Strep-tag II fusion technology for the immobilization of lipase B from Candida antarctica

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Strep-tag II fusion technology for the immobilization of lipase B from *Candida antarctica*

by

Sumreet Singh Johar

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Joey N. Talbert, Major Professor
Tong Wang
Surya K. Mallapragada

Iowa State University
Ames, Iowa
2016

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DEDICATION

To all the enlightened and saintly beings, Dhamma, Sangha, my revered Dhamma teachers (Satya Narayan Goenka, Shyam Sunder Taparia and Dr. Neena Lakhani), my Dhamma sister (Sumegha Kapoor Ohri), my parents, grandparents, brother, sister-in-law and niece whose countless blessings and unconditional support provided me an invaluable opportunity to work for my venerable professor Dr. Joey N. Talbert
TABLE OF CONTENTS

NOMENCLATURE ........................................................................................................... v

ACKNOWLEDGEMENTS .............................................................................................. vi

ABSTRACT .................................................................................................................... ix

CHAPTER 1: GENERAL INTRODUCTION ...................................................................... 1
  1.1. Introduction ........................................................................................................... 1
  1.2. Thesis Organization ............................................................................................ 1
  1.3. Literature Review ................................................................................................ 2
      • Enzyme Immobilization
      • Potential of Fusion Tags for Enzyme Immobilization
      • Lipases
  1.4. Research Objectives ............................................................................................ 15
  1.5. References .......................................................................................................... 17

CHAPTER 2: EXPRESSION, PURIFICATION AND CHARACTERIZATION
OF STREP – TAG II LIPASE B FROM Candida antarctica (CALB) .................................. 22
  2.1 Abstract ................................................................................................................ 22
  2.2 Introduction .......................................................................................................... 23
  2.3 Materials and Methods ...................................................................................... 27
      2.3.1 Materials ........................................................................................................ 27
      2.3.2 Construction of Plasmids ............................................................................ 28
      2.3.3 Culture Media ............................................................................................... 31
      2.3.4 Expression of Recombinant Strep-tag II CALB .......................................... 31
      2.3.5 Purification of Recombinant Strep-tag II CALB from E. coli ...................... 32
      2.3.6 Lipase Assay ................................................................................................. 33
  2.4 Results and Discussion ...................................................................................... 34
  2.5 Conclusion ............................................................................................................ 44
  2.6 Acknowledgement ............................................................................................... 44
  2.7 References .......................................................................................................... 46
CHAPTER 3: STREP-TAG II FUSION TECHNOLOGY FOR THE
IMMOBILIZATION OF LIPASE B FROM *Candida antarctica* (CALB) ...................... 48

3.1 Abstract ............................................................................................................... 48

3.2 Introduction ....................................................................................................... 49

3.3 Materials and Methods .................................................................................... 53

  3.3.1 Materials ....................................................................................................... 53
  3.3.2 Affinity Purification of Strep-tag II CALB from *E. coli* ......................... 53
  3.3.3 Immobilization of Strep-tag II CALB Using Strep-Tactin Resin .......... 54
  3.3.4 Protein Leaching ........................................................................................ 55
  3.3.5 Enzyme Kinetics ......................................................................................... 56
  3.3.6 CALB Activity and Stability ................................................................. 56
  3.3.7 Statistical Analysis ..................................................................................... 57

3.4 Results and Discussion ..................................................................................... 57

3.5 Conclusions ....................................................................................................... 64

3.6 Acknowledgement ............................................................................................ 65

3.7 References ....................................................................................................... 66

CHAPTER 4: CONCLUSIONS AND FUTURE WORK ........................................... 70
# NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amp (Ap)</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>CALB</td>
<td><em>Candida antarctica</em> lipase B</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-b-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>MES</td>
<td>4-morpholineethanesulfonic</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>p-NPB</td>
<td>p-nitrophenyl butyrate</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific Broth</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my advisor Dr. Joey N. Talbert for the continuous support of my Masters study and related research and for his virtuous qualities of faith, patience, generosity, compassion, unconditional support, motivation, and immense knowledge. His supreme guidance helped me throughout the course of my research and writing of this thesis. The door to his office was always open whenever I faced any troubles or had a question about my courses, research or writing. He consistently allowed papers from this research to be my own work, but steered me in the right direction whenever he thought I needed it. It is my great privilege to be his first graduate student and contribute towards the progress of his research work and laboratory organization. I could not have imagined having a better advisor and mentor for my Masters study. In addition, I would also like to offer my deep appreciation to my advisor’s wife, Megan Sloat for her immense kindness that assisted me in making a smooth transition in Ames.

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challenges in my personal as well as professional life and emerge out as a stronger person than before. I am highly fortunate to come in touch with such noble persons early in my life who have played an integral role in strengthening my morality, concentration and experiential wisdom. I would not have reached so far without you all. Thank you doesn't seem enough for the highest form of generous deed that you all have done for me.

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ABSTRACT

Fusion tags are genetically coded protein or peptides that can be expressed as attached moieties to the desired enzyme to improve downstream purification of the target protein. Fusion tags have the potential to provide specific immobilization with repeatable attachment of the enzyme, eliminate the need for purification and post-modification and enable dissociation of spent enzyme and regeneration of the support. Lipase is an industry-relevant enzyme utilized for the production of natural flavors, biodegradable polyesters for food packaging, structured lipids, antioxidant esters, sugar esters and several other important compounds used in food and agriculture industries. In this research work, we are targeting the immobilization of lipase B from *Candida antarctica* (CALB) using novel Strep-tag II fusion technology as a means to enhance its functional properties.

The gene encoding CALB was codon-optimized and synthesized for expression in *E. coli*. Multiple plasmid constructs were designed to allow for the direct fusion of a Strep-tag II to lipase. The corresponding bacterial expression systems were optimized to promote lipase expression. SDS-PAGE was employed to monitor protein expression. The expressed recombinant lipase having Strep-tag II was purified using Strep-Tactin (engineered streptavidin) column and further used for immobilization to Strep-Tactin based supports. Enzyme specific activity and kinetics were evaluated using colorimetric assays.

The optimized bacterial expression system yielded a recombinant CALB with a Strep-II fusion tag. The expressed enzyme displayed specific affinity to Strep-Tactin and could be attached to a Strep-Tactin coated support. The resulting enzyme was catalytically active and was purified and characterized for immobilization studies using bio-affinity interaction.
CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

While the potential uses of immobilized enzymes in food processing and biomanufacturing are well known, practical applications have remained elusive. Current approaches to immobilization are limited in terms of economics, efficiency, and efficacy. Ideally, an expressed enzyme would be rapidly immobilized in a strong and specific fashion without involving advanced expertise in bioconjugate chemistry. Further, the ability to remove spent enzyme and reuse a support is desirable to enable cost reduction. Fusion tags have been employed in molecular biology as a means to purify a desired protein from other proteins associated with the fermentation product through the use of affinity interactions. However, there are ample opportunities to employ this technology to improve enzyme immobilization. There is, however, limited data regarding the use of the affinity tags for enzyme immobilization in bioprocessing applications. Here, we focus on the development of a Strep-tag II fusion technology for the immobilization of the industry-relevant lipase B from *Candida antarctica* (CALB).

1.1 Thesis Organization

This thesis begins with a review of literature focusing on enzyme immobilization, potential of fusion tags for enzyme immobilization and lipases applications and immobilization followed by research objectives.
Two manuscripts follow the literature review. Dr. Joey N. Talbert is the author for correspondence for both manuscripts. A general conclusion forms the climax of this thesis.

1.2 Literature Review

Enzyme Immobilization

Enzyme technology is attracting wide attention for industrial applications owing to the current demands for establishing sustainable “green” chemistry based methodologies. Biocatalysis using enzymes confers several benefits over chemical catalysis such as mild reaction conditions, high specificity, high catalytic efficiency and environmentally friendly procedure [1, 2]. However, enzymes are prone to destabilization and inactivation under process conditions, thereby restricting their use in industries. In order to enhance the feasibility of utilizing enzymes for biotechnological processes, the technology of enzyme immobilization is currently being explored.

Immobilization of enzymes involves physical confinement or localization of the enzyme in a distinct phase, separated from the bulk phase yet allowing it to react with substrate in the reaction medium [1-3]. An ideal immobilized enzyme system is expected to offer several advantages such as convenient handling of the enzyme, easy separation of the enzyme from the product, increased enzyme stability under storage as well as operational conditions, easy manipulation of the reaction process and efficient recovery and reuse of enzymes to enable cost reduction [1-3]. Thus, enzyme immobilization has the potential to improve the catalytic properties and long term operational stability of enzymes, making them suitable for diverse industrial applications.
An immobilized enzyme system comprises of three principal components – the enzyme, material support and the mode of attachment. There are three key factors underlying the development of immobilized biocatalysts: selection of immobilization supports, conditions and methods of immobilization. The characteristic features of material support play an integral role in developing an effective immobilized system [1, 4]. An ideal support should possess certain essential properties such as hydrophilicity, inertness towards enzymes, high affinity for protein, presence of reactive functional group, resistance to microbial attack, mechanical stability, rigidity, feasibility of regeneration, non-toxicity, biodegradability and low cost [1, 2]. Various natural polymers (such as alginate, chitosan, collagen, gelatin, cellulose, pectin, sepharose, etc.) and synthetic polymers (such as zeolites, ceramics, celite, silica, glass, activated carbon, charcoal, etc.) have been developed as material supports to improve enzyme immobilization [1, 2].

Enzyme immobilization methods can be broadly divided into two main categories – physical and chemical methods (Figure 1). Physical methods involve attachment of the enzyme to the support using weaker, non-covalent interactions such as hydrogen bonds, hydrophobic interactions, van der Waals forces and ionic interaction in a process known as adsorption or mechanical entrapment within the support [1, 2]. Chemical methods involve formation of covalent bonds between the enzyme molecules (cross-linking) or between the enzyme and the material support (covalent binding) using bi- or multi-functional cross linking reagents such as glutaradehyde [1, 2]. Given the complex nature of protein structure, not one method is suitable for all enzymes or purposes and each method has its share of merits and demerits as listed in Table 1.
Currently, very few immobilized enzymes are being implemented for industrial applications on a commercial scale [5]. Immobilized glucose isomerase has been used in the production of high fructose corn syrup and to enhance the conversion of sugars derived from cellulosic biomass to ethanol [6]. Lipase enzymes immobilized on different supports are practiced commercially to carry out the enzymatic interesterification of fats and oils to produce different kinds of lipids for food and fuel industries [7]. Other emerging immobilized enzyme applications include pectin hydrolysis by immobilized pectinases to reduce turbidity in fruit juices, reduction of bitter components in fruit juices by treatment with immobilized naringinase enzyme, absorption of post combustion CO₂ by immobilized carbonic anhydrase and several others [8-11].

**Figure 1. Enzyme Immobilization Methods** (Reproduced from Analyst 2013, 138, Iqbal, J., Iqbal, S., and Müller, C.E., Advances in immobilized enzyme microbioreactors in capillary electrophoresis, 3104-3116 with permission of The Royal Society of Chemistry) [http://dx.doi.org/10.1039/C3AN00031A](http://dx.doi.org/10.1039/C3AN00031A)
Table 1: Commonly Used Strategies for Enzyme Immobilization (Adapted from [3])

<table>
<thead>
<tr>
<th>Immobilization Method</th>
<th>Binding Nature</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption</td>
<td>Weak bonds: hydrophobic, Van der Walls or ionic interactions</td>
<td>Usually does not involve enzyme modification, cheap, less disruptive to enzyme, easy to carry out, absence of expensive and toxic chemicals, may not require surface functionalization, enzyme is substrate accessible</td>
<td>Desorption, non-specific adsorption, varying immobilized enzyme orientations, ionic or hydrophobic interactions may lead to surface denaturation</td>
</tr>
<tr>
<td>Entrapment</td>
<td>Inclusion of enzyme within a polymeric network</td>
<td>Higher loading compared to other methods, does not require enzyme or surface modification, not dependent on enzyme orientation</td>
<td>Mass transfer limitations, enzyme leakage, mechanical stability of the immobilized system</td>
</tr>
<tr>
<td>Covalent Attachment</td>
<td>Chemical binding between functional groups of the enzyme and support</td>
<td>No enzyme leakage, potential for enzyme stabilization, enzyme is substrate accessible</td>
<td>Requires surface and/or enzyme functionalization, typically non-specific orientation, more expensive than other immobilization methods, matrix and enzyme cannot be not regenerated, major loss of activity due to enzyme denaturation during immobilization</td>
</tr>
<tr>
<td>Cross-Linking</td>
<td>Enzymes molecules are cross-linked by a functional reactant</td>
<td>Very little desorption, biocatalyst stabilization</td>
<td>Requires enzyme functionalization, mass transfer limitations, loss of enzyme activity</td>
</tr>
</tbody>
</table>
Potential of Fusion Tags for Enzyme Immobilization

Current methodologies for enzyme immobilization suffer from several limitations such as enzyme inactivation during immobilization, reduced mass transfer, enzyme leakage from the support and inability to reuse the material support once the enzyme has been spent [13, 14]. This suggests that there is continuous need to develop viable and effective enzyme immobilization systems. Recent advances in protein engineering and biochemistry have resulted in the discovery of versatile array of affinity binding proteins and peptides that have specific binding interaction with biomolecules such as proteins, carbohydrates, and more recently, metals, silica, and synthetic polymers [15-17]. Fusion protein strategy involves the genetic insertion of one or more affinity proteins or peptide at the N – or C – terminus of the gene coding for the target protein. Fusion tags are incorporated to enhance protein solubility, improve recombinant protein expression yields, enable protein purification and detection, and accelerate protein characterization studies [15, 17-19]. Thus, protein fusion tags are requisite tools used to facilitate efficient identification, production, and isolation of the proteins of interest from the host system. Various fusion tags are in use to achieve the purpose of enhancing protein expression and/or solubility including glutathione S-transferase (GST), maltose-binding protein (MBP), thioredoxin A (TrxA), small ubiquitin related modifier (SUMO), N-utilization substance A (NusA) and Protein disulfide isomerase I (DsbA) [18, 20]. Other fusion tags that are commonly used for protein detection, characterization, and purification include c-myc, Flag, hemaglutinin antigen (HA), Streptavidin binding tags, polyhistidine, polyarginine, calmodulin-binding peptide (CBP), chitin-binding domain, cellulose-binding domain [20]. The advantages and disadvantages of some of these fusion tags are outlined in Table 2.
<table>
<thead>
<tr>
<th>Tag</th>
<th>Size (aa)</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-tag</td>
<td>5-15</td>
<td>Low metabolic burden, inexpensive, Mild elution conditions, works under both native and denaturing conditions</td>
<td>Specificity of IMAC is low than other affinity methods</td>
</tr>
<tr>
<td>GST</td>
<td>201</td>
<td>Efficient translation initiation, inexpensive, mild elution conditions</td>
<td>High metabolic burden</td>
</tr>
<tr>
<td>MBP</td>
<td>396</td>
<td>Efficient translation initiation, inexpensive, mild elution conditions, enhances solubility, mild elution conditions</td>
<td>High metabolic burden</td>
</tr>
<tr>
<td>NusA</td>
<td>495</td>
<td>Efficient translation initiation, enhances solubility, not an affinity tag</td>
<td>High metabolic burden</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>109</td>
<td>Efficient translation initiation, enhances solubility</td>
<td>Except few derivatives, does not act as an affinity tag</td>
</tr>
<tr>
<td>FLAG</td>
<td>8</td>
<td>Low metabolic burden</td>
<td>Expensive, harsh elution conditions</td>
</tr>
<tr>
<td>SUMO</td>
<td>~ 100</td>
<td>Enhances solubility</td>
<td>Not an affinity tag</td>
</tr>
<tr>
<td>CBP</td>
<td>27</td>
<td>Low metabolic burden, high specificity</td>
<td>Expensive, does not enhance solubility</td>
</tr>
<tr>
<td>Strep-II</td>
<td>8</td>
<td>Low metabolic burden, high specificity</td>
<td>Expensive, does not enhance solubility</td>
</tr>
<tr>
<td>CBD</td>
<td>51</td>
<td>No exogenous proteolytic</td>
<td>Does not enhance solubility</td>
</tr>
</tbody>
</table>

His-tag: poly-histidine; GST: Glutathione-S-transferase; MBP: Maltose Binding Protein; NusA: N-Utilization Sample; FLAG: FLAG-tag peptide; SUMO: Small Ubiquitin Modifier; CBP: Calmodulin Binding Peptide; Strep-II: Streptavidin binding peptide; CBD: chitin binding domain
While primarily designed and applied to facilitate the production and single step purification of recombinant proteins as well as biochemical characterization of their structural and functional properties, fusion tags have the potential to serve as useful and gentle tools for enzyme immobilization [15, 22, 23]. Fusion tag based immobilization relies on the specificity of enzyme to its material support under different physiological conditions. It is achieved by genetic engineering of the enzyme to insert fusion or affinity tag at N– or C– terminus of the enzyme and then allowing the target enzyme to bind to a matrix pre-coupled with a ligand possessing affinity for the fusion tag on the target enzyme. This method for immobilization has several potential benefits [22]. Specificity of the fusion tag on the enzyme for a material support enables directed immobilization. Due to the same connection binding site or specific group of the support, selective and repeatable oriented immobilization similar for all biomolecules is possible provided that only the fusion tag is taking part in interacting with the polymeric support. In addition, the affinity of the tag for its binding partner has the potential to reduce the need for purification and post-modification prior to immobilization. Chemical modification of the enzyme to add additional functional groups on enzyme is not required, thereby minimizing the conformational changes induced in the enzyme structure. Spent enzyme can be dissociated from the material support under tailored conditions and the same activated material support displaying affinity ligand can be reloaded with new enzyme allowing support reuse according to different demands.

There are various factors that influence the development of an efficient and robust fusion tag based enzyme immobilization system including solubility and activity of the expressed protein, specificity, binding affinity, leaching, support regeneration, and cost. It is imperative that the fusion tag used for expression of the recombinant enzyme results in the
production of soluble and catalytically active enzyme. Fusion tags such as glutathione-transferase (GST) and maltose-binding protein (MBP) have long been used for increasing the solubility of recombinant enzymes [24]. However, the large size of these tags can negatively affect enzyme activity as well as may put a heavy metabolic load on the host cell thereby driving the recombinant protein majorly into the insoluble fraction [16]. In addition, other factors such as the location on the protein, presence of linker region between the target protein and fusion tag and hydrophilicity/hydrophobicity of the target protein can also exert an influence on the folding, solubility and activity of the protein [25]. Thus, to determine the effect of fusion tags on enzyme solubility and catalytic activity, preliminary screening is crucial.

An effective fusion tag based immobilization system requires the association between the recombinant protein and the affinity ligand to be strong enough to minimize the leaching of recombinant enzyme from the matrix during the use of the biocatalyst [3]. Several factors influence binding of a fusion tag protein such as binding specificity, affinity, desorption kinetics, and induced leaching. Specificity of the fusion tag to the binding partner is a critical factor to ensure increased loading of the target protein from an impure preparation as well as to prevent dissociation during processing. Immobilized metal affinity adsorption technique that relies on the specific interaction of polyhistidine residues on the target protein with metal ions on the matrix support possesses some complications that restrict its use in immobilization processes [23, 26]. For instance, interference of large number of metal-binding proteins in the impure sample can result in non-specific attachment of unwanted proteins thereby affecting the purity of immobilized enzyme system. Additionally, metal
ions from the matrix support can undergo leaching resulting in product contamination which could be of serious concern in food applications.

Binding affinity is often characterized by a dissociation constant (K_D). To promote association of the protein to its binding partner, a K_D of 1 µM and less is often desirable [27]. Dissociation constants of most of the fusion-tags are in the nM to µM range, although some affinity partners such as biotin-streptavidin may have dissociation constants in the fM range [28-31]. However, binding affinity alone does not reflect the entire scenario with respect to attachment. Kinetic barriers to desorption have been observed for some fusion tags including certain Carbohydrate Binding Modules (CBM) even with K_D values in excess of 1 µM [31, 32]. Also, removal of co-factors (e.g. CaCl_2 in CBP-tag systems), addition of common compounds (e.g. chelators to His-tag systems, NaOH in Strep-tag systems) or even the bioprocessing reaction conditions (e.g. presence of denaturing organic solvents) can lead to leaching of the protein from the support [33].

Another factor of paramount importance for economic viability of immobilized enzyme system is support regeneration that involves removal of the used enzyme while keeping the binding properties of the support intact for reuse. In order to enable support recovery and reuse, the interaction between the fusion tag and binding partner should be reversible enough to be disrupted under controlled conditions without altering the properties of binding partner to allow subsequent bindings. Several fusion-tagged systems can be regenerated by elution. For example, elution of CBM-3 tagged proteins from cellulose supports is carried out by applying ethyl glycol or glycerol, and 2-[4′-hydroxy-benzeneazo] benzoic acid (HABA) is used for regenerating strep-tag binding supports [33-36]. The cost of the support is also a concern for an immobilized system. Small tags such as Flag, c-myc
and HA utilize bound antibodies as material supports, which, though efficient, are expensive – restricting the potential applications of such a support [37].

A limited number of fusion-tag immobilized enzymes systems have been explored for bioprocessing applications. Industrially relevant enzymes have been successfully immobilized using streptavidin affinity binding [33, 38-42]. However, it is important to distinguish attachment of biotin to the enzyme (which requires purified protein for chemical modification) from genetic expression of fusion tags [43]. Several researchers have demonstrated the application of CBM-tags for enzyme immobilization [34, 44-47]. Research studies have also been conducted using Halo-tag, DNA-binding tag, calmodulin-tag, maltose-binding tag and charged peptides [48-52]. These studies highlight several aspects of the potential for fusion tags to provide simple directed immobilization, specific purification, consistent orientation, and regeneration. However, there remains a significant knowledge gap regarding the broad application of fusion tagged enzymes for bioprocessing applications, as well as the specific application of the technology for lipase-catalyzed reactions. Thus, utilization of affinity interactions for protein immobilization needs further investigation in order to improve its feasibility for industrial applications.

**Lipases**

Lipases (triacylglycerol acyl-hydrolase) belong to class of hydrolase enzymes that act on carboxylic ester bonds. They play an essential physiological role in lipid digestion by breaking down complex triglycerides (esters derived from glycerol and three fatty acids) into simpler forms such as diglycerides, monoglycerides, fatty acids and glycerol.
In addition to their natural role of hydrolyzing ester bonds, lipases can catalyse a broad spectrum of synthesis reactions under thermodynamically favored conditions of low water activity [53]. Such reactions can be classified into two main types: esterification and transesterification. As seen in Figure 2, esterification reactions involve the formation of covalent linkage between a fatty acid and alcohol resulting in the formation of an ester and release of a water molecule. Thio-esterification and amidation are similar reactions but involve a thiol or an amine as substrates. Transesterification reactions include alcoholysis, acidolysis, aminolysis, and interesterification that involve exchange of alkyl groups between substrates to produce new ester compounds.

**Figure 2. Reactions catalyzed by Lipases** (Reproduced from Lipases and Phospholipases Methods and Protocols, Chapter 1 – Lipases: An Overview, Volume: 861, Year: 2012, Page # 5, Editor : Georgina Sandoval, Copyright 2012, “with permission of Springer”)

Although fatty acid esters can be synthesized chemically, chemical catalysis involves problems such as high energy consumption, generation of non-specific and unwanted byproducts, harsh reaction conditions (high temperature, pressure and alkaline catalyst) and frequent regeneration of catalyst. Esterification using lipases confers several benefits over
chemical routes such as milder conditions, enhanced specificity resulting in fewer side products, high selectivity, environmentally friendlier, low-energy demanding operation and easier downstream processing leading to reduction in overall operation costs.

The mechanism underlying lipase catalysis is illustrated in Figure 3 [55]. Lipases possess catalytic triad composed of serine, histidine and aspartate or glutamate. In the first step, activation of serine occurs by deprotonation, for which histidine and aspartate are required (Figure 3a). This increases the nucleophilicity of the hydroxyl residue of serine and allows it to attack the carbonyl group of the incoming substrate forming an acyl-enzyme intermediate (Figure 3b). In the next step, deacylation occurs due to attack of a nucleophile (such as H₂O or monoglyceride) on the acylated enzyme resulting in product release and regeneration of the catalytic site for next series of reaction.

![Mechanism of Lipase Catalysis](image)

**Figure 3.** Mechanism of Lipase Catalysis (Reprinted from Advances in Colloid and Interface Science 2009, 147-48, Reis, P., Holmberg, K., Watzke, H., Leser, M. E. and Miller, R., Lipases at interfaces: A review, 237-250, Copyright (2008), with permission from Elsevier)
High versatility of lipases owing to their wide substrate acceptance, selectivity, stability in many organic solvents, ability to catalyze reactions without the need for expensive co-factors, easy production and activity in a wide range of conditions makes them attractive biocatalysts for the production of several industry relevant compounds such as free fatty acids, glycerol, flavors, structured lipids, phytosterol esters, antioxidant esters, improved biofuels, biodegradable polyesters and many others [7, 56, 57].

Though lipases present a great potential for use in industries, commercial implementation of lipase-catalyzed processes is still far from satisfactory. Various issues need to be addressed to optimize the processes for lipase based bio-catalysis such as solvent recycling, separation and reusability of lipases, water and side products removal from the reaction and cost estimation of the overall process. In addition, several strategies need to be developed to improve biochemical properties of lipases such as regioselectivity, substrate specificity, solvent tolerance, productivity, catalytic efficiency and pH and temperature stability for industrial applications. To address these challenges, use of genetic engineering, development of novel supports and enzyme immobilization methods represent a major area of research in this field.

Immobilization of various lipases has been performed on different material supports to enhance the catalytic activity of lipases, especially in organic media [14]. Commonly used lipase immobilization systems are based on the adsorption or covalent attachment of lipase to solid supports, or entrapment in gel matrices [14, 58]. Although some of these approaches have shown considerable lipase activation effects, maximal activation potential of immobilized lipase system is yet to be achieved. An ideal immobilized lipase preparation should show no enzyme leakage after immobilization, no lipase inactivation during and after
immobilization and negligible mass transfer limitations [14]. Additional factors that influence the development of efficient lipase immobilization system for large scale industrial applications include cost and methods of enzyme preparation, unique properties of each lipase, cost of material supports and long term operational stability in bioreactors. In view of the development of an efficient lipase immobilized system, it is essential that continuous research work needs to be performed in order to optimize immobilization techniques and procedures and gain fundamental knowledge on the physicochemical factors affecting the activity and stability of immobilized lipases.

1.4 Research Objectives

The overall goal of this research work is to develop an immobilized enzyme system using fusion technology and to gain an understanding about various factors affecting the performance of fusion tag based enzyme immobilization system. The specific focus of this research is the immobilization of lipase (Candida antarctica lipase B; CALB) using Strep-tag II fusion technology. This system has been targeted due to the versatility of CALB for the production of industry relevant compounds, the current hurdles to commercial application of lipase-catalyzed production of bio-based compounds and potential benefits associated with the use of Strep-tag II for recombinant protein purification and immobilization. The specific objectives of this research are outlined below.

1. Develop a Strep-tag II construct for the active expression of Strep-tag II Lipase B from Candida antarctica (CALB). Plasmid for the expression of lipase (C. antarctica Lipase B) with Strep-tag II at C-terminus was constructed and analyzed by
DNA sequencing. Expressed enzyme was characterized with respect to expression level, purified using Strep-tag II affinity chromatography and tested for enzyme activity. Successful completion of this objective resulted in the production of recombinant Strep-tag II CALB in pure and catalytically-active form.

2. **Immobilize Strep-tag II CALB on Strep-Tactin material support and define system capabilities.** Purified recombinant Strep-tag II CALB was immobilized on Strep-Tactin matrix using the principle of affinity interaction and protein loading, leaching, enzyme kinetics and stability of activity were evaluated. Successful completion of this objective resulted in lipase that can be selectively immobilized to affinity support material and is catalytically active in the immobilized form.

Collectively, these research outcomes yield new insights about fundamental knowledge of fusion tags for protein immobilization. This is expected to contribute to advances in the development of fusion tag based enzyme immobilization systems for industrial applications.
1.5 References


[38] Huang, X. L., Walsh, M. K., Swaisgood, H. E., Simultaneous isolation and immobilization of streptavidin-beta-galactosidase: Some kinetic characteristics of the


CHAPTER 2

EXPRESSION, PURIFICATION AND CHARACTERIZATION OF STREP-TAG II LIPASE B FROM Candida antarctica (CALB)

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2.1 Abstract

Recombinant extracellular Strep-tag II Candida antarctica lipase B (CALB) was produced in an Escherichia coli expression system. The mature portion of the CALB gene was cloned into pASG-IBA2 vector to generate pASG-IBA2 CALB construct with OmpA signal sequence at the N-terminus and Strep-tag II at the C-terminus. Strep-tag II CALB was successfully expressed in E. coli NEB (New England BioLabs) Express cells using a tetracycline promoter (tet P) system. The recombinant CALB was found to be released into the culture supernatant and showed the highest specific activity in the culture supernatant in comparison to the soluble and insoluble fractions. Catalytically active recombinant Strep-tag II CALB was purified from the culture supernatant using Strep-Tactin affinity chromatography, yielding 140 µg of purified recombinant CALB from 500 mL of culture. Under the standardized conditions, the Strep-tag II CALB in the culture supernatant was
found to be ~ 3 mg/L. The kinetic parameters of the recombinant Strep-tag II CALB towards p-nitrophenyl butyrate were compared with those of the commercial CALB. Purified Strep-tag II CALB was found to have higher catalytic efficiency than the commercial CALB under the conditions tested. This work represents the first illustration of extracellular production of Strep-tag II CALB enzyme using an *E. coli* expression system.

### 2.2 Introduction

Lipases (triacylglycerol acyl-hydrolases) belong to class of hydrolase enzymes that act on carboxylic ester bonds. They play an essential physiological role in lipid digestion by breaking down complex triglycerides (esters derived from glycerol and three fatty acids) into simpler forms such as diglycerides, monoglycerides, fatty acids and glycerol. In addition to their natural role of hydrolyzing ester bonds, lipases can catalyse a broad spectrum of synthesis reactions (esterification and transesterification) under thermodynamically favored conditions of low water activity [1]. Production of industry relevant compounds using lipases confers several benefits over chemical routes such as milder conditions, enhanced specificity resulting in fewer side products, high selectivity, environmentally friendlier, low-energy demanding operation and easier downstream processing leading to reduction in overall operation costs [2]. High versatility of lipases owing to their wide substrate acceptance, selectivity, stability in many organic solvents, ability to catalyze reactions without the need for expensive co-factors, convenient production and activity in a wide range of conditions makes them highly attractive for potential applications in food, dairy,
pharmaceutical, agrochemical, oleochemical, detergent, cosmetics, paper and textile industries [3, 4].

Due to its excellent features such as broad substrate specificity, high stereoselectivity in aqueous as well as organic media, enantio preference and high thermostability, Lipase B from yeast *Candida antarctica* (CALB) represents one of the most widely used lipases in industrial biotransformation applications over other lipases [5]. Thus, the use of genetic engineering approaches to further enhance the properties of CALB is a promising and challenging area of research. One such strategy is the inclusion of fusion tag in the protein of interest. As the name suggests, fusion tags are genetically coded protein or peptides that can be expressed as attached moieties to the target protein or enzyme. These tags usually display a high affinity for a specific biological or chemical ligand. The use of fusion tags for recombinant protein expression and purification has been observed to confer several advantages such as improvement in protein yield, preventing proteolysis, facilitating protein refolding and enhancing protein solubility [6, 7]. Fusion tags also have the potential to provide specific immobilization with repeatable attachment of the enzyme, eliminate the need for purification and post-modification and enable dissociation of spent enzyme and regeneration of the material support [8, 9].

Several fusion tags have been successfully employed for the production of recombinant CALB. Larsen, M.W. et al., have demonstrated the purification of polyhistidine tagged recombinant CALB from different *E. coli* strains by Immobilized Metal Affinity Chromatography (IMAC) [10]. However, they also detected several other proteins in the purified protein samples, which were not completely removed by the IMAC purification procedure. The reason behind this observation could be that the presence of naturally
occurring histidine rich regions in host proteins may result in non-specific protein binding during IMAC purification [11]. CALB fusion with large protein affinity tags such as Glutathione-S-transferase (GST), Cellulose Binding Domain (CBD) and Maltose Binding Protein (MBP) has been observed to improve the enzyme solubility in E. coli, yet it negatively affected the activity of the recombinant enzyme [12-14]. This suggests that large size fusion tags can lead to conformational changes in the recombinant enzyme thereby interfering with its substrate accessibility [13]. In addition, large fusion tags may also put heavy metabolic burden on the host organism during overproduction of the recombinant protein [7]. Therefore, it is essential to explore various other fusion tags to improve the production of catalytically active CALB in E. coli expression system.

Previous research has shown successful purification of thermostable and organic solvent stable lipase from Bacillus sp. strain 42 in a single step using Strep-tag II affinity chromatography system [15]. The resulting Strep-tag II lipase 42 was found to be most active at 70 °C and pH 8.0, and stable in a broad pH range 7-10 and organic solvents with low partition coefficient. This research work highlights that, with an improved recovery of purification, Strep-tag II fusion system gives better specificity than His-tag system. Strep-tag II is a short synthetic peptide comprising eight amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys). This sequence can be fused to recombinant proteins at the N- or C-terminus and displays intrinsic affinity towards Strep-Tactin, a specifically engineered form of streptavidin [16]. There are several potential benefits associated with the use of Strep-tag II over other fusion tags that make it an attractive choice for the generation of recombinant proteins [17]. Strep-tagged proteins can be isolated in one step from crude cell lysates or culture media by utilizing the highly specific interaction of Strep-tag II and streptavidin. This is carried out
with competitive elution using D-desthiobiotin that results in low background. Strep-tag II elutes under gentle, physiological conditions, thus making it suitable for the production of functional proteins. The proteins obtained are biologically active and exhibit high purity levels of above 95%. In addition, mild elution conditions and low washing volumes allow isolation of protein complexes and thus are favorable for protein:protein interaction studies. Being a small tag with neutral pI, it does not affect protein folding or function and thus removal of the tag from the recombinant protein is not essential. Unlike His-tag, Strep-tag II is independent from metal ions in the purification and thus is of interest when metalloproteins have to be studied. Robust Strep-tactin resins can also be regenerated and reused several times for purification. Also, the Strep-tag system enables protein detection in various assays. Strep-tag proteins can be also used for immobilization with a specific high affinity antibody or Strep-Tactin resin on microplates or biochips for qualitative and quantitative assays.

Given the above stated advantages, we employed Strep-tag fusion technology for the first time to establish a simple *E. coli* based expression system for the expression and production of recombinant Strep-tag II CALB. We demonstrate that expression of the mature portion of the CALB gene fused with an OmpA signal sequence at N-terminus and Strep-tag II at C-terminus enables the extracellular production of the recombinant CALB enzyme. The simple shaking-flask *E. coli* cultivation system followed by Strep-tactin resin based affinity purification procedure yields the recombinant lipase in highly pure and catalytically active form. We also compared the kinetic properties of the recombination Strep-tag II CALB with commercially available CALB and found the recombinant Strep-tag II enzyme to possess much higher catalytic ability than the commercial CALB. With these
features, this Strep-tag II lipase construct could represent an example of recombinant enzyme that is amenable to future rational design and scale up strategy, for potential applications in industrial biocatalysis.

2.3 Materials and Methods

2.3.1 Materials

pASG-IBA2 Star Gate Acceptor Vector, Strep-Tactin Superflow (high capacity) resin and Strep-tag protein purification buffer set were purchased from IBA Life Sciences. p-Nitrophenyl butyrate (p-NPB) was from Sigma. pET 20b(+) vector was a kind gift from Professor Robert S. Haltiwanger at Complex Carbohydrate Research Center, The University of Georgia. Oligonucleotides were from Integrated DNA Technologies. Fast digest Restriction endonucleases (Esp3I, Hind III, NdeI, Xho I) and T4 DNA Ligase enzymes were from Thermo Fisher Scientific. Q5 Hot Start High-Fidelity DNA Polymerase, Deoxynucleotide (dNTP) Solution Mix, Shrimp Alkaline phosphatase, NEB® 5-alpha Competent E. coli (Subcloning Efficiency), NEB® Express Competent E. coli (High Efficiency) and T7 Express lysY/Iq Competent E. coli (High Efficiency) were purchased from New England BioLabs. Arctic Express (DE3) Competent Cells were from Agilent Technologies. Ampicillin was from G-Biosciences. Anhydrotetracycline hydrochloride, 4-morpholineethanesulfonic (MES) acid monohydrate and MES sodium salt were from Acros Organics. Isopropyl-b-D-thiogalactopyranoside (IPTG), gentamycin sulfate and Blue-Clean Protein Stain were purchased from IBI Scientific. Coomassie G-250 (Bradford) dye and bovine serum albumin were purchased from Thermo Scientific. 12% precast polyacrylamide
gels for use with Mini-PROTEAN Electrophoresis Cells, Poly-Prep Chromatography Columns (2 ml bed volume and 10 ml reservoir), Precision Plus Protein All Blue Standards and Precision Plus Protein Dual Color Standards were from Bio-Rad. Commercial CALB was from Chiral Vision.

2.3.2 Construction of plasmids

CALB gene was custom-synthesized by GeneScript (USA) based on the amino acid sequence of mature portion of original CALB gene from *C. antarctica* (LF 058, GenBank accession no.: Z30645.1) and was codon-optimized by taking into account the codon usage of the *E. coli* B strain. This gene was amplified by Polymerase Chain Reaction (PCR) using two phosphorothioate protected primers: Forward Primer - 5’-AGC GCG TCT CCA ATG CTG CCG TCT GGT AGC G*A-3’ and Reverse Primer - 5’AGC GCG TCT CCT CCC CGG CGT CAC GAT ACC A*G-3’ where * indicates phosphorothioate bond between the last two nucleotides. The amplified PCR product and pASG-IBA2 empty vector were digested with Esp3I restriction endonuclease. Esp3I digested PCR product and vector were further digested with DpnI and Shrimp Alkaline Phosphatase, respectively. The digested PCR product and vector from these reactions were then ligated together using T4 DNA ligase to generate pASG-IBA2-CALB construct. Figure 1A shows the structure of this construct. The plasmid contains the mature portion of the CALB gene with OmpA signal sequence at its N-terminus and Strep-tag II at its C-terminus. The sequence of the construct was confirmed using Sanger DNA Sequencing performed at the Iowa State University DNA Facility.
For IPTG inducible expression of Strep-tag II CALB in *E. coli* cells, the mature CALB gene with OmpA signal sequence at its N-terminus and Strep-tag II at its C-terminus from the above generated pASG-IBA2-CALB was amplified by PCR using the primers: Forward Primer 5’ – ATC TAG CATATG AAA AAG ACA GCT ATC – 3’ and Reverse Primer 5’ – ATC TTA CTC GAG TTA TTT TTC GAA CTG CG – 3’. The amplified PCR product and pET20b(+) empty vector were then digested with restriction endonucleases NdeI and XhoI. The digested PCR product and vector were further digested with DpnI and Shrimp Alkaline Phosphatase respectively. The products from these reactions were then ligated to produce pET20b(+) CALB construct containing the mature portion of the CALB gene with OmpA signal sequence at its N-terminus and Strep-tag II at its C-terminus (Figure 1B). The construct sequence was verified using DNA Sanger Sequencing.

**Figure 1.** Structure of Strep-tag II CALB-expressing plasmid. (A) Schematic diagram of the expression cassette of pASG-IBA2-CALB-Strep-tag II. Only the part from the tet promoter (tetP) to Strep-tag II is shown. Esp3I restriction sites were used for cloning, however, these sites are not retained after cloning. (B) Schematic diagram of the expression cassette of pET20b (+)-CALB-Strep-tag II. Only the part from the T7 promoter (T7P) to Strep-tag II is shown. (C) Nucleotide sequences of OmpA signal-mature CALB gene-Strep-tag II with the corresponding amino acid sequence.
Figure 1. (Continued)
2.3.3 Culture Media

For cultivation of the recombinant *E. coli* strains, the following sterilized culture media were used: Luria–Bertani (LB; 1% tryptone, 0.5% yeast extract, 1% NaCl) for plasmid extraction and Terrific Broth (TB; 1.2% tryptone, 2.4% yeast extract, 0.5% glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄) for expression of Strep-tag II CALB. Ampicillin (Ap) was used as a selection antibiotic at 100 µg/mL. For the overnight culture of Arctic Express (DE3) *E. coli* cells, gentamycin sulfate was used at 20 µg/mL in addition to Ap.

2.3.4 Expression of recombinant Strep-tag II CALB

A sample (60 µL) of an overnight growth culture of the recombinant strain NEB® Express *E. coli* harboring pASG-CALB was inoculated into 6 mL of fresh TB medium supplemented with 100 µg/mL Ap. The cells were cultivated aerobically at 37°C until reaching an O.D. of 0.5-0.6 at 550 nm. Anhydrotetracycline hydrochloride was added to give a final concentration of 200 ng/ml, and the cultivation continued at 20°C for 24, 48, or 72 h. The culture was centrifuged to separate the supernatant and the cell pellet. The cell pellet was re-suspended in 0.6 mL of Buffer P (100 mM Tris/HCl pH 8.0, 500 mM sucrose and 1 mM EDTA), disrupted by sonication on ice with 10 seconds pulses for 5 times at 50 Amplitude using ultrasonic processor (QSonica Sonicators Part No. Q55) and centrifuged at 14000 X g for 10 minutes, and the supernatant containing cellular soluble proteins was recovered. The precipitate containing cellular insoluble proteins was re-suspended in 0.6 mL of Buffer P. The obtained fractions (i.e., the culture supernatant, the cellular soluble fraction and the cellular insoluble fraction) were used for further analysis.
Similar procedure was carried out for the expression of Strep-tag II CALB in the recombinant strains T7 Express lysY/l and Arctic Express (DE3) *E. coli* harboring pET20b(+)CALB construct with the modifications as follow. For both of these strains, Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 0.5 mM for the induction of expression. In case of Arctic Express (DE3) cells, overnight culture used for inoculation was grown in TB media containing Ap and gentamycin sulfate at concentrations stated before. After induction with IPTG, the cultivation of Arctic Express (DE3) *E. coli* cells was carried out at 10°C.

### 2.3.5 Purification of recombinant Strep-tag II CALB from *E. coli*

The recombinant NEB® Express *E. coli* strain harboring pASG-CALB was cultivated, and expression of the target gene was induced at 20°C for 24 h in 1500 mL of TB. The cells were removed by centrifugation and filtration, and the culture supernatant was recovered. 500 ml of the culture supernatant was, subsequently, used for purification. The supernatant was concentrated to approximately 20 mL with a centrifugal filtration device (Centricon® Plus-70 Centrifugal Filter Units, molecular weight cut off 10 kDa, EMD Millipore). Purification was performed under refrigeration at 4°C by selective binding of engineered streptavidin (i.e. Strep-Tactin) to Strep-tag II fusion protein. For this purification, 800 μl of 50% suspension of Strep-Tactin Superflow (high capacity) resin was taken to get a Strep-Tactin gravity column with a bed volume of 400 μl. The Strep-Tactin column was equilibrated with 2 CVs (column bed volumes) of Buffer W (100 mM Tris/HCl pH 8.0, 150 mM NaCl and 1 mM EDTA). The concentrated culture supernatant expressing Strep-tag II CALB was filtered using 0.45 μm PVDF syringe filter to remove insoluble aggregates and then added to the column. The column was washed 5 times with 1 CV Buffer W, after the
culture supernatant completely entered the column. The washes were collected in fractions having a size of 1 CV. After washing the column eight times, 0.5 CVs Buffer E (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA and 2.5 mM D-des thiobiotin) was added to the column and the eluate was collected in 0.5 CV fractions. The culture supernatant, wash fractions, and elution fractions were analyzed by SDS-PAGE. Elution fractions containing purified Strep-tag II CALB were pooled and Bradford protein estimation was done on pooled eluate. Further, D-des thiobiotin and EDTA were removed by passing the purified sample through centrifugal filtration device (Amicon® Ultra 0.5mL Filters, molecular weight cut off 10 kDa, EMD Millipore) and bringing it back to its original volume with Buffer W. Both non-desalted and desalted purified Strep-tag II CALB were used for subsequent experiments. Commercial CALB in Buffer W was purified using centrifugal filter device and used as a positive control for lipase assay experiments. Protein concentration was estimated by Bradford method.

### 2.3.6 Lipase Assay

Lipase activity was measured using p-NPB as a substrate at 0.5 mM final concentration in 10 mM MES Buffer pH 7. The progress of the hydrolysis was monitored spectrophotometrically at 400 nm at 37°C for 15 min. Kinetic analysis of the lipase was performed similarly with varying substrate concentrations (0.1 – 2 mM p-NPB). The substrate-versus-velocity graphs were plotted and fitted to the Michaelis-Menten equation by a non-linear regression algorithm using Prism software (Graph Pad, USA) to determine Michaelis constant ($K_m$) and maximum velocity ($V_{max}$). To calculate turnover number ($k_{cat}$), $V_{max}$ was divided by the initial enzyme concentration assuming a molecular weight of 33 kDa for the enzyme.
2.4 Results and Discussion

2.4.1 Expression of Strep-tag II CALB

The expression of Strep-tag II CALB in the recombinant Arctic Express (DE3) *E. coli* cells in TB medium was evaluated before and after induction with IPTG at 10°C for 24, 48 and 72 hours (Figures 2A and 2B). For all cultivation times, non-induced cells yielded target protein in both the intracellular insoluble fraction and the soluble fraction suggesting that there was leaky expression of recombinant CALB. The possible reason for leaky expression in these cells is that expression of the recombinant CALB in these cells is under the control of strong T7 promoter that could result in high level transcription of the target gene even under basal conditions [18]. In case of induced cells, similar pattern was observed with a portion of recombinant CALB detected in the culture supernatant only after 72-hour induction. In addition, for all cultivation times a thick band of ca MW 60 kDa was seen in all the fractions for both non-induced and induced cells. It is expected that this protein band corresponds to cold adapted chaperonin protein Cpn 60 that gets co-expressed in Arctic Express (DE3) *E. coli* cells to improve the processing of recombinant protein at low induction temperatures [19, 20]. Since leaky expression of Strep-tag II CALB was found along with high levels of co-expression of Cpn 60 in the recombinant Arctic Express (DE3) *E. coli* cells, these cells were not employed for subsequent purification of Strep-tag II CALB. Similarly, leaky expression of Strep-tag II CALB was also detected in non-induced T7 Express lysY/I* E. coli* (Figure 3A). In addition, a majority of the target protein was seen in the insoluble fraction under both non-induced and induced conditions when using this system (Figures 3A and 3B). This result is attributed to high level of T7 driven expression of the target protein that puts a heavy metabolic burden on the recombinant host and may lead to
improper folding of the recombinant protein, thereby directing it to inclusion bodies [21].

For these reasons, these cells were also not used for further study.

**Figure 2.** Effect of Induction Time on Strep-tag II CALB Expression in recombinant Arctic Express (DE3) *E. coli* cells. Recombinant Arctic Express (DE3) *E. coli* cells harboring pET20b(+)–CALB–Strep tag II plasmid were grown in Terrific Broth media without (A) and with induction (B) by IPTG (at final concentration of 0.5 mM) at 10°C for 24, 48 and 72 hours. SDS-PAGE analysis for expression of Strep-tag II CALB was done on different fractions – culture supernatant (C), cellular soluble fraction (S) and cellular insoluble fraction (I) on a 12% polyacrylamide gel along with the molecular size marker (lane M) and commercial CALB (lane *). Protein bands were visualized with Blue-Clean Protein Stain. The arrows indicate the position of CALB (MW ~ 33 kDa) and chaperonin Cpn60 (MW ~ 60 kDa) on the gels.
Figure 3. Effect of Induction Time on Strep-tag II CALB Expression in recombinant T7 Express lysY/Iq E. coli cells. Recombinant T7 Express lysY/Iq E. coli cells harboring pET20b(+)CALB- Strep tag II plasmid were grown in Terrific Broth media with (A) and without induction (B) by IPTG (at final concentration of 0.5 mM) at 20°C for 24, 48 and 72 hours. SDS-PAGE analysis for expression of Strep-tag II CALB was done on different fractions – culture supernatant (C), cellular soluble fraction (S) and cellular insoluble fraction (I) on a 12% polyacrylamide gel along with the molecular size marker (lane M) and commercial CALB (lane *). Protein bands were visualized with Blue-Clean Protein Stain. The arrows indicate the position of CALB (MW ~ 33 kDa) on the gels.

Figure 4 shows the expression of Strep-tag II CALB by the recombinant strain NEB express E. coli in TB medium before and after induction with anhydrotetracycline hydrochloride at 20°C for 24, 48 and 72 hours. For all cultivation times, it was observed that non-induced cells yielded target protein exclusively in the intracellular insoluble fraction whereas induced cells produced target protein in the soluble fraction as well as in the culture
supernatant (Figures 4A and 4B). However, prolonging the induction time did not lead to increase in expression levels of the recombinant Strep-tag II CALB in different fractions. Previous research suggests that although periplasmic signal sequence is expected to direct CALB in the periplasm, the enzyme may also appear in the culture supernatant [22]. This phenomenon is expected to occur when the amount of the accumulated overexpressed protein in *E. coli* surpasses the threshold amount that can be retained inside the periplasm, resulting in escape of the enzyme from the periplasm into the culture medium with the rupturing of the outer periplasmic membrane. Unlike previously used *E. coli* systems, some fraction of Strep-tag II CALB in the soluble form (soluble fraction as well as culture supernatant) without leaky expression and co-expression of large amount of chaperone proteins was observed when NEB express *E. coli* system was used. Therefore, this system was employed for subsequent enzyme activity studies.

The activity of the expressed Strep-tag II CALB protein was confirmed by determining the enzyme activity in all the three fractions from NEB express *E. coli* cells. Figure 5 shows that the target protein was active in all the fractions induced for different times. We observed that specific activity of Strep-tag II CALB increased to some extent in the culture supernatant and decreased in the insoluble fraction with not much change in specific activity in the soluble fraction upon prolonging the induction time to 48 h and 72 h. However, since the increase in specific activity of the culture supernatant was minimal on enhancing the induction time, the induction time of 24 hours was selected for expression of Strep-tag II CALB in NEB Express *E. coli* cells for subsequent experiments.
Figure 4. Effect of Induction Time on Strep-tag II CALB Expression in recombinant NEB (New England Biolabs) Express E. coli cells. Recombinant NEB express E. coli cells harboring pSAG-IBA2-CALB-Strep tag II plasmid were grown in Terrific Broth media without (A) and with induction (B) by anhydrotetracycline (at final concentration of 200 ng/ml) at 20°C for 24, 48 and 72 hours. SDS-PAGE analysis for expression of Strep-tag II CALB was done on different fractions – culture supernatant (C), cellular soluble fraction (S) and cellular insoluble fraction (I) on a 12% polyacrylamide gel along with the molecular size marker (lane M) and commercial CALB (lane *). Protein bands were visualized with Blue-Clean Protein Stain. The arrows indicate the position of CALB (MW ~ 33 kDa) on the gels.
Figure 5. Effect of Induction Time on Strep-tag II CALB Specific Activity in recombinant NEB (New England Biolabs) Express E. coli cells. Strep-tag II CALB expression was induced in recombinant NEB express E. coli cells harboring pSAG-IBA2-CALB- Strep tag II plasmid by anhydrotetracycline (at final concentration of 200 ng/ml) at 20°C for 24, 48 and 72 hours. Different fractions – culture supernatant (C), cellular soluble fraction (S) and cellular insoluble fraction (I) were tested for activity using 0.5 mM p-Nitrophenyl butyrate at 37 °C in 10 mM MES buffer (pH 7.0) for 15 minutes. Protein estimation was done using Bradford method with bovine serum albumin as a standard. Enzyme activity values were converted to specific activity values. Specific activity shown is an average from triplicate measurements of each sample. Results are representative of duplicated experiments.

2.4.2 Purification of Strep-tag II CALB from E. coli culture

Strep-tag II CALB protein was purified from the culture supernatant of the recombinant NEB Express E. coli cells using Strep-Tactin affinity chromatography. The use of Strep-Tactin column was effective in enabling recovery of the target protein in pure form while the impurities passed in the flow through and wash fractions (Figures 6A and 6B). The purified Strep-tag II CALB gave a single band on SDS-PAGE with a molecular mass of ca 33 kDa (Figure 6B). From a 500 mL culture concentrated to ca 20 mL, Strep-tactin affinity purification procedure yielded 140 µg of purified Strep-tag II CALB, when a 400 µl column
bed volume was utilized. The summary of the purification procedure is outlined in Table 1. Since the target protein was detected in the flow-through fraction, it can be reasoned that the volume of the Strep-Tactin resin used was not sufficient for complete recovery of the recombinant Strep-tag II CALB from the culture supernatant. The purified Strep-tag II CALB showed specific activity of 301.26 µmoles/min/mg of protein (Table 1). Total enzyme activity in the culture supernatant was found to be 1359.45 µmoles/min (Table 1). Using these values, the amount of CALB in the crude culture supernatant was estimated to be ca 3 mg of Strep-tag II CALB per liter of culture supernatant under the conditions described above.

Figure 6. Purification of Strep-tag II CALB from recombinant NEB Express E. coli cells culture media. Recombinant NEB express E. coli cells harboring pASG-CALB- Strep tag II
plasmid were grown in Terrific Broth media with induction by anhydrotetracycline (at final concentration of 200 ng/ml) at 20°C for 24 hours. Culture Supernatant was collected for purification of Strep tag II CALB using Strep Tactin affinity chromatography. Protein samples in each of the fractions collected during purification were analyzed by SDS-PAGE on a 12% polyacrylamide gel along with the molecular size marker (lane M) and commercial CALB (lane *). Protein bands were visualized with Blue-Clean Protein Stain. The arrows indicate the position of CALB (MW ~ 33 kDa) on the gels. Lanes in (A): 1 – Culture Supernatant, 2 – Filtered Culture Supernatant, 3 – Concentrated Culture Supernatant, 4 – Flow Through after concentration, 5 – Flow Through from column purification, 6 to 10 – Wash fractions 1 to 5. Lanes in (B): 1 to 8 – Elution Fractions 1 to 8.

**Table 1.** Summary of purification of Strep-tag II CALB from recombinant *E. coli*

Units = µmoles/min

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<th>Purification Step</th>
<th>Total Activity (Units)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (Units/mg)</th>
<th>Activity Yield (%)</th>
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</table>

**2.4.3 Catalytic Properties of Purified Strep-tag II CALB**

Figure 7 demonstrates that specific activity of purified Strep-tag II CALB (both before and after desalting) was greater than that of commercial CALB. Minimal difference
was detected in the specific activity of purified Strep-tag II CALB before and after desalting (removal of D-desthiobiotin) indicating that presence of D-desthiobiotin does not have much influence on the specific activity of the purified recombinant enzyme. Further, Table 2 shows the comparison of kinetic parameters of commercial CALB and purified Strep-tag II CALB (before and after removal of D-desthiobiotin) on the hydrolysis of p-NPB. The data suggests that the apparent $K_m$ of purified Strep-tag II CALB (before and after desalting) was comparable to that of commercial CALB. The apparent turnover number ($k_{cat}$) and catalytic efficiency of purified Strep-tag II (for both before desalting and after desalting) were higher than that of commercial CALB. These results confirmed that the recombinant Strep-tag II CALB had greater catalytic ability than that of the commercial CALB. The reason for this greater activity could be attributed to the presence of Strep-tag II in the recombinant CALB that allows elution of the recombinant protein in highly purified form under gentle and physiological purification conditions that may aid in preserving or improving protein functionality [17, 23]. Additionally, commercial CALB is available in dry form and thus its downstream processing may also affect its catalytic activity.
Figure 7. Comparison of Specific activity of Commercial CALB and Strep-tag II CALB purified from recombinant NEB Express *E. coli* cells culture media. Purified commercial CALB and Strep-tag II CALB (before and after desalting) were tested for activity using 0.5 mM p-Nitrophenyl butyrate at 37 °C in 10 mM MES buffer (pH 7.0) for 15 minutes. Protein estimation was done using Bradford method with bovine serum albumin as a standard. Enzyme activity values were converted to specific activity values. Specific activity shown is an average from triplicate measurements of each sample. Results are representative of duplicated experiments.

Table 2. Apparent Kinetic Parameters of Commercial CALB and purified Strep-tag II CALB. Values represent average of triplicate measurements. Results are representative of duplicated experiments.

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat}/K_m$ (x 10$^3$ M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial CALB</td>
<td>0.35</td>
<td>49.43</td>
<td>141.23</td>
</tr>
<tr>
<td>Purified Strep-tag II CALB</td>
<td>0.32</td>
<td>215.33</td>
<td>672.91</td>
</tr>
<tr>
<td>Purified desalted Strep-tag II CALB</td>
<td>0.16</td>
<td>148.73</td>
<td>929.56</td>
</tr>
</tbody>
</table>
2.5 Conclusion

In summary, we have described the extracellular production of Strep-tag II CALB from a recombinant strain of *E. coli*. The recombinant Strep-tag II CALB could be recovered from the culture supernatant and purified using Strep-Tactin affinity chromatography, producing ca 140 µg of purified Strep-tag II CALB per 500 mL of culture when 400 µl Strep-Tactin resin bed volume was used. The amount of Strep-tag II CALB in the crude culture supernatant was estimated to ~ 3 mg/L under the conditions used. The purified Strep-tag II CALB showed a higher catalytic efficiency when compared to the commercial enzyme though the difference in their $K_m$ values was not significant. The combination of the following five features were employed to yield successful Strep-tag II CALB production from our expression system: (i) the OmpA signal sequence preceding the mature portion of CALB for periplasmic localization of the enzyme; (ii) a codon optimized CALB gene for expression in *E. coli* [22]; (iii) a low temperature for inducing expression; (iv) a good culture medium (i.e., TB) [22] and; (v) presence of C-terminal Strep-tag II. Given these features, this system represents a promising approach that can be optimized for future rational design to conduct lipase engineering studies to improve biochemical properties of lipases and thus for the large-scale production of catalytically active recombinant lipases for potential applications in food and biotechnological industries.

2.6 Acknowledgement

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and his laboratory members for providing pET 20b(+) as a kind gift. The authors gratefully acknowledge Deepika Vasudevan for assistance with molecular cloning protocol.
2.7 References


CHAPTER 3

STREP-TAG II FUSION TECHNOLOGY FOR THE IMMOBILIZATION OF
LIPASE B FROM Candida antarctica (CALB)

A paper to be submitted

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3.1 Abstract

While the potential uses of immobilized enzymes in food processing and biomanufacturing are well known, practical applications have remained elusive. Current approaches to immobilization are limited in terms of economics, efficiency, and efficacy. Ideally, an expressed enzyme would be rapidly immobilized in a strong and specific fashion without involving advanced expertise in bioconjugate chemistry. Further, the ability to remove spent enzyme and reuse a support is desirable to enable cost reduction. Fusion tags have been employed in molecular biology as a means to purify a desired protein from other proteins associated with the fermentation product though the use of affinity interactions. However, there are ample opportunities to employ this technology to improve enzyme immobilization. There is, however, limited data regarding the use of the fusion tags for enzyme immobilization in bioprocessing applications. In this work, we demonstrate the
use of a Strep-tag II fusion technology for the immobilization of the industry-relevant lipase B from *Candida antarctica* (CALB). The gene encoding mature portion of CALB was codon-optimized and cloned in pASG-IBA2 plasmid for expression in *E. coli*. The expressed recombinant lipase with Strep-tag II at C-terminus was purified using Strep-Tactin (engineered streptavidin) column and further used for immobilization to Strep-Tactin based support using the principle of affinity interaction. Enzyme specific activity and kinetics were evaluated using colorimetric assay. Immobilized enzyme exhibited reduced specific activity and apparent turnover number ($k_{cat}$) in comparison to free Strep-tag II enzyme. Apparent $K_m$ of immobilized lipase was comparable to that of free lipase. In addition, immobilization of the enzyme did not result in enhanced stability providing ca 13 % activity retention after 25 h at 37 °C (pH 7.0). However, immobilized Strep-tag II CALB showed no considerable leakage from the support at 37 °C and pH 7.0. This work provides interesting insights about fusion tag technology for enzyme immobilization, a strategy that is amenable to future rational design and optimization to yield successful immobilized enzyme system for industry relevant enzymes that could surmount the limitations underlying current immobilization technologies.

### 3.2 Introduction

Lipases are one of the most widely used enzymes in industrial biocatalysis for the production of free fatty acids, natural flavors and fragrances, structured lipids, antioxidant esters, sugar esters, polyesters and biofuels [1-6]. Owing to their ability to catalyze diverse reactions under mild conditions, general ease of handling, tolerance to wide variety of
substrates and solvents, thermostability and convenient commercial availability, lipases have attracted significant attention worldwide for bioprocessing applications [7]. However, commercial implementation of lipase-catalyzed processes is still far from satisfactory as the native enzyme is not stable and susceptible to inactivation under processing conditions [8-11]. In order to improve functional properties and specificity of lipases and enable their reusability for industrial applications, various strategies for lipase immobilization have been employed such as physical methods (adsorption, entrapment, encapsulation) and chemical methods (covalent bonding and cross-linking) [2, 8]. However, these current methodologies suffer from one or more of the drawbacks such as mass transfer limitations, chemical modification of the enzyme, reduction in enzyme activity and stability, enzyme leakage and an inability to reuse the support once the enzyme is spent [2, 8]. To address these limitations, development of novel enzyme immobilized systems represents a major area of research in this field.

With significant advances in DNA recombinant technology, a variety of fusion tags with affinity towards a specific biological or chemical ligand have been discovered [12-15]. Commonly used fusion tags include the polyhistidine tag (His-tag), FLAG-tag, Strep-tag, Streptavidin Binding Peptide, GST-tag, Calmodulin-tag, and S-tag, and Carbohydrate Binding Modules (CBMs) [16]. Although originally designed to improve protein production yields, solubility and folding, and to facilitate the detection and purification of recombinant proteins, fusion tags also provide a strategy to improve enzyme immobilization systems [12, 17-19]. Fusion tags have the potential to provide specifically directed immobilization of the target enzyme with repeatable attachment of the enzyme, eliminate the need for purification and post-modification and enable dissociation of spent enzyme and regeneration of the
support [12]. Several factors govern the use of a fusion tag for enzyme immobilization such as solubility and activity of the expressed protein, specificity, binding affinity, leaching, nature of the material support and its regeneration, and cost.

To develop a viable fusion tag based enzyme immobilization system, expression of recombinant enzymes that are soluble and catalytically active is essential. Fusion tags such as Glutathione S-Transferase (GST) and maltose binding protein can improve the solubility of enzymes; however, the larger size of these tags can reduce catalytic activity due to conformational changes and present a high metabolic burden during expression [20, 21]. In addition, specificity of the fusion tag to the binding partner is important to enhance loading of the desired protein from an impure sample as well as to prevent dissociation during processing. Some supports, such as an Immobilized Metal Affinity Chromatography (IMAC) support used for His-tag immobilization, can result in the attachment of undesired proteins [13, 22, 23].

Regeneration (i.e. removal of the enzyme while maintaining the binding properties of the support) is important should the support need to be reused. Several fusion-tagged systems can be regenerated by elution. For example, CBM-3 can be eluted from cellulose supports by ethyl glycol or glycerol, and strep-binding supports can be regenerated using 2-[4'-hydroxy-benzeneazo] benzoic acid (HABA) [24-27]. Leaching of the protein from the support is also a concern for enzymes immobilized by affinity binding. Leaching can be attributed to the binding kinetics between enzyme and support, the presence/absence of cofactors or presence of interfering compounds (e.g. chelators to His-tag systems) [26, 28]
A limited number of fusion-tag immobilized enzymes systems have been explored for bioprocessing applications. Streptavidin affinity binding has been employed to successfully immobilize industrial-relevant enzymes [26, 29-33]. Pioneering work by Kilburn along with subsequent studies by others have demonstrated application of CBM-tags for enzyme immobilization [25, 34-37]. Studies have also been conducted using Halo-tag, calmodulin-tag, maltose-binding tag and charged peptides [38-42]. While these studies demonstrate the potential for fusion tags to provide simple immobilization, specific purification, consistent orientation, and regeneration, there remains a significant knowledge gap regarding the broad application of tagged enzymes for bioprocessing applications, as well as the specific application of the technology for lipase-catalyzed reactions.

In this research work, we focus on utilizing Strep-tag II fusion technology for the immobilization of lipase B from *Candida antarctica*. Strep-tag II was selected due to its various inherent features that are desirable for successful enzyme immobilization [24]. Strep-tag II affinity purification relies on highly specific interaction between Strep-tag II and Strep-tactin resin, thereby minimizing non-specific interaction with other host proteins and enabling the recombinant protein to be obtained in highly pure form [24, 43]. The tag allows the elution of recombinant protein under gentle, physiological conditions, thus making it suitable for the production of biologically active proteins [44]. Strep-tag II is a small peptide tag with neutral pI that is expected to have limited influence on protein folding or function, and thus does not require removal from the recombinant protein after purification. The tag exhibits a dissociation constant (K_D) of ~ 1µM towards Strep-tactin resin, which is desirable for efficient binding to the support [24, 26, 45]. In addition, Strep-tactin resins are robust and their regeneration and reuse can be attained several times for purification [24, 46]. The
objective of this work was to immobilize CALB by employing a Strep-II fusion tag and to evaluate the resulting properties of the immobilized conjugate.

3.3 Materials and Methods

3.3.1 Materials

pASG-IBA2 Star Gate Acceptor Vector, Strep-Tactin Superflow (high capacity) resin and Strep-tag protein purification buffer set were purchased from IBA Life Sciences. p-Nitrophenyl butyrate (p-NPB) was from Sigma. NEB® Express Competent *E. coli* (High Efficiency) cells were purchased from New England Biolabs. Ampicillin was from G- Biosciences. Anhydrotetracycline hydrochloride, 4-morpholineethanesulfonic (MES) acid monohydrate and MES sodium salt were purchased from Acros Organics. Blue-Clean Protein Stain was purchased from IBI Scientific. Coomassie G-250 (Bradford) dye and bovine serum albumin were purchased from Thermo Scientific. 12% precast polyacrylamide gels for use with Mini-PROTEAN Electrophoresis Cells, Poly-Prep Chromatography Columns (2 ml bed volume and 10 ml reservoir), Precision Plus Protein All Blue Standards and Precision Plus Protein Dual Color Standards were from Bio-Rad. Commercial CALB was from Chiral Vision.

3.3.2 Affinity Purification of Strep-tag II CALB from *E. coli*

The mature portion of the CALB gene was cloned into pASG-IBA2 vector to generate a pASG-IBA2 CALB construct with an OmpA signal sequence at the N-terminus and Strep-tag II at the C-terminus. The resulting plasmid was transformed into NEB
Express *E. coli* cells, which were then grown in Terrific Broth (TB) media containing ampicillin (100 μg/mL) for overnight. Then, 1% inoculum was transferred into a fresh TB medium. At an OD$_{550}$ of 0.5–0.6, lipase expression was induced by adding anhydrotetracycline hydrochloride at final concentration of 200 ng/mL. The induced culture was grown at 20 °C, and the culture media was separated from cells by centrifugation and then concentrated using a centrifugal filtration device (Centricon® Plus-70 Centrifugal Filter Units, molecular weight cut off 10 kDa, EMD Millipore). Concentrated culture media was loaded onto Strep-Tactin packed column (with resin bed volume of 4 ml) to purify the Strep-tag II CALB. The purified lipase was quantified with Bradford reagent using bovine serum albumin (BSA) as a standard. The molecular mass and purity were evaluated using 12% precast polyacrylamide gels for sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). Purified Strep-tag II CALB was further concentrated down to ~ 3 mg/ml using centrifugal filtration device (Corning® Spin-X® UF 20mL Centrifugal Concentrator, molecular weight cut off 10 kDa). This was also performed to facilitate desalting by removal of D-desthiobiotin and EDTA from the purified lipase sample. Concentrated and desalted purified Strep-tag II CALB was stored separately in 10 mM MES Buffer (pH 7.0) and Buffer W at –20 °C until further use.

### 3.3.3 Immobilization of Strep-tag II CALB using Strep-Tactin Resin

Enzyme loading was assessed using 40 μL of 50% suspension of Strep-Tactin resin (beads). The beads were centrifuged at 5000 X g for 2 minutes and the supernatant was removed using a syringe needle. Beads were washed with 2 volumes of Buffer W (100 mM Tris/HCl pH 8.0, 150 mM NaCl and 1 mM EDTA) and the buffer was removed after centrifugation using a syringe needle. 25 μL of seven different concentrations (0.1, 0.2, 0.4,
0.8, 3, 6 and 12 µg/µL) of purified Strep-tag II CALB were applied to washed 20 µL beads. The samples were incubated on ice for 30 minutes and supernatant containing the unbound fraction of purified Strep-tag II CALB was recovered using centrifugation as described previously. Bradford protein estimation was done on supernatant to determine the amount of unbound Strep-tag II CALB. This amount was subtracted from the initial amount applied to the beads to calculate the amount of Strep-tag II CALB bound to the beads. The percentage of bound Strep-tag II CALB was plotted against the initial enzyme concentration applied to the beads.

### 3.3.4 Protein Leaching

Protein leaching was determined for the immobilized Strep-tag II CALB sample at 37°C and pH 7.0 as a function of time. Immobilized Strep-tag II CALB samples were prepared as described above using 6 µg/µL of purified enzyme. After recovering the supernatant, beads with immobilized Strep-tag II CALB were suspended in 25 µL of 10 mM MES buffer pH 7.0 and incubated at 37°C. Samples were taken out at different times (0, 3, 6, 9 and 24 h) and supernatants were recovered. Bradford protein assay was performed on the supernatant recovered before the incubation to determine the initial amount of Strep-tag II CALB bound to beads and on the supernatant recovered after the incubation to estimate the amount of Strep-tag II CALB leached from the beads. These values were used to calculate the remaining amount of bound Strep-tag II. Residual amount of bound Strep-tag II CALB and amount of Strep-tag II CALB leached were plotted against the time of incubation.
3.3.5 Enzyme Kinetics

Purified commercial CALB, free Strep-tag II CALB and immobilized Strep-tag II CALB samples were tested for activity using 0.1 – 2 mM p-NPB at 37°C in 10 mM MES buffer (pH 7.0) under constant shaking (Biotek Synergy H4; medium speed). Immobilized Strep-tag II CALB samples were prepared as described above using 6 µg/µL of purified enzyme. Absorbance values were read at 400 nm excitation for 15 minutes (Biotek Synergy H4). A standard curve of p-nitrophenol was used to determine substrate conversion and subsequent rates. The Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) were extrapolated from nonlinear regression of a plot of velocity versus substrate concentration using Michaelis–Menten enzyme kinetics (Graph Pad Prism Software, U.S.A.). $V_{max}$ was divided by the enzyme concentration to obtain the apparent turnover number ($k_{cat}$).

3.3.6 CALB Activity and Stability

Purified commercial CALB, free Strep-tag II CALB and immobilized Strep-tag II CALB in 10 mM MES Buffer (pH 7.0) were incubated at 37 °C. Immobilized Strep-tag II CALB samples were prepared as described above using 6 µg/µL of purified enzyme. Supernatant from immobilized Strep-tag II CALB (containing unbound Strep-tag II CALB in Buffer W) was also incubated at 37°C. These samples were pulled at 3, 6, 9, and 25 h. At each time interval, commercial lipase, Strep-tag II CALB and supernatant (from immobilized Strep-tag II CALB) samples were diluted to a concentration of 3.13 µg/mL, and immobilized Strep-tag II CALB samples were diluted to a concentration of 25 µg/mL in 10 mM MES buffer (pH 7.0) and tested for activity using 0.5 mM p-NPB at 37 °C. Absorbance values, substrate conversion, and subsequent rates were determined as described previously.
Activity was expressed as a percentage of retained activity relative to the initial activity of each sample.

3.3.7 Statistical Analysis

To determine statistical differences at $p < 0.05$, curve fitting and one-way analysis of variance (ANOVA) with Tukey’s pairwise comparison were performed using Prism software (Graph Pad, USA).

3.4 Results and Discussion

3.4.1 Affinity Purification of Strep-tag II CALB

Recombinant Strep-tag II CALB was successfully expressed in NEB Express *E. coli* cells and purified from the culture supernatant using Strep-tag II affinity chromatography. Both crude culture supernatant (Figure 1A) and purified protein elution fractions (Figure 1B) were visualized on a 12% SDS-PAGE gel. It was observed that Strep-tag II purification system resulted in highly pure recombinant Strep-tag II CALB corresponding to a single band of size ca 33 kDa. Using Braidford protein quantification, the amount of purified lipase was estimated to be ca 9 mg when 850 ml of culture supernatant was loaded in concentrated form on 4 ml of Strep-Tactin resin column. The purified Strep-tag II CALB was found to be catalytically active with specific activity of ca 122 µmoles/min/mg of protein.
Figure 1. Purification of Strep-tag II CALB from recombinant NEB Express *E. coli* cells culture media using Strep-Tactin affinity Chromatography. Protein samples in each of the fractions collected during purification were analyzed by SDS-PAGE on a 12% polyacrylamide gel along with the molecular size marker (lane M) and commercial CALB (lane *). Protein bands were visualized with Blue-Clean Protein Stain. The arrows indicate the position of CALB (MW ~ 33 kDa) on the gels. Lanes in (A): 1 – Concentrated Culture Supernatant, 2 – Filtered Culture Supernatant, 3 – Flow Through after concentration, 4 – Flow Through from column purification, 5 to 9 – Wash fractions 1 to 5. Lanes in (B): 1 to 10 – Elution Fractions 1 to 10.

3.4.2 Immobilization of Strep-tag II CALB on Strep-Tactin resin

In order to determine the optimal condition for the immobilization of purified Strep-tag II CALB to Strep-Tactin resin, the binding ability of Strep-Tactin resin was studied. When different concentrations of purified Strep-tag II CALB were applied to 20 µL of resin, minimal amount of protein (< 20% of the initial amount applied) was detected in the
supernatant under the described conditions – suggesting almost complete enzyme (ranging from > 80% to 100% of the initial applied amount) binding at every concentration evaluated (Figure 2). Thus, it can be concluded that there was a linear relationship between the amount of bound Strep-tag II CALB and initial enzyme concentration (up to 12 µg/µL) applied when 20 µL resin volume was used. However, greater than 12 µg/µL concentrations of enzyme was not tested due to experimental limitation in getting sufficient volume of concentrated stock of purified Strep-tag II CALB. For subsequent immobilization experiments, 25 µL of 6 µg/µL stock concentration of Strep-tag II CALB was used with 20 µL of Strep-tactin beads. This corresponds to lipase concentration of ca 101 µM on the beads which is ca 100 times higher than the reported K_D [24, 26].

![Figure 2. Immobilization of different concentrations of purified Strep-tag II CALB on 20 µL of Strep-Tactin Superflow (high capacity) resin in Buffer W on ice. Values represent an average ± standard deviation from triplicate independent measurements of each sample.](image-url)
3.4.3 Protein Leaching

An ideal immobilized enzyme system should have no leakage of the enzyme as this limits the feasibility of immobilized enzyme in industrial applications that involve recycling and reusability. As such, protein leaching from our immobilized Strep-tag II CALB system was determined. Figure 3 shows that no measurable amount of enzyme loss was detected when immobilized Strep-tag II CALB was incubated at 37 °C in 10 mM MES Buffer (pH 7.0). This result suggests that the immobilized lipase is strongly associated with Strep-Tactin matrix support and is not readily removed up to 24 hours of incubation under the conditions described, which is a crucial parameter for a successful immobilized enzyme system.

Figure 3. Protein leaching from Immobilized Strep-tag II CALB incubated in 10 mM MES Buffer (pH 7.0) at 37°C for different times. No measurable protein loss corresponds to <10% protein loss where 10% is the detection limit of the assay. Initial amount of bound lipase on 20 µl of Strep-Tactin resin before incubation was ca 107 µg on an average. Values represent average ± standard deviation of n = 3 independent determinations.
3.4.4 Enzyme Activity and Kinetics

Immobilized Strep-tag II CALB was characterized for retained activity and change in kinetics constants in comparison to free Strep-tag II CALB and commercial CALB controls. Specific activity of immobilized Strep-tag II CALB was found to be comparable to that of commercial CALB but less than Strep-tag II CALB (Figure 4). The apparent turnover number \((k_{cat})\) of Strep-tag II lipase decreased from 70.67 s\(^{-1}\) to 6.5 s\(^{-1}\) following immobilization (Table 1), indicating a decrease in the maximum velocity of substrate conversion compared to free Strep-tag II lipase. Potential reasons for decrease in specific activity and \(k_{cat}\) of immobilized lipase can be attributed to: (i) immobilization leading to alteration in enzyme conformation due to surface denaturation through interaction with Strep-Tactin protein on resin, (ii) micro-environmental enzyme-substrate interactions that take place in an environment different from that in free solution, and/or (iii) crowding of the enzyme on the surface of material support resulting in surface denaturation through lateral contacts with neighboring enzyme molecules [47, 48].

The apparent \(K_m\) value of free Strep-tag II increased from 0.18 mM to 0.27 mM following immobilization; however, this change was not determined to be statistically significant (Table 1). The apparent \(K_m\) of the immobilized Strep-tag II enzyme, being comparable to that of the free and commercial enzyme, suggests that immobilized enzyme is probably confined to the surface of the Strep-Tactin resin particles in a manner that limits mass transfer restrictions, thereby allowing the enzyme to freely react with the substrate [8]. The apparent catalytic efficiency \((k_{cat}/K_m)\) of Strep-tag II lipase decreased from 392.61 X 10\(^3\) M\(^{-1}\) s\(^{-1}\) to 24.19 X 10\(^3\) M\(^{-1}\) s\(^{-1}\) (Table 1). Given that there was minimal difference between \(K_m\) of free and immobilized lipase, change in catalytic efficiency was the outcome of
decrease in apparent $k_{cat}$ of the immobilized lipase system due to the possible reasons listed above.

Figure 4. Comparison of Specific activity of Commercial CALB, free and immobilized Strep-tag II CALB. Purified commercial CALB and Strep-tag II CALB (before and after immobilization) were tested for activity using 0.5 mM p-Nitrophenyl butyrate at 37 °C in 10 mM MES buffer (pH 7.0) for 15 minutes. Protein estimation was done using Bradford method with bovine serum albumin as a standard. Enzyme activity values were converted to specific activity values. Specific activity shown is an average from triplicate measurements of each sample. Results are representative of duplicated experiments.

Table 1. Apparent Kinetic Parameters of Commercial CALB, free and Immobilized Strep-tag II CALB. Values represent average of triplicate measurements. Results are representative of duplicated experiments.
3.4.5 Stability of CALB Activity

Stability of commercial, free Strep-tag II CALB and immobilized Strep-tag II CALB were characterized at 37 °C in 10 mM MES Buffer (pH 7.0) to further quantify the effect of Strep-tag II CALB immobilization on enzyme stability. Figure 5 shows that there was significant drop (p < 0.05) in activity retention for all the samples within first three hours of incubation in comparison to no incubation. It has been observed that when the free enzyme is stored at –20°C under the same buffer conditions, it retains complete activity (data not shown). Also, decrease in specific activity of free Strep-tag II CALB was also observed upon repeated freeze thawing (data not shown). This suggests that temperature plays a critical role in influencing the stability of enzyme [11]. Immobilization has been employed to enhance the stability of native CALB with significant improvements in stability being demonstrated [8, 11, 49-51]. Compared to these other forms of lipase immobilization, attachment of the enzyme through affinity interaction with Strep-tactin resin did not promote stability. This could be because immobilization using Strep-tag II and Strep-Tactin affinity interaction may not be strong enough to impose physical restrictions and rigidity on the immobilized enzyme, thereby giving the enzyme freedom for molecular motion and conformational changes leading to unfolding in response to incubation at 37 °C such as in case of free enzyme [11, 48].
Figure 5. Stability of Commercial CALB, Free and Immobilized Strep-tag II CALB at pH 7.0 (10 mM MES buffer) and 37 °C using p-Nitrophenyl butyrate as the substrate. Values represent average ± standard deviation of n = 3 independent determinations.

3.5 Conclusion

In summary, we have demonstrated the immobilization of lipase (CALB) using a novel Strep-tag II fusion technology. Strep-tag II CALB could be rapidly immobilized to Strep-tactin matrix support through the principle of affinity interaction under mild conditions favorable to enzyme structure retention. The enzyme was strongly immobilized under the conditions used, showing no considerable leakage from the support. The immobilized enzyme showed decreased catalytic efficiency when compared to the free Strep-tag II CALB. The immobilized Strep-tag II lipase displayed decreased catalytic activity and stability under the temperature and pH conditions used. This research work suggests that the rational design of protein engineering approaches is needed to manipulate the functional characteristics of enzyme for an efficient binding and enhanced stability on affinity supports using fusion tag
technology. Nonetheless, fusion tag technology represents a promising eco-friendly approach that can be tailored for the immobilization of industrially important enzymes onto biopolymeric supports to produce highly stable and active biocatalysts without requiring any chemical modification.

3.6 Acknowledgement

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3.7 References


CHAPTER 4

GENERAL CONCLUSION AND FUTURE WORK

The overall goal of this research work was to utilize Strep-tag II fusion technology for the immobilization of lipase B from *Candida antarctica*. To accomplish this, production of CALB with Strep-tag was required. The first study (Chapter 2) focused on the expression, purification and characterization of Strep-tag II CALB. Plasmid harboring Strep-tag II CALB was constructed using genetic engineering and used for expression studies in *E. coli*. Recombinant Strep-tag II CALB was successfully expressed in *E. coli* followed by purification using Strep-Tactin affinity chromatography column. Strep-tag II CALB was obtained in highly pure form as shown by SDS-PAGE and its kinetic properties were further characterized using commercial CALB as a control. Strep-tag II CALB was found to be catalytically active. The determination of purity and activity of Strep-tag II CALB was critical for further immobilization study detailed in Chapter 3.

The second study (Chapter 3) investigated the conditions for optimal immobilization of Strep-tag II CALB on Strep-Tactin resin and the activity behavior of the resulting immobilized enzyme under a given set of conditions. It was observed that 25 µl of ~ 6 mg/mL stock of purified Strep-tag II CALB showed almost complete binding when 20 µl of Strep-Tactin resin was used. Under these conditions, no considerable leakage of the enzyme was seen after incubation at 37 °C and pH 7.0 for up to 24 hours. Immobilized Strep-tag II CALB was found to be catalytically active, although less than the free Strep-tag II CALB. Free Strep-tag II CALB was not also found to be very stable in response to repeated freeze thawing. Both immobilized and free Strep-tag II CALB were not found to be stable in terms
of activity after incubation at 37 °C and pH 7.0 for up to 24 hours. These results suggest that though immobilization of lipase using Strep-tag II fusion technology proved efficient in terms of enzyme binding under the conditions tested, it did not confer any additional benefits to the enzyme in terms of activity. Nevertheless, this research still sheds light on various factors such as activity of free recombinant enzyme, protein binding, leaching and stability of immobilized enzyme activity that need to be considered while designing a robust and efficient enzyme immobilization system.

Future work pertaining to this research should include the following objectives:

1. Generation of a library of constructs for the expression of different lipase enzymes in heterologous systems and further screening the effect of specific fusion tag on the expression, solubility and activity of the enzyme
2. Immobilization of soluble and catalytically active fusion tagged lipase variants on different types of material supports and evaluation of various factors that affect the performance of immobilized system such as protein loading, binding, leaching and immobilization efficiency under varying conditions of temperature, pH and solvents
3. Testing enzyme recycling and support regeneration to determine if the enzyme and support may be re-used across multiple cycles.
4. Analyzing the ability of the developed fusion tag based lipase immobilization system for the production of industry relevant compounds using both hydrolytic and esterification reaction conditions

Getting a deeper and systematic understanding of the above outlined objectives will enable the discovery of next generation lipase immobilized systems that can be commercially
implemented to improve the production efficiency of bio-based products for food and agriculture industries.