbial engineering of AATases in yeast.

**FIGURE 2-1:** Biosynthesis of short and medium chain length acetate esters from an alcohol and acetyl-CoA by AATase.
2.2 MATERIALS AND METHODS

2.2.1 Chemicals

Methanol, ethanol, isopropanol, and dithiothreitol (DTT) were purchased from Fisher (Fairlawn, NJ, USA). Propanol, butanol, isobutanol, pentanol, isopentanol, methyl acetate, ethyl acetate, propyl acetate, isopropyl acetate, butyl acetate, isobutyl acetate, pentyl acetate, isopentyl acetate, sulfuric acid, Bradford Reagent and acetyl-CoA were obtained from Sigma-Aldrich (St. Louis, MO., USA). All chemicals were of high grade.

2.2.2 Strains, media, and culturing conditions

All plasmids, yeast and fruit species strains used in this study are listed in Table 2-1. The *S. cerevisiae* strain BY4742 was used in all experiments of this study. Yeast cultures were routinely grown at 30°C in YPD medium (2% glucose, 2% peptone (Difco) and 1% yeast extract (Difco)). Yeast harboring overexpression plasmids were grown in synthetic minimal medium containing 0.67% nitrogen base without amino acids (Difco) and 2% glucose, supplemented with 1.92g/L of yeast synthetic dropout medium supplement without uracil (Sigma). All cultures were shaken in an incubator orbital shaker at 220 rpm for both test tubes and 125ml Erlenmeyer flasks.
### TABLE 2-1: Microbial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/strain description</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>YS5 (S. cerevisiae)</em></td>
<td>hisΔ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
<td>BY4742 (Open Biosystem)</td>
</tr>
<tr>
<td><em>YS21 (pIW7): BY4742</em></td>
<td>hisΔ1 leu2Δ0 lys2Δ0 ura3Δ0 pRS426</td>
<td>This study</td>
</tr>
<tr>
<td><em>YS202 (S. cerevisiae)</em></td>
<td>BY4742 + pIW107 (ATF1-myc Sc)</td>
<td>This study</td>
</tr>
<tr>
<td><em>YS204 (S. cerevisiae)</em></td>
<td>BY4742 + pIW108 (ATF2-myc Sc)</td>
<td>This study</td>
</tr>
<tr>
<td><em>YS212 (S. pastorianus)</em></td>
<td>BY4742 + pIW112 (ATF1-myc Sp)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>non-saccharomyces</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>YS208 (K. lactis)</em></td>
<td>BY4742 + pIW110 (ATF1-myc Kl)</td>
<td>This Study</td>
</tr>
<tr>
<td><em>YS210 (P. anomala)</em></td>
<td>BY4742 + pIW111 (ATF1-myc Pa)</td>
<td>This Study</td>
</tr>
<tr>
<td><strong>fruit species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>YS206 (S. lycopersicum)</em></td>
<td>BY4742 + pIW109 (ATF1-myc Sl)</td>
<td>This Study</td>
</tr>
<tr>
<td><em>YS214 (C. melo)</em></td>
<td>BY4742 + pIW113 (ATF1-myc Cm)</td>
<td>This Study</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Inserted genes</th>
<th>Marker</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIW7</td>
<td>Vector (pRS426)</td>
<td>Amp</td>
<td>This Study</td>
</tr>
<tr>
<td>pIW107</td>
<td>PGK1p-ATF1-myc-PGK1t (S. cerevisiae)</td>
<td>Amp/URA</td>
<td>This Study</td>
</tr>
<tr>
<td>pIW108</td>
<td>PGK1p-ATF2-myc-PGK1t (S. cerevisiae)</td>
<td>Amp/URA</td>
<td>This Study</td>
</tr>
<tr>
<td>pIW109</td>
<td>PGK1p-ATF1-myc-PGK1t (S. lycopersicum)</td>
<td>Amp/URA</td>
<td>This Study</td>
</tr>
<tr>
<td>pIW110</td>
<td>PGK1p-ATF1-myc-PGK1t (K. lactis)</td>
<td>Amp/URA</td>
<td>This Study</td>
</tr>
<tr>
<td>pIW111</td>
<td>PGK1p-ATF1-myc-PGK1t (P. anomala)</td>
<td>Amp/URA</td>
<td>This Study</td>
</tr>
<tr>
<td>pIW112</td>
<td>PGK1p-ATF1-myc-PGK1t (S. pastorianus)</td>
<td>Amp/URA</td>
<td>This Study</td>
</tr>
<tr>
<td>pIW113</td>
<td>PGK1p-ATF1-myc-PGK1t (C. melo)</td>
<td>Amp/URA</td>
<td>This Study</td>
</tr>
</tbody>
</table>

### 2.2.3 Overexpression plasmids

Overexpression plasmids for the AATase production in *S. cerevisiae* were accomplished with the help of a lab member, Aaron Lin. Briefly, An ATF overexpression cassette yeast promoter phosphoglycerate kinase 1 (PGK1p) and terminated by PGK1t was constructed (Figure 2-2). The promoter and terminator were amplified from genomic DNA of BY4742 and cloned into pRS426 (vector). PGK1p was inserted using SacI and SacII sites. PGK1t was inserted using SpeI and KpnI sites while ATF-myc was inserted at SacII and NotI. Figure 2-2 represented the sites of the inserted AATase overexpressions.
FIGURE 2-2: pR426-PGK1p-ATF-myc-PGK1t plasmid used for this study. The promoter and terminator were amplified from genomic DNA of BY4742 and cloned into pR426 (vector). PGK1p was inserted using SacI and SacII sites. PGK1t was inserted using SpeI and KpnI sites while ATF-myc was inserted at SacII and NotI.

2.2.4 Alcohol acetyltransferase activity

Precultures were shaken at 30°C in 15ml test tubes containing 1ml of SD/-U medium. After 8h of growth the cultures were used to inoculate 50 ml of SD/-U medium in a 125 ml Erlenmeyer flaks, and these second cultures were shaken at 30°C for 16h overnight period (Figure 2-3A).

The cultures were used to produce whole cell lysate for activity screening. Cells were harvested from overnight precultures by centrifugation at 3500 rpm at 4°C for 5
min. Cells were washed twice, once with 10 ml of distilled water, centrifuged at 3000 rpm at 4°C for 5 min, and then washed with 5 ml of disruption buffer (ice cold). Cells were resuspended in 1ml lysis buffer containing 2 mM MgCl₂, 5mM dithiothreitol (DTT), protease inhibitor (Roche) and 100mM potassium phosphate (pH 7.4). Cell suspensions were vigorously vortexed with 1:1 ratio of glass beads (sigma) 0.5 mm in diameter for 30 sec and cooled on ice for 1.5 min. Vortexing was repeated 10 times. Glass beads and cell debris was removed by centrifugation at 500 rcf at 4°C for 5 min and the supernatant was collected. The supernatant was used as crude extract to measure AATase whole cell lysate activities, 100 µL in each reaction. The protein concentration in the crude extract was determined by Bradford reagent (Sigma) samples using SD/-U medium as a standard (Figure 2-3B). Thermo Scientific NanoDrop 2000c spectrophotometer was used by Bradford reagent samples.

The activity of AATases for the synthesis of acetate esters was determined in a 1 ml reaction mixture containing either 500mM (Methanol, Ethanol, Propanol or Isopropanol) or 200 mM (Butanol, Isobutanol, Pentanol or Isopentanol) alcohol, 0.25 mM acetyl coenzyme A and 100mM potassium phosphate buffer (pH 7.4). The reaction mixtures were incubated in a 10 ml capped glass vial at 30°C for 30 min (Figure 2-3C). The reaction was stopped by adding 20 µl 3M H₂SO₄. Volatiles were increased by adding 1 g NaCl.
2.2.5 Headspace gas chromatography (GC)

Headspace gas chromatography was used for the measurement of volatile acetate esters and alcohols present in sample vials (Figure 2-3C). Volatiles were analyzed using Agilent 7890 gas chromatography equipped with a CombiPAL autosampler and were separated in a RESTEK Rtx-1 column (length 30 m, internal diameter 0.32 mm, layer thickness 5 µm) (Figure 2-3D). Samples were heated for 10 min at 30°C in the
autosampler. The oven and FID detector temperatures were kept constant at 75 and 275°C respectively. Helium was used as the carrier gas. The following temperature program was used: 7 min hold at 75°C; temperature increase at 30 °C/min to 175°C; 2 min hold at 175 °C; temperature increase 50 °C/min to 275 °C. Results were analyzed with Agilent GC ChemStation software. Identification of volatiles was confirmed using retention time of calibration mixtures. Eight calibration mixtures (corresponding to the eight acetate ester products) of increasing compound concentration were analyzed by the same method mentioned above. The resulting peaks were used for setting up calibration curves.

2.2.6 Acetate ester toxicity assays

Toxicity assays were with *Saccharomyces cerevisiae* (BY4742) yeast strain. Precultures were shaken overnight at 30 °C in 15 ml tubes containing 5 ml of YPD medium for 16h. Precultures were inoculated into 25 ml YPD medium in 125 Erlenmeyer flasks to with 1/100 % volume of culture to fresh media with the following conditions: sample 1 (0% glucose, 0% acetate ester), sample 2 (0% glucose, 1% acetate ester), sample 3 (2% glucose, 0% acetate ester), sample 4 (2% glucose, 0.5% acetate ester), sample 5 (2% glucose, 2.5% acetate ester) and sample 6 (2% glucose, 5% acetate ester). Sample 1 and 2 were used a negative controls. Sample 3 was used as the positive control. Samples were collected for analysis at 0, 2, 4, 6, 8, 24 and 48h of fermentation. Cell count in each sample was determined by optical density (OD) measurements at 600 nm by the Thermo Scientific NanoDrop 2000c spectrophotometer. Each sample was diluted
as necessary by corresponding YDP medium to obtain a reading maximum limit of 1.3 OD at 600 nm. The initial cell count present in each trial was 0.15 OD at 600 nm.

### 2.3 RESULTS

#### 2.3.1 Multiple alignment of amino acid sequences for AATase orthologs

A sequence comparison was performed between the primary sequences of AATases from different orthologs used in this study. The sequence alignments shown in Figure 2-4 reveal the presence the conserved putative active site H-X-X-X-D in all AATase sequences. This active site motif including the histidine and the aspartate residues has been shown to be conserved across acyl- and acetyl-transferases (Van Laere et al., 2008).

**FIGURE 2-4:** Multiple alignment of the amino acid sequence of Atf1 and Atf2 from *S. cerevisiae* (sc) and their corresponding orthologs from *S. lycopersicum* (sl), *S. pastorianus* (sp), *K. lactis* (kl), *C. melo* (cm), and *P. anomala*. The alignments were performed using T-coffee online software. Non-conserved residues are shaded in green and yellow, similar residues are shaded in orange, and conserved and identical residues are shaded in red. The conserved residues H-X-X-X-D, possibly essential for catalytic activity and considered the putative active site, is boxed and marked by arrows.
The AATase orthologs were analyzed using pairwise alignment with the amino acid sequences of Atf1 and Atf2 from *S. cerevisiae*. The alignments were accomplished by TCOFFEE and NCBI Blast online programs and used to estimate the relatedness among the amino acid sequences of the orthologs. The percentages of similarities and identity between the orthologs are presented in Table 2-1. Similarities in multiple alignments are the amino acids that represent similar physico-chemical properties and behave alike. Identity in multiple alignments is derived by a mathematical formulation that describes the matched identical amino acids and gaps involved in the sequence comparison (NCBI-Blast). In comparison to Atf1, the variant with the greatest similarity is the corresponding *S. cerevisiae* Atf2 with a 95% and an identity value of 37%. It was observed that the greatest similarity with is among the yeast orthologs, *S. pastorianus* Atf1 with a 99% similarity. *K. lactis* and *P. anamola* are also similar with a 95% and 86% similarity, respectively. The divergent orthologs to Atf1 *S. cerevisiae* correspond to the fruit species with a similarity percentage of only 14% to *S. lycopersicum* Atf1 and 14% to *C. melo* Atf1. In comparison to Atf2 *S. cerevisiae*, the similarity percentages include 96% to *S. pastorianus* Atf1, a 96% to *K. lactis* Atf1 and 77% to *P. anamola* Atf1. The divergent orthologs to Atf2 *S. cerevisiae* correspond to the fruit species with a similarity percentage of 3% to *S. lycopersicum* Atf1 and 3% to *C. melo* Atf1. The percentage identity for *S. cerevisiae* Atf1 ranges from 80% for *S. pastorianus* Atf1 to 26% for *P. anamala* Atf1. For Atf2 *S. cerevisiae*, the percentage identity ranges from 39% for the *C. melo* Atf1 to 25% for the *P. anamala* Atf1.
TABLE 2-2: Protein sequence similarity and identity of *S. cerevisiae* Atf1 and all orthologs protein sequence found.

<table>
<thead>
<tr>
<th>S. cerevisiae Atf1</th>
<th>S. cerevisiae Atf2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Similarity %</td>
<td>Identity %</td>
</tr>
<tr>
<td><strong>S. cerevisiae Atf1</strong></td>
<td>100</td>
</tr>
<tr>
<td><strong>S. cerevisiae Atf2</strong></td>
<td>95</td>
</tr>
<tr>
<td><strong>S. lycopersicum Atf1</strong></td>
<td>14</td>
</tr>
<tr>
<td><strong>S. pastorianus Atf1</strong></td>
<td>99</td>
</tr>
<tr>
<td><strong>K. lacti Atf1</strong></td>
<td>95</td>
</tr>
<tr>
<td><strong>C. melo Atf1</strong></td>
<td>14</td>
</tr>
<tr>
<td><strong>P. anomala Atf1</strong></td>
<td>86</td>
</tr>
</tbody>
</table>

2.3.2 AATase activity screening

In this study, eight different alcohol substrates including methanol, ethanol, propanol, isopropanol, butanol, isobutanol, pentanol, and isopentanol were screened against ATFs from *S. cerevisiae*, *S. pastorianus*, *K. lactis*, *P. anomala*, *S. lycopersicum*, and *C. melo* for the production of the corresponding acetate ester. Acetate ester production was determined via GC headspace analysis. Figure 2-5 demonstrates a GC analysis for the Atf1 from *S. cerevisiae* with different alcohols and their production of corresponding acetate esters. In each case the first peak correspond to alcohol substrate and the second peak corresponds to the ester product. For example, the top chromatograph in figure 2-5 shows a peak at ~2.5 minutes for the methanol substrate and a second peak at ~4 minutes corresponding to methyl acetate. The peaks of all ester products were confirmed using standard acetate ester samples.

Whole cell lysate activity screening revealed that Atf1 from *S. cerevisiae* has the highest overall activity (Figure 2-6). Atf1 *S. cerevisiae* (Atf1-S.c.) was most active
towards ethanol (0.88 µmol/(min•mg)), was fairly active towards methanol (0.031 µmol/(min•mg)) and propanol (0.052 µmol/(min•mg)), and least active towards isopropanol (0.013 µmol/(min•mg)), butanol (0.014 µmol/(min•mg)), isobutanol (0.0067 µmol/(min•mg)), pentanol (0.0030 µmol/(min•mg)) and isopentanol (0.0041 µmol/(min•mg)). In comparison, Atf2-S.c. was most active towards ethanol (0.025 µmol/(min•mg)) and isobutanol (0.0105 µmol/(min•mg)) but had low activity towards propanol (0.0049 µmol/(min•mg)), butanol (0.0011 µmol/(min•mg)), pentanol (0.0012 µmol/(min•mg)) and isopentanol (0.0012 µmol/(min•mg)) and no activity towards methanol and isopropanol.

The activity of Atf1 from *S. lycopersicum* (Atf1-S.l.) exhibit high activity towards ethanol with an activity of 0.24 µmol/(min•mg), a value of 27.3% of Atf1-S.c. Atf-S.l. was fairly selective towards propanol (0.010 µmol/(min•mg)) and least selective towards methanol (0.0007 µmol/(min•mg)), isobutanol (0.0003 µmol/(min•mg)), pentanol (0.0011 µmol/(min•mg)) and isopentanol (0.0004 µmol/(min•mg)), and no activity towards isopropanol and butanol. The activity of Atf1-S.l. is interesting because it is a fruit species with a similarity percentage of 14% towards Atf1-S.c. and 3% towards Atf2-S.c., yet it has the second (after Atf1-S.c.) highest selective activity towards an ethanol.
FIGURE 2-5: GC analysis of the acetate ester products formed by Atfl-S.c. enzyme with acetyl Co-A and a range of short chain alcohol substrate.
In contrast, all the other Atfs including those from, *S. pastorianus* (Atf1-S.p.), *K. lactis* (Atf1-K.l), *P. anomala* (Atf1-P.a), *S. lycopersicum* (Atf1-S.l), and *C. melo* (Atf-C.m.), had comparative low activity. For example, Atf2-S.c. had activity towards ethanol (0.025 \( \mu \text{mol/(min\text{•}mg)} \)) and showed low activity for the other alcohols. Atf1-C.m. was active toward isopentanol (0.012 \( \mu \text{mol/(min\text{•}mg)} \)), was slightly active towards propanol (0.0005 \( \mu \text{mol/(min\text{•}mg)} \)), and showed no activity with the rest of the alcohols. Atf1-S.p. was slightly active towards propanol (0.0006 \( \mu \text{mol/(min\text{•}mg)} \)) and isobutanol (0.0008 \( \mu \text{mol/(min\text{•}mg)} \)), and showed no activity with the rest of the alcohols. Atf1-P.a was slightly active towards propanol (0.0003 \( \mu \text{mol/(min\text{•}mg)} \)) and pentanol (0.0006 \( \mu \text{mol/(min\text{•}mg)} \)), and showed no activity with the rest of the alcohols. Atf1-K.l. was slightly active toward ethanol (0.0040 \( \mu \text{mol/(min\text{•}mg)} \)), propanol (0.00040 \( \mu \text{mol/(min\text{•}mg)} \)), pentanol (0.0023 \( \mu \text{mol/(min\text{•}mg)} \)) and isopentanol (0.0016 \( \mu \text{mol/(min\text{•}mg)} \)), but showed no activity with the rest of the alcohols.

**FIGURE 2-6:** Enzymatic ester synthesis of Atf1 overexpressed transformant by combining alcohols and acetyl-CoA. Substrate specificity of Atf activity in cell-free extract is demonstrated. Substrate concentration was of 500mM (methanol, ethanol, propanol and isopropanol) and 200 mM (butanol, isobutanol, pentanol and isopentanol). Ester formation was measured utilizing the kinetic assay procedure described under materials and methods.
2.3.3 Acetate ester toxicity

Experiments were conducted to study the effect of acetate ester on *S. cerevisiae* (BY4742). Growth cultures were sampled at regular intervals over a 48h period and analyzed for OD at 600 nm. The results of these experiments are shown in Figure 2-7. In all cases ester acetate concentration greater than 5% in the medium was inhibitory to yeast growth; however, growth with lower concentration was dependent on the type and amount of each acetate ester present in media conditions.

The final OD_{600} observed for methyl acetate was 36.0 at 0.5%, decreasing to 31 and 13.1 for 2.5% and 5%, respectively. For ethyl acetate, the final OD_{600} was 29.8 at 0.5%, decreasing to 26.0 and ~0 for 2.5% and 5%, respectively. In comparison, the final OD_{600} for propyl and isopropyl was 40.3 and 33.5 at 0.5%, respectively, but decreased to ~0 for 2.5% and 5%. For higher chained alcohols starting with butanol, a sample of 0.1% acetate esters was also included. The final OD_{600} observed for butyl acetate was 32.9 at 0.1%, decreasing to 11.6 for 0.5% and to ~0 at both 2.5% and 5%. The final OD_{600} observed for isobutyl acetate was 32.1 at 0.1%, decreasing to 27.9 for 0.5% and to ~0 at both 2.5% and 5%. In comparison, the final OD_{600} for both pentyl and isopentyl was 26.1

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Whole Cell Lysate Alcohol Acetyltransferase Activity (µmol/(min•mg))</th>
<th>Atf1 (S.c.)</th>
<th>Atf2 (S.c.)</th>
<th>Atf1 (S.l.)</th>
<th>Atf1 (C.m.)</th>
<th>Atf1 (S.p.)</th>
<th>Atf1 (P.a.)</th>
<th>Atf1 (K.l.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>0.0311 n.d. 0.0007 n.d. n.d. n.d. n.d. n.d.</td>
<td>0.0007</td>
<td>0.0007</td>
<td>0.0007</td>
<td>0.0007</td>
<td>0.0007</td>
<td>0.0007</td>
<td>0.0007</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.8795 0.0245 0.2406 n.d. n.d. n.d.</td>
<td>0.0245</td>
<td>0.2406</td>
<td>0.0245</td>
<td>0.2406</td>
<td>0.0245</td>
<td>0.2406</td>
<td>0.0245</td>
</tr>
<tr>
<td>Propanol</td>
<td>0.0522 0.0049 0.0104 0.0005 0.0006 0.0003 0.0004</td>
<td>0.0049</td>
<td>0.0104</td>
<td>0.0005</td>
<td>0.0006</td>
<td>0.0003</td>
<td>0.0004</td>
<td>0.0004</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>0.0067 0.0105 0.0003 n.d. 0.0008 n.d. n.d.</td>
<td>0.0105</td>
<td>0.0003</td>
<td>n.d.</td>
<td>0.0008</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Pentanol</td>
<td>0.0030 0.0012 0.0011 n.d. 0.0006 0.0003 0.0023</td>
<td>0.0012</td>
<td>0.0011</td>
<td>n.d.</td>
<td>0.0006</td>
<td>0.0003</td>
<td>0.0023</td>
<td>0.0016</td>
</tr>
<tr>
<td>Isopentanol</td>
<td>0.0041 0.0012 0.0004 0.0121 n.d. n.d. n.d.</td>
<td>0.0012</td>
<td>0.0004</td>
<td>0.0121</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.0016</td>
</tr>
</tbody>
</table>
and 30.3 at 0.1%, respectively, but decreased to ~0 for 0.5%, 2.5% and 5%. Methyl acetate ester manifests itself as the leading tolerable acetate ester present in the medium environment. The presence of larger chain acetate esters like butyl, isobutyl, pentyl and isopentyl acetate in the medium environment results in a rapid toxic condition.

**FIGURE 2-7:** Cell growth of *S. cerevisiae* in a spectrum of acetate ester toxic environments. Cells were cultured in 2% YPD medium and a corresponding acetate ester percentage (0, 0.5, 2.5 or 5%). Cultures were grown at 30°C in an incubated orbital shaker. Samples were taken at 0, 2, 4, 6, 8, 24 and 48h.

Growth rates were estimated by analyzing the log phase growth of each cell culture (Figure 2-8). Significant differences were observed in the growth rate with the addition of high ester concentrations. The higher-chain ester acetates (butyl acetate-isopropyl acetate) were observed to have a rapid decrease in growth rates as ester
concentration increases. It was determined that a higher toxicity is preserved in the presence of higher chain acetate esters.

**FIGURE 2-8:** Cell growth rate of *S. cerevisiae* in a spectrum of acetate ester toxic environments. The cell growth rate (OD/hr) is calculated from the growth rate analysis and the corresponding log phase region. Cells were cultured in 2% YPD medium and a corresponding acetate ester percentage (0, 0.5, 2.5 or 5%). Cultures were grown at 30°C in an incubated orbital shaker. Samples were taken at 0, 2, 4, 6, 8, 24 and 48h.

**2.4 DISCUSSION**

The widespread application of acetate esters along with the innovative and technological advances in research of metabolic engineering, present a potential option for AATase enzymes as biocatalysts for industrial synthesis of acetate esters. Acetate ester formation in yeast is controlled by alcohol-O-acetyltransferase enzyme (Verstrepen, Derdelinckx, et al., 2003), and as such, AATase variants of yeast and fruit species were
studied for the development of biosynthetic pathways for the production of short chain acetate esters.

Bioinformatics tools include the NCBI and TCOFFEE programs were used to create an amino acid sequence alignments and compare AATases. The seven AATase orthologs presented in this study are members of the pfam07247 family, described as a “family containing a number of alcohol acetyltransferase (EC 2.3.1.84) enzymes approximately 500 amino acids long, catalyzing the esterification of isoamyl alcohol by acetyl coenzyme A” (Van Laere et al., 2008). This family was originally based on the Atf1 and Atf2 of *S. cerevisiae*, but gradually increased in size as more protein genomes were sequenced and added. The analysis of AATase orthologs in yeast and fruit species from this family reveals a consistent H-X-X-X-D motif. However, some of these AATases have low identity to *S. cerevisiae* Atf1 and Atf2, most likely due to the larger evolutionary distance of the species relative to *S. cerevisiae* (Van Laere et al., 2008). The similarity percentages in comparison to Atf1 *S. cerevisiae* between the other AATases from yeast is higher with a maximum similarity of 99% to *S. pastorianus* and a range of 85-95% with the other variants of yeast. Atf1 *S. cerevisiae* amino acid sequence comparison to fruit species exhibits a smaller range of similarity percentage with a value of 14% for both *S. lycopersicum* and *C. melo*.

The substrate specificity of overexpressed of AATase variants (*S. cerevisiae*, *S. pastorianus*, *K. lactis*, *P. anomala*, *S. lycopersicum*, and *C. melo*) in *S. cerevisiae* from yeast and fruit species was examined with C1-C5 (methanol, ethanol, propanol, isopropanol, butanol, isobutanol, pentanol, and isopentanol) alcohols for the production
of acetate esters. Gas chromatography analysis of the enzymatic activity of each sample of AATase variants showed a significantly higher ester production in the overexpressed Atf1 S. cerevisiae with ethanol (0.8795 µmol/(min•mg)). In comparison, activity screening for the other variants revealed lower activity with all alcohol substrates. Atf1 S. cerevisiae, Atf 2 S. cerevisiae, Atf1 S. lycopersicum and Atf1 K. lactis were most active towards ethanol. Atf1 C.melo, Atf1 S. pastorianus and Atf1 P. anomala were most active towards longer-chain alcohols (isopentanol, isobutanol and pentanol, respectively). Atf1 from both S. cerevisiae (a yeast) and S. lycopersicum (a fruit species) revealed the highest specificity for ethanol, yet they have a sequence similarity of 14%, an interesting comparison that can be attributed the putative H-X-X-X-D motif.

The ester production of Atf1 S. cerevisiae with isopentanol (isoamyl/isopentyl acetate; 0.0043 µmol/(min•mg)) for this study, was compared with external sources (Table 2-4). The experiments reported in Table 2-4 differ in expression levels of Atf1-S.c.; however, this comparison serves as a validation of the experiments presented here. For example, in a study by Vestrepen, K. J. et al (2004), the production of isoamyl acetate was of 0.0034 µmol/(min•mg) in overexpressed Atf1, an activity level consistent with the value determined in this work.
Fermentation experiments were conducted to study the effect of acetate ester toxicity on *S. cerevisiae* (BY4742). These samples demonstrated that a high level of ester acetate in the medium was inhibitory to yeast growth. The growth of *S. cerevisiae* culture is dependent on the type and amount of each acetate ester present in media conditions. As the number of cell increase, cells growth decelerated due to the limiting nutrients available and the accumulation of waste products. The presence of acetate esters in cell cultures are considered “waste products” that, at certain levels, begin to have a toxic effect on the yeast cells. The leading tolerable acetate ester in the cell culture was methyl acetate and ethyl acetate. The presence of larger chain acetate esters like butyl, isobutyl, pentyl and isopentyl acetate in the medium environment results in a rapid toxic condition.

These results may be advantageous parameters used in the design of future AATase overexpression strains and their expected growth rate based on acetate ester yields and their corresponding toxicity levels.

**TABLE 2-4:** Atf from *S. cerevisiae* activity with acetyl Co-A and isopentanol substrate

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Alcohol Substrate</th>
<th>Expression</th>
<th>Alcohol Concentration</th>
<th>Ester Product</th>
<th>Activity (µmolar/min/mg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Isopentanol</td>
<td>overexpressed ATF</td>
<td>200 mM</td>
<td>Isopentyl Acetate</td>
<td>0.0043</td>
<td>This Study</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Isopentanol</td>
<td>overexpressed ATF</td>
<td>15 mM</td>
<td>Isopentyl Acetate</td>
<td>0.0034</td>
<td>Verstrepen, K. J. et al (2004)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Isopentanol</td>
<td>overexpressed ATF:GFP</td>
<td>15 mM</td>
<td>Isopentyl Acetate</td>
<td>0.0031</td>
<td>Verstrepen, K. J. et al (2004)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Isopentanol</td>
<td>wild type</td>
<td>15 mM</td>
<td>Isopentyl Acetate</td>
<td>0.0003</td>
<td>C. Plata et al. (2003)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Isopentanol</td>
<td>wild type</td>
<td>1 mM</td>
<td>Isopentyl Acetate</td>
<td>0.0001</td>
<td>T. Fujii et al. (1994)</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Isopentanol</td>
<td>wild type</td>
<td>15 mM</td>
<td>Isopentyl Acetate</td>
<td>0.0040</td>
<td>T. Fujii et al. (1994)</td>
</tr>
</tbody>
</table>
This work analyzed the amino acid sequence, substrate specificity and acetate ester toxicity of a range of yeast and fruit species AATase overexpressed variants for the development of biosynthetic pathways for the production of short chain acetate esters. Further research is needed for strain development and process optimization to improve acetate ester production. However, these presented results stand as a technological platform for future production of cost effective acetate esters.
3.1 INTRODUCTION

Substrate channeling is the process in which the reaction intermediate \( I \) from the active site of one enzyme \( E_1 \) is transferred directly to the active site of a second enzyme \( E_2 \) without dissociation into the bulk solution (Elcock, Huber, & McCammon, 1997; Geck & Kirsch, 1999; Huang, Holden, & Raushel, 2001; Miles, Rhee, & Davies, 1999; Spivey & Ova, 1999). This is an important phenomenon that can result in increasing pathway flux and protection of pathway intermediates from parasitic side reactions (Lin, Palomec, & Wheeldon, 2014). The channeling effect has been demonstrated in various settings, both with naturally occurring bifunctional enzymes, engineered fusion proteins, and scaffold multi-enzyme complexes (Elcock et al., 1997). Substrate channeling can also occur within multifunctional enzymes, tightly associated multienzyme complexes or transient enzyme complexes (Cheng et al., 2008; Huang et al., 2001).

Substrate channeling has many potential advantages over the free diffusion of the reaction intermediate through the bulk solvent, which include: (1) isolating the intermediate; (2) circumventing unfavorable metabolites; (3) protecting intermediates; (4) enhancing catalysis by avoiding unfavorable reactions; (5) reducing transients time; and (6) providing new means of metabolic regulation (Cheng et al., 2008; Spivey & Ova, 1999). There have been many interesting discoveries related to substrate channeling, nevertheless there has also been considerable controversy in the analysis of experimental evidence and the existence of different mechanism (Lin et al., 2014; Miles et al., 1999).
Considering the potential advantages of substrate channeling and the uncertainties of the experimental results, in this chapter, computational studies of the channeling process are studied in an attempt to work towards a development of biosynthetic pathways for the production of short chain acetate esters. Specifically, numerical solution method to solve a previously developed enzyme reaction mechanism for coupled reactions with substrate channeling was developed. In addition, the model was validated using three well-known coupled reactions and later used to analyze the final step of acetate ester synthesis by AATase. Parts of the modeling efforts described in this chapter contributed to an invited perspective article published in ACS Catalysis. The article was peer reviewed and accepted for publication. The entire content of the article is presented in the appendix of this thesis.

3.2 ENGINEERING SUBSTRATE CHANNELING IN MULTI-ENZYME STRUCTURES

Inspired by natural metabolic pathways and bifunctional enzymes, researchers are working to develop and optimize new colocalized and spatially organized multienzyme structures (Lin et al., 2014). These research focuses on enhancing overall reaction cascade kinetics by using protein, nucleic acid and polymer scaffolds to create multienzyme structures. For example, a protein scaffold used to assemble a three enzyme pathway to produce mevalonate resulted in a 77-fold increase in yield over the unstructured pathway (Dueber et al., 2009). The colocalization of a two enzyme system with protein and RNA scaffolds has produced up to 50-fold increased in production of
biohydrogen in *E. coli* (Agapakis et al., 2010). The most common explanation for kinetic enhancements is the optimization of the colocalization of enzymes assembled at close proximity, which leads to increases in catalysis (Lin et al., 2014). At low intermediate concentrations, the second enzyme of a coupled reaction is pseudo-first-order with respect to the intermediate and thus increasing local concentrations leads to a direct increase in reaction velocity (Elcock et al., 1997; Lin et al., 2014). Despite the strong potential for enhanced catalysis by engineering multienzyme structures, many challenges remain. For example, orientation of enzymes at the nanometer scale remains challenging and prevents further advancements; also the vast diversity of enzyme structures prevents direct a generalized solution to creating multienzyme cascade assembly.

3.3 SUBSTRATE CHANNELING IN NATURE AND TRANSFER MECHANISMS

Numerous examples of substrate channeling in nature have been reported including coupled reaction in amino acid metabolism, lipid metabolism, glycolysis, the tricarboxylic acid cycle, DNA replication, RNA synthesis, and protein biosynthesis (Cheng et al., 2008; Huang et al., 2001). The transfer of reaction intermediates in multifunctional enzymes or multienzyme complexes has been investigated by a variety of kinetic approaches which include, the measurement of transient time, isotope dilution or enhancement, competing reaction effects and transient state kinetics (Huang et al., 2001; Spivey & Ova, 1999). For example, if the intermediate is directly transferred (“perfect” channel) from one active site (*E1*) to another (*E2*) (Figure 3-1A), the transient time, will approach zero. However, if only some of the intermediate is transferred (“leaky”
channel), then the transient time is less than that corresponding to free diffusion, but not entirely equaled to zero (Huang et al., 2001).

A second example for a kinetic approach to investigating sub-channeled is demonstrated in figure 3-1B, where the isotope dilution method is illustrated (Huang et al., 2001). Here, the presence of an isotopic external intermediate \((I^*)\) competes with the reaction intermediate \((I)\). In a free diffusion system, the presence of \(I^*\) dilutes the availability of \(I\) and lowers the specific activity with \(E2\). In a channeling system, the intermediate is transferred directly from \(E1\) to \(E2\), reducing the effect of the external \(I^*\).

A third enzyme \((E3)\) can also be added to the reaction mixture to interfere with the intermediate \(I\) availability (Figure 3-1C) (Huang et al., 2001). Here, the presence of \(E3\) competes with \(E2\) for the reaction intermediate. However, in substrate channeling, \(E3\) is assumed to not interfere with the \(E1E2\) complex but rather remains in the bulk solvent.

![Substrate channeling schematics](image)

**FIGURE 3-1:** Substrate channeling schematics of (A) General process for the direct delivery of a reaction intermediate \((I)\) from the active site of one enzyme \((E1)\) to the active site of a second enzyme \((E2)\) without dissociation into bulk solvent. (B) The general process with the presence of an inhibitor intermediate \((I^*)\). (C) The general process with the presence of a third competing enzyme \((E3)\) (Huang et al., 2001).
Substrate channeling, can be achieved by several different mechanisms (Spivey & Ova, 1999). Detecting and characterizing substrate channeling for an enzyme pair depends on the nature of the interaction of enzymes and on the method of intermediate transfer (Geck & Kirsch, 1999; Huang et al., 2001; Spivey & Ova, 1999). Studies on several enzymes have revealed two main molecular mechanisms for channeling (Miles et al., 1999; Spivey & Ova, 1999). The two different channeling mechanism of the reaction intermediate proposed are (1) tunneling and (2) electrostatic channeling (Cheng et al., 2008; Miles et al., 1999; Spivey & Ova, 1999). Examples of tunneling and electrostatic channeling, respectively, are demonstrated by tryptophan synthase (TS) and thymidylate synthase-dihydrofolate reductase (TS-DHFR), respectively (Cheng et al., 2008; Elcock et al., 1997; Geck & Kirsch, 1999; Huang et al., 2001; Lin et al., 2014; Miles et al., 1999; Spivey & Ova, 1999; Zhang, 2011). The bifunctional enzyme tryptophan synthase is an example of channeling capable by natural optimized spatial organization that creates optimal local conditions to protect cells from toxic intermediates and drive a coupled reaction (Figure 3-2A) (Huang et al., 2001; Lin et al., 2014; Miles et al., 1999). In tryptophan synthase, two active sites on the different α- and β- subunits are connect by a tunnel ~25 Å long through which the reaction intermediate (indole) passes (Figure 3-3A) (Geck & Kirsch, 1999; Lin et al., 2014).
FIGURE 3-2: Natural bifunctional enzyme that exhibit substrate channeling. Channeled intermediates are boxed. The represented examples include (A) Tryptophan Synthase, (B) Dihydrofolate Reductase-Thymidylate Synthase. Abbreviations definition: IGP, indole-3-glycerol phosphate; G3P, D-glyceraldehyde 3-phosphate; H2folate, dihydrofolate; H4folate, tetrahydrofolate; C2H4folate, methylene tetrahydrofolate; (Huang et al., 2001; Lin et al., 2014; Miles et al., 1999). Figure adapted from (Miles et al., 1999).
FIGURE 3-3: Natural examples of tunneling and electrostatic channeling. **Tunneling:** (A) One αβ-heterodimer of Tryptophan Synthase (TS) with a α-subunit that contains the indole-3-propanol phosphate and the β-subunit that contains the L-serine. This tunnel is ~25 Å long. The cyan/blue section and the yellow section represent the different active sites of each enzyme. The orange/yellow spheres represent the course of the tunnel from the different subunits (active sites). **Electrostatic channeling:** (B) Dihydrofolate Reductase-Thymidylate synthase (DHFR-TS) reveals an electrostatic potential distribution (negative charge shown in red, positive charge shown in blue, active sites are yellow), and connects the two active sites by a positive pathway. Figure adapted from reference (Huang et al., 2001; Miles et al., 1999).
One example that has influenced this study is the bifunctional enzyme thymidylate synthase-dihydrofolate reductase (TS-DHFR). The reaction catalyzed by TS enzyme produces 2-deoxythymidylate (dTMP) and dihydrofolate (H$_2$folate), with the latter acting as the substrate for the second reaction catalyzed by the DHFR active site (Figure 3-2B) (Elcock et al., 1997; Elcock, Potter, Matthews, Knighton, & McCammon, 1996; Lin et al., 2014). Experimental results have revealed a higher efficiency in H$_2$folate intermediate transfer by the bifunctional enzyme versus the monofunctional enzymes: An estimated ~80% of substrate leaving TS active site reaches the DFHR active site in the bifunctional case (Elcock et al., 1996). The TS-DHFR structures reveal no obvious means for substrate channeling, for example, no tunnel connecting active sites (Elcock et al., 1997, 1996). Instead, expected behavior would be for the intermediate to be exposed to external solvent and surroundings. Examination of the region between the two active sites revealed strong positive electrostatic potential, which interacts with the -2 net charge of H$_2$folate intermediate (Figure 3-3B) (Elcock et al., 1997, 1996). The electrostatic potential has been suggested to induce the channeling of the intermediate between the active sites (Elcock et al., 1997, 1996). Electrostatic channeling in TS-DHFR is further supported by Brownian dynamics simulations and experimental kinetics studies (Elcock et al., 1997; Lin et al., 2014; Miles et al., 1999). Recent transient time and steady-state kinetics analysis of TS-DHFR from L. major (Elcock et al., 1997; Miles et al., 1999) have demonstrated that the negatively charged H$_2$folate intermediate diffuses between active sites with high efficiency. The TS-DHFR example is an important one for the engineering of new spatially organized pathways (Lin et al., 2014). The structure and kinetic data of
TS-DHFR are used together to further study the mechanism of substrate channeling and indentify potential kinetic enhancements.

### 3.4 KINETIC ANALYSIS OF SUBSTATE CHANNELING

There have been attempts to study and understand the kinetics of coupled enzyme reactions, both with and without substrate channeling (Lin et al., 2014). The analysis of substrate channeling often focuses on one of more of the following; (1) transient time of overall reaction; (2) pathway resistance to competing side reactions; and (3) enhancement to the initial pathway reaction rate (Lin et al., 2014). This study attempts to demonstrate and test the common methods of evaluating coupled enzyme reactions in the context of bifunctional enzymes and a comparison between experimental and simulation observables.

The transient time, \( \tau \), of a coupled enzyme reaction is defined, as the time (lag phase) needed for the intermediate produced by the first enzyme to reach levels needed to maintain a steady state flux (Elcock et al., 1997; Lin et al., 2014). Scheme 3-1, adapted from Elcock et al. and Lin et al., demonstrates a basic enzyme coupled reaction. Here, Michaelis-Menten kinetics are applied, where the first enzyme operates at constant velocity, \( v_1 \), to generate the intermediate I, which is then used by the second enzyme with a higher velocity (\( V_{\text{max}2} \) and \( K_{m2} \)) to make the product, which leads to a steady state concentration of intermediate to be attained (Elcock et al., 1997; Lin et al., 2014).
A decrease in transient time is often an indication of substrate channeling. Equation 3-1 was adapted to include channeling parameters (equation 3-2), with the inclusion of the channeling probability ($p_c$) and the reaction probability ($p_r$) (Elcock et al., 1997; Lin et al., 2014). The channeling probability, $p_c$, is the channeling probability where an intermediate is transferred from active site to active site without dissociation into the bulk solution. The reaction probability, $p_r$, which is given by $p_r = k_{cat}/k_{cat} + k_{-1}$, is the reaction probability after the substrate-enzyme complex is formed at the second enzyme, reaction occurs to give products (Elcock et al., 1997; Lin et al., 2014). In equation 3-2, the $p_c p_r$ gives the combined probability that an intermediate is channeled and reacts with the second enzyme. From the application of equation 3-2, it can be observed that for any value of $p_r$, $\tau$ is linearly decreasing as $p_c$ increases (Figure 3-4). When $p_r$ is changed, there are interesting results, for example, when $p_r$ is decreased, $p_c$ has a smaller impact on $\tau$ (Elcock et al., 1997). The closer $p_r$ is to zero, the less effect $P_c$ will have on transient time by $p_c$. Enzymes that channel efficiently have a value of $p_c p_r$ close to one (Elcock et al., 1997).

**Equation 3-2:**

$$\tau = \frac{K_{M,2}(1 - p_c p_r)}{V_{max,2}}$$

**Scheme 3-1:**

$$S \overset{v_1}{\rightarrow} I \overset{K_{M,2}V_{max,2}}{\rightarrow} P$$
Equations 3-3 to 3-5 were developed from the application of scheme 3-1 and the following assumptions: (1) the first enzyme has a constant velocity of $v_1$; (2) the steady state concentration of the intermediate is denoted as $[I_{ss}]$; (3) $[I_{ss}]$ is separated into channeled intermediate $[I_{i}]_{ss}$ and escaped intermediate $[I_{e}]_{ss}$, where both their concentrations change over time are required to equal zero to be at steady state; (4) the product of $p_c v_o$ is the rate of formation of $[I_i]$; (5) the rate of disappearance of $[I_i]$ is given by the rate of formation of product ($k_{cat}[I_i]$) and the dissociation of the second enzyme complex ($k_1[I_i]$); (6) $k_1$ for $[I_i]$ is much faster than its corresponding $k_{cat}$ and $k_{-1}$; (7) the rate of formation of $[I_e]$ is for un-channeled molecules (e.g. $[1-p_c]v_o$); and (8) the product of $p_cp_r$ provides the probabilities that an intermediate is both channeled and reacted upon.

**FIGURE 3-4:** Transient time as a function of $p_c$ values as described by equation 3-2.
The product formation, based on the applications of equations 3-3 to 3-5, was determined using a MATLAB ODE simulation with the experimental kinetic parameters (presented in Appendix C) from the known coupled reaction of malate dehydrogenase-citrate synthase (MDH-CS) (Figure 3-5). The effect of channeling on transient time is demonstrated by figure 3-5 for MDH-CS. Here, the simulation gives the expected results that as the channeling probability, $p_c$, increases the transient time, $\tau$, decreases. A linear fit to the product concentration as a function of time at steady state crosses x-axis at $\tau$.

For example, in the case on MDH-CS, transient time decreases from 22.50 seconds to 6.67 seconds, based on the $p_c$ values of 0.01 to 0.95 respectively. This suggested that at high channeling probability, the lag time involved in reaching steady state conditions is lower thus the intermediate is more efficiently channeled across active sites.
FIGURE 3-5: Graphical calculation of the transient time, \( \tau \), in MDH-CS coupled-enzyme reaction with varied channeling probability (Pc). A linear fit to the product concentration as a function of time at steady state crosses x-axis at \( \tau \). Simulations values as described by equation 3-3 to 3-5.

3.5 NUMERICAL SOLUTIONS OF KINETICS EQUATIONS

Equations 3-2 to 3-5 are analytical and derived by many assumptions, which limit the range of application (Elcock et al., 1997). For a more appropriate numerical description of the channeling pathway, a time evolution of the system is applied (Elcock et al., 1997). The kinetic scheme, adapted from Elcock et al., is presented in figure 3-6. The reaction pathways in scheme 2 describes the process of product (P) formation from an initial substrate component (S) catalyzed by the first enzyme (E’) to form a reaction intermediate (I), which is then catalyzed by the second enzyme (E”). Both enzymes follow the Michaelis-Menten kinetics, in which each contain a reversible binding step rate followed by an irreversible catalytic step (Elcock et al., 1997). The reaction
intermediate is allowed to follow two different routes, a channeling pathway \((I_i)\) or the dissociation into the bulk solution \((I_e)\). The rate constants \(k_c\) and \(k_e\) determine the separation of the reaction intermediate and reflect the channeling probability through the relationship \(p_c = k_c/(k_c + k_e)\). These rate constants are fictitious and set to a high value of \(~1\times10^{20} \text{ s}^{-1}\), to allow the rate constant \(k_c\)’ sole representation of channeling. Scheme 2 is applied using known or estimated values for rate constant. For example, \(k_1\), \(k_{-1}\) and \(k_{\text{cat}}\) are calculated from the Michaelis-Menten kinetic parameter \(K_m\) through the relationship

\[
K_m = \frac{k_{\text{cat}} + k_{-1}}{k_1} \quad \text{(or} \quad k_{-1} = k_1 K_m - k_{\text{cat}}).\]

Given values for all rate constants and the initial concentrations of substrate, intermediate, \(E'\) and \(E''\), the predicted time evolution of the system is solved through a set of ordinary differential equations (ODE) represented in figure 3-7. Due to the nature of this system these equations are solved using an ODE stiff integration technique in MATLAB (Appendix C). Scheme 3-2 also provides two possible extensions to the basic scheme that includes the presence of a third enzyme \((E''')\) or the competition of the second enzyme with an external inhibitor. Either competitive route is easily included in the differential equations presented in figure 3-7 without the involvement of any new concepts.
Scheme 3-2:

\[ E' + S \xrightleftharpoons[k_i']{k_i} E'S \xrightarrow[k_{cat}']{k_{cat}'} I \]
\[ \xrightarrow[k_i''']{k_i''} \]
\[ \text{E''} \cdot \text{I} \xrightarrow[k_{cat}'']{k_{cat}''} \text{P} \]
\[ \xrightarrow[k_i''']{k_i''} \]
\[ \text{E''} \cdot \text{I} \xrightarrow[k_i''']{k_i''} \text{E''-Inhibitor} \]

\[ \text{Scavenged} \]

**FIGURE 3-6:** Reaction scheme used in numerical solution of differential equations. Figure adapted from reference (Elcock et al., 1997).

\[
\begin{align*}
\frac{d[E']}{dt} &= -k_i'[E'][S] + k_i'[E'S] + k_{cat}'[E'S] \\
\frac{d[E'']}{dt} &= -k_i'''[E''][I_e] - k_i'[I_e] + k_i'''[E'']I_e \\
&\quad + k_{cat}'' [E''I_e] \\
\frac{d[S]}{dt} &= -k_i'[E'][S] + k_i'[E'S] \\
\frac{d[E'S]}{dt} &= -k_i'[E'S] - k_{cat}'[E'S] + k_i'[E'][S] \\
\frac{d[I]}{dt} &= -k_i[I] - k_i[I] + k_{cat}'[E'S] \\
\frac{d[I_e]}{dt} &= -k_i'[I_e] + k_i[I] \\
\frac{d[I]_e}{dt} &= -k_i'''[E''][I_e] + k_i'''[E''I_e] + k_i[I] \\
\frac{d[E''I]}{dt} &= -k_i'''[E''I] - k_{cat}'' [E''I] + k_i'''[E''][I_e] + k_i'[I_e] \\
\frac{d[P]}{dt} &= +k_{cat}'' [E''I]
\end{align*}
\]

**FIGURE 3-7:** First-order differential equations describing time evolution of the reaction scheme shown in Figure 3-6. Figure adapted from (Elcock et al., 1997).
Analysis of the equation set reveals that the overall rate of a coupled enzyme is greatly affected by $k_{\text{cat}}$, $K_m$ and the concentrations of the enzyme and substrate. These effects can be seen in $k_{\text{cat}}$ values for $E'$ and $E''$, presented in figure 3-8. This comparison represents a range of $k_{\text{cat}}'$ and $k_{\text{cat}}''$ ratios and their effect on substrate and product concentration over time. For example, in figure 3-8, the product concentration is greatest when the ratio of $k_{\text{cat}}'/k_{\text{cat}}''$ equals 1000, primarily due to the high intermediate availability due to the direct substrate catalysis motivated by the higher $k_{\text{cat}}'$ value.
3.6 GOx-HRP: EFFECTS OF ADDING A COMPETITIVE ENZYME

A relatively direct way to test the extent of channeling in a coupled enzyme system is challenging the pathway with an orthogonal reaction (Lin et al., 2014). It is assumed that the presence of a third enzyme will have no effect on the channeled reaction intermediate but rather affect the intermediate present in the bulk solution (Elcock et al., 1997). For example, if a bifunctional enzyme exhibits “leaky” channeling or no channeling at all, product from the third enzyme reaction will be observed (Elcock et al., 1997; Lin et al., 2014). Transient time is also affected by the addition of a third enzyme. Transient time is predicted to decrease as the concentration of the third enzyme increases.
This is due to the fact that the third enzyme reacts with the intermediate in the bulk solution resulting in a decrease in the time needed to reach steady state (i.e. transient time) (Elcock et al., 1997; Lin et al., 2014).

For the simulation of a competing reaction, the glucose oxidase-horseradish peroxide (GOx-HRP) coupled reaction was used. Here, the first enzyme (GOx) catalyses the substrate glucose into gluconolactone, which is then transferred as the reaction intermediate to the second enzyme (HRP) and it is then transformed into the product hydrogen peroxide (Delvaux, Walcarius, & Demoustier-Champagne, 2005; Mackey, Killard, Ambrosi, & Smyth, 2007). In this system, the competitive third enzyme is catalase that reacts with hydrogen peroxide to produce water. MATLAB simulations of the addition of the third enzyme to the GOx-HRP bienzyme complex are demonstrated in figure 3-9. These simulations showed that the addition of increasing concentration of catalase caused a dramatic decrease in the activity of the coupled reaction for the free monofunctional enzyme (represented by the pc=0.01). In comparison, when the concentration of catalase is increased for a coupled enzyme (Pc=0.95), the activity was again decreased but to a lesser extent. For example, the relative activity difference between the un-channeled and channeled systems demonstrates a ~3.5x greater difference with changes in E3 concentration from 1e-6M to 1e-10M.
FIGURE 3-9: Simulated product concentration behavior as a function of time for bifunctional GOx-HRP at various competitive third enzymes (catalase) concentrations including $[E_1]=1\times10^{-8}$ M and $[E_2]=1\times10^{-8}$ M with $[E_3]=1\times10^{-6}$ M, $[E_3]=1\times10^{-7}$ M, $[E_3]=1\times10^{-8}$ M, $[E_3]=1\times10^{-9}$ M and $[E_3]=1\times10^{-10}$ M. A mono-functional system is represented by (A) with $P_c=0.01$. A substrate channeling bi-functional system is represented by (B) with $P_c=0.95$.

3.7 TS-DHFR: EFFECTS OF ADDING A COMPETITIVE INHIBITOR

For the simulation of a competitive inhibitor, the bifunctional enzyme thymidylate synthase-dihydrofolate reductase (TS-DHFR) was used. Here, the dihydrofolate is the reaction intermediate produced by the first enzyme TS and used by the second enzyme DHFR to produce tetrahydrofolate (Elcock et al., 1997; Miles et al., 1999). In this system, the competitive inhibitor for the bifunctional enzymes is demonstrated by the addition of trimethoprim (TMP) (Elcock et al., 1997). The MATLAB simulation of the addition of the competitive inhibitor to the TS-DHFR bienzyme complex is demonstrated by figure 3-10. These simulations showed that the activity of monofunctional ($P_c=0.01$) DHFR in
the coupled enzyme was strongly diminished by the addition of the competitive inhibitor TMP. In comparison, when the bifunctional enzyme was used, the activity was again reduced but to a lesser extent. For example, the relative activity difference between the un-channeled and channeled values demonstrates a ~3x greater difference with changes inhibitor [TMP] concentration from 1µM to 30µM.

**FIGURE 3-10:** Simulated production concentration behavior as a function of time for bifunctional DHFR-TS at various competitive inhibitor substrates concentrations including [TMP]=1µM, [TMP]=5µM, [TMP]=10µM, [TMP]=30µM, [E1]=2e-8M and [E2]=2e-8M. Images (A) and (B) are validations of the model obtained from the external reference. (A) represent a mono-functional system with no channeling of Pc=0.01. (B) represent a bi-functional system with channeling of Pc=0.95. Figure adapted by (Elcock et al., 1997).
3.8 AATASE COUPLED ENZYME CHANNELING APPLICATION

Substrate channeling simulations were performed for the case of Atf1-S.c. and with ethanol and actyl-CoA as substrates. Under normal fermentation conditions, ethanol is produced at high concentration, thus we focused on the acetyl-CoA substrate produced by the acetyl coenzyme-A synthase enzyme (ACS1). The coupled enzyme ACS1-AATase (or ACS1-AAT or ACS1-ATF1) is demonstrated in Figure 3-11. This coupled enzyme reaction is simulated to work towards the development of acetate ester synthesis pathway by microbial engineering. The first enzyme, ACS1, converts acetate into the acetyl-CoA, which is the reaction intermediate channeled to the second enzyme, AATase, and together with alcohol, the acetate ester product is formed. In the coupled enzyme reaction of ACS1-ATF1, both catalysts have different kinetics and thus reaction simulations were performed to determine best enzyme stoichiometry for optimal results. The MATLAB simulation for product formation is demonstrated in figure 3-12(A) with various E1:E2 ratios. In the mono-functional enzyme complex (Pc=0.01) this simulation demonstrates a higher formation of product where the ratio of the E2 is greater than E1, and an insignificant formation of product where the ratio of the E2 equals E1. These simulations demonstrate ATF1 to be the rate-limiting enzyme with a need to have a higher concentration of E2 than E1.
FIGURE 3-11: Natural bifunctional enzyme that exhibit substrate channeling. The channeled intermediate is boxed. The coupled enzyme complex is acetyl coenzyme-A synthase-alcohol acetyltransferase (ACS1-AAT).

FIGURE 3-12: For the coupled enzyme acetyl-coenzyme A synthase-alcohol acetyltransferase is simulated to demonstrate the product formation with E1:E2 ratios and a Pc value of 0.01 (A).
3.9 DISCUSSION

In this study, a numerical method of solving a set of ODEs describing a substrate channeling reaction mechanism was developed and analyzed (Elcock et al., 1997). This theoretical approach to substrate channeling included the effects of adding a competitive enzyme, a competitive inhibitor, transient time variations, and the application of an AATase coupled channeling pathway for acetate ester production.

The model was validated using three different known coupled enzyme reactions 1) MDH-CS, 2) GOx-HRP, and 3) TS-DHFR. The MDH-CS coupled enzyme was used to simulate the change in transient time based on the probability of channeling. This simulation demonstrated that transient time is dependent in the probability of channeling, decreases from 22.50 seconds to 6.67 seconds, based on the \( p_c \) values of 0.01 to 0.95 respectively. The GOx-HRP coupled enzyme was used to simulate the effect of a competitive third enzyme addition, represented by catalase. The addition of increasing concentration of catalase caused a dramatic decrease in the activity of the coupled reaction for both the free monofunctional enzyme \((Pc=0.01)\) and the coupled enzyme \((Pc=0.95)\), but to a lesser extent in the coupled enzyme. The TS-DHFR coupled enzyme was used to simulate the affect of a competitive inhibitor, represented by the addition of TMP. The addition of increasing amounts of TMP results in a dramatic decrease in the overall rate when the system of monofunctional enzymes is used. In comparison, the inhibitor affects much less for the bifunctional enzyme complex with the same experiment parameters (Elcock et al., 1997). Further simulation with AATase coupled enzyme reaction was performed to study the kinetics of an acetate ester-producing
pathway. These simulations demonstrate observable behavior of substrate electrostatic channeling in the given sampled complex enzyme systems. These results are in good agreement with referenced experimental estimates of coupled enzymes and further support electrostatic channeling.
CHAPTER 4: CONCLUSION & RECOMMENDATIONS

Acetate esters are ubiquitous in nature and broadly used in industry for a wide range of applications. In yeast fermentation and fruit ripening, alcohol-o-acetyltransferases (AATase) are responsible for the biosynthesis of short chain acetate esters from an alcohol and an acetyl-CoA (Barney, 2014; Park et al., 2009; Rodriguez et al., 2014; Shalit et al., 2001). Despite some success in metabolic engineering, poor understanding of kinetic characteristics and limited knowledge of substrate specificity of AATase has limited works to low yields and titers. The overall goal of this study was to work towards development of a biosynthesis pathway for the short chain acetate ester production.

In this study, seven different yeast and fruit species of AATases were overexpressed in *S. cerevisiae* and experimented on for further our understanding of their substrate selectivity. The enzyme activity was determined for each AATases presented and the specificity of substrate short chain alcohols was determined. Gas chromatography analysis showed high enzymatic activity for Atf1 from *S. cerevisiae* with ethanol (0.88 µmol/min•mg)). Multiple sequence alignment were performed for the different AATases in this study and results demonstrated a conserved H-X-X-X-D motif in all AATase. Cell growth of *S. cerevisiae* was observed overtime in the presence of a range of acetate ester concentrations in order to determine the toxic effects. Results demonstrated a lower toxicity and higher cell growth for shorter chain acetates ester (C2, C3 and C4).

Substrate channeling of a coupled enzyme complex was simulated with MATLAB and the model was validated using the kinetic parameters of known coupled
enzymes. The behavior of transient time corresponds to changes in channeling probability. Transient time was observed to decrease as the channeling probability increase by the simulations through the coupled enzyme MDH-CS and ACS1-AAT. The coupled enzyme reaction, GOx-HRP, simulated the effect of a competing third enzyme addition, catalase. Results demonstrated that increasing the concentration of a third enzyme decreased the activity coupled enzyme complex, but the effect was reduced with high channeling. The coupled enzyme, TS-DHFR, simulated the effect of a competing inhibitor addition, TMP. Results demonstrated that increasing the concentration of TMP decreased overall rate, but the effect was again reduce with high channeling. Further simulations were performed with the coupled enzyme, ACS1-AAT, to study the effect of product formation with variable enzyme concentrations. These simulations are a baseline for future metabolic engineering of the ACS1-AAT coupled enzyme complex, which works to develop more efficient biosynthesis forms of acetate ester production.

4.1 RECOMMENDATIONS

This study analyzed the amino acid sequence, substrate specificity, acetate ester toxicity, and substrate channeling simulations to work towards the development of acetate ester synthesis pathways by microbial engineering of AATase in yeast. Further research is needed for strain development and process optimization to improve acetate ester synthesis. Future study recommendations include: (1) testing the enzyme expression levels of AATase variants from whole cell lysate with western blot experimentation; (2) fermentation experiments with AATase overexpression variants; and (3) engineering
spatial organized AATase pathway to promote substrate channeling. Characterizing AATase expression levels will provide accurate quantification of the specific activity of each enzyme. Fermentation experiments with the most activity AATase will demonstrate and quantify the ability of engineered microorganism to convert glucose into bioesters. Finally, developing and testing spatially organized bioester synthesis pathway will increase the yields and titers of synthesized bioesters. These suggested studies along with the presented results and the substrate channeling simulations of a possible coupled enzyme complex, stand as a platform for future research and acetate ester production.
REFERENCES


Mason, A. B., & Dufour, J. (2000). Alcohol acetyltransferases and the significance of ester synthesis in yeast., *(Figure 1)*, 1287–1298.


# APPENDIX A: ALCOHOLS AND ESTERS

## TABLE A-1: Properties of acetate esters ester produced during enzyme kinetics and fermentation experiments.

<table>
<thead>
<tr>
<th>Acetate Ester Name</th>
<th>Structure</th>
<th>Molecular Formula</th>
<th>Molar Mass (g/mol)</th>
<th>Density (g/ml)</th>
<th>Boiling Point</th>
<th>Solubility in Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl acetate</td>
<td><img src="structure1.png" alt="Structure" /></td>
<td>C₃H₆O₂</td>
<td>74.08</td>
<td>0.93</td>
<td>56.9</td>
<td>~25% (20 °C)</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td><img src="structure2.png" alt="Structure" /></td>
<td>C₄H₈O₂</td>
<td>88.11</td>
<td>0.90</td>
<td>77.1</td>
<td>8.3 g/100 mL (20 °C)</td>
</tr>
<tr>
<td>Propyl acetate</td>
<td><img src="structure3.png" alt="Structure" /></td>
<td>C₅H₁₀O₂</td>
<td>102.13</td>
<td>0.89</td>
<td>102</td>
<td>18.9 g/L</td>
</tr>
<tr>
<td>Isopropyl acetate</td>
<td><img src="structure4.png" alt="Structure" /></td>
<td>C₅H₁₀O₂</td>
<td>102.13</td>
<td>0.87</td>
<td>89</td>
<td>4.3 g/100 mL (27 °C)</td>
</tr>
<tr>
<td>Butyl acetate</td>
<td><img src="structure5.png" alt="Structure" /></td>
<td>C₆H₁₂O₂</td>
<td>116.16</td>
<td>0.88</td>
<td>127</td>
<td>10 g/L (20.0 °C)</td>
</tr>
<tr>
<td>Isobutyl acetate</td>
<td><img src="structure6.png" alt="Structure" /></td>
<td>C₆H₁₂O₂</td>
<td>116.16</td>
<td>0.88</td>
<td>118</td>
<td>0.63-0.7g/100g at 20 °C</td>
</tr>
<tr>
<td>Pentyl acetate</td>
<td><img src="structure7.png" alt="Structure" /></td>
<td>C₇H₁₄O₂</td>
<td>130.19</td>
<td>0.88</td>
<td>149</td>
<td>10 g/l (20 °C)</td>
</tr>
<tr>
<td>Isopentyl acetate</td>
<td><img src="structure8.png" alt="Structure" /></td>
<td>C₇H₁₄O₂</td>
<td>130.19</td>
<td>0.88</td>
<td>142</td>
<td>0.20 g/100 mL. Slightly soluble</td>
</tr>
</tbody>
</table>
### TABLE A-2: Properties of alcohol used during enzyme kinetics and fermentation experiments.

<table>
<thead>
<tr>
<th>Alcohol Name</th>
<th>Structure</th>
<th>Molecular Formula</th>
<th>Molar Mass (g/mol)</th>
<th>Density (g/ml)</th>
<th>Boiling Point °C</th>
<th>Solubility in Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td><img src="https://via.placeholder.com/15" alt="Structure" /></td>
<td>CH4O</td>
<td>32.04</td>
<td>0.79</td>
<td>64.7</td>
<td>Soluble</td>
</tr>
<tr>
<td>Ethanol</td>
<td><img src="https://via.placeholder.com/15" alt="Structure" /></td>
<td>C2H6O</td>
<td>46.07</td>
<td>0.79</td>
<td>78.37</td>
<td>Soluble</td>
</tr>
<tr>
<td>Propanol</td>
<td><img src="https://via.placeholder.com/15" alt="Structure" /></td>
<td>C3H8O</td>
<td>60.10</td>
<td>0.80</td>
<td>97 to 98</td>
<td>Soluble</td>
</tr>
<tr>
<td>Isopropanol</td>
<td><img src="https://via.placeholder.com/15" alt="Structure" /></td>
<td>C3H8O</td>
<td>60.10</td>
<td>0.79</td>
<td>82.6</td>
<td>Soluble</td>
</tr>
<tr>
<td>Butanol</td>
<td><img src="https://via.placeholder.com/15" alt="Structure" /></td>
<td>C4H10O</td>
<td>74.12</td>
<td>0.81</td>
<td>117.7</td>
<td>73 g/L at 25°C</td>
</tr>
<tr>
<td>Isobutanol</td>
<td><img src="https://via.placeholder.com/15" alt="Structure" /></td>
<td>C4H10O</td>
<td>74.12</td>
<td>0.80</td>
<td>107.89</td>
<td>8.7 mL/100 mL</td>
</tr>
<tr>
<td>Pentanol</td>
<td><img src="https://via.placeholder.com/15" alt="Structure" /></td>
<td>C5H12O</td>
<td>88.15</td>
<td>0.81</td>
<td>137 to 139</td>
<td>22 g/L</td>
</tr>
<tr>
<td>Isopentanol</td>
<td><img src="https://via.placeholder.com/15" alt="Structure" /></td>
<td>C5H12O</td>
<td>88.15</td>
<td>0.81</td>
<td>131.1</td>
<td>28 g/L</td>
</tr>
</tbody>
</table>
## APPENDIX B: KINETIC PARAMETERS OF COUPLED ENZYME SYSTEMS

<table>
<thead>
<tr>
<th>TS-DHFR kinetic parameters</th>
<th>MDH-CS kinetic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Channeling Parameters</strong></td>
<td><strong>Channeling Parameters</strong></td>
</tr>
<tr>
<td>$P_c$= [.01, .25, .50, .75, 1]</td>
<td>$P_c$= [.01, .25, .50, .75, 1]</td>
</tr>
<tr>
<td>$k_{c2}$= 1.00E+08 1/s</td>
<td>$V_o$= 7.20E-07 M/sec</td>
</tr>
<tr>
<td>$k_{c1}$= 1.00E+20 1/s</td>
<td>$k_{c1}$= 7.50 1/s</td>
</tr>
<tr>
<td>$ke$= $(k_{c1}-k_{c1}<em>P_c)</em>(1/P_c)$ 1/s</td>
<td>$k_{m1}$ = 3.30E-05 mol/L</td>
</tr>
<tr>
<td>$k_{c2}$= 1.00E+08 1/s</td>
<td>$k_{FWD1}$= 1.00E+10 mol/L/s</td>
</tr>
<tr>
<td>$k_{m1}$= 3.00E-05 mol/L</td>
<td>$k_{RV1}$= $k_{FWD1}^<em>k_{m1}^</em>-k_{c1}$ mol/L/s</td>
</tr>
<tr>
<td>$k_{FWD1}$= 1.00E+10 mol/L/s</td>
<td><strong>Kinetic Parameters for E2</strong></td>
</tr>
<tr>
<td>$k_{RV1}$= $k_{FWD1}^<em>k_{m1}^</em>-k_{c1}$ mol/L/s</td>
<td>$Pr$= 0.82</td>
</tr>
<tr>
<td><strong>Kinetic Parameters for E1</strong></td>
<td>$k_{c2}$= 9.00 1/s</td>
</tr>
<tr>
<td>$Pr$= 0.82</td>
<td>$K_m2$ = 7.50E-06 mol/L</td>
</tr>
<tr>
<td>$k_{c2}$= 13 1/s</td>
<td>$V_{max2}$= $k_{c2}*[E2_0]$ M/sec</td>
</tr>
<tr>
<td>$K_m2$= 6.00E-07 mol/L</td>
<td>$E2_0$= 1.00E-08 M</td>
</tr>
<tr>
<td>$k_{RV2}$= $(k_{c2}/Pr)-k_{c2}$ mol/L/s</td>
<td><strong>Initial Concentrations</strong></td>
</tr>
<tr>
<td>$k_{FWD2}$= $(k_{RV2}+k_{c2})/K_m2$ mol/L/s</td>
<td>$P_0$= 0.00 M</td>
</tr>
<tr>
<td>$k_{RV2}$= $(k_{c2}/Pr)-k_{c2}$ mol/L/s</td>
<td>$Ic_0$= 0.00 M</td>
</tr>
<tr>
<td>$k_{FWD2}$= $(k_{RV2}+k_{c2})/K_m2$ mol/L/s</td>
<td>$Ie_0$= 0.00 M</td>
</tr>
<tr>
<td>$S1_0$= 2.80E-05 M</td>
<td><strong>Kinetic Parameters for Competitive Inhibitor</strong></td>
</tr>
<tr>
<td>$E1_0$= 1.74E-08 M</td>
<td>$k_{c3}= 18.00$ 1/s</td>
</tr>
<tr>
<td>$E2_0$= 1.89E-08 M</td>
<td>$K_m3$ = 5.00E-06 mol/L</td>
</tr>
<tr>
<td><strong>Kinetic Parameters for Competitive Inhibitor</strong></td>
<td>$k_{RV3}$= $k_{FWD1}^<em>k_{m1}^</em>-k_{c1}$ mol/L/s</td>
</tr>
<tr>
<td>$k_{FWD4}$= $k_{FWD2}$ mol/L/s</td>
<td>$V_{max3}$= 1.84E-07 M/sec</td>
</tr>
<tr>
<td>$k_{RV4}$= $k_{RV2}$ mol/L/s</td>
<td>$E3_0$= 1.00E-08 M</td>
</tr>
<tr>
<td>$A_0$= Varies M</td>
<td><strong>Channeling Parameters</strong></td>
</tr>
</tbody>
</table>

### GOx-HRP kinetic parameters

<table>
<thead>
<tr>
<th><strong>Channeling Parameters</strong></th>
<th><strong>Channeling Parameters</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_c$= [.01, .25, .50, .75, 1]</td>
<td>$P_c$= [.01, .25, .50, .75, 1]</td>
</tr>
<tr>
<td>$k_{c2}$= 1.00E+08 1/s</td>
<td>$k_{c2}$= 1.00E+08 1/s</td>
</tr>
<tr>
<td>$k_{c1}$= 1.00E+20 1/s</td>
<td>$k_{c1}$= 1.00E+20 1/s</td>
</tr>
<tr>
<td>$ke$= $(k_{c1}-k_{c1}<em>P_c)</em>(1/P_c)$ 1/s</td>
<td>$ke$= $(k_{c1}-k_{c1}<em>P_c)</em>(1/P_c)$ 1/s</td>
</tr>
<tr>
<td>$k_{m1}$= 1.00E+03 mol/L</td>
<td>$k_{c1}$= 4.2 1/s</td>
</tr>
<tr>
<td>$k_{FWD1}$= 1.00E+10 mol/L/s</td>
<td>$K_m1$ = 1.00E+02 mol/L</td>
</tr>
<tr>
<td>$k_{RV1}$= $k_{FWD1}^<em>k_{m1}^</em>-k_{c1}$ mol/L/s</td>
<td>$k_{FWD1}$= 1.00E+10 mol/L/s</td>
</tr>
<tr>
<td><strong>Kinetic Parameters for E2</strong></td>
<td><strong>Kinetic Parameters for E2</strong></td>
</tr>
<tr>
<td>$Pr$= 0.82</td>
<td>$Pr$= 0.82</td>
</tr>
<tr>
<td>$k_{c2}$= 1000 1/s</td>
<td>$k_{c2}$= 0.84 1/s</td>
</tr>
<tr>
<td>$K_m2$= 1.00E+06 mol/L</td>
<td>$K_m2$= 3.00E+01 mol/L</td>
</tr>
<tr>
<td>$k_{RV2}$= $(k_{c2}/Pr)-k_{c2}$ mol/L/s</td>
<td>$k_{RV2}$= $(k_{c2}/Pr)-k_{c2}$ mol/L/s</td>
</tr>
<tr>
<td>$k_{FWD2}$= $(k_{RV2}+k_{c2})/K_m2$ mol/L/s</td>
<td>$k_{FWD2}$= $(k_{RV2}+k_{c2})/K_m2$ mol/L/s</td>
</tr>
<tr>
<td><strong>Initial Concentrations</strong></td>
<td><strong>Initial Concentrations</strong></td>
</tr>
<tr>
<td>$S1_0$= 1.00E-05 M</td>
<td>$S1_0$= 1.00E-03 M</td>
</tr>
<tr>
<td>$E1_0$= 1.00E-08 M</td>
<td>$E1_0$= 1.00E-08 M</td>
</tr>
<tr>
<td>$E2_0$= 1.00E-08 M</td>
<td>$E2_0$= Varies M</td>
</tr>
<tr>
<td>$E3_0$= Varies M</td>
<td><strong>Kinetic Parameters for E3 Competitive Enzyme</strong></td>
</tr>
<tr>
<td>$k_{c3}= 4300$ 1/s</td>
<td>$K_m3$ = 3.70E-03 mol/L</td>
</tr>
<tr>
<td>$k_{FWD3}$= 1.00E+10 mol/L/s</td>
<td>$k_{FWD3}$= 1.00E+10 mol/L/s</td>
</tr>
<tr>
<td>$k_{RV3}$= $k_{FWD1}^<em>k_{m1}^</em>-k_{c1}$ mol/L/s</td>
<td>$k_{RV3}$= $k_{FWD1}^<em>k_{m1}^</em>-k_{c1}$ mol/L/s</td>
</tr>
</tbody>
</table>
APPENDIX C: MATLAB CODE

C.1 TRANSIENT TIME VERSUS Pc VARIATIONS

% The effect of Pc variations on Transient time calculations
% Kinetic Parameters based on the enzyme complex: GOx-HRP
% Equation adapted from Elcock A.H. et al (1997)

clear;clc;
fl = figure(); hold all

for Pc=[.01 .25 .5 .75 .95]; %Channeling Probability
    Pr=.82; %Probability of Reaction for second enzyme

    %Parameters for E2
    Km_2=1e-6; %M
    kcat_2=1000; %s-1
    E2_0=1e-8; %M
    Vmax_2=kcat_2*E2_0; %M/sec

    %Transient time calculations
    tau=((Km_2)*(1-Pc*Pr))/(Vmax_2); %Equation 3-2

    %Figure(1)Transient time
    figure(fl)
    plot(Pc,tau,'*', 'markersize', 18);
    legend('Pc=.01','Pc=.25','Pc=.5','Pc=.75','Pc=.95');
    title('Transient Time (\(\tau\)) Behavior with Pc variations', 'fontsize', 18);
    ylabel('Transient Time', 'fontsize', 18);
    xlabel('Channeling Probability (Pc)', 'Fontsize', 18);
end

C.2 NUMERICAL COMPARISON OF \(k_{cat1}\) AND \(k_{cat2}\) RATIOS

% Modeling the time course of a coupled reaction with MM kinetics
% Model Adapted from Elcock A.H. et al (1997)
% Kinetic Parameters based on the enzyme complex: GOx-HRP

clear;clc;
global kFWD_1 kRV_1 kcat_1 Km_1 kFWD_2 kRV_2 kcat_2 Km_2 kc1 kc2 ke1 Pc;
kcat_2=1000; %units of 1/s approx 200 to 1000 1/s for HRP
This is to vary the colors in between the plots

```matlab
m1 = {'r', 'g', 'b', 'k', 'm', 'c', 'y'};
m2 = {'r', 'g', 'b', 'k', 'm', 'c', 'y'};
m3 = {'r', 'g', 'b', 'k', 'm', 'c', 'y'};
c1=1;
c2=1;
c3=1;
```

This Script will include each single line of the kcat ratios in each

```matlab
f1 = figure(); hold on
f2 = figure(); hold on
for x=[.1 1 10 100 1000]
kcat_1= x* kcat_2; %units of 1/s approx 300 1/s for GOx

%kinetic parameters for E1
Km_1= 1e-3; %unit of mol/L Km for GOx = 1 mM
kFWD_1=1e10; %units of mol/L/s
kRV_1=kFWD_1*Km_1-kcat_1; %units of mol/L/s

%kinetic parameters for E2
Km_2= 1e-4; %unit of mol/L Km for HRP (H2O2) = 0.1 mM
kFWD_2=1e10; %units of mol/L/s
kRV_2=kFWD_2*Km_2-kcat_2; %units of mol/L/s

%Initial conditions
S1_0=1e-3; %Initial [Substrate] mol/L
E1_0=2e-10; %Initial [Enzyme one] mol/L
E1S1_0=0; %Initial [Substrate-Enzyme one complex]
I_0=0; %Initial [Reaction Intermediate] mol/L
Ic_0=0; %Initial [Channeled Intermediate]
e_0=0; %Initial [Escaped Intermediate]
E2_0=2e-10; %Initial [Second Enzyme] in mol/L
E2I_0=0; %Initial [Intermediate-Enzyme two complex]
P_0=0; %Initial [Product]

% Time span
t_max = 500; % Maximum Given Time
t_span=[0:1:t_max];

%Channeling parameters
Pc= 1e-10; %Probability of channeling
kc2=1e8; % Second channeling rate constant
kc1=1e20; % First channeling rate constant
ke1=(kc1-kc1*Pc)*(1/Pc);

%Ordinary Differential Equations application
[t,y]=ode15s(@function_kcatratios,t_span,[S1_0 E1_0 E1S1_0 I_0 Ic_0 Ie_0 E2_0 E2I_0 P_0]);

%outputs from
S1=y(:,1); % [Substrate] mol/L
E1=y(:,2); % [Enzyme one] mol/L
E1S1=y(:,3); % [Substrate-Enzyme one complex] mol/L
l=y(:,4); % [Reaction Intermediate] mol/L
lc=y(:,5); % [Channeled Intermediate]
le=y(:,6); % [Escaped Intermediate]
E2=y(:,7); % [Second enzyme] mol/L
E2I=y(:,8); % [Intermediate-Enzyme two complex]
P=y(:,9); % [Product]

%Figure 1-Substrate concentration change over time
figure(f1);
plot(S1,m1{c1});
c1=1+c1;
legend('(kcat_1/kcat_2)=.1','(kcat_1/kcat_2)=1','(kcat_1/kcat_2)=10','(kcat_1/kcat_2)=100','(kcat_1/kcat_2)=1000');
title('Substrate Behavior', 'fontsize', 18);
ylabel('Concentration (mol/L)', 'fontsize', 18);
xlabel('Time (s)', 'Fontsize', 18);

%Figure 2- Product concentration change over time
figure(f2);
plot(P,m3{c3});
c3=1+c3;
legend('(kcat_1/kcat_2)=.1','(kcat_1/kcat_2)=1','(kcat_1/kcat_2)=10','(kcat_1/kcat_2)=100','(kcat_1/kcat_2)=1000');
max_x = t_max *1.05;
min_x = -(t_max * .05);
xlim([min_x max_x]);
top_y=(S1_0*.01);
ylim([0 top_y]);
title('Product Behavior', 'fontsize', 18);
ylabel('Concentration (mol/L)', 'fontsize', 18);
xlabel('Time (s)', 'Fontsize', 18);

end
C.3 NUMERICAL COMPARISON OF $K_{\text{CAT}1}$ AND $K_{\text{CAT}2}$ RATIOS – FUNCTION

% Michaelis-Menten kinetics for a coupled enzyme reaction: FUNCTION SCRIPT

function dydt = function_kcatratios(t,y)
global kFWD_1 kRV_1 kcat_1 kFWD_2 kRV_2 kcat_2 kc1 kc2 ke1;
dydt=zeros(9,1);

S1=y(1); E1=y(2); E1S1=y(3); I=y(4); Ic=y(5); Ie=y(6); E2=y(7); E2I=y(8); P=y(9);

dydt(2) = -kFWD_1*S1*E1 + kRV_1*E1S1 + kcat_1*E1S1; %balance of [E1]
dydt(7) = -kFWD_2*E2*Ie + kRV_2*E2I - kc2*Ic*E2 + kcat_2*E2I; %balance of [E2]
dydt(1) = -kFWD_1*E1*S1 + kRV_1*E1S1; %balance of [S1]
dydt(3) = -kRV_1*E1S1 + kFWD_1*E1S1 - kcat_1*E1S1; %balance of [E1S1]
dydt(4) = -ke1*I - kc1*I + kcat_1*E1S1; %balance of [I]
dydt(5) = -kc2*Ic*E2 + kc1*I; %balance of [Ic]
dydt(6) = -kFWD_2*Ie*E2 + kRV_2*E2I + ke1*I; %balance of [Ie]
dydt(8) = -kRV_2*E2I + kFWD_2*Ie*E2 + kc2*Ic*E2 - kcat_2*E2I; %balance of [E2I]
dydt(9) = kcat_2*E2I; %balance of [P]
end

C.4 GOx-HRP: EFFECTS OF ADDING A THIRD ENZYME

% Bifunctional Enzyme channeling
% Model Adapted from Elcock A.H. et al (1997) and McCammon
% Kinetic Parameters based on the enzyme complex: GOx-HRP

clear;clc;
global kFWD_1 kRV_1 kcat_1 kFWD_2 kRV_2 kcat_2 kc1 kc2 ke kFWD_3 kRV_3 kcat_3;

%This is to vary the colors in between the plots
m1 = {'r', 'g', 'b', 'k', 'm', 'c', 'y'};
c1=1;
f1 = figure(); hold on
for E3_0=[1e-10,1e-9,1e-8,1e-7,1e-6]; %Probability of channeling

%channeling parameters
Pc=0.95;
kc2=1e8;
kc1=1e20;
ke=(kc1-kc1*Pc)*(1/Pc); %Pc=.95 --> ke~5.26e18
%kinetic parameters for E1  
kcat_1 = 100; %units of 1/s  
Km_1 = 1e-3; %unit of mol/L  
kFWD_1 = 1e10; %units of mol/L/s  
kRV_1 = kFWD_1*Km_1-kcat_1; %units of mol/L/s --> kRV_1 = 3.3e-5

%kinetic parameters for E2  
Pr = 0.82;  
%Pr = 0.78/Pc; %Probability of reaction with E2 (PcPr = 0.78)  
kcat_2 = 1000; %units of 1/s  
Km_2 = 1e-6; %unit of mol/L  
kRV_2 = (kcat_2/Pr)-kcat_2; %units of mol/L/s --> kFWD_2 = 2.833  
kFWD_2 = (kRV_2+kcat_2)/Km_2; %units of mol/L/s --> kRV_2 = 4.4e13

%kinetic parameters for E3 and by-product=B  
kcat_3 = 4300; %units of 1/s  
Km_3 = 3.7e-3; %unit of mol/L  
kFWD_3 = 1e10; %units of mol/L/s  
kRV_3 = kFWD_3*Km_3-kcat_3; %units of mol/L/s

% Time span  
t_max = 500; %time in secs  
t_span = [0:25:t_max];

%Initial conditions  
S1_0 = 1e-5; %Initial [S] mol/L  
E1_0 = 1e-8; %Initial [E1] mol/L  
E1S1_0 = 0; %Initial [E1S1]  
I_0 = 0; %Initial [I] mol/L  
Ic_0 = 0; %Initial [Ic]  
e_0 = 0; %Initial [Ie]  
E2_0 = 1e-8; %Initial [E2] mol/L  
E2I_0 = 0; %Initial [E2I]  
%E3_0 = 1e-10; % Vary this  
E3e_0 = 0; %Initial [E3e]  
B_0 = 0; %Initial [B]  
P_0 = 0; %Initial [P]

%Solve ODE equation for final product and bifunctional Enzyme channeling  
[t,y] = ode23t(@function_Pc_vary_third_enzyme,t_span,[S1_0 E1_0 E1S1_0 I_0 Ic_0 Ie_0 E2_0 E2I_0 E3e_0 B_0 P_0]);

% Output Concentrations  
S1 = y(:,1); %Progressing [S] mol/L
E1=y(:,2); %Progressing [E1] mol/L
E1S1=y(:,3); %Progressing [E1S] mol/L
l=y(:,4); %Progressing [I] mol/L
le=y(:,5); %Progressing [Ic] mol/L
le=y(:,6); %Progressing [Ie] mol/L
E2=y(:,7); %Progressing [E2] mol/L
E2I=y(:,8); %Progressing [E2I] mol/L
E3=y(:,9); %Progressing [E3]
E3Ie=y(:,10); %Progressing [E3Ie]
B=y(:,11); %Progressing [B]
P=y(:,12); %Progressing [P] mol/L

%Figure(1)---Product (P)
figure(f1)
plot(t,P,m1{c1});
c1=1+c1;
axis tight
title('Product Behavior w/Competitive Enzyme', 'fontsize', 18);
ylabel('Concentration (mol/L)', 'fontsize', 18);
xlabel('Time (s)', 'fontsize', 18);
text(25, 3e-6,'Pc=0.95', 'fontsize', 18);
end
%---------------------------------------------------------------
alldata = [t,P];
xlswrite('Pc_vary_third_enzyme',alldata);

C.5 GOx-HRP: EFFECTS OF ADDING A THIRD ENZYME - FUNCTION
% Michaelis-Menten kinetics for a coupled enzyme reaction: FUNCTION SCRIPT
% Presence of competitive enzyme

function dydt = function_Pc_vary_third_enzyme(t,y)
global kFWD_1 kRV_1 kcat_1 kFWD_2 kRV_2 kcat_2 kc1 kc2 ke kFWD_3 kRV_3 kcat_3;
dydt=zeros(9,1);
S1=y(1); E1=y(2); E1S1=y(3); l=y(4); Ic=y(5); le=y(6); E2=y(7); E2I=y(8); E3=y(9)
E3Ie=y(10); B=y(11); P=y(12);

dydt(1) = -kFWD_1*E1*S1 + kRV_1*E1S1; %balance of [S1]
dydt(2) = -kFWD_1*S1*E1 + kRV_1*E1S1 + kcat_1*Ic; %balance of [E1]
\[
\frac{dy}{dt}(3) = -k_{RV_1}E_1S_1 + k_{FWD_1}E_1S_1 - k_{cat_1}E_1S_1; \text{ %balance of } [E_1S_1]
\]
\[
\frac{dy}{dt}(4) = -k_eI - k_{c1}I + k_{cat_1}E_1I; \text{ %balance of } [I]
\]
\[
\frac{dy}{dt}(5) = -k_{c2}Ic + k_{c1}I; \text{ %balance of } [Ic]
\]
\[
\frac{dy}{dt}(6) = -k_{FWD_2}I_2E_2 + k_{RV_2}E_2I_2 + k_eI - k_{FWD_3}E_3I_2 + k_{RV_3}E_3E_3I_2; \text{ %balance of } [I_2]
\]
\[
\frac{dy}{dt}(7) = -k_{FWD_2}E_2I_2 + k_{RV_2}E_2I_2 - k_{c2}Ic + k_{cat_2}E_2I; \text{ %balance of } [E_2]
\]
\[
\frac{dy}{dt}(8) = -k_{RV_2}E_2I + k_{FWD_2}E_2I + k_{c2}Ic - k_{cat_2}E_2I; \text{ %balance of } [E_2I]
\]
\[
\frac{dy}{dt}(9) = -k_{FWD_3}E_3I_2 + k_{RV_3}E_3I_2 + k_{cat_3}E_3I_2; \text{ %balance of } [E_3]
\]
\[
\frac{dy}{dt}(10) = k_{FWD_3}E_3I_2 + k_{RV_3}E_3E_3I_2 - k_{cat_3}E_3I_2; \text{ %balance of } [E_3I_2]
\]
\[
\frac{dy}{dt}(11) = k_{cat_2}E_2I; \text{ %balance of } [P]
\]
\[
\frac{dy}{dt}(12) = k_{cat_3}E_3I_2;
\]
end

C.6 TS-DHFR: EFFECTS OF ADDING A COMPETITIVE INHIBITOR

% Modeling the time course of a coupled reaction with MM kinetics
% Model Adapted from Elcock A.H. et al (1997)
% Kinetic Parameters based on the enzyme complex: TS-DHFR
% Effect of competitive inhibitor on Product concentration

clear;clc;
global k_{FWD_1} k_{RV_1} k_{cat_1} k_{FWD_2} k_{RV_2} k_{cat_2} k_{c1} k_{c2} k_e k_{FWD_4} k_{RV_4};
%This is to vary the colors in between the plots
m1 = {'r', 'g', 'b', 'k', 'm', 'c', 'y'};
c1=1;

f1 = figure(); hold on
for A_0= [1e-6, 5e-6, 10e-6, 20e-6, 40e-6];       %Probability of channeling

%channeling parameters
Pc=.01;
k_{c2}=1e8;
k_{c1}=1e20;
ke=(k_c1-k_{c1}*Pc)*(1/Pc); %Pc=.95 --> ke~5.26e18

%kinetic parameters for E1
k_{cat_1}=7.5;  %units of 1/s
K_{m_1}= 3.3e-5;  %unit of mol/L
k_{FWD_1}=1e10;  %units of mol/L/s
k_{RV_1}=k_{FWD_1}*K_{m_1}-k_{cat_1};  %units of mol/L/s --> k_{RV_1}=3.3e-5

%kinetic parameters for E2
Pr=.82;
%Pr=.78/Pc;
%Probability of reaction with E2 (PcPr=.78)
kcat_2=13;
%units of 1/s
Km_2=6e-7;
%unit of mol/L
kRV_2=(kcat_2/Pr)-kcat_2;
%units of mol/L/s --> kFWD_2=2.833
kFWD_2=(kRV_2+kcat_2)/Km_2;
%units of mol/L/s --> kRV_2=4.4e13

%kinetic paramets for E2 and Inhibitor=A
%kFWD_4=0;
%kRV_4=0;
kFWD_4=kFWD_2;
kRV_4=kRV_2;

% Time span
 t_max= 500;
%time in secs
t_span=[0:25:t_max];

%Initial conditions
S1_0=28e-6;
%Initial [S] mol/L
E1_0= 2e-8;
%Initial [E1] mol/L
E1S1_0=0;
%Initial [E1S1]
I_0=0;
%Initial [I] mol/L
Ic_0=0;
%Initial [Ic]
le_0=0;
%Initial [le]
E2_0= 2e-8;
%Initial [E2] mol/L
%A_0=30e-6;
%----Inhibitor
E2A_0=0;
%----Inhibitor & E2
E2I_0=0;
%Initial [E2I]
P_0=0;
%Initial [P]

%Solve ODE equation for final product and bifunctional Enzyme channeling
[t,y]=ode23t(@function_Pc_vary_inhibitor,t_span,[S1_0 E1_0 E1S1_0 I_0 Ic_0 le_0 E2_0 A_0 E2A_0 E2I_0 P_0]);

% Output Concentraions
S1=y(:,1);
%Progressing [S] mol/L
E1=y(:,2);
%Progressing [E1] mol/L
E1S1=y(:,3);
%Progressing [E1S] mol/L
l=y(:,4);
%Progressing [I] mol/L
le=y(:,5);
%Progressing [le] mol/L
E2=y(:,7);
%Progressing [E2] mol/L
A=y(:,8);
E2A=y(:,9);
E2I=y(:,10); %Progressing [E2I] mol/L
P=y(:,11); %Progressing [P] mol/L

%Figure(1)---Product (P)
figure(f1)
plot(t,P,m1{c1});
c1=1+c1;
legend('[TMP]=1\mu M', '[TMP]=5\mu M','[TMP]=10\mu M',
'[TMP]=20\mu M','[TMP]=40\mu M');
axis tight
title('Product Behavior w/Inhibitor', 'fontsize', 18);
ylabel('Concentration (mol/L)', 'fontsize', 18);
xlabel('Time (s)', 'Fontsize', 18);
text(50, 1e-5,'Pc=0.01','fontsize', 18);
end
alldata = [t,P];
xlswrite('third_enzyme_vary',alldata);

C.7 TS-DHFR: EFFECTS OF ADDING A COMPETITIVE INHIBITOR -
FUNCTION
% Modeling the time course of a coupled reaction with MM kinetics -FUNCTION
% Effect of competitive inhibitor on Product concentration

function dydt = function_Pc_vary_inhibitor(t,y)
global kFWD_1 kRV_1 kcat_1 kFWD_2 kRV_2 kcat_2 kc1 kc2 ke kFWD_4 kRV_4;
dydt=zeros(9,1);
S1=y(1); E1=y(2); E1S1=y(3); I=y(4); Ic=y(5); Ie=y(6); E2=y(7); A=y(8); E2A=y(9);
E2I=y(10); P=y(11);

dydt(2) = -kFWD_1*S1*E1 + kRV_1*E1S1 + kcat_1*E1S1; %balance of [E1]
dydt(7) = -kFWD_2*E2*Ie + kRV_2*E2I - kc2*Ic + kcat_2*E2I - kFWD_4*E2*A +
kRV_4*E2A ; %balance of [E2]
dydt(1) = -kFWD_1*E1S1 + kRV_1*E1S1; %balance of [S1]
dydt(3) = -kRV_1*E1S1 + kFWD_1*E1S1 + kcat_1*E1S1; %balance of [E1S1]
dydt(4) = -ke*I - kc1*I + kcat_1*E1S1; %balance of [I]
dydt(5) = -kc2*Ic + kc1*I; %balance of [Ic]
dydt(6) = -kFWD_2*Ie*E2 + kRV_2*E2I + ke*I; %balance of [Ie]
dydt(8) = -kFWD_4*E2*A + kRV_4*E2A; %---- Inhibitor
dydt(9) = kFWD_4*E2*A - kRV_4*E2A; %---- inhibitor & E2
dydt(10) = -kRV_2*E2I + kFWD_2*Ie*E2 + kc2*Ic - kcat_2*E2I; %balance of [E2I]
dydt(11) = kcat_2*E2I; %balance of [P]
end

C.8 MDH-CS: PRODUCT FORMATION VIA KINETIC EQUATIONS
% Modeling the time course of a coupled reaction with MM kinetic EQUATIONS
% Model EQUATIONS Adapted from Elcock A.H. et al (1997)
% Kinetic Parameters based on the enzyme complex: MDH-CS
% Effect of competitive inhibitor on Product concentration

clear;clc;

global Pr Pc V0 Vmax_2 Km_2 Vmax_3 Km_3 tau kRV_2 kcat_2;

%This is to vary the colors in between the plots
m1 = {'r', 'g', 'b', 'k', 'm', 'c', 'y'};
c1=1;

f1 = figure(); hold on

for Pc=[.01,.25,.50,.75,1];

% Probability values
Pr=.82; %Reaction (of second enzyme and intermediate) Probability

% Initial Velocity of the couple reaction
V0=7.2e-7; %M/sec

% Parameters for E2
Km_2=7.5e-6; %M
kcat_2=9; %s-1
E2_0=1e-8; %M
Vmax_2=kcat_2*E2_0; %M/sec
kFWD_2=1e10;
kRV_2=kFWD_2*Km_2*-kcat_2;

% Parameters for E3
Km_3=5e-6; %M
kcat_3=18; %s-1
E3_0=1e-8; %M
Vmax_3=kcat_3*E3_0; %M/sec

% Transient Time Calculation
tau= (Vmax_2/Km_2);

% Initial Product Concentration
P0=0;   %M
Ii_0=0; %M
le_0=0; %M

% Time span
\[ t_{\text{max}} = 0.05; \] %Time in secs
\[ t_{\text{span}} = [0:0.001:t_{\text{max}}]; \]

% Transient time calculations
\[ [t,y] = \text{ode23t}(@(\text{function} \_\_\_\_\_\_\_\_\_\_\_Product \_14 \_Pc\_\text{Vary},t_{\text{span}},[P0,Ii\_0,Ie\_0]); \]

% Output Concentrations
P=y(:,1); Ii=y(:,2); Ie=y(:,3);

% Figure(1)--Transient time
figure(f1)
polt(t,P,m1{c1}, 'markersize', 16);
c1=1+c1;
legend('Pc=.01','Pc=.25','Pc=.50','Pc=.75','Pc=1');
title('Product Behavior w/Competitive Enzyme', 'fontsize', 18);
ylabel('Product (M)', 'fontsize', 18);
xlabel('Time (sec)', 'Fontsize', 18);
end

C.9 MDH-CS: PRODUCT FORMATION VIA KINETIC EQUATIONS - FUNCTION
% The effect of Pc variation on Product Formation: FUNCTION

function dydt = function_Product_14_PcVary(t,y)
global Pr Pc V0 Vmax_2 Km_2 Vmax_3 Km_3 tau kRV_2 kcat_2;
dydt=zeros(3,1);

P=y(:,1); li=y(:,2); le=y(:,3);

dydt(1) = (Pc*Pr*V0) + (tau*Ie); %balance of [P]
dydt(2) = (Pc*V0) - (kcat_2*Ii) - (kRV_2*Ii); %balance of [Ii]
dydt(3) = ((1-Pc)*V0) + (kRV_2*Ii) - ((Vmax_2*Ie)/(Km_2+Ie)) - ((Vmax_3*Ie)/(Km_3+Ie));
C.10 TRANSIENT TIME VERSUS Pc VARIATIONS FOR ACS1-AAT COUPLED COMPLEX

% ACS1-ATF1 coupled enzyme system
clear;clc;
f1 = figure(); hold all
for Pc=[.01 .25 .5 .75 .95]; %Probability of channeling
  %Probability values
  Pr=.82; %Reaction Probability
  %Parameters for E2
  Km_2=30; %M
  kcat_2=.84; %s^{-1}
  E2_0=1e-8; %M
  Vmax_2=kcat_2*E2_0; %M/sec
  %Transient time calculations
  tau=((Km_2)*(1-Pc*Pr))/(Vmax_2);
figure(f1)
plot(Pc,tau,'*', 'markersize', 18);
legend('Pc=.01','Pc=.25','Pc=.5','Pc=.75','Pc=.95');
title('Transient Time (\tau) Behavior with Pc variations', 'fontsize', 18);
ylabel('Transient Time', 'fontsize', 18);
xlabel('Channeling Probability (Pc)', 'Fontsize', 18);

end

C.11 NUMERICAL COMPARISON OF E1 AND E2 RATIOS FOR ACS1-AAT COUPLED COMPLEX AT Pc = 0.01

% Modeling the time course of a coupled reaction with MM kinetics
clear;clc;
global kFWD_1 kRV_1 kcat_1 Km_1 kFWD_2 kRV_2 kcat_2 Km_2 kc1 kc2 ke1 Pc;
E1_0=1e-8; %units of 1/s
%This is to vary the colors in between the plots
m1 = {'r', 'g', 'b', 'k', 'm', 'c', 'y'};

m2 = {'r', 'g', 'b', 'k', 'm', 'c', 'y'};
c1=1;
c2=1;

%figure of the plots
f1 = figure(); hold on
f2 = figure(); hold on

for x=[1 5 25 50];
    E2_0= x * E1_0;    %units of 1/s approx 300 1/s for ACS-AAT

%channeling parameters
Pc= .01;            %probability of channeling
kc2=1e8;
kc1=1e20;
ke1=(kc1-kc1*Pc)/(1/Pc);

%kinetic parameters for E1
Km_1= 100;         %unit of M
kFWD_1=1e10;       %units of M/s
kcat_1=4.2;        %units of s-1
kRV_1=kFWD_1*Km_1-kcat_1;  %units of M/s

%kinetic parameters for E2
Km_2= 30;          %unit of M
kFWD_2=1e10;       %units of M/s
kcat_2=.84;        %units of s-1
kRV_2=kFWD_2*Km_2-kcat_2;  %units of M/s

%Initial conditions
S1_0=1e-3;        %Initial [S] M
E1S1_0=0;         %Initial [E1S1]
I_0=0;            %Initial [I] M
Ic_0=0;           %Initial [Ic]
le_0=0;           %Initial [le]
E2I_0=0;          %Initial [E2I]
P_0=0;            %Initial [P]

% Time span
t_max = 100;
t_span=[0:1:t_max];
%call to equation set with Initial Conditions of the coupled reaction
[t,y]=ode15s(@function_ACS1_AAT,t_span,[S1_0 E1_0 E1S1_0 I_0 Ic_0 Ie_0 E2_0 E2I_0 P_0]);

%outputs from
S1=y(:,1); E1=y(:,2); E1S1=y(:,3); I=y(:,4); Ic=y(:,5); Ie=y(:,6); E2=y(:,7); E2I=y(:,8); P=y(:,9);

%Figure 1- substrate
figure(f1);
plot(S1,m1{c1});
c1=1+c1;
legend('E2/E1=1','E2/E1=10','E2/E1=100','E2/E1=1000');
title('Substrate Behavior', 'fontsize', 18);
ylabel('Concentration (mol/L)', 'fontsize', 18);
xlabel('Time (s)', 'Fontsize', 18);

%Figure 2- product
figure(f2);
plot(P,m2{c2});
c2=1+c2;
legend('E2/E1=1','E2/E1=5','E2/E1=25','E2/E1=50');
title('Product Behavior', 'fontsize', 18);
ylabel('Concentration (mol/L)', 'fontsize', 18);
xlabel('Time (s)', 'Fontsize', 18);
text(5, 3e-17,'Channeling Probability (Pc)=0.01','fontsize', 18);
end

C.12 NUMERICAL COMPARISON OF E1 AND E2 RATIOS FOR ACS1-AAT COUPLED COMPLEX AT Pc = 0.01 -FUNCTION

% Michaelis-Menten kinetics
function dydt = function_ACS1_AAT(t,y)
global kFWD_1 kRV_1 kcat_1 kFWD_2 kRV_2 kcat_2 kc1 kc2 ke1;
dydt=zeros(9,1);

S1=y(1); E1=y(2); E1S1=y(3); I=y(4); Ic=y(5); Ie=y(6); E2=y(7); E2I=y(8); P=y(9);

dydt(2) = -kFWD_1*S1*E1 + kRV_1*E1S1 + kcat_1*E1S1; %balance of [E1]
dydt(7) = -kFWD_2*E2*Ie + kRV_2*E2I - kc2*Ic*E2 + kcat_2*E2I; %balance of [E2]
dydt(1) = -kFWD_1*E1*S1 + kRV_1*E1S1; %balance of [S1]
dydt(3) = -kRV_1*E1S1 + kFWD_1*E1*I1 - kcat_1*E1S1; %balance of [E1S1]
dydt(4) = -ke1*I - kc1*I + kcat_1*E1S1; %balance of [I]
dydt(5) = -kc2*Ic*E2 + kc1*I; %balance of [Ic]
\[
dydt(6) = -kFWD_2*le*E2 + kRV_2*E2I + ke1*I; \text{ %balance of [le]}
\]
\[
dydt(8) = -kRV_2*E2I + kFWD_2*le*E2 + kc2*le*E2 - kcat_2*E2I; \text{ %balance of [E2I]}
\]
\[
dydt(9) = kcat_2*E2I; \text{ %balance of [P]}
\]
end
APPENDIX D: GAS CHROMATOGRAPHY STANDARDS

- **Methyl acetate**
  - \[ y = 2636.3x \]
  - \[ R^2 = 0.99881 \]

- **Ethyl acetate**
  - \[ y = 241.13x \]
  - \[ R^2 = 0.99606 \]

- **Propyl acetate**
  - \[ y = 26550x \]
  - \[ R^2 = 0.99973 \]

- **Isopropyl acetate**
  - \[ y = 7263.8x \]
  - \[ R^2 = 0.93717 \]

- **Butyl acetate**
  - \[ y = 12544x \]
  - \[ R^2 = 0.97493 \]

- **Isobutyl acetate**
  - \[ y = 5481.4x \]
  - \[ R^2 = 0.81752 \]

- **Pentyl acetate**
  - \[ y = 13890x \]
  - \[ R^2 = 0.9890x \]

- **Isopentyl acetate**
  - \[ y = 21303x \]
  - \[ R^2 = 0.99876 \]
Design and Analysis of Enhanced Catalysis in Scaffolded Multi-Enzyme Cascade Reactions.

Jyun-Liang Lin, Leidy Palomec, and Ian Wheeldon*

Department of Chemical and Environmental Engineering, University of California, Riverside, Bourns Hall, 900 University Avenue, Riverside, CA 92521, United States

ABSTRACT

New developments in nucleic acid nanotechnology and protein scaffold designs have enabled unparalleled control over the spatial organization of synthetic multi-enzyme cascade reactions. One of the goals of these new technologies is to create nanostructured enzyme cascade reactions that promote substrate channeling along the cascade, and in doing so enhance cascade catalysis. The concept of substrate channeling has a long and rich history in biochemistry and has established methods of evaluation and quantification. In this perspective, we review the most common of these methods and discuss them in the context of engineered multi-enzyme systems and natural bifunctional enzymes with known mechanisms of substrate channeling. Additionally, we use experimental data and the results of simulations of coupled-enzyme reactions to develop a set of preliminary design rules for engineering multi-enzyme nanostructures. The design rules address the limitations on interenzyme distance and active site orientation in substrate channeling and suggest designs for promoting enhanced catalysis. Specifically, that enzyme orientation
should minimize interenzyme distance and that at distances greater then 1 nm between active sites significant channeling occurs only if diffusion of the intermediate is bounded through interactions with the surface or scaffold between active sites. This field is rapidly developing and promises to create many more new and exciting technologies.

1. Introduction

The multi-step reaction cascades of cellular metabolism are highly coordinated, simultaneously processing of a battery of biosynthetic and oxidative pathways to build essential biomolecules and catabolize energy sources. One strategy that has evolved to allow for this to occur within the confined environment of a single cell is substrate channeling along spatially organized multi-enzyme structures. Substrate channeling is the transfer of a reaction intermediate from the active site of one enzyme to the active site of a downstream enzyme without first diffusing to the bulk solution. This is an important phenomenon that can result in sequestering substrates and intermediates along specific pathways, thus increasing pathway flux and minimizing cross-talk between separate pathways.

Substrate channeling has a long history in biochemistry with many interesting discoveries, and like many fields it is not without controversy in the analysis of experimental evidence and in the existence of different mechanisms.\textsuperscript{1-3} The past five years have seen a re-emergence of this topic with a focus on engineering nanostructured assemblies for coupled-enzyme reactions and co-localization scaffolds for biosynthetic
At the root of these efforts is the desire to engineer enzyme reaction cascades with enhanced catalysis. Systems are designed to promote substrate channeling and control the local concentration and flux of reaction intermediates and in doing so increase reaction rates, prevent undesired side reactions from consuming intermediates, and drive reactions counter to unfavorable thermodynamics in the bulk environment.

Inspired by natural metabolic pathways and bifunctional enzymes, researchers are developing new strategies to create co-localized and spatially organized multi-enzyme structures (Figure 1). Using protein, nucleic acid, and polymer scaffolds to position enzymes in multi-enzyme structures, enhancements in overall reaction cascade kinetics have been demonstrated both in vivo and in vitro. For example, a modular DNA scaffold expressed in *E. coli* has produced a 5-fold increase in trans-resveratrol yield from a coupled enzyme reaction; co-localization of a two-enzyme system using protein and RNA scaffolds have produced up to 50-fold increases in the production of biohydrogen in *E. coli*; a protein scaffold used to assemble a three-enzyme mevalonate pathway resulted in a 77-fold increase in yield; and, two separate examples of DNA scaffolds used to co-localize a model coupled-enzyme reaction in vitro demonstrate greater than 15-fold increase in initial pathway reaction rate. The prevailing reasoning used to explain enhancements due to co-localization is that when enzymes are assembled in close proximity, enhancements in catalysis are due to increased local concentration of reaction intermediates. At low intermediate concentration, the second enzyme of a coupled reaction is pseudo-first order with respect to the intermediate; thus, increases in local concentrations increase the reaction velocity. An alternative mechanism of yield
enhancement may be at work in vivo: in pathways with toxic intermediates it is possible that high local enzyme concentrations ensure that intermediates are largely consumed prior to diffusing from the co-localized pathway, thus conferring a fitness benefit in comparison to microorganisms with freely diffusing pathway enzymes.⁴

Figure 1. Natural strategies of substrate channeling in bifunctional enzymes and engineering approaches to co-localizing enzymes in a cascade reaction. (top, left) Crystal structure of tryptophan synthase (TRPS) with intra-molecular tunnel (dashed lines) that connects the active site of the a-subunit to the active site in the b-subunit.²⁰ (bottom, left) Crystal structure of thimidylate synthase-dihydrofolate reductase (TS-DHFR) bifunctional enzyme. The structure is shown with an overlay of the electrostatic map (negative charge shown in red, positive charge shown in blue). The electrostatic interactions between enzyme and negatively charged cascade intermediate promote diffusion between active sites. Adapted with permission from Biochemistry 2013, 52 (41) 7305-7317. (top, right) Examples of enzymes attached to nucleic acid scaffolds including, from left to right, a DNA binding protein fused to an enzyme, aptamer attachment, and chemical conjugation of an enzyme to a nucleic acid strand. (bottom, right) Two enzymes
attached to a protein scaffold by unique protein binding domains fused to the enzyme termini.

These new examples of enhanced catalysis in engineered multi-enzyme structures are important and encouraging, but many challenges remain in developing generalizable systems for assembling multi-enzyme pathways or cascades with optimized kinetics. For example, accurate positioning and orientation of enzymes at the nanometer scale remains technically challenging and the diversity of enzyme structures, from primary amino acid sequence to quaternary structure, can prevent broad application of a single strategy for assembly of arbitrary multi-enzyme cascades. These and more challenges continue to be defined and are being addressed with new strategies to create spatially organized, multi-enzyme systems with well-defined nanoscale architectures (reviewed in 15 and 16).

In this perspective, we describe recent advances in engineering spatially organized, multi-enzyme systems in the context of methods of evaluating changes in coupled-enzyme catalysis and by extension cascade reactions. We focus on established methods from the biochemistry community to observe and quantify substrate channeling and coupled-enzyme kinetics, and we apply these methods to newly developed spatially organized, multi-enzyme systems. As a point of comparison, we discuss selected natural examples of bifunctional enzymes with known mechanisms of substrate channeling. Our discussion focuses on in vitro examples, because the complexity of the intracellular environment often prevents detailed kinetic analysis and a full understanding of the system architecture is difficult to obtain. Based on published experimental data and
simulations of such systems, we describe a preliminary set of design rules (in terms of spatial organization, active site orientation, and scaffold design) for engineering multi-enzyme systems with enhanced catalysis.

2. Substrate Channeling in Nature

Substrate channeling in natural metabolic pathways is not uncommon. In plant biochemistry many secondary metabolites including, isoprenoids, alkaloids, and flavonoids are produced by spatially organized pathways assembled, sometimes transiently, on lipid membranes.\(^\text{17}\) These enzyme complexes create multi-step reaction cascades with optimized ratios of rates and overall structures that create optimal local conditions to protect cells from toxic intermediates and drive pathway catalysis.\(^\text{18}\) Channeling and spatial organization also occur at the protein level. The canonical example is the bifunctional enzyme tryptophan synthase, whose active sites are linked by an intra-enzyme molecular tunnel through which the reaction intermediate can pass (Figure 1, top, left). Similar intra-molecular channels are also found in carbamoylphosphate synthetase and glutamine phosphoribosylpyrophosphate amidotransferase (GPTase).\(^\text{19,20}\) Bifunctional aldolase-dehydrogenase complexes are another example of coupling active sites through intra-enzyme tunnels.\(^\text{21}\) In this case, the open-ended barrels of each enzyme are genetically fused sequestering the toxic aldehyde intermediate from the bulk solution.

One example that has inspired our research group is the bifunctional enzyme thymidylate synthase-dihydrofolate reductase (TS-DHFR). TS methylates the RNA base
dUMP to complete the synthesis of the DNA base dTMP. The synthesis requires a methylated tetrahydrofolate co-factor (Figure 2).

**Figure 2.** The coupled reaction of thymidylate synthase-dihydrofolate reductase (TS-DHFR). The dihydrofolate intermediate is boxed for emphasis.

Recycling of the co-factor begins with the reduction of the dihydrofolate product from the TS reaction to tetrahydrofolate at the DHRF active site. The co-factor product of the coupled reaction is subsequently methylated by a third enzyme, serine hydroxymethyltransferase, to complete the cycle. It is in the first step of co-factor recycling that channeling occurs. The TS-DHFR crystal structure of *Leishmania major* reveals a positively charged region on the outer enzyme surface that spans the ~4 nm distance between active sites (Figure 1, bottom, left). This distance is too far for direct transfer of the intermediate from active site to active site to account for the kinetic behavior of the enzyme and limited conformational change in protein structure prevents intermediate transfer by a dynamic enzyme-substrate complex mechanism. Brownian dynamic simulations show that the negatively charged dihydrofolate intermediate
diffuses between the active sites with high efficiency.\textsuperscript{23} Under simulated physiological conditions, the electrostatic interactions between intermediate and enzyme create a zone of bounded diffusion resulting in greater than 50\% channeling, increasing to greater than 90\% under reduced ionic strength. These simulations are supported by transient and steady-state kinetics that provide evidence of fast intermediate transfer between active sites.\textsuperscript{24} A recent work investigating the TS-DHFR bifunctional enzyme from different species reveal that this mechanism of substrate channeling is common among different parasitic protozoa.\textsuperscript{25}

The TS-DHFR example is an important one for engineering of new spatially organized pathways. Structural and kinetic data come together to create a compelling picture of the extent and mechanism of channeling. As substrate channeling occurs via a bounded diffusion mechanism, channeling is dependent on the chemical and physical environment and careful analysis was required to clearly identify a mechanism of substrate channeling and the kinetic enhancements due to that mechanism.

3. Kinetic Analysis of Substrate Channeling

There have been substantial efforts made to understand the kinetics of coupled enzyme reactions, both with and without the potential occurrence of substrate channeling.\textsuperscript{26-28} Analysis of channeling often focuses on one or more of the following: 1) transient time of the overall reaction, 2) pathway resistance to a competing side reactions, and 3) enhancement in the initial pathway reaction rate. Isotope dilution/enrichment studies and pre-steady state kinetic analysis can also be used to evaluate possible channeling but can
be technically challenging and may not be applicable to all coupled-enzyme systems. Here, we discuss the more common methods of evaluating coupled-enzyme reactions in the context of bifunctional enzymes and engineered multi-enzyme systems. We direct readers to a comprehensive review on substrate channeling for a full discussion of all evaluation methods.29

3.1 Transient time of a coupled-enzyme reaction. Transient time, t, is the time required to reach steady-state flux of an intermediate in a coupled reaction, and is an observable lag phase prior to reaching steady state velocity in a coupled reaction. If we consider the simple reaction of substrate, S, to product, P, via intermediate, I, (Scheme 1) when Michaelis-Menten kinetics apply t is dependent on the maximum velocity and the Michaelis constant of the second reaction ($V_{\text{max},2}$ and $K_{\text{m},2}$, respectively) and the reaction velocity of the first reaction ($n_1$) in the relationship$^{14,29}$:

$$\tau = \frac{K_{\text{M},2} V_{\text{max},2}}{V_{\text{max},2} - n_1}$$

[1]

The transient time, t, is of interest as it is readily observable in time course kinetic assays and decreases in t can be an indication of increased substrate channeling. To this end, equation 1 has been extended to include a condition of channeling.

$$\tau = \frac{K_{\text{M},2} (1 - p_c p_r)}{V_{\text{max},2}}$$

[2]

This equation was developed with the condition that a relatively small concentration of the intermediate escapes to the bulk in comparing to the value of $K_{\text{m},2}$.14 The channeling
probability, \( p_c \), is the probability that the intermediate is transferred from active site to active site without first escaping to the bulk. The reaction probability, \( p_r \), is the probability that a reaction occurs once a substrate-enzyme complex is formed at the second active site prior to dissociation of the complex. Figure 3 shows the graphical calculation of \( t \) from a time course of a coupled reaction. A linear fit to the data crosses the x-axis at \( t \), and by equation 1, the concentration of intermediate at steady state is the negative of the y-intercept.

**Figure 3.** Graphical calculation of the transient time, \( t \), in a coupled-enzyme reaction. A linear fit to the product concentration as a function of time at steady state crosses the x-axis at \( t \). the y-intercept is equal to the negative of the steady state concentration of the cascade reaction intermediate, \([I]_{ss}\).

Analysis of the TS-DHFR reaction cascade shows a decrease in \( t \) from 22 seconds with monofunction DHFR and TS to a \( t \) approaching zero for the bifunctional enzyme under the same experimental conditions (Figure 4).\(^{30}\) By equation 2, a decrease in \( t \) is indicative
of an increase in the extent of substrate channeling in the bifunctional enzyme in comparison to the freely diffusing enzyme pair.

Figure 4. Experimental data of the TS-DHFR bifunctional enzyme and freely diffusing DHFR and TS. Adapted with permission from Biochemistry, 1996 35 (20) 6366-6374. A clear decrease in the transient time can be observed in the bifunctional enzyme in comparison with the freely diffusing enzyme pair.

Changes in t have also been experimentally observed in an engineered system where glucose oxidase (GOx) and horseradish peroxidase (HRP) were co-localized on a hexagonal DNA scaffold. This model enzyme pair has been used in a number of DNA scaffold technologies that control co-localization of the coupled reaction. Both GOx and HRP are robust, maintain high activity when modified with chemical crosslinking agents, and make for an excellent model cascade for developing new scaffolds and for exploring the effects of spatial organization and scaffold design on the kinetics of coupled-enzyme reactions (Figure 5). GOx converts glucose and oxygen to gluconolactone and hydrogen
peroxide. Hydrogen peroxide is the intermediate of the coupled reaction and is oxidized by HRP with the concomitant reduction of an electron acceptor (in this case the colorimetric substrate ABTS). A change in scaffold design produces a decrease in $t$ from 65 to 46 seconds. When GOx and HRP are co-localized on the surface of ~10 nm diameter micelles, $t$ is reduced from ~25 to <1 second indicating a substantial increase in peroxide intermediate directly accessing the downstream enzyme prior to diffusing to the bulk.\textsuperscript{31}

Figure 5. Coupled reaction of glucose oxidase (GOx) and horseradish peroxidase (HRP) and examples of DNA scaffolding of the GOx-HRP model cascade. (Top, left) Reaction scheme of the coupled GOx-HRP reaction. This model cascade is commonly used to explore new scaffold designs for spatially organized multi-enzyme structures. (Top, right) A cartoon representation of the controlled assembly of GOx and HRP on a DNA origami tile.\textsuperscript{13} (Bottom, left) An active DNA scaffold that, with the addition of an DNA “fuel” strand, assembles and dis-assembles the GOx-HRP cascade. Adapted with permission from reference 39. (Bottom, right) Immobilization of the GOx-HRP with DNA strands
for the controlled assembly of the GOx-HRP cascade. Adapted with permission from reference 38.

Given well-defined kinetic parameters, for both freely diffusing enzymes and modified enzymes assembled into spatially organized structures, t-analysis can be a powerful method of evaluating substrate channeling and enhancements in the kinetics of coupled reactions. Equation 2, as well as similar derivations of kinetic expressions of substrate channelings\textsuperscript{28, 32}, can be used to quantify channeling and changes in channeling in a given system.

### 3.2 Competition for Pathway Intermediates from a Competing Reaction.

A relatively straightforward and robust method of evaluating coupled-enzyme reactions is challenging the cascade with a parasitic side reaction whose substrate is the intermediate of interest. The observation of any product from the competing reaction indicates the presence of intermediate in the bulk solution, and therefore less than perfect channeling in the coupled-enzyme system. The effect of the competing reaction can also be observed in t. Reaction with the competing enzyme decreases the bulk concentration of intermediate, and consequently the time required to reach steady state concentrations. These evaluations of channeling (i.e., decreased t and side product formation) do not require a detailed knowledge of kinetic parameters; however, given such data and an extension of equation 2 to include the third, competing enzyme can be used to quantify the extent of channeling.\textsuperscript{14}
Challenge from a competing reaction can also affect overall pathway activity and yield. Figure 6 shows the residual activity of a coupled-enzyme reaction when genetically fused into a single bifunctional enzyme in comparison to a freely diffusing enzyme pair. In this experiment mitochondrial malate dehydrogenase (mMD) and citrate synthase (CS), a coupled reaction from the Kerbs cycle that converts malate to citrate via an oxaloacetate intermediate, are fused N- to C-terminus with a three amino acid linker. A model of the mMD-CS fusion protein derived from crystal structures of each individual enzyme shows that an electrostatic patch bridges the protein surface from the mMD active site to the CS active site, a distance of approximately 6 nm. With high concentration of aspartate transaminase (AAT) competing for the oxaloacetate intermediate, the mMD-CS fusion maintains upwards of 60% activity. Under the same conditions freely diffusing enzymes maintain no more than 20% of the maximum activity, indicating that channeling occurs in the enzyme fusion. Combined with Brownian dynamics simulations showing the importance of electrostatics in mMD-CS channeling, the structural data and competition assays present compelling evidence of substrate channeling in the fusion protein.
Figure 6. Residual activity of the coupled-enzyme reaction of malate dehydrogenase (mMD) and citrate synthase (CS) in the presence of a freely diffusing enzyme that competes for the coupled reaction intermediate. (top) The coupled reaction of mMD and CS with a competing reaction from aspartate transaminase (AAT). (bottom) A fusion between mMD and CS maintains nearly 60% of maximum activity in the presence of 10 units of competing enzyme while the freely diffusing enzyme pair is reduced to approximately 20% activity. Adapted with permission from Biochemistry, 1999 38 (3) 881-889.

The presence of a competing reaction can also affect the yield of the desired cascade. For example, an aptamer-based DNA scaffold that assembles GOx and HRP in close proximity protects the peroxide intermediate from a competing reaction with
catalase. In the presence of catalase the reaction yield of the assembled GOx-HRP structure is decreased by 20%, while the decrease is upwards of 50% under the same conditions with freely diffusing, unassembled GOx and HRP. The resistance of the assembled pathway to the competing reaction is consistent with the concept of channeling by close proximity.

Similarly, the yield of the coupled reaction of bifunctional aldolase-dehydrogenase complexes was used to evaluated substrate channeling. The aldolase cleaves 4-hydroxy-2-oxoacids producing pyruvate and an aldehyde. The aldehyde intermediate travels through an intra-molecular tunnel and is converted to acyl-CoA at the active site of the coupled dehydrogenase. A freely diffusing aldehyde dehydrogenase that converts aldehydes to carboxylates competes for the aldehyde intermediate. The competition assays revealed that upwards of 90% of various chain length aldehyde intermediates are channeled from aldolase to the dehydrogenase. This natural bifunctional enzyme is another important example of kinetic and structural data combining to reveal the extent and mechanism of substrate channeling.

3.3 Enhancement in Initial Reaction Rate. The overall reaction rate of a coupled-enzyme reaction is the sum of rates from reaction with intermediates that take a direct route from active site to active site (i.e., intermediates that are channeled) and from intermediates that diffuse to the bulk prior to reaching a downstream active site. At steady state, in the absence of side reactions, and when intermediate decomposition is negligible, this overall rate is not affected by substrate channeling.
overall rate of a multi-enzyme cascade is not affected by substrate channeling and is limited by the maximum rate of the slowest reaction step. The data presented in Figure 4 from the TS-DHFR example demonstrates this point: the slope of the time course data at times greater than t are equal, corresponding to a steady-state rate of approximately 0.25 mM per min.\textsuperscript{30} Similarly, in analyzing GOx-HRP experimental data we estimate the rates resulting from two different DNA scaffolds to be equal (approximately 8 mM/s) at times greater than t (46 and 65 seconds).\textsuperscript{12}

The situation is more complex at times less than t when a coupled-enzyme reaction has yet to reach steady state. At times less than t, the contributions of channeling towards the overall rate can dominate and can result in significant enhancements to the observed overall reaction rate. Modeling and simulation of coupled-enzyme reactions supports this idea and demonstrate that the initial enhancement in overall rate can range from <1s to tens of minutes depending on system architecture and reaction volume.\textsuperscript{37}

Enhancements in overall initial rate have also been experimentally observed. For example, a DNA origami tile that co-localizes GOx and HRP at ~10 nm interenzyme distance results in a greater than 15-fold increase in initial pathway reaction rate (Figure 5, top, right).\textsuperscript{13} A 3-fold enhancement in initial rate was demonstrated with the same enzyme pair assembled in close proximity with surface tethered linear DNA scaffolds (Figure 5, bottom, right).\textsuperscript{38} Enhancements in overall rate have also been demonstrated with a dynamic scaffold that repeatedly closes (high activity) and opens (low activity) the GOx-HRP reaction cascade (Figure 5, bottom, left).\textsuperscript{39} Finally, the GOx-HRP coupled
reaction shows significant enhancement in overall rate when co-localized inside of a DNA nanotube.  

In the emerging field of DNA scaffolds the predominant model cascade has been GOx-HRP; however, a number of different multi-enzyme cascades have been assembled using protein scaffolds (Figure 7). For example, three enzymes from the glycolysis pathway (triosephosphate isomerase, aldolase, and fructose 1,6-biphosphatase) were assembled with a dockerin/cohesin-based protein scaffold. The assembled multi-enzyme cascade produced a greater than 20-fold increase in initial rate in comparison to freely diffusing enzymes.  
A similar scaffold was used to assemble a cascade of dehydrogenases to oxidize methanol to CO$_2$.  
Catalytic enhancement of the assembled cascade was observed by a 5-fold increase in the rate of enzyme co-factor production in comparison to an un-assembled control. Finally, a trimeric ring-shaped protein made from proliferating cell nuclear antigens (PCNAs) was used as a scaffold to assemble a redox reaction cascade of cytochrome P450 with P450 electron transfer proteins ferredoxin and ferredoxin reductase. The assembled cascade exhibited 50-fold increase in initial activity over the un-assembled control. In each of these protein scaffolding examples the overall structure of the scaffolded cascade is not well known. Flexible protein scaffolds allow enzymes to aggregate and form a cluster of enzymes in close proximity. Interenzyme distance and orientation are likely variable, thus preventing clear identification of a mechanism of potential substrate channeling.
4. Preliminary Design Rules for Engineering Enhanced Catalysis in Nanostructured Multi-Enzyme Cascades

Newly developed nucleic acid, protein, and polymer scaffolding technologies have enabled the design and engineering of multi-enzyme cascade reactions with control over the positioning of cascade enzymes and the overall architecture of the multi-enzyme structure. From the kinetic analysis of these multi-enzyme structures and of natural bifunctional enzymes that exhibit substrate channeling, we aim to extract a preliminary set of rules for designing new multi-enzyme cascades. This set of design rules addresses interenzyme distance, active site orientation, and overall architecture of coupled-enzyme structures for promoting substrate channeling. We present these guidelines and design rules as a preliminary set and do not expect them to account for differences in specific reaction mechanisms. We recognize that there remains significant technical challenges to the precise the positioning of multiple enzymes at the <1 to 10 nm scale, and in the
control of active site orientation. As such, we expect that as the set of available tools used to create spatially organized multi-enzyme structures improves new experiments will reveal refinements to the design rules as well as reveal new guidelines not proposed here.

4.1 Interenzyme Distance. Both experimental observations and molecular simulations of coupled-enzyme reactions show us that the distance between enzymes has a significant effect of the proportion of intermediate that take a direct path from upstream to downstream active sites. Studies of the TS-DHFR and mMD-CS bifunctional enzymes show that channeling can occur with inter-active site distances of up to 6 nm when aided by electrostatic interactions that promote a diffusional path.\textsuperscript{14, 34} In the absence of electrostatic interactions in the intervening space between enzymes, Brownian dynamics simulations of two fixed position enzymes suggest that the probability that the products of the first reaction channels to the second active site is >90\% at a 0.5 nm, decreasing to <10\% at a distance of 4 nm, given optimal orientations of actives sites.\textsuperscript{44} Many experimental examples that bring enzymes in close proximity (i.e., that are assembled in a structure where upstream and downstream enzymes are essentially in contact) are consistent with these simulation results. For example, when the GOx-HRP cascade is assembled with 10 nm between flexible tethers on a DNA origami tile, a 15-fold increase in the initial rate is observed. The flexibility of the tethers allows for GOx and HRP to come into contact.\textsuperscript{13} Similarly, flexible protein scaffolds allow cascade enzymes to cluster resulting in enhanced initial rates.\textsuperscript{41-43} When taken together, the experimental and simulation data describing substrate channeling as a function of the distance between active sites suggests that at fixed distances between active sites greater 1-2 nm, substrate
channeling occurs to a significant extent only if diffusion between active sites is promoted by interactions between the cascade intermediate and the surface or scaffold between enzymes. This distance can be extended with bounded diffusion between active sites, as is demonstrated in the case of the natural bifunctional enzyme TS-DHFR (~4 nm between active sites) and the fusion of mMD and CS (~6 nm between active sites).

4.2 Enzyme orientation. Intimately coupled to the effects of interenzyme distance on substrate channeling is the orientation of active sites within a cascade. Natural examples of bifunctional enzymes have inherent control over the positioning of active sites, an ability that still remains as a significant technological challenge in engineering multi-enzyme structures. Recent works have demonstrated the importance of active site orientation in single enzyme systems immobilized on a surface\textsuperscript{45} and in electron transfer reactions with electrode-immobilized redox enzymes\textsuperscript{46}. Specific to cascade reactions, Brownian dynamics simulations have been used to describe a relationship between orientation of active sites and the probability of the downstream reaction.\textsuperscript{44} At a fixed interenzyme distance of 0.5 nm, inward facing active sites can limit substrate access to the first enzyme in the cascade, thus reducing the overall throughput of the cascade. At 1 nm spacing direct alignment of the active sites is optimal and leads to the highest probability that the second reaction in the cascade occurs. Experimental descriptions of the effects of active site and enzyme orientation on cascade reactions has yet to be developed and represents an important area of future research.
4.3 Multi-Enzyme Architectures. Newly developed enzyme co-localization techniques have only just begun to enable the analysis of spatial organized coupled reactions. As such, there is not an extensive data set from which we can distill detailed, generalizable design rules for assembling multi-enzyme structures with optimized channeling. However, from established t-analysis and from simulations and modeling of an engineered GOx-HRP system we can draw two preliminary guidelines. Firstly, equation 1 above provides the simple principle that the rate of the downstream enzyme must be greater than the velocity of the first reaction. Under these conditions the concentration of intermediate will attain a steady state value. If the production of the intermediate is greater than the maximum value of its consumption (i.e. $V_{\text{max},2}$) then a steady state will not be reached and, regardless of channeling, the concentration of bulk intermediate will increase. With respect to the design of multi-enzyme nanostructures, this suggests that the ratio of the upstream enzyme to the downstream enzyme within the cascade should be balanced so that $V_{\text{max},2}$ is greater that then velocity of the upstream enzyme. Secondly, modeling and simulation work that describes the kinetic enhancements in an experimental systems that couples GOx and HRP in a hexagonal DNA structure shows that the overall structure of the multi-enzyme systems has a significant effect on system catalysis. Such modeling and simulation efforts are important in understanding these systems and help in our understanding of the kinetic of the systems and the relationships between the system architecture and catalysis.

5. Conclusions
In this perspective we aim to connect new progress in engineering nanoscale multi-enzyme structures to the methods and analysis techniques that the biochemistry community has established over the past 20 or more years to evaluate the kinetics of coupled-enzyme reactions and substrate channeling. Recent progress in the development DNA nanotechnologies that create programmable, well-defined structures through Watson-Crick base pairing have enabled the creation of multi-enzyme nanostructures with a high degree of spatial organization. The enzymes of a coupled reaction can be tethered at known distances down to ~10 nm\(^1\), organized into aggregate structures with high enzyme density\(^2\), assembled on the inside of nanoscale DNA tubes,\(^3\) and dynamically push together and pulled apart\(^4\). Engineerable protein scaffolds that can assemble multi-enzyme structures with high densities of enzymes and with tunable enzyme ratios compliment these new DNA nanotechnology tools.\(^5, 6\)

Detailed analysis of the kinetics of coupled-enzyme reactions assembled with these DNA and protein technologies is important and promises to reveal how far we have come in mimicking natural bifunctional enzymes and metabolic cascades that rely on substrate channeling to drive pathway flux and protect cascade intermediates from reaction in alternate pathways. Transient time (t) analysis along with a detailed knowledge of the enzyme kinetic parameters can be used to quantitatively evaluate the extent of substrate channeling in a given system. Challenge from a competing reaction in the bulk solution that uses cascade intermediates as substrates can also be used to evaluate channeling and the extent of protection that a multi-enzyme structure provides to
its cascade intermediates. And finally, under pre-steady state conditions increases in the overall reaction rate can reveal enhanced catalysis and possibly substrate channeling.

A second goal of this Perspective is to develop a preliminary set of design rules or guidelines for promoting substrate channeling in engineered multi-enzyme systems. To do so, we evaluate published data on natural bifunctional enzyme systems with strong experimental and modeling evidence supporting the existence of substrate channeling as well as data from new engineered multi-enzyme systems that demonstrate enhanced catalysis. The preliminary design rules are, 1) in the absence of bounded diffusion, interenzyme distances in which substrate channeling can occur to a significant extent is limited to approximately 1 nm; 2) significant substrate channeling can be achieved at interenzyme distances of 5-6 nm if diffusion between active sites is promoted through interactions between the surface in between the active sites and the cascade intermediate; 3) at close proximity, active site orientation can block substrate access to the coupled reaction; 4) when substrates can access active sites from the bulk solution, active site orientation should minimize interenzyme distance; and 5) to achieve a steady state concentration of cascade intermediate the ratio of enzymes should be balanced so that the velocity of the first reaction is less than the maximum velocity of the second reaction. These general design rules are preliminary and we expect that as the capabilities of the engineering tools for multi-enzyme nanostructures improve that these guidelines will be refined and new rules will be discovered.
This set of guidelines, and the data sets from which they were drawn, also highlight the technological challenges that remain in engineering generalizable tools for the assembly of multi-enzyme nanostructures with optimized catalysis. New protein and biomolecular engineering tools are needed to create multi-enzyme systems with precise interenzyme distances in the <1 to 10 nm range. Similar tools are needed to accurately control the orientations of enzyme active sites. And importantly, many more examples of nanostructured coupled-enzyme reactions are needed so that we can evaluate the effects of new multi-enzyme architectures as well as the behavior of different enzyme reaction mechanisms in such systems.

Engineering new multi-enzyme nanostructures with optimized kinetics is a complex and multi-disciplinary problem that includes aspects of biophysics and biochemistry, as well as protein, biomolecular, and nanoscale engineering. Recent progress in technology development is encouraging and promises to produce many more interesting discoveries.

AUTHOR INFORMATION

Corresponding Author

*E-mail: iwheeldon@engr.ucr.edu

ACKNOWLEDGMENT

This work was supported by the Air Force Office of Scientific Research Young Investigator Program (FA9550-13-1-0184) and the Bourns College of Engineering at the University of California, Riverside.
REFERENCES


