Synthesis of enantio-pure amides by reversal of the *Geobacillus pallidus* RAPc8 amidase hydrolysis reaction in non-aqueous media

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Abstract

Synthesis of enantiopure amides by reversal of the thermostable *Geobacillus pallidus* RAPc8 amidase in non-aqueous media

Amidases are hydrolytic enzymes that catalyze the hydrolysis of amides to their corresponding carboxylic acids and ammonia. Amidases are ubiquitous in nature, and they have been isolated from a wide range of microorganisms, the most common source being bacteria. Amidases are recognized as potential industrial biocatalysts in processes that involve the synthesis of chiral compounds, mostly used in the pharmaceutical, agrochemical and food industries. The discovery of amidases from extremophiles has increased the potential for application of these enzymes for the development of new processes. In non-aqueous media, amidases have the ability to synthesize enantiopure amides due to the shift in thermodynamic equilibrium towards synthesis. For synthesis to occur, an acyl donor and an acyl acceptor are required, in which the acyl acceptor acts as a nucleophile. The applicability of amidases in non-aqueous media opens new possibilities for processes in which the enzyme can be used for the industrial synthesis of commercially relevant new products.

A novel amidase was previously isolated from a thermophilic *Geobacillus* species, and the amidase was cloned and expressed in an *Escherichia coli* BL21 strain. Also in previous studies, it was shown that the enzyme exhibits both amide hydrolysis and acyl transfer activities. The highest activity of the *G. pallidus* RAPc8 amidase was observed at 50°C in the presence of acetamide and substrate preference was towards aliphatic, short chain amides. Furthermore, the enzyme displayed enantioselectivity towards lactamide, which is a chiral compound. The amidase compound showed selectivity towards the D-isomer of lactamide and no detectable activity on the L-isomer.

This study presents the investigation and development of a novel biocatalytic process that involves the synthesis of enantiopure amides in non-aqueous media, using the *G. pallidus* RAPc8 amidase. The amidase was produced and expressed in *E. coli* BL21. The crude extract, which had a specific activity of 70 U/mg, was subsequently heat treated at 75°C, (the amidase retained over 90% of its activity, while the *E. coli* proteins were denatured). The purified amidase was immobilized onto Eupergit C beads followed by cross-linking with

ethylenediaminetetraacetic acid (EDAC). The Eupergit-immobilized amidase was applied in batch synthesis reactions in organic solvents and room temperature ionic liquids, using the acetamide synthesis reaction with acetic acid and ammonium carbamate as the model reaction. The organic solvents tested in amide synthesis were: acetonitrile, tetrahydrofuran, toluene and heptane. The ionic liquids investigated were: 1-methyl-3-butylimidazolium tetrafluoroborate, 1-methyl-3-butylimidazolium hexafluorophosphate and 1-octyle-3-methylimidazolium hexafluorophosphate. The optimal water content in all of these solvents was investigated, by varying the water concentration between 0.1 and 10% (v/v). The stability of the enzyme in the solvents used was determined through the incubation of the amidase in the solvents at 50°C, for 96 h. Additional studies were conducted to optimize the acetamide synthesis reaction by varying substrate concentrations and the amount of the biocatalyst added to the reaction mix. To study the effects of the different solvents on the enantioselectivity of the amidase, various chiral separation techniques were tested. Both direct and indirect methods, which include liquid chromatography and derivatization with chiral derivatizing agents, were investigated.

From the batch synthesis of amides in organic solvents, the product yields obtained were 90, 41, 29 and 11% in acetonitrile, tetrahydrofuran, toluene and heptane respectively. For synthesis in ionic liquids, the yields in 1-methyl-3-butylimidazolium tetrafluoroborate, 1-methyl-3-butylimidazolium hexafluorophosphate and 1-octyl-3-methylimidazolium hexafluorophosphate, were 95, 51 and 0% respectively. The trend observed was that the yield increased with increasing solvent polarity. The results obtained from the studies conducted to determine the amidase stability in organic solvents and ionic liquids showed similar trends, where 40-50% of the enzyme activity was retained after 96 h of incubation in the solvents.

The methods tested in the separation of the lactamide enantiomers were not successful. Direct and indirect methods for chiral separation were investigated. The direct method used for the separation of the lactamide enantiomers was through the injection of the racemic lactamide mix into chiral columns. The separations were optimized by modifying the mobile phase as well as the column temperatures. These tests were conducted at Macherey Nagel in Karlsruhe, Germany. The second direct method tested was separating the lactamide enantiomers on a chiral TLC plate, supplied by Macherey Nagel. The indirect method investigated was the derivatization of the lactamide enantiomers with the derivatization agent, *O*-phthaldialdehyde (OPA) and *N*-Isobutyryl-L-Cysteine (IBLC). The diastereomers formed

were then injected into a reverse phase HPLC column for separation. The derivatization test was done as it had previously worked in the separation of amines, which have an amino group that partakes in the derivatization reaction. Amides also have the amino group in their structure, therefore, the assumption was that the reaction would work with amides, however, the separation of the lactamide enantiomers was unsuccessful.

In conclusion, this study showed that the *G. pallidus* RAPc8 amidase can be used for the synthesis of enantiopure amides, as an alternative to the traditional chemical synthesis methods. With further studies, a process at industrial scale could be developed, based on the results obtained in this study.

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List of Abbreviations

A	Absorbance
a_w	Thermodynamic water activity
[bmim][BF_4]	1-butyl-3-methylimidazolium tetrafluoroborate
[bmim][PF_6]	1-butyl-3-methylimidazolium hexafluorophosphate
BSA	Bovine Serum Albumin
CDA	Chiral Derivatization Agent
CSP	Chiral Stationary Phase
E. coli	*Escherichia coli*
ee	*enantiomeric excess*
EDAC	Ethylenediaminetetraacetic acid
HPLC	High Pressure Liquid Chromatography
HIC	Hydrophobic Interaction Chromatography
IBLC	*N*-Isobutyryl-L-Cysteine
IEX	Ion Exchange Chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria Broth
LogP	Logarithmic Partition Coefficient
[omim][PF_6]	1-octyl-3-methylimidazolium hexafluorophosphate
OD	Optical Density
OPA	*O*-phthaldialdehyde
PAGE	Polyacrylamide Gel Electrophoresis
SDS	Sodium Dodecyl Sulphate
TLC	Thin Layer Chromatography

DECLARATION

I declare that the synthesis of enantiopure amides by the reversal of thermostable *Geobacillus pallidus* RAPc8 amidase is my own work.

Anne O Gabathuse September 2012

Signature:.....

Chapter 1 : Introduction

1.1 Introduction and Problem Statement

This dissertation reports an investigation into the development of a batch reaction system for the synthesis of enantiopure amides in non-aqueous media, through the reversal of the hydrolytic reaction of the *Geobacillus pallidus* RAPc8 amidase. The motivation for this research project was based on the interesting properties displayed by the *G. pallidus* RAPc8 amidase such as its ability to retain high activity at temperatures higher than those at which other biocatalysts function, and its ability to retain activity in the presence of organic solvents (Makhongela *et al.*, 2007). In addition, the motivation for carrying out this study was also because this type of investigation involving the application of the *G. pallidus* RAPc8 amidase in non-aqueous media synthesis has not been done previously, therefore, making it novel.

Furthermore, studies involving the use of biocatalysts in non-aqueous media have revealed that enzyme specificities and selectivities are altered from those they display in aqueous media (Klibanov, 2001; Milner & Maguire, 2012), and these findings present an opportunity for the development of new processes for this biocatalyst in the synthesis of homochiral compounds. The increasing need for methods for the synthesis of new and optically active compounds in the chemical and pharmaceutical industries has led to an increase in the demand for biocatalysts with enhanced enantioselective properties for application at industrial scale (Antipov *et al.*, 2008). According to technical market research reports, the global market for enzymes is expected to increase to over $ 2.7 billion by the year 2012 (www.allaboutfeed.net/news; www.business-market-research.info) and this figure is expected to increase rapidly in future years. The anticipated growth in the need for enzymes has prompted researchers in academia and industry to search for novel biocatalysts and also to develop new methods for biocatalysis and process development. The development of biology-based processes can play an important role in relieving the effects of energy expenditure and industrial pollution on the environment. Conventional chemical processes generally operate under harsh and hazardous conditions such as high pH, temperatures and pressures, which require high energy and other renewable resources such as water. Alternatively, biocatalytic processes function under ambient conditions and generally do not require high energy inputs (Johannes *et al.*, 2006).

The importance of biocatalytic processes and the value they add to industrial chemical processes is easily identifiable, but only a few processes utilize biocatalysts for organic synthesis at industrial scale. This is in part due to the difficulty of identifying a biocatalyst that is suitable for a particular process and whose production will not contribute immensely to the costs of the process (Pollard *et al.*, 2006; Kaul & Asano, 2012). Although enzymes present many advantages over chemical synthesis methods, biocatalysts are often unable to catalyze the synthesis of unnatural products at levels high enough for practical application at industrial scale. Also, the lack of thermal stability of mesophilic organisms contributes to the low number of biocatalytic processes currently in use. Numerous techniques have been developed, however, that assist scientists in identifying suitable biocatalysts for new processes (Schmid *et al.*, 2001; Kaul & Asano, 2012).

Biocatalytic reactions sometimes require the presence of non-aqueous solutions, particularly for reactions involving hydrophobic substrates. Reaction rates may be higher in non-aqueous media due to higher substrate solubilities. Furthermore hydrolytic reactions may be reversed under these conditions, resulting in the favouring of synthesis reactions. One important factor to bear in mind is that, in non-aqueous media, enzyme specificities may be modified: they may exhibit altered characteristics otherwise achievable only through techniques such as protein engineering. Despite all these advantages, the major disadvantage of using enzymes in non-aqueous media is that their activities are low compared to their activities in aqueous media, due to a number of factors that come into effect when enzymes are exposed to solvents (Kim *et al.*, 2000; Milner & Maguire, 2012). These factors may include, but are not limited to, the denaturation of the biocatalyst by solvents if they penetrate the enzyme structure and disrupt the molecular bonds kept intact by water or the inability of the substrates to dissolve in the solvents. A number of techniques have been developed to counteract these, for example, Subtilisin Carlsberg, prepared by lyophilizing the enzyme in the presence of non-buffer salts had an enhanced yield rate that was 20 000-fold higher than the yield rate obtained from the non-lyophilized enzyme. This is mainly because the salts protected the enzyme by substituting the water molecules inside the enzyme structure, preventing the structure from collapsing through the formation of hydrogen bonds with the amino acid residues (Schmid *et al.*, 2001).

There are a number of chemicals, such as amides, amines and peptides, which are of importance to the pharmaceutical industry as they are used as intermediates in the production

of pharmacologically active compounds (Pilissao & Nascimento, 2006). These chemicals can be synthesized in organic solvents by different enzymes, mainly from the enzyme class hydrolases (Gotor-Fernandez *et al.*, 2006; Slotema *et al.*, 2002; Litjens *et al.*, 1999; Ebert *et al.*, 1998; Zoete *et al.*, 1996). The majority of the amides are produced through chemical processes, under harsh conditions. However, these processes are accompanied by low yields and poor stereoselectivities. In addition, heat labile amides cannot be synthesized through such processes. The production of amides at an industrial scale is also achieved through the conversion of nitriles to their corresponding amides by nitrile hydratases. For example, the industrially important amide, acrylamide is successfully produced by the hydration of acrylonitrile by a nitrile hydratase from a *Rhodococcus* species (Nagasawa *et al.*, 1993). At present, there are no amides produced in non-aqueous media by biocatalysts. Certain hydrolases, such as lipases and amidases have shown to have the ability to synthesize amides from a carboxylic acid and ammonia, in non-aqueous media, at laboratory scale (Al-Muller *et al.*, 2010).

Experimental studies have shown that most amidases (EC 3.1.5.4), which catalyze the conversion of amides to carboxylic acids and water, also possess acyl transferase properties (Fournand *et al.*, 1998; Sharma *et al.*, 2012). In non-aqueous media, their hydrolytic reactions can be reversed towards synthesis, taking advantage of their acyl transferase activity. This acyl transferase activity is the property that makes amidases good candidates for amide synthesis in non-aqueous media, as an alternative to the chemical methods (Perreux *et al.*, 2002). At industrial scale, amidases are often used in the synthesis of natural and non-natural amino acids from amino amides, as well as in bioremediation and biodegradation processes, such as the treatment of industrial waste containing nitriles (Kobayashi & Shimizu, 1998; Nel *et al.*, 2011). The most commonly applied amidase is Penicillin amidase, for the synthesis of 6-aminopenicillanic acid, which is an intermediate in the synthesis of penicillins (Gabor *et al.*, 2004).

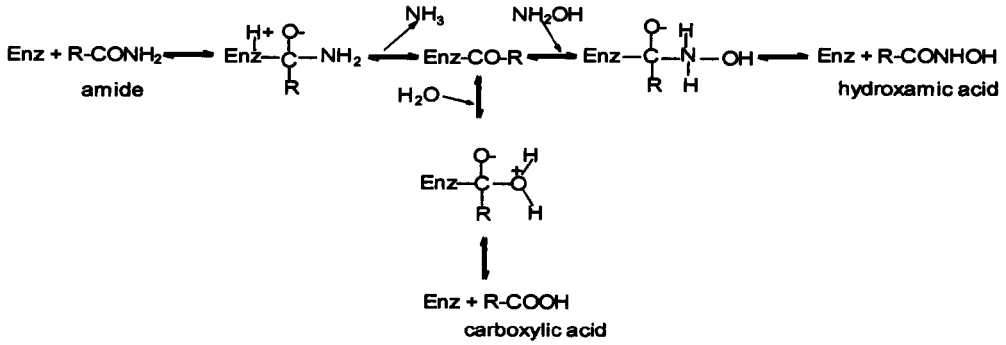


Figure 1.1: The hydrolytic and acyl transfer reactions catalysed by amidases (Fournand *et al.*, 1998).

1.2 Motivation

An amidase enzyme was previously isolated from the thermophilic *Geobacillus pallidus* RAPc8 strain that was isolated from a thermal environment, in New Zealand. The enzyme was isolated, cloned and expressed in *Escherichia coli* BL21 by our collaborators at the Institute for Microbial Biotechnology and Metagenomics (IMBM) (Pereira *et al.*, 1998). The production of the amidase in *E. coli* BL21 cells was optimised (Olaofe, 2009). Growing the cells in a bioreactor resulted in a larger biomass and volumetric enzyme production. This study was undertaken to investigate the synthesis of enantiopure amides by the immobilized *G. pallidus* RAPc8 amidase in non-aqueous media.

1.3 Aims and Objectives

The aim of this project was to develop a novel process which exploits the reversal of the hydrolysis reaction catalyzed by the thermostable *Geobacillus pallidus* RAPc8 amidase, to synthesize enantio-pure amides, in non-aqueous media. To achieve this aim, the specific objectives were as follow:-

1. To determine the most suitable organic solvent (hydrophobic or hydrophilic) in which the amidase was sufficiently active, to catalyze the reverse reaction.
2. To determine the most suitable room temperature ionic liquid (water miscible or immiscible) in which the amidase was sufficiently active, to catalyze the reverse reaction.
3. To compare the effects both organic solvents and room temperature ionic liquids on the activity of the *G. pallidus* RAPc8 amidase.

4. To study the activity of the amidase in low aqueous systems, at different water concentrations to determine the optimum water content for the acetamide synthesis reaction system.
5. To identify a suitable chiral separation method for the separation of the D,L-lactamide enantiomers.

1.4 Dissertation Structure

Chapter 2 presents the extensive literature of the study, which is composed of information relating to the application of biocatalysts in non-aqueous media; the effects of the media on enzyme activity as well as stability. The enzymology of the thermostable *G. pallidus* RAPc8 amidase is also reviewed and the application of other amidases in non-aqueous media and their potential application in industrial synthesis.

A detailed description of the materials and methods used in this study are discussed in **Chapter 3.**

The results obtained from the investigations involving the growth of the recombinant *Escherichia coli* BL21 strain, enzyme production, purification and immobilization of the *G. pallidus* RAPc8 amidase are discussed in detail in **Chapter 4.**

In **Chapter 5**, the results obtained from the synthesis of acetamide and lactamide in non-aqueous media are presented and discussed. The effect of the type of solvent on acetamide synthesis is discussed in detail. Furthermore, the batch acetamide synthesis reaction conditions, such as the effect of substrate concentration on product yield and the effect of the biocatalyst mass on the product yield are discussed. Lastly, results obtained from the identification of a chiral enantio-separation method, to determine the enantioselectivity of the *G. pallidus* RAPc8 amidase, are presented and discussed.

A summary of the conclusions from Chapters 4 and 5, as well as a general discussion are presented in **Chapter 6.** Furthermore, for future work, recommendations are also listed; to assist in further understanding of the *G. pallidus* RAPc8 amidase catalyzed acetamide synthesis reaction in non-aqueous medium.

Chapter 2 : Literature Review

2.1 Introduction

This chapter presents previous and recent literature relevant to this study, including the application of biocatalysts in non-aqueous media in the synthesis of enantiopure amides. Biochemical studies of the *G. pallidus* RAPc8 amidase had revealed that the amidase possesses some acyl transfer properties in the presence of hydroxylamine to form hydroxamic acid (Makhongela *et al.*, 2005). This acyl transferase property was exploited in this study in a novel reaction for the synthesis of enantiopure amides in organic solvents and room temperature ionic liquids. In non-aqueous media, some of the biocatalyst properties such as its thermostability and enantioselectivity towards compounds that exist as enantiomers may be enhanced (Vermue & Tramper, 1995; Milner & Maguire, 2012).

2.2 Amidases

Structural studies have shown that amidases belong to the thiol nitrilase family. This group includes aliphatic amidases which catalyze the hydrolysis of short chain aliphatic amides (Makhongela *et al.*, 2007). Structural studies have revealed that amidases have a cysteine residue present in each of the subunits. This cysteine acts as an active nucleophile in the active site, within the catalytic triad; Glu-Lys-Cys, as confirmed by Farnaud and his colleagues (1999), through site-directed mutagenesis of the cysteine residue (Cys166) of the *Pseudomonas aeruginosa* amidase. This cysteine residue has been identified in all the members of the nitrilase superfamily (Andrade *et al.*, 2007; Novo *et al.*, 1995). The amino acid sequence of these amidases does not have the conserved GGSS signature which is a characteristic of most amidases (Makhongela *et al.*, 2007). Bacterial amidases have been extensively studied such as those isolated from *Pseudomonas aeruginosa* (Farnaud *et al.*, 1999; Andrade *et al.*, 2007), *Rhodococcus* sp. R312 (Pertsovich *et al.*, 2005) and *Geobacillus pallidus* RAPc8 (Pereira *et al.*, 1998).

2.2.1 Amidases from thermostable bacteria

In nature, there is a group of microorganisms that have evolved and as a result have adapted to extreme conditions. These organisms are found in extreme environments where their mesophilic counterparts cannot survive. These extremophiles also produce enzymes that can function in these extreme conditions. Extremophiles are categorized into groups depending on the type of extreme environment they can survive in. These include: thermophiles which

are tolerant of high temperatures, halophiles which are tolerant of high salt concentrations and piezophiles which are tolerant of high pressures (Bommarius & Riebel, 2004).

Given that these organisms produce enzymes that are active at extreme conditions, these enzymes are good candidates for use in industrial processes due to their enhanced thermostability especially since the efficiency and productivity of an industrial process depends on the stability of the catalyst used (Dermirjian *et al.*, 2001).

2.2.2 *Geobacillus pallidus* RAPc8 amidase

Pereira *et al.* (1998) identified an amidase from a thermophilic *Geobacillus pallidus* RAPc8 strain. This *G. pallidus* RAPc8 amidase, which is of interest to this particular study, was found to belong to branch 2 of the nitrilase superfamily. An open reading frame (ORF) encoding a 348 amino acid amidase was located downstream of the ORF encoding α and β subunits of the nitrile hydratases. The amidase gene was cloned and over-expressed in *Escherichia coli BL21* (Cameron *et al.*, 2005). The structure of the *G. pallidus* RAPc8 amidase was resolved by Kimani *et al.* (2007). (Figure 2.1).

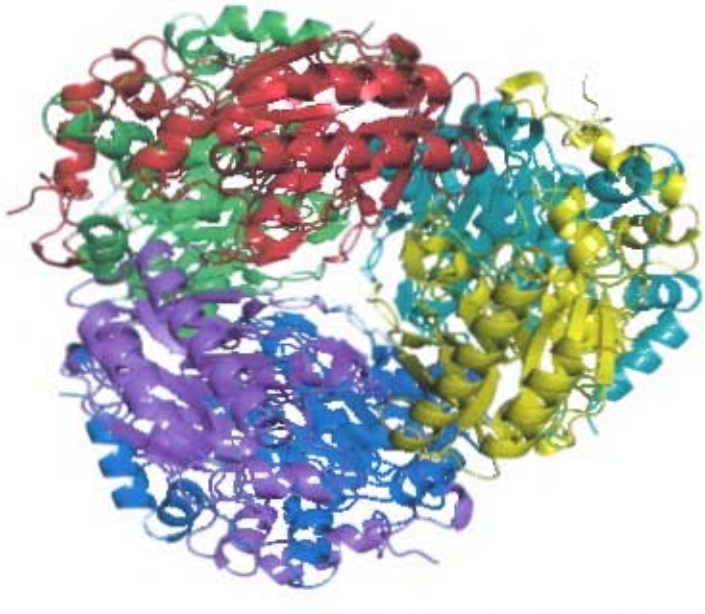


Figure 2.1: The structure of the *G. pallidus* RAPc8 amidase. The different colours represent the six subunits that form the enzyme structure. Picture reproduced from Kimani *et al.* (2006).

Biochemical studies showed that the *G. pallidus* RAPc8 amidase is a thermostable enzyme as it exhibited activity between 50°C and 60°C at a pH of 7.0. Analysis of the primary sequence showed the presence of the catalytic triad Glu, Lys, Cys; which is characteristic of the nitrilase superfamily. The amidase rapidly hydrolyzed low molecular weight aliphatic amides and it was not able to hydrolyze aromatic or long-chain aliphatic amides. Inhibition studies revealed the presence of a catalytic thiol group in the active site and acyl transfer activities were revealed from the biochemical studies. The amidase was immobilized onto Eupergit C® beads at 25°C and subsequently cross-linked with EDAC to improve the yield of the protein bound to the beads, as recommended by Makhongela *et al.* (2007). Following immobilization, the amidase had improved thermal stability and showed activity in a broad pH range (Makhongela *et al.*, 2007).

Preliminary results also showed that the *G. pallidus* RAPc8 amidase retained activity in organic solvents and that the hydrolytic reaction of the amidase can be reversed and hence applied in the synthesis of carboxylic amides from an organic carboxylic acid and ammonia (Glowacka *et al.*, 2007). The enzyme also exhibited enhanced selectivity towards the D- and L- enantiomers of lactamide, in a hydrolytic reaction (Makhongela *et al.*, 2007). These characteristics exhibited by the amidase are advantageous for the synthesis of pure products required by the food and pharmaceutical industries.

2.3 Biocatalysis in non-aqueous media

Prior to Klibanov *et al.* (1988) establishing that enzymes can be catalytically active in non-aqueous organic solvents, the general consensus within the scientific community was that enzymes are only active in aqueous media. In reality, enzymes can function in non-aqueous media with very little water, as long as there is sufficient water to keep the enzyme hydrated, thereby preserving the optimal catalytic conformation. With this breakthrough, a number of reactions that were not possible in aqueous media can now be conducted in non-aqueous media (Clarke, 2004).

There are a numerous advantages associated with non-aqueous or low water biocatalysis, such as the ability of the solvents to dissolve hydrophobic substrates that are not soluble in aqueous media. In non-aqueous media, the thermodynamic equilibrium of a hydrolytic

enzyme reaction may be shifted from hydrolysis towards synthesis, therefore allowing for thermodynamically limited reactions that cannot take place in aqueous media, to be achieved. In addition, high product yields may be obtained by the elimination of side reactions which result in the formation of by-products (Cabral *et al.*, 1997; Milner & Maguire, 2012). However, in non-aqueous media, most enzymatic reaction rates are lowered by magnitudes of about 4 to 5, as compared to their aqueous counterparts. This trend was observed in the transesterification reaction catalyzed by the protease subtilisin in a variety of organic solvents (Clark, 2004; Solanki & Gupta, 2008). Nevertheless, there are techniques that can be applied to improve the enzymatic catalytic rates in non-aqueous media, such as the addition of lyoprotectants prior to the lyophilization or freeze drying process (Mattos & Ringe, 2001). Before enzymes are placed in non-aqueous media, the biocatalyst must be prepared prior the start of the catalytic reactions. Such preparations may include the drying of the biocatalyst through a lyophilization process. During the lyophilization, denaturation of the protein may occur due to the rapid removal of water molecules from the protein structure, which may contribute to the reduction of the enzymatic activity. The denaturation process may be reversed when the protein is placed in aqueous media; however, the same does not apply in organic solvents. When placed in an organic solvent, the protein may form inactive aggregates (Mattos & Ringe, 2001). To prevent this inactivation from occurring, salts and lyoprotectants can be added to the protein solution before lyophilization is done. The lyoprotectants reduce enzyme inactivation by acting as water replacements through the formation of hydrogen bonds with the protein residues, when water is reduced during drying (Mattos & Ringe, 2001; Gupta & Roy, 2004).

2.3.1 Types of Solvents

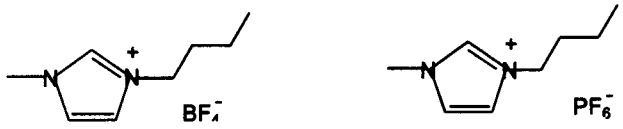
A number of non-conventional media have been tested at laboratory scale as potential reaction media for biocatalytic processes; the most commonly used non-conventional media in biocatalytic reactions being organic solvents and room temperature ionic liquids. Other alternative reaction media that have been used are reverse micelles and supercritical fluids (Vermue & Tramper, 1995; Andrade & Alves, 2005; Illanes *et al.*, 2012). Supercritical fluids (SCF) are substances that exist above their critical temperatures (T_c) and pressures (P_c). The critical point of a substance represents the temperatures and pressures at which the substance can exist as a vapour and a liquid in equilibrium (Adams *et al.*, 2004; Illanes *et al.*, 2012). The properties of a SCF lie in between those of its liquid and gas phases, that is, the densities

and the dissolving powers of the SCF resemble those of a liquid while the viscosities resemble those of gases. The SCF most commonly used as a reaction medium is carbon dioxide ($scCO_2$). Using SCFs as reaction media has numerous advantages such as the fact that they are non-flammable and less toxic than organic solvents (Andrade & Alves, 2005; Sheldon, 2005; Bornsheuer & Kazlauskas, 2005).

Reverse micelles are made up of a bulky organic phase which contains water droplets which are stabilized by a surfactant. When reverse micelles are used as the medium in a biocatalytic reaction, the enzyme remains soluble in the aqueous phase, while the substrates and products are dissolved in the organic phase (Bornsheuer & Kazlauskas, 2005). The advantages associated with reverse micelles as reaction media is that the small amount of water allows for condensation reactions to take place. Also, mass transfer limitations are diminished due to the large interfacial surface between the micelles and the organic phase. Reverse micelles systems are important for biocatalysis since it is assumed that they mimic the natural environment in which enzymes are found inside living cells (Krieger *et al.*, 2004; Kolisis & Stamatis, 2010). For the purpose of this study, the non-conventional media investigated in detail are room temperature ionic liquids and organic solvents.

2.3.1.1 Room temperature ionic liquids (RTILs) as reaction medium

Another group of solvents that are gaining recognition as potential environmentally benign, non-aqueous reaction media for application in biocatalytic processes are room temperature ionic liquids (RTILs). RTILs are salts with low melting points which exist in a liquid phase at room temperature. They are composed solely of ions, i.e. cations and anions. The most commonly used ionic liquids are 1-butyl-3 methylimidazolium tetrafluoroborate (bmim-BF_4) and hexafluorophosphate (bmim-PF_6), (Figure 2.2) (Park & Kazlauskas, 2003; Krieger *et al.*, 2004; Kim *et al.*, 2001). A number of processes that involve whole cell or enzyme reactions in ionic liquids have been reported (Table 2.1). The first synthetic enzymatic reaction in ionic liquids reported involved the synthesis of Z-aspartame in [Bmim][PF_6] by thermolysin. The product yield obtained was found to be comparable to that in conventional organic solvents such as ethyl acetate. The thermolysin displayed some enhanced thermostability properties in this ionic liquid (Wenyong *et al.*, 2004).



Bmim-BF_4 Bmim-PF_6

Figure 2.2: The molecular structure of the most commonly used room temperature ionic liquids: 1-butyl-3methylimidazolium tetrafluoroborate [Bmim-BF_4] and 1-butyl-3-methylimidazolium hexafluorophosphate [Bmim-PF_6], used as alternative media in organic synthesis (Kim *et al.*, 2001).

Table 2.1: Examples of organic synthetic reactions carried out in different ionic liquids at commercial scale.

Biocatalyst	Reaction
Lipases (*Candida antarctica* and *Mucor miehei*)	Synthesis of glycidyl esters
Recombinant free epoxide hydrolase	Stereoselective hydrolysis of epoxides
Immobilized lipase: *Pseudomonas cepacia*	Resolution of racemic alcohols
Immobilized lipase: *Candida antarctica* B lipase	Amine synthesis
Commercial lipases	Production of glycidyl esters by transesterification in ILs-scCO_2
Commercial protease	Resolution of *N*-acetyl amino acid esters
Thermolysin	Synthesis of *Z*-aspartame
Whole cells of *Rhodococcus* R312	Biotransofromation of 1,3-dicyanobenzene
Whole cells of baker's yeast	Reduction of ketones

Table adopted from (Kragl *et al.*, 2003; Moon *et al.*, 2006)

The unique properties of RTILs are listed below, emphasizing the reason why RTILs are more attractive as reaction media for non-aqueous catalysis. These properties also make the ionic liquids more environmentally attractive compared to the use of organic solvents (Moon *et al.*, 2006; Lau *et al.*, 2000; Rantwijk & Sheldon, 2007; Adams *et al.*, 2005; Wenyong *et al.*, 2004):

i). the lack of vapor pressure and the thermal stability of RTILs eliminates the emission of volatile compounds into the atmosphere.

ii). Their non-flammable properties makes them safe to use in bioprocesses as this reduces the threat of fire in a bioprocessing plant.

iii). Their ability to dissolve a wide spectrum of organic, inorganic, highly polar, non-polar and polymer compounds, facilitates the production of a broad range of useful compounds.
iv). The potential to fine tune their chemical and physical (hydrophobicity, miscibility, viscosity and polarity) properties to suit a particular biocatalytic reaction, by altering the anion or the substitute group on the cation, means that the RTILs can be selected to suit a particular biocatalytic process.

v). their recoverability from the reaction mixture makes it possible to re-use the RTILs for a few reaction cycles.

2.3.1.2 Organic solvents as reaction medium

There is currently a broad range of alternative non-conventional media that can be applied as reaction media instead of conventional organic solvents, however, organic solvents are still the most commonly used. This is due to the fact that organic solvents do not require specific and costly equipment such as those required for supercritical fluids, and they are also not as expensive as ionic liquids, which cost about 800 times more than the molecular solvents. Ionic liquids are therefore often only used for processes that involve the synthesis of high value commodities such as therapeutics for the medical industry (Krieger *et al.*, 2005).

A number of reactions have been carried out successfully in organic solvents, largely due to the fact that organic solvent systems have been studied in detail, including their physical and chemical characteristics and the effects they have on biocatalysts. There is a vast amount of literature documenting issues that need to be considered when performing biotransformations in organic solvents which include: the effect of the solvent on the reaction rate, biocatalyst thermostability and enantioselectivity, and the role of water in low water systems (Adamczak

& Krishna, 2004; Gupta, 1992; Vermue & Tramper, 1995; Bornscheuer & Kazlauskas, 2005). All these factors are discussed in detail in sections (2.3.2-2.3.3).

2.3.2 Solvent Effects on enzyme activity

The type of solvent used in a biocatalytic reaction can have significant effects on the reaction, especially in terms of the rate at which the substrates are converted to their corresponding products. The stability and selectivity of a biocatalyst are therefore dependent on the type of solvent used (Andrade & Alves, 2005). In non-aqueous media, the catalytic reaction can be influenced either by direct interaction of the solvent with the biocatalyst or through the extent to which the substrates and products are dissolved and interact with the solvent. For example, the solvent can alter the catalytic properties of the enzyme or even deactivate it through interfering with the water layer around the enzyme and thereby penetrating and reaching the hydrophobic residues within the enzyme structure. Highly polar solvents are known to strip the essential water layer around the enzyme, thereby interfering with its catalytic ability. Hydrophobic solvents do not have the ability to pull away the water layer and therefore they do not interfere with the enzyme activity in this manner (Diaz-Garcia & Valencia-Gonzalez, 1995; Verma & Ghosh, 2010).

The solvent can also have an effect on the reaction rate depending on the extent to which the substrates and products are dissolved in the reaction medium. If the solvent solvates the substrates efficiently, (that is, if the interaction between the solvent and the substrate are particularly favorable), the substrate may not be available to the enzyme (Verma & Ghosh, 2010). Thus, the choice of solvent to be used in a biocatalytic process is very important. Although there are no set rules to follow when choosing a solvent, some parameters are commonly used to aid this process. These include partition coefficients of solvents, logP (the logarithmic partition coefficient of a solvent in a bi-phasic system consisting of 1-octanol and water, also a measure of the hydrophobicity of a solvent) and dielectric constants (Vermue & Tramper, 1995). The most frequently applied parameter for the selection is the partition coefficient, logP, which is used as an indicator of the polarity of the solvent. Solvents with a logP of less than 2 are considered to be highly polar and therefore tend to inactivate enzymes. Solvents having logP values between 2 and 4 are moderate and those with a logP value higher than 4 are hydrophobic. Therefore, in organic synthesis, solvents with logP values greater than 4 are recommended for processes utilizing enzymes as catalysts. This is because hydrophobic solvents do not strip the enzyme of the essential water shell around it, which is

required to keep the enzyme active (Gupta, 1992). Selection of a solvent is also dependent on the ability of the solvent to dissolve the substrates and products by stabilizing the molecules, thereby lowering their free energies of activation which in turn improves the binding of the substrate to the protein (Diaz-Garcia & Valencia-Gonzalez, 1995).

2.3.2.1 Solvent effects on the thermostability of biocatalysts

The thermostability of enzymes in low water environments is enhanced by the rigidity of the protein structure brought about by the presence of organic solvents (Gupta, 1992; Clarke, 2004). In aqueous media, inactivation of enzymes occurs due to the alteration of the enzyme protein structure and also due to partial unfolding of the enzyme structure, through inactivation processes such as deamidation, peptide hydrolysis and cysteine decomposition. Water is required for these mechanisms to take place. In organic solvents, these reactions are slowed down (Krishna, 2002). Zaks & Klibanov, (1984) demonstrated that the thermostability of porcine pancreatic lipase was enhanced substantially when the enzyme was transferred from aqueous media to non-aqueous media. In an aqueous solution at 100°C, the enzyme was inactivated in a matter of seconds, while in a dry mixture of tributyrin and heptanol, the half-life was increased to 12 hours at the same temperature, due to the stabilization of the protein structure through rigidification, which prevents water accessing the hydrophobic moieties in the protein structure. The elimination of water from enzymes' hydrophobic residues prevents the denaturation reactions from occurring, since these reactions require water.

The thermostability of the enzyme can, however, be diminished in organic solvents if there is accumulation of water which is formed as a by-product. Therefore in reactions where water is one of the products, it is important to apply water removal techniques such as equilibrating with salts or by addition of molecular sieves in the reaction mixture (Diaz-Garcia & Valencia-Gonzalez, 1995). In condensation reactions, water is formed as one of the products; this can cause a complication in the reaction medium because not only will the reaction equilibrium be affected, but the stability of the enzyme as well. Therefore the process should be designed in such a way that the water concentration in the reaction mixture can be controlled (Klibanov, 2001; Krishna, 2002).

Despite the enhanced thermostability of enzymes in organic solvents, their enzymatic activity is often still lower compared to that in water. This is because of other factors in non-aqueous media that act on the biocatalyst, therefore interfering with its activity through alteration of the protein structure or with the active site. For example, if the organic solvent used as the medium is polar, it may interact with the hydroxyl groups of the biocatalyst, thereby accelerating the denaturation process. Some techniques have been developed to counteract the denaturation process in non-aqueous media, Table 2.2 (Klibanov, 1997).

Table 2.2: Possible causes of loss of enzymatic activity in non-aqueous media and the possible solutions to prevent or lessen the loss of activity (Klibanov, 1997).

CAUSES	SOLUTIONS
• Diffusion limitation of substrate	• The enzyme suspensions should be vigorously agitated. Small enzyme particles can be used.
• Altered protein conformation structure	• During lyophilization, lyoprotectants, e.g. carbohydrates should be used.
• Stabilized ground state energy of substrate	• The type of solvent used should result in an unfavourable interaction between the substrate and solvent.
• Destabilization of the transition state	• Choice of solvent should allow for favourable interactions between the transition state and the solvent.
• Reduced conformational flexibility	• The water activity should be at its optimum concentration. Additives that mimic water can be used.
• Non-optimum pH	• The dehydration process in the aqueous medium should be carried out at the enzyme's optimum pH.

2.3.2.2 Solvent effects on the enantioselectivity of the biocatalyst

Application of an enzyme in non-aqueous medium can have an effect on the specificity of the enzyme towards certain substrates, by altering the active site of the enzyme. In solvents, the enzyme may assume a rigid conformation due to the elimination of water around the enzyme by the solvent, thereby reducing the enzyme's flexibility. Due to this rigidity, the enzyme's

active site may not accept a broad range of substrates; the site is able to accommodate specific substrates only, therefore making the enzyme specific towards certain substrates. The solvent used can also have an effect on the enzyme's selectivity by modifying the binding affinity of the substrate (K_m) to the active site. In polar solvents or aqueous media, a driving force behind the substrate binding to the active site is the unfavorable interaction of the substrate with the media. The substrate is forced into the active site due to the less suitable interactions with the reaction media. In non-polar media, however, the substrate is not forced into the enzyme's active site due to complete dissolving of the substrate, and therefore the K_m value of the substrate is increased (Diaz-Garcia & Valencia-Gonzalez, 1995).

With respect to enantioselectivity, an enantioselective enzyme is usually able to utilize one of the enantiomers of a chiral molecule as a substrate and not the other. This is a result of the different conformations by the two enantiomers and therefore possible steric hindrances with one of the enantiomers. The increasing demand of single enantiomer compounds by the pharmaceutical and agrochemical industries has led to the extensive investigation on hydrolases and solvents effects on their enantioselective properties. However, in bio-processes that involve the synthesis of non-natural compounds, the enantioselectivity of the biocatalyst is often affected and often not sufficient. The application of enzymes in non-aqueous medium greatly influences the enantioselectivity of the enzyme, even reversing it at times. For example, in a reaction that involves the synthesis of 3-hydroxy-2-phenylpropionate (a pharmacologically important compound) by α-chymotrypsin, the enantioselectivity of the enzyme is inclined towards the *S*-enantiomer of the substrate; however, in other solvents the enantioselectivity is inclined towards the *R*-enantiomer of the substrate (Klibanov, 2001). The enantioselectivity of enzymes is applied in industry, amongst others, in the synthesis of aspartame (a low calorie sweetener) which is a food additive, a compound of high purity that is produced at low cost (Bornsheuer & Kazlauskas, 2005). The enantioselectivity of an enzyme can be determined using the enantiomeric ratio, *E*. This parameter measures the ratio of the one enantiomer to the other at the completion of the reaction (Carrea & Riva, 2000).

2.3.3 Effects of water on enzymatic activity

Water activity (a_w) plays a crucial role in biocatalytic reactions. It is plays a vital role on the biological structure and function of a biocatalyst, in a non-aqueous biocatalytic process. The term water activity is used to describe the water available in the reaction mixture, required to

keep the keep the enzyme active in the reaction medium (Carrea *et al.*, 1995). Several studies have shown that in non-aqueous media, water plays a significant role on the activity, stability and conformational flexibility of the enzyme (Carrea *et al.*, 1995). In low water systems, addition of water increases the enzyme's activity; however, in the case of bio-processes that involve hydrolases, a high increase of water leads to a reduction of product yield as the reaction equilibrium will be shifted from synthesis to hydrolysis (Yang *et al.*, 2004).

The amount of water present in the medium should be controlled and kept low as it also influences the reaction equilibrium, too little water reduces the enzyme activity by reducing its flexibility and too much of it may result in a shift of the reaction equilibrium. Therefore, it is vital that the optimal amount of water required in a non-aqueous reaction system is known so that precautions can be taken to keep the water activity at the required level (Kaur *et al.*, 1997, Krishna, 2002).

The water activity varies with the type of solvents used. For example, polar solvents require a higher water content to reach a specific water activity as compared to non-polar solvents, because polar solvents are water miscible, while non-polar solvents are not (Adamczak & Krishna, 2004). In a non-aqueous reaction system, it is important the water content is controlled and kept at the optimum to avoid interference with the reaction equilibrium. A number of methods have been investigated for controlling the water activity in a system by equilibrating the reaction components. These methods include the equilibration with salt solutions of known a_w. For example, the salt pairs $CuSO_4.5H_2O/CuSO_4.3H_2O$ have a_w of 0.33 at 25°C. The salt hydrates act as water buffers in a reaction mixture by absorbing or releasing water to the reaction (Bornscheuer & Kazlauskas, 2005).

2.3.4. Effect of pH on enzymatic activity

In order to obtain a biocatalyst with its optimum activity in organic solvent reactions, the lyophilization process should be performed in an aqueous media at a pH value where the enzyme exhibits optimum activity. In addition to that, during lyophilization, lyoprotectants such as crown ethers (macrocyclic organic molecules) and cyclodextrins (glucose oligomers) should be included in the aqueous buffer prior to the lyophilization (Mine *et al.*, 2003). This is because enzyme molecules have a 'memory' of the aqueous conditions from which they were lyophilized, termed 'pH memory'. Hence, when placed in non-aqueous media, the

activity of the enzyme will be subject to the conformation they exhibited while in the media from which they were lyophilized (Bommarius & Riebel, 2004). The pH of the media from which they were lyophilized will pre-determine the activity of the enzyme in the solvents, because the ionizable amino acid residues of the protein molecules retain their last ionic form during dehydration (Adamczak & Krishna, 2004). Therefore, for the enzyme to work at its optimum in the solvent, the pH of previous medium should be at the optimal working pH of the enzyme (Klibanov, 1997, 2001).

In aqueous media, the flexibility of enzymes in their native conformations is due to conformational mobility, which is a result of the presence of water molecules. However, in organic solvents, there is no free water present to allow an enzyme to change its conformation, and therefore the enzyme stays locked in the conformation it was in during lyophilization. Therefore, in non-aqueous media, enzymes are unable to unfold due to the rigidity, thereby preventing the inactivation of the enzyme by the solvents (Ke & Klibanov, 1997; Gupta & Roy, 2004). It is vital to lyophilize enzymes in a medium whose pH value is that which the enzyme operates optimally. In aqueous media, the pH can be altered by adjusting the pH values with salt buffer solutions. However, the same cannot be done in organic solvents. In non-aqueous media, the use of buffering systems containing highly hydrophobic acids and their hydrochlorides has been documented (Carrea & Riva, 2000). The method most often applied is making sure that the medium from which the enzyme was recovered was at the optimum pH of the biocatalyst.

2.3.5 Enzyme structure in non-aqueous media

The tertiary structure of a protein is maintained by a number of weak, non-covalent bonds such as hydrogen bonds, van der Waals interactions, hydrophobic interactions and ionic bonds. These bonds are promoted by water, and therefore in the absence of water the organic solvents can disrupt these weak interactions and result in the unfolding of the protein tertiary structure (Iyer & Ananthanarayan, 2008). The unfolding will result in the denaturation of the protein and therefore a loss in activity. Polar solvents are more capable of causing protein unfolding than their non-polar counterparts because the former are able to penetrate into the protein's interior and strip off the essential water molecules required to keep the hydrogen bonds in place. Furthermore, polar solvents can have an effect on the transition state for

activity of the enzyme will be subject to the conformation they exhibited while in the media from which they were lyophilized (Bommarius & Riebel, 2004). The pH of the media from which they were lyophilized will pre-determine the activity of the enzyme in the solvents, because the ionizable amino acid residues of the protein molecules retain their last ionic form during dehydration (Adamczak & Krishna, 2004). Therefore, for the enzyme to work at its optimum in the solvent, the pH of previous medium should be at the optimal working pH of the enzyme (Klibanov, 1997, 2001).

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enzymes with a very polar transition state if they are able to penetrate into the active site of the enzyme (Serdakowski & Dordick, 2007).

Some research has suggested that it is not only the organic solvents that are responsible for the disruption of the protein structure but that the lyophilization step contributes. The most commonly form of biocatalyst used in non-aqueous reactions system is the powder form obtained by the lyophilization process. Lyophilization (freeze drying), is a dehydration procedure performed to prepare the enzyme before transferring it to a solvent medium (Secundo & Carrea, 2003). The freeze-drying is a three step process. In the freezing step, the protein temperature is quickly reduced to a temperature lower than its lowest freezing point; this is so that in the following step, sublimation occurs rather than melting. This step is then followed by a primary freezing step where the pressure is reduced and some heat is applied to allow for sublimation to take place. In the final drying step, the temperature is increased to evaporate any water molecules that remain attached to the protein molecule (Serdakowski & Dordick, 2007, Klibanov, 2001). In order to keep the conformation of the enzyme at the optimal form during lyophilization, there are some procedures that can be followed in order to achieve this. These include the addition of some chemicals during lyophilization, such as glycerol, which preserve the structure of the biocatalyst by replacing water molecules within the protein structure (Adamczak & Krishna, 2004).

2.4 Application of hydrolases in non-aqueous biotransformations

In aqueous media, hydrolases catalyze the hydrolytic cleavage of the C-C, C-N and C-O bonds. Hydrolases are the class of enzymes that is most often applied in industrial organic biotransformations, particularly the enzymes, proteases and lipases. It is estimated that some 80% of the biocatalysts used in industry are from the hydrolase class. This is due to the fact that hydrolases are a versatile group of catalysts and are easily obtainable in large quantities at reasonable prices (Krishna, 2002; Klibanov, 1990). In organic synthesis, the hydrolases, especially lipases have been widely applied in the synthesis of esters such as fatty acid aliphatic alcohol esters and fatty acid polyol esters which are incorporated in the manufacture of drugs, cosmetics and food stuffs, through condensation reactions. The majority of esters manufactured currently are prepared through conventional methods. However, this is limiting due to the fact that harsh conditions are required for these processes. This limits the versatility of esters that can be produced, because heat unstable esters cannot be produced

through this method. In chemical synthesis, harsh chemicals are also used; therefore some esters to be used in food stuffs cannot be manufactured with conventional methods because these chemicals are restricted in food (Kobayashi & Adachi, 2004). Due to these limitations, there is a need for the development of biocatalytic processes which can be used instead of the conventional method for the synthesis of these compounds, and hence the increased interest in the development of biocatalytic processes which utilizes hydrolases.

The amidation reaction has not been well studied and for exploitation of this type of reaction, the majority of processes use proteases and lipases as the biocatalysts rather than amidases (Figure 2.3) (Gotor, 1999). Like esters, amides are important compounds, and they are used in the same industries as esters. They are also used in important processes such as the synthesis of peptides and lactams (Varma *et al.*, 2007). A method has been developed for the synthesis of a very important amide called pyrrole-amide which is of importance in the pharmaceutical industry. This compound is used as an intermediate in the synthesis of a compound that regulates plasma levels of the insulinotropic pro-glucagon, called dipeptidyl peptidase IV (Gotor-Fernandez *et al.*, 2006). Although lipases are used for this purpose and are known to accept a wide range of substrates, there are, however, some substrates that they would not be able to accept which amidases can accept. Therefore, it is advisable that research on amidases is exploited to improve the variety of products that can be synthesized. Penicillin G acylase (PGA, E.C. 3.5.1.11) is a commercially relevant enzyme and the most industrially applied amidase in organic synthesis (Lindsay *et al.*, 2002). It is used in the synthesis of 6-aminopenicillanic acid (6-APA) through the hydrolysis of Penicillin G. It is also used for the hydrolysis and synthesis of esters and amides of phenylacetic acid (Ebert *et al.*, 1998). Ebert *et al.* (1998) and her colleagues also demonstrated that immobilized Penicillin G acylase can be applied in the synthesis of amides in toluene. The hydrolytic enzyme used in the current study, for the synthesis of amides, is an amidase from *Geobacillus pallidus* RAPc8; therefore amidases will be discussed in detail for the purpose of this study.

Figure 2.3: The formation of an amide bond through the amidation of a carboxylic acid with ammonia.

2.5 Amide bond formation

The condensation of carboxylic acids and amines is the most commonly used method for the synthesis of amides, with ammonia as the nucleophile. The most commonly used group of enzymes for the formation of the amide bond are hydrolases as they are naturally involved in the hydrolysis of compounds in possession of the amide linkage. The most used enzymes in this group are proteases and lipases (Gotor-Fernandez *et al.*, 2006). Two different types of strategies can be used for the formation of the amide bond, which are thermodynamic synthesis and kinetic synthesis. In thermodynamic synthesis, the reaction conditions are altered to shift the reaction equilibrium towards synthesis. The most commonly used method of shifting the reaction equilibrium is by applying medium engineering techniques such as replacing the water in the reaction medium with an organic solvent. The organic solvent suppresses the hydrolytic reaction by preventing the ionization of the substrates (Bornsheuer & Kazlauskas, 2005). An example of a thermodynamically controlled synthesis of the amide linkage is the large scale synthesis of the low calorie sweetener, aspartame by thermolysin, which produces about hundreds to thousands of tons of aspartame. The reaction catalyzed by thermolysin is depicted in Figure 2.4. The precipitation of the product, aspartame, drives the thermodynamically controlled synthesis (Oyama, 1992).

Figure 2.4: Thermodynamic control for the large scale synthesis of the sweetener, aspartame by the protease, thermolysin.

In the kinetically controlled synthesis of amides, an activated carboxyl compound such as an ester is used. The activated compound reacts with the enzyme to form an acyl-enzyme intermediate complex. The acyl-enzyme complex then reacts with an amine to form the corresponding amide. Kinetically controlled reactions often occur at a faster rate than the thermodynamically controlled reactions because they involve an activated substrate, therefore less energy will be required to start the reaction. For kinetically controlled reactions, only enzymes whose mechanisms are the same as the serine hydrolases can be used since there is a need for the formation of an acyl enzyme complex. Therefore, kinetically controlled synthesis can only be applied in reactions that require subtilisin and lipase as biocatalysts and does not apply to amidases, because amidases are cysteine hydrolases (Bornsheuer & Kazlauskas, 2005). An example of a kinetically controlled synthesis at large scale is the synthesis of the

dipeptide, kyotorphin (Tyr-Arg) by β-carboxyltransferase (β-CT), depicted in Figure 2.5. High concentrations of the nucleophile are used to minimize the hydrolysis of the acyl enzyme intermediate (Fischer *et al.*, 1994). Therefore, in this study, for the synthesis of amides the thermodynamic control strategy will be applied.

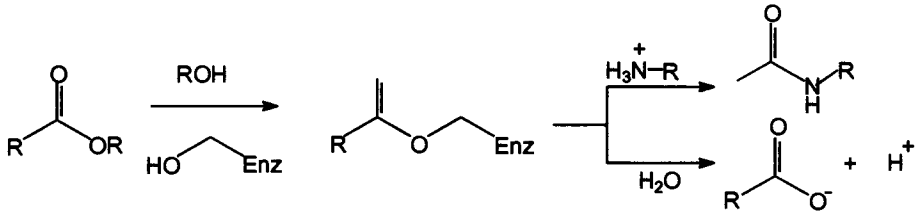

Figure 2.5: Kinetic control for the large scale synthesis of the dipeptide, kyotorphin (Tyr-Arg) by β-CT.

2.6 Enzyme stabilization techniques

The operational stability of biocatalysts plays an important role in the success of any bioprocess. Therefore, it is imperative to find a biocatalyst that will be stable for the time period it would take for a catalytic reaction to reach completion. This, however, is a difficult task because the native environment in which enzymes catalyse reactions is quite different from that it is expected to perform in bioprocesses, and enzymes cannot always function optimally under these conditions (Zhao *et al.*, 2002). Much attention is given in finding techniques that can be used to improve the catalytic stability of the enzymes, due to the fact that their lack of stability poses as a stumbling block in the development of biocatalytic driven processes. Several stabilization strategies have been developed and applied, such as: chemical modification of the enzyme structure, use of stabilization additives, alteration of the enzyme's primary structure by protein engineering methods, the use of immobilization techniques and medium engineering (Adamczak & Krishna, 2004).

2.6.1 Protein engineering

Protein engineering, which involves the direct alteration of an enzymes primary structure, is receiving attention as a strategy to enhance the biocatalyst properties. Advances in the development of protein engineering techniques for gene diversification and also new screening methods have led to the optimization of various enzyme properties such as the thermostability, activity and selectivity. These techniques allow for 'tailor made' biocatalysts, suited for a specific process and also for the ability to alter various enzymatic traits at once (Zhao *et al.*, 2002; Böttcher & Bornscheuer, 2010).

One strategy applied in the altering of the enzyme structure is a technique called rational design, which involves the engineering of specific amino acids on the enzyme's primary structure. For this method to be applied, information describing the protein structure of the enzyme, its function and catalytic mechanism are of importance and should be available. However, an increase in information regarding the structure of a large group of enzymes makes this method applicable (Rubin-Pitel and Zhao, 2006; Milner & Maguire, 2012). The alteration of specific amino acid residues in the genome sequence are expected to change certain properties of the enzyme to suit a particular process.

Another technique used in protein engineering is the directed evolution method which involves the application of a number of cycles of random mutagenesis to alter the genetic sequence of the enzyme, through the use of different methods. This procedure is then followed by recombination and high throughput screening of a library of the various mutants from which strains with the desired improved traits will be selected. The protein with one of the desired traits is then exposed to a number of mutagenesis cycles, to accumulate other traits required (Zhao *et al.*, 2002; Milner & Maguire, 2012). The directed evolution method has been used to alter various properties of enzymes such as stability, activity, selectivity and substrate specificity (Rubin-Pitel & Zhao, 2006). This technique mimics the natural evolution process that occurs in cells for the selection of proteins that will keep the cells alive in changing environments, during the asexual and sexual processes.

2.6.2 Immobilization

Immobilization is the most commonly applied enzyme stabilization technique used to improve operational stability of biocatalysts. Immobilization is a process where a catalyst is linked or attached to a support material resulting in a situation where the enzymes movement is confined to a specified area (Iyer & Ananthanarayan, 2008; Illanes *et al.*, 2012). In industry, enzymes or cells are usually used in an immobilized form because it increases the catalyst's longevity in chemical synthesis or in bioremediation. Not only does it result in improved stability of the biocatalyst, it also allows for the recycling of the catalyst (especially if it is expensive) and flexibility of the reactor design. The enhanced thermostability post-immobilization of biocatalysts is due to the molecular rigidity and the protected microenvironment (Graham *et al.*, 2000; Illanes, 1998). Although there are numerous

advantages that come with the immobilization of biocatalysts, there are, however, few industrial processes that use immobilized enzymes. This is because, during the immobilization process, the enzyme's activity might be lost due to pH changes or high temperature. Secondly, the immobilization step adds extra costs to a process. Lastly, immobilized enzymes occupy more space in the reactor because of the support material as compared to free enzymes and also there could be diffusion limitations due to the presence of the solid support (Wiseman, 1983).

Designing an immobilization system for an enzyme to be applied in a biotransformation process is therefore not an easy task. The choice of the immobilization technique and the support material are dependent on a number of factors such as: the type of enzyme to be used, the reaction system (whether it is an aqueous or organic solvent system), the process conditions, which include pH, temperature and pressure, and also the reason for immobilization (Knezevic *et al.*, 2006). Immobilization techniques fall under four categories: adsorption, covalent binding, cross-linking and entrapment.

2.6.2.1 Adsorption

Adsorption is the easiest enzyme immobilization technique to perform as it only involves the mixing of the enzyme solution with the support material under suitable conditions (pH, ionic strength and temperature), which will promote binding. The interaction between the enzyme and the support is reversible due to the weak forces formed on interaction, such as van der Waal's, hydrogen bonds and hydrophobic interactions (Knezevic *et al.*, 2004). The adsorption method takes advantage of the natural properties of the enzyme and the carrier and their tendency to interact, no enzyme modifications occur, hence the enzyme conformation is slightly altered or not at all. Adsorption can be executed through physical or ionic interactions. With physical adsorption, the enzyme is physically attached to the support material and ionic adsorption used electrostatic forces where the enzyme is attached onto a charged material. Desorption of the enzyme from the carrier can easily occur if the conditions of the medium are changed (Adamczak & Krishna, 2004).

2.6.2.2 Covalent Immobilization

In covalent immobilization, a covalent bond is formed between the enzyme and the support material. There is a wide range of inorganic and organic supports available for the

immobilization; this shows that there is no ideal support that offers the best possible immobilization system. The choice of support plays a crucial role and the more hydrophilic it is, the higher the immobilization efficiency and enzyme activity (Mateo *et al.*, 2007). The functional groups on the enzyme surface commonly used for covalent binding are: the amino group (NH_2) of lysine and arginine, the carboxyl group (COOH) of aspartic and glutamic acid, the hydroxyl group (OH) of serine and threonine, and the sulfhydryl group (SH) of cysteine. The covalent immobilization of proteins requires short spacer arms which promote rigidification of the protein molecules (Mateo *et al.*, 2007).

2.6.2.3 Cross-linked enzyme crystals and aggregates

Cross-linked Enzyme Aggregates (CLEAs) or Cross-linked Enzyme Crystals (CLECs) can be made depending on the form in which the enzyme is in, but generally these methods are used for highly purified enzymes (Sheldon, 2007). CLECs are made by crystallization and the subsequent cross-linking to preserve the crystal structure. Enzyme activity can be lost during this procedure because it is performed under harsh as it has to be performed in the presence of organic solvents such as acetone (Lopez-Serano *et al.*, 2002). Cross-linking can be done through chemical or physical methods. With the chemical methods, reagents are used to form covalent bonds. Glutaraldehyde is the most commonly used reagent. The advantage of using this method is that it is cheap and simple to perform and the high stability of CLECs in organic solvents has been reported (Illanes, 1998; Noritomi *et al.*, 1998; Illanes *et al.*, 2012). There are problems associated with this method which include the reactivity between the reagents and the functional groups in the active site of the enzyme as well as poor mechanical stability. These are some of the reasons why cross-linking is never used solely as a means of immobilization, but is often used in combination with the other immobilization methods to enhance them (Roy & Abraham, 2003).

2.6.2.4 Entrapment of the enzyme

In entrapment, the enzyme is localized within a porous matrix or membrane. The enzymes are free in solution but their movement is restricted by the membrane or matrix. The porosity of the membrane is essential as it allows substrates and products to diffuse freely in and out but must not allow the enzyme to leak out. The advantage of this method is that it does not lead to modification of the enzyme, so the enzyme activity is used to its maximum potential (Spahn & Minteer, 2008). There are drawbacks with this method however, such as limited

diffusion for the substrate and the products and the supports cannot be renewed. The most commonly used material for the preparation of entrapment gels includes: cellulose, agar, agarose, collagen, polyacrylamide, sodium alginate, e.t.c. The most commonly used material is sodium alginate due to the mild conditions required for its formation into a hydrogel (Jovanovi-Malinovska *et al.*, 2006).

2.7 Chiral separations

Enantiomers are a pair of compounds that exist as non-superimposable mirror images of each other. The chirality of enantiomers is caused by the presence in their molecular structure of one or more chiral element, such as an asymmetric carbon atom. Enantiomeric molecules are chemically and physically identical to each other in an achiral environment, thereby making it difficult for chemists specializing in stereochemistry to separate the enantiomers in a racemic mixture. The spatial difference in the structures of enantiomers can only manifest itself when the chiral compounds interact with a chiral phase or structure. Pure enantiomers can be obtained through different modes, either by the asymmetric synthesis route and kinetic resolutions using enzymes or separation techniques of racemates (Turner, 2003).

Over the past 20 years, there has been a shift towards obtaining optically pure compounds mainly because of the realization of the importance of these compounds in the pharmaceutical, agrochemical, food and flavor, and fragrance industries. The biomolecules that make up living organisms, amino acids, sugars and proteins (including enzymes), are chiral in nature. Because of this phenomenon, organisms respond differently to each of the enantiomers, whether they are drugs or pesticides (Toyo'oka, 2002). Racemic drugs can have varying pharmacodynamic and pharmacokinetic effects when ingested by a living organism. The enantiomers in the body are metabolized through different pathways, hence their varying effects in the body. While one of the enantiomers may produce the desired therapeutic effects, the other may not have any kind of effect or worse, produce undesired effects (may be toxic). Therefore, the two enantiomers of a drug should be considered as two different drug moieties, unless proven by studies that the two molecules are similar even in a chiral environment (McConathy & Owens, 2003). One good example of this effect was observed through the thalidomide disaster experienced in the 1950s. The drug was administered as a racemate mixture of both the *R* and the *S* isomers, to pregnant women for morning sickness. The *R*-enantiomer produced the desired effects as a powerful tranquilizer, however, the *S*-

enantiomer was discovered to be a teratogenic after a number of abnormal infants were born (Toyo'oka, 2002).

In 1992, the U.S Food and Drug (FDA) issued a policy that stated that the pharmacokinetic effects which encompass: absorption, distribution, metabolism and excretion and its pharmacodynamic effects which include: activity, efficacy and toxicity of each of the isomers of the racemic drug should be studied and reported before the drug can be approved (http://www.fda.gov; Toyo'oka, 2002). This has therefore contributed to the efforts made by researchers in the pharmaceutical industry to develop separation techniques that will be used in the separation of racemic mixtures. For enantiomeric analysis, the currently used techniques include non-chromatographic and chromatographic methods. The non-chromatographic methods include techniques such as polarimetry, nuclear magnetic resonance (NMR), isotopic dilution, calorimetry and some enzyme techniques (Bojarski *et al.*, 2005). The main disadvantage with these methods is that they can only be used for the quantification of the enantiomers and separation cannot be achieved. Therefore, pure samples of the enantiomers are required. Often, for quantification of enantiomers to be done, prior separation is required. The methods commonly used for such application; are separation and quantitative analysis, and chromatographic methods. These include: liquid chromatography, gas chromatography, thin-layer chromatography, supercritical fluid chromatography, liquid-liquid extractions, electromigration and membrane based methods (Davankov, 1997). For the purpose of this study, only the chromatography methods will be discussed.

2.7.1 Chiral separation by chromatography

Chromatographic methods are most often applied for the separation of enantiomers in a racemic mixture. These methods are divided into Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) and Supercritical Fluid Chromatography (Godel & Ross, 2008). Chiral HPLC, however, has proven to be more efficient and versatile than chiral GC, because with chiral HPLC a variety of non-volatile and thermolabile compounds can be separated. The chromatographic methods have been applied in enantio-separation by researchers for over a decade. Therefore, this area has grown tremendously, as shown by the large variety of equipment and methods available commercially. Chromatographic methods can act as replacements of organic synthetic methods or in combination (Shaikh *et al.*, 2009). The main advantage associated with the

application of chromatographic methods is that they can be used for both quantitative and qualitative analysis of compounds. The chromatographic enantio-separation of enantiomers can proceed through direct or indirect methods. The direct method uses chiral stationary phases (CSPs) and chiral selectors as additives in the mobile phase, while the indirect method employs chiral derivatization agents (CDAs). See Tables 2.3 and 2.4 for the advantages and disadvantages of direct and indirect methods (Bojarski *et al.*, 2005).

Table 2.3: The advantages of direct and indirect methods used for the separation of the enantiomers of chiral compounds (Ilisz *et al.*, 2008).

INDIRECT METHODS	**DIRECT METHODS**
Advantages	
• Elution sequence of the enantiomers is predictable	• The purity of the chiral selector is not important
• Good chromatographic properties of the Derivatives	• Similar molar absorptivities of enantiomers
• Good chromophoric and fluorophoric properties of the reagent, which improves detection sensitivity	• No chances of racemization and kinetic resolution
• Low cost of achiral columns	• Racemates without functional groups can be separated
• Easy development of methods	• Availability of preparative applications
• Selectivity of enantiomers is increased (better separation achieved with the indirect method)	• Altering temperatures may favour the enantio-resolution
• Opportunity to select the elution sequence of the derivatives	• Simple preparation of analytes and simple chromatographic runs

Table 2.4: The disadvantages of direct and indirect methods used for the separation of the enantiomers of chiral compounds (Ilisz et al., 2008).

INDIRECT METHODS	DIRECT METHODS
Disadvantages	
• The purity of the chiral derivatization reagents is crucial	• The theoretical plate number of the chiral stationary phase may be small
• The molar absorptivities of the diastereomers may differ	• The slow kinetics of desorption of the enantiomers by the CSP
• There is a possibility of racemization	• The elution sequence of the enantiomers is not clear
• There is a possibility of kinetic resolution	• Unavailability of a universal column
• The excess reagent and side products may interfere with the resolution of the enantiomers	• CSPs are sensitive to chromatographic conditions (temperatures, pH)
• Preparative application is restricted	• High cost of chiral columns
• The derivatization process may be time consuming	

2.7.1.1 Stereo-specific Interactions of enantiomers and chiral stationary phase

The success of enantio-separations relies solely on enantiomeric recognition, for the formation of the enantiomer and chiral selector complex, without the recognition, the resolution process will not be feasible (Berthod, 2006). Complexes between the enantiomers and the chiral selector are formed through intermolecular interactions such as hydrogen bonds, π-π interactions, van der Waal's, covalent bonds, ion-pairing and dipole-dipole forces. The interaction between the enantiomers and the chiral selector is explained by the three point attachment (TPA) model, called the Easson- Stedman three-point attachment model (Bentley, 2003) (Figure 2.6). Easson and Stedman proposed that a minimum of three points of attachment were required in order to observe the differential binding of two enantiomers as they bind to the chiral selector (Berthod, 2006).

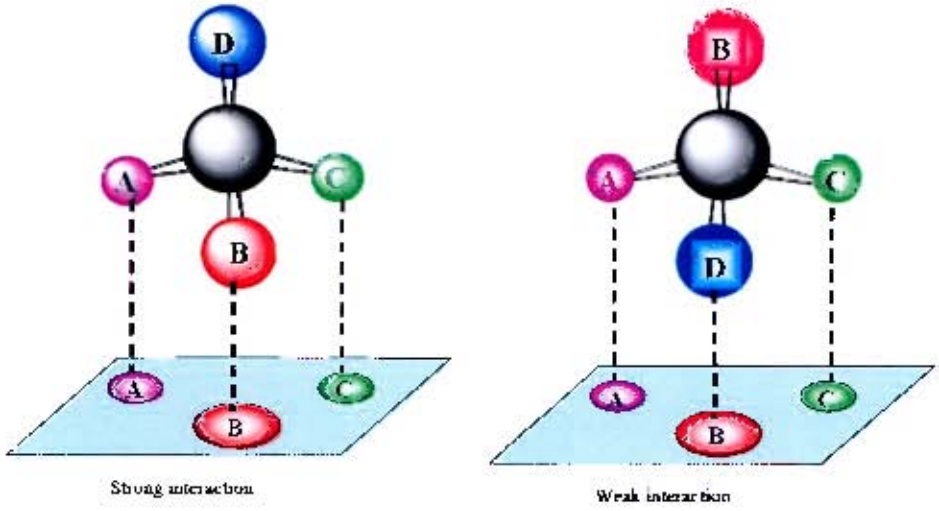

Figure 2.6: The difference in interaction with the chiral selector of the two enantiomers, as explained by the Easson-Stedman three-point attachment model (Berthod, 2006).

2.7.1.2 Chiral separation of enantiomers by indirect methods

The indirect enantiomeric resolution involves the formation of diastereomers through the pairing of the enantiomers with chiral derivatizing reagents, where a second chiral centre is introduced into the analyte molecule. The formed diastereomers can then be subsequently separated by an achiral column because of their differing chemical and physical properties (Davankov, 1997). For the derivatization reaction to work, a functional group on the enantiomers is required which will be compatible to the group on the derivatization reagent and for the separation to be efficient, the functional group should be closer to the chiral centre of the molecule. There are certain procedures to follow when selecting a chiral derivatizing agent (CDA) which will be used for the derivatization of the enantiomers, because the success of the chiral resolution depends on the reaction between the analyte and the CDA. The points to be considered are (according to Bhushan & Bruckner, 2004; Gorog, 2000).

1. The enantiomers and the chiral derivatizing agent should have compatible functional groups (amino, hydroxyl, epoxy,thiol, carboxyl) on their structure which can form covalent interactions with the reactive groups on the analyte chiral molecules.

2. The chiral purity of the CDA should be known and taken into consideration when determining the chiral purity of the enantiomers to prevent any false positives, which could

occur through the impurity with the analyte. Therefore, the CDA should be of high purity, preferably >99% purity.

3. The CDA used should be chemically stable to avoid any loss or change in composition of the CDA, so as to ensure that the reaction reaches completion with all the reactants present. The CDA used should allow for the derivatization reaction to take place at mild temperatures, and the resulting compounds should be directly injected into the column.

4. The CDA used should be compatible with the chromatographic method used, for example, for gas chromatography; the CDA should be volatile in order to avoid the use of high temperatures in the chiral resolution process. In liquid chromatography, the most suitable CDAs should be one that enhances the detectability of the separated enantiomers by introducing chromophoric or fluorophoric groups into their structures.

5. The CDA chosen should react with a functional group that is the closest to the chiral centre, for better resolution. Introduction of bulky groups also contributes to an enhanced chiral resolution.

6. The derivatization reaction between the CDA and the enantiomers should be fast, to prevent a disparity in the diastereomer formation rate, which could lead to kinetic resolution.

The derivatization method is one of the most commonly used methods of enantio-separations because of the large number of commercially available CDAs and well established methods and literature, for the derivatization of a variety of compounds available. About 300 publications have been published between the 1970s and 2004, documenting the successful separation of a large group of compounds.

2.7.1.3 Chiral separation of enantiomers by direct methods

Direct separation methods do not require prior derivatization of enantiomers. The resolution of enantiomers occurs through the reversible formation of diastereomers between the chiral selectors immobilized onto the column and the analyte enantiomers (Shaikh *et al.*, 2009; Bhuchan & Bruckner, 2006). The use of chiral stationary phase (CSP) is the most commonly applied direct method because of its convenience; however, there is no available universal

stationary phase that is applicable to all types of compounds. Selecting a suitable CSP for the resolution of a chiral compound is a difficult task, but prior empirical data about the enantiomers is very useful for this process. Therefore a lot of research has been conducted over the years to develop more CSPs which are better than the previous ones and hence, the large number of CSPs available commercially.

2.8 Conclusion

This literature review has described the shift towards organic synthesis using biocatalysts to synthesize a broad range of compounds, the majority of which are of importance to the pharmaceutical industry. The advantages associated with organic synthesis in terms of process optimization and improvements of the biocatalyst properties have been outlined. The different techniques that can be used to improve the biocatalyst properties, therefore making it compatible for a particular process, are also discussed. These techniques range from stabilization techniques such as protein engineering, immobilization, and medium engineering.

This chapter also discusses the importance of obtaining enantiopure compounds, which are vital in the pharmaceutical, food and agrichemical industries. The importance of obtaining optically pure compounds is discussed and the consequences of using impure or racemic compounds are also outlined, with given examples. There are different techniques used to obtain pure compounds, which range from asymmetric synthesis through the use of biocatalysts, to chromatographic methods that can separate chiral compounds. There are a variety of enzymes used at industrial scale in organic synthesis, most of which are hydrolases. Most of these processes use lipase because of its versatility in terms of the number of substrates it can use as substrates. However, few of these processes use amidases as catalysts for the production of fine chemicals. Amidases have also shown to exhibit broad substrate selectivities.

Through preliminary studies, the *Geobacillus pallidus* RAPc8 amidase, isolated from a thermostable *Geobacillus* strain, was for the first time, found to have the ability to synthesize enantiopure amides in non-aqueous media. This reaction has never been done using the *G. pallidus* RAPc8 amidase, therefore making it novel.

Chapter 3: Materials and Methods

3.1 Introduction

Chapter 3 gives a detailed description of the experimental methodologies and materials used in this study. The experimental approach involves the production, purification and immobilization of the *Geobacillus pallidus* RAPc8 amidase and the subsequent application of the biocatalyst in the synthesis of enantiopure amides in non-aqueous media.

3.2 Materials

Chemicals were supplied by Sigma-Aldrich (SA), Merck Chemicals and Fluka. All solvents were purchased from Merck-SA. All chemicals and solvents used were of the highest grade available. The Eupergit C® beads were kindly donated by Degussa, Germany. The ionic liquids, 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4], 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF_6] and 1-octyl-3-methylimidazolium hexafluorophosphate [omim][PF_6] were purchased from ROTH (Germany) and used as supplied.

3.3 Bacterial Strains and stock maintenance

The recombinant *Escherichia coli* BL21 strain containing the vector with the *G. pallidus* RAPc8 amidase gene was kindly donated by our collaborators at the Institute for Microbial Biotechnology and Metagenomics (IMBM), University of the Western Cape. The gene for the target protein, amidase, was coded on the expression vector PNH223, which is expressed under the control of the isopropyl-thiogalactopyranoside (IPTG) inducible T7 DNA polymerase promoter. This recombinant strain was resistant to ampicillin and cabernicillin (Cameron, 2005). Stocks of the recombinant *E. coli* BL21 strain were stored at -20°C in 20% (v/v) glycerol. The *E. coli* was sub-cultured every two weeks in fresh Luria agar, supplemented with ampicillin (Olaofe, 2009).

3.4 Methodology

3.4.1 Media Preparation

Two types of media, namely complex and defined media, were used to cultivate the recombinant *E. coli* BL21strain at the initial stage of this study. Both media were prepared and sterilized by autoclaving at 121°C for 20 min.

3.4.1.1 Complex medium (Luria Bertani broth and agar)

The complex medium consisted of 10.0 g/l of Tryptone, 10.0 g/l sodium chloride (NaCl) and 5.0 g/l yeast extract, for broth and for Luria Bertani agar, 15.0 g/l of agar was added in addition to the Tryptone, yeast extract and NaCl. On cooling, the medium was supplemented with filter-sterilized ampicillin, to a final concentration of 100 µg/l. Addition of the ampicillin serves to maintain plasmid stability and to ensure that only the recombinant *E. coli* clones grow.

3.4.1.2 Defined medium

The defined medium used was adapted from Kortz *et al.* (1995), (Table A3). The defined medium composition was as follow (g/l): 25.0 glucose, 13.3 KH_2PO_4, 4.0 $(NH_4)SO_4$, 1.2 $MgSO_4.7H_2O$, 1.7 citric acid, 0.0084 EDTA, 0.0025 $CoCl_2.6H_2O$, 0.015 $MnCl_2.4H_2O$, 0.0015 $CuCl_2.2H_2O$, 0.003 H_3BO_3, 0.0025 $Na_2MoO_4.2H_2O$, 0.013 $Zn(CH_3COO)_2.2H_2O$, 0.11 $FeCl_3$. The pH of the medium was adjusted to 6.3 with 1 M NaOH, prior to sterilization by autoclaving. In order to prevent the reaction of glucose with the trace elements, glucose and magnesium sulphate were autoclaved separately from the rest of the medium, at 121°C for 20 min. After cooling, all components of the medium were combined under sterile conditions and the pH was subsequently adjusted to 6.7 using ammonia (25% v/v). Filter sterilized ampicillin was added to a final concentration of 100 µg/l (Olaofe, 2009).

3.4.2 Culture conditions

3.4.2.1 Plate cultures

The *E. coli* BL21 glycerol stocks stored at -20°C, were streaked onto agar plates supplemented with ampicillin, under sterile conditions. The streaked plates were incubated at 37°C overnight and stored at 4°C. Pre-cultures were prepared every two weeks.

3.4.2.2 Pre-cultures of *E. coli* BL21

Pre-cultures for both the complex and defined media, were cultivated in LB broth supplemented with ampicillin. A single colony of *E. coli* BL21 was picked from LB plates and inoculated into 30 ml of LB broth. The inoculum was incubated in a 100ml Erlenmeyer flask at 37°C with shaking at 180rpm overnight on an orbital shaker. The inoculum was then transferred into fresh medium to a final volume of 100 ml (10%, v/v) in a 500 ml flask to allow for adequate aeration of the cells. The 100 ml inoculum was incubated overnight at 37°C, with shaking at 180 rpm on an orbital shaker.

3.4.2.3 Growth of *E. coli* BL21 strain in shake flasks

The 100ml pre-cultures grown overnight was used to inoculate fresh LB broth or defined media, to a final working volume of 1 L in a 2 L Erlenmeyer flask stoppered with cotton wool. The flasks were incubated at 37°C for complex media and 30°C for defined media (Olaofe, 2009) on an orbital shaker at 180 rpm. During incubation, 1ml volumes of culture were removed every 30 min and the optical density (OD) values measured at A_{660} nm. At OD values of A_{660}nm = 0.4, IPTG was added to a final concentration of 0.4 mM, to induce protein expression in the cells.

3.4.2.4 Growth of the *E. coli* BL21 strain in a batch bioreactor

Bioreactor batch growth studies were conducted in a 7 L New Brunswick bioreactor with a 5 L working volume. The growth medium was sterilized in the bioreactor at 121°C for 20 min. A 10% (v/v) inoculum was added to the medium in the bioreactor to achieve a final volume of 5 L. The inoculum was prepared as described above (section 3.4.2.2). The temperature of the medium was measured by a temperature probe and maintained at a set value by a heat jacket and cooling water. The pH was measured using a pH probe (Mettler Toledo) and maintained at a constant value by titration of NaOH and H_3PO_4 for LB medium and 25% (v/v) ammonia for defined medium. Dissolved oxygen was measured using an oxygen probe. Oxygen was made available to the microbes through sparging of compressed filtered air (filtered with a 25 µm Express® PES membrane filter, Millipore, Corp.), into the bioreactor. The temperature, oxygen and pH were monitored online through a primary control unit. The bioreactor is equipped with six-blade Rushton impellers which allows for the mixing of the contents in the bioreactor. Values set on the controller were as follows: temperature: 30°C (both media), agitation: 500 rpm, initial dissolved oxygen: 3.0 vvm and pH: 6.7 (Olaofe, 2009).

Figure 3.1: BioFLO 110 series bioreactor used in this study (Picture reproduced from Olaofe, 2009).

3.4.2.5 Harvesting of *E. coli* BL21 cells and disruption using ultrasonification

Once OD values of 2 and 9 were achieved in complex and defined media respectively, the cells were harvested by centrifugation at 8000 rpm for 15 min at 4°C. The pellet was resuspended and washed with 50 mM potassium phosphate buffer (pH 7.0). Cells suspended in phosphate buffer (see appendix B for buffer composition) were frozen at -20°C overnight prior to lysis by sonication (Virsonic ultrasonic). The cells were allowed to thaw at room temperature, followed by 8 cycles of sonication (1 min burst and 30 s breaks). The disrupted cells were centrifuged (8000 rpm, 15 min, 4°C). The cell free supernatant was stored at 4°C until required.

3.4.3 Purification of the *G. pallidus* RAPc8 amidase

The cell free supernatant was purified by different methods, namely: heat treatment, ammonium sulphate precipitation, hydrophobic interaction chromatography and ion exchange chromatography. Samples from each purification method were tested for amidase activity through the ammonia assay (section 3.5.1) and the protein concentration was determined

through the Bradford assay (section 3.5.4). The samples were further analyzed for degree of purity using 12% SDS polyacrylamide gel electrophoresis.

3.4.3.1 Heat shock purification

The soluble fraction from the crude extract obtained through the ultrasonification and centrifugation of the recombinant *E. coli* BL21 cells, was initially purified by heat shock treatment. The fraction was incubated in a water bath at 70°C for 45 min. The debris, consisting of precipitated *E. coli* proteins, was separated from the soluble fraction by centrifugation (8000 rpm, 15 min, and 4°C). The resulting semi-purified soluble amidase was stored in a fresh tube at -20°C (Makhongela *et al.*, 2005; Olaofe, 2009).

3.4.3.2 Ammonium sulphate precipitation

The second step of purification following heat treatment (section 3.4.3.1) was precipitation of the heat treated amidase extract in an ammonium sulphate salt solution. The heat treated fraction (25 ml) was mixed with ammonium sulphate salt to give a saturation concentration of 20% at 4°C, with gentle stirring. The solution was left on ice for 1 h. The precipitated proteins were removed by centrifugation (8000 rpm, 15 min). The soluble fraction from the ammonium precipitated sample was stored at -20°C until further use (Agarkar *et al.*, 2006; Chiyanzu *et al.*, 2009).

3.4.3.3 Hydrophobic Interaction Chromatography (HIC)

The ammonium sulphate saturated protein solution was further purified by loading onto a pre-packed Hi-Load 16/10 Phenyl-Sepharose column (Amersham Biosciences), pre-equilibrated with 50 mM potassium phosphate buffer containing 1.7 M ammonium sulphate and 5 mM DTT, pH 7.4. Protein elution of bound proteins was achieved using a linear gradient of decreasing ammonium sulphate concentration (1.7-0 M) in 50 mM potassium phosphate buffer containing 5 mM DTT. The eluent was collected as 2 ml fractions. Fractions containing high amidase concentrations (as shown by the UV detector), were pooled. These pooled fractions were then dialyzed against 20 mM potassium phosphate buffer containing 5 mM DTT and 100 mM NaCl, pH 7.4 (Agarkar *et al.*, 2006; Chiyanzu *et al.*, 2009).

3.4.3.4 Ion Exchange Chromatography (IEX)

A HiPrep 16/10 Q-Sepharose FF column (Amersham Biosciences) was pre-equilibrated with 20 mM phosphate buffer containing 5 mM DTT and 100 mM NaCl, pH 7.4. The dialyzed protein fraction was loaded onto the column and eluted with a linear gradient of increasing NaCl concentration (0.1-1 M) in 20 mM potassium phosphate buffer containing 5 mM DTT and 1 M NaCl, pH 7.4. Fractions containing the amidase protein were pooled and stored at -20°C (Agarkar *et al.*, 2006; Chiyanzu *et al.*, 2009).

3.4.4 Immobilization of *G. pallidus* RAPc8 amidase on Eupergit C® by covalent binding and cross-linking

For the immobilization experiments, 25 ml of purified amidase solution in phosphate buffer, pH 7.0, was added to 5 g of dry Eupergit C beads. The mixture was incubated at room temperature (25°C) with gentle shaking (100 rpm) for 72 h. After incubation, the immobilized enzyme mixture was cross-linked with 0.5% (v/v) 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDAC) for 3 h (Makhongela *et al.*, 2007). The beads were recovered by filtration using a vacuum system. The beads were subsequently washed three times with 15 ml of 50 mM potassium phosphate buffer, pH 7.0. To determine the immobilization efficiency, amidase activity of the beads (section 3.5.1) and protein concentration (section 3.5.4) were determined in the filtrate and washings, taking into consideration the dilution factor, during the washes. The immobilized enzyme was lyophilized by freezing with liquid nitrogen and then drying in a vacuum freeze drier at -54°C for 24 h. Beads without immobilized enzyme were used as controls (Makhongela *et al.*, 2005).

The amount of protein loaded onto the carrier material was calculated by determining the amount of protein present in the washes. The protein loading was calculated in terms of percentage of the protein loaded onto the beads, as follows:

$$\text{Protein loading (\%)} = \frac{Ci - Cf}{Ci} x\ 100 \qquad 3.1$$

C_i (mg/ml) represents the initial protein concentration before immobilization, C_f (mg/ml) is the total protein concentration in the filtrate.

The amount of enzyme immobilized was determined by calculating the enzyme activity before and after immobilization. The protein immobilized was expressed in terms of the immobilization efficiency, as a percentage:

$$\text{Immobilization efficiency (\%)} = \frac{Aimm}{Asol} x\ 100 \qquad 3.2$$

Where A_{imm} (U/mg) represents the specific activity of the immobilized biocatalyst. A_{sol} (U/mg) represents the specific activity of the soluble enzyme.

3.5 Analytical procedures

3.5.1 Measurement of amidase activity

The phenol-hypochlorite ammonia detection assay described by Weatherburn, (1976), was used to determine the amidase activity by quantifying the ammonia released on the hydrolysis of amides to their corresponding carboxylic acids and ammonia. The ammonia produced reacts with sodium hypochlorite and phenol to form indophenols, which produce a blue colour. Unless stated otherwise, amidase activity was determined as follows: 100 µl aliquots of the reaction mixture (initially containing 25 mM substrate and 25 µl of amidase solution) were added to 500 µl of reagent A (1 mM sodium nitroprusside and 0.59 M phenol). Reagent B (0.11 M sodium hypochlorite and 2.0 M sodium hydroxide) was added to the mixture. The samples were vortexed and incubated at room temperature for 20 min. Amidase activity was measured at an absorbance of 600 nm using a Novasina, UV-VIS spectrophotometer.

Ammonium chloride solution was used to prepare the standard solutions, with concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1 mM). The standard samples were treated in the same manner as the reaction mixture samples. One unit of activity is defined as the amount of enzyme required to produce 1 µmol of ammonia per minute under standard assay conditions (pH 7.0 and 50°C). The reagent preparation and standard curve are given in Appendix B.

3.5.2 Determination of activity of immobilized amidase

Activity assays for immobilized amidase were performed as described in section 3.5.1, except that 100 mg of immobilized enzyme biocatalyst was used instead of the 100 µl of reaction mixture. The reaction mixture was subjected to gentle shaking as described in section 3.5.1.

3.5.3 Acyl transfer activity measurement

The method for acyl transfer reactions was adapted from Fournand *et al.* (1998). Reaction mixture (475 µl), containing 95 mM acetamide and 0.5 mM of freshly prepared hydroxylamine in 50mM potassium phosphate buffer, was preincubated at 50°C for 15 min. An aliquot containing 25 µl of amidase or 100 mg of immobilized amidase biocatalyst was added to start the reaction (Makhongela *et al.*, 2005). The reaction mixture was incubated for 30 min. Samples of 500 µl were then added to 1 ml of $FeCl_3$ acidic solution (356 mM in 0.68 M HCl). To quantify the hydroxamic acid produced, readings were taken using a Novasina, UV-Vis spectrophotometer at a wavelength of 500 nm, using $\varepsilon = 996\ M^{-1}cm^{-1}$. One unit of activity is defined as the amount of enzyme required to produce 1 µmol of hydroxamic acid per minute under standard conditions (pH 7.0 and 50°C).

3.5.4 Protein concentration assay

The Bradford Coomassie Brilliant Blue dye assay (Bradford, 1976), was used to determine total soluble protein concentrations. The assay was performed by adding a 20 µl aliquot of the sample to 1 ml of Bradford solution (BioRad) (Makhongela *et al.*, 2005). The solution was vortexed and incubated at room temperature for 5 min. The protein concentration was determined using a spectrophotometer. Absorbance was determined at 595 nm. Protein standards were prepared using Bovine Serum Albumin (BSA) and treated in the same manner as the amidase reaction mixture samples. The reagent preparation and standard curve are given in Appendix B.

3.5.5 Sodium Dodecyl Sulphate-polyacrylamide electrophoresis (SDS-PAGE)

The samples (50 µl) obtained from each of the purification steps (sections 3.5.3.1- 3.5.3.4) were separated on a 12% SDS-PAGE to determine the purity and size of the amidase protein. Samples were prepared by mixing with SDS-PAGE loading buffer, and boiled at 100°C for 5min prior to loading onto the gel. The gels were run for 2 h at a constant current of 50 mA in running buffer. Gel staining was done with 0.25% (w/v) Coomassie Brilliant Blue-R250 solution containing 10% (v/v) glacial acetic acid and 50% (v/v) methanol, for 1 h. The gel was de-stained overnight to remove excess dye using a destaining solution containing 10% and 50% glacial acetic acid and methanol respectively (Laemmli, 1970). Activity and protein concentrations were determined for each of the samples using the methods described below (see sections 3.6.2 and 3.6.4). The reagents preparations are given in Appendix C.

3.6 Synthesis of amides in non-aqueous media

3.6.1 Measurement of water activity in acetonitrile and [Bmim][BF_4] using a hygrometer

Water activity was determined using a hygrometer (Novasina, Switzerland), equipped with a humidity sensor. The hygrometer was calibrated between 0 and 1, using standard salt solutions. The water activity was measured when the humidity sensor was equilibrated with the sample, until constant values were reached. Prior to testing, samples were equilibrated 24 h at 50°C (Ebert *et al.*, 1998).

3.6.2 Amide Synthesis reactions

3.6.2.1 Amide synthesis in organic solvents

Synthesis of amides was performed in the following organic solvents: Acetonitrile, toluene, tetrahydrofuran and *n*-heptane. The reaction mixtures of 5 ml containing 25 mM acetic acid as the acyl donor, was pre-incubated at 50°C, for 24 h. The reaction was initiated by adding the lyophilized biocatalyst (20 mg, 2.36 U/mg of beads) and the ammonium carbamate to the reaction medium containing acetic acid. The reactions were incubated in an incubator equipped with a shaker, using agitation at 200 rpm at 50°C (Ebert *et al.*, 1998). Samples were periodically withdrawn from the reaction mixture, filtered and dried for 24 h. The product was resuspended in 1 ml HPLC mobile phase, 30:70 v/v (Methanol: water) and assayed for product by HPLC. Qualitative and quantitative analysis of the product was performed by a LaChrome L-7400 HPLC system (Merck, Hitachi, Germany), equipped with a UV/VIS detector and an Autosampler. Samples were eluted with a mobile phase of methanol/water with volumetric ratios of 30:70, using a C18 (250mm x 4.6mm) reverse phase column. Samples were run under the following conditions: flow rate 0.8 ml/min, temperature 20°C and detection at 210 nm. Acetamide standards were treated in the same manner as the samples, refer to Appendix B for standard curve and reagent preparation.

All control reactions were performed in the absence of the amidase enzyme, under the same conditions, in duplicates.

3.6.2.2 Amide synthesis in room temperature ionic liquids (RTILs)

Synthesis of amides was performed in the following room temperature ionic liquids: 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4], 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF_6] and 1-methyl-3-octylimidazolium hexafluorophosphate [omim][PF_6]. The 1ml reaction mixtures were preincubated under the same conditions described in section 3.6.2.1. The product from the ionic liquids was extracted through a liquid-liquid extraction method by adding 500 µl of hexane three times, to 1 ml of the reaction mixture sample. For the quantitative analysis of the product, the method described in section 3.6.2.1 was used.

3.6.2.3 Product isolation and analysis in ionic liquid reaction systems

For synthesis reactions in ionic liquids, the product was recovered from the ionic liquid via liquid extraction, by washing twice with 500 µl hexane. The hexane was subsequently evaporated, leaving the product, which was resuspended in the mobile phase, 30:70% v/v (methanol: water) used for HPLC analysis. Qualitative and quantitative analysis of the product was performed by a La-Chrome L-7400 HPLC system (Merck, Hitachi, Germany), equipped with a UV/VIS detector and an Autosampler. Samples were eluted with a mobile phase of methanol/water with volumetric ratios of 30:70, using a C18 (250mm x 4.6mm) reverse phase column. Samples were run under the following conditions: flow rate 0.8 ml/min, temperature 20°C and detection at 210 nm.

One unit of enzyme activity was defined as the amount of enzyme that catalyzed the production of 1 µmol of product per minute under standard conditions.

3.6.2.4 Determination of the optimal water content for amide synthesis

To the reaction mixtures, consisting of one of a range of organic solvents (with logP values from -0.33 to 4.0) and ionic liquids (water miscible and water immiscible), water was added to give final water concentrations between 0.1 and 10%, v/v. The organic solvents were previously dried in 3Å molecular sieves prior to the start of the synthesis reactions. The reaction mixtures were preincubated at 50°C for 24 h. The reactions were initiated by adding ammonium carbamate, acetic acid and biocatalyst to the reaction mixture, and performed as described in sections 3.6.2.1 and 3.6.2.2. Samples were taken at 6 and 24 h.

3.6.2.5 Determination of stability of the amidase in organic solvents and room temperature ionic liquids

The lyophilized, immobilized amidase biocatalyst (20 mg, 2.36 U/mg) was incubated in each of the organic solvents and ionic liquids (1 ml) at 50°C and agitated at 200 rpm. Samples were withdrawn at time intervals for a period of 96 h. The biocatalyst was carefully separated from the solvent, making sure that there was minimal loss of the enzyme, by centrifugation. The biocatalyst was washed with 50 mM phosphate buffer. Ionic liquids were removed by pippeting. Amidase activity was assayed by the phenol-hypochlorite assay as described in section 3.5.1.

3.6.2.6 Optimization of the reaction conditions for acetamide synthesis

3.6.2.6.1 Determination of the effects of initial substrate concentration on acetamide yield

Using the method described in section 3.6.2.1, for the synthesis of acetamide in organic solvents, the effects of the substrate concentration on the synthesis reaction was determined by varying equimolar substrate concentrations of acetic acid. The acetic acid concentration was varied between 25 mM and 150 mM, keeping the equimolar ratio of acetic acid/ammonium carbamate at 1. The substrates, with 20 mg of the immobilized biocatalyst were added to 5 ml of organic solvent. The reactions were performed at 50°C in acetonitrile. The biocatalyst mass and equimolar substrate ratio was kept constant. The reaction conditions at which the reactions were carried out and the product yield were determined by following the methods described in section 3.6.2.1.

3.6.2.6.2 Effects of molar ratio of substrates on the product yield of acetamide in acetonitrile

The effect of the increase of the ammonium carbamate/acetic acid ratio on the substrate conversion was determined by varying the molar concentration of ammonium carbamate. The acetic acid concentration used was the one that gave the highest product yield in section 3.6.2.6.1, of 100 mM. Reactions were performed at 50°C in acetonitrile. The molar ratio values were varied between 0.5 and 4. The effect was determined by monitoring the yield of the product, through analysis by HPLC (as described in section 3.6.2.1). All other parameters such as acetic acid concentration and biocatalyst mass were kept constant at 100 mM and 20 mg of immobilized biocatalyst (2.36 U/mg) respectively.

3.6.2.6.3 Effect of the amount of enzyme used in the acetamide synthesis reaction determined by varying the biocatalyst mass

The effect of the amount of enzyme on the acetamide yield in acetonitrile was determined. To the reaction mixture, differing masses of the immobilized amidase, between 20 mg and 500 mg were added. The acetic acid concentration and the molar ratio between the two substrates (acetic acid/ammonium carbamate), were kept constant at 100 mM and 0.5 respectively. The mixture was incubated at 50°C with shaking for 24 h. The product yield was determined by HPLC as described in section 3.6.2.1.

3.6.2.6.4 Synthesis of lactamide using amidase in acetonitrile and [bmim][PF_6]

For the synthesis of lactamide, acetonitrile and [bmim][PF_6] respectively were used as the reaction media, in the presence of 8% (v/v) water. The substrates used for the synthesis reactions were D,L-lactic acid and ammonium carbamate. The substrate concentrations and biocatalyst mass used were those determined in the optimization studies described above, which were 100 mM lactic acid at a molar ratio of 0.5 of lactic acid/ammonium carbamate and 20 mg (2.36 U/mg). The reaction conditions used were the same to those applied above for the synthesis of acetamide, (50°C with agitation at 200 rpm). Analysis of the lactamide product was determined on a LaChrome L-7400 HPLC system (Merck, Hitachi, Germany), equipped with a UV/VIS detector and an Autosampler. Samples were eluted with a mobile phase of methanol/water with volumetric ratios of 30:70, using a C18 (250mm x 4.6mm) reverse phase column. The following conditions were used: flow rate 0.8ml/min, temperature 20°C and detection at 210 nm. Lactamide standards were treated with the same manner as the samples, see Appendix B for standard curve.

3.7 Effects of solvent on the enantioselectivity of the *G. pallidus* RAPc8 amidase

This section of the work, involving the investigation of different chiral separation techniques to determine a method suitable for the separation of lactamide enantiomers, was carried out in Karlsruhe Institute of Technology (Department of technical Biology), Germany. This work was done to determine the yield of the two lactamide enantiomers in the reaction mixture, and hence to determine the effects of the different solvents on the enantioselectivity of the amidase. The solvent effects on the selectivity of *G. pallidus* RAPc8 amidase were tested using the lactamide synthesis reaction. The effect of the solvent on the enantioselectivity was quantified in terms of enantiomeric excess. The enantiomeric excess was quantified using a

suitable chiral separation technique for the separation of lactamide enantiomers, determined in this study. The following equation was used to determine the enantio-purity of the end product:

$$Enantiomeric\ excess\ (\%ee) = \frac{major-minor}{major+minor} x100 \qquad 3.3$$

3.7.1 Indirect methods

3.7.1.1 Preparation of solutions for derivatization

The *o*-phthaldialdehyde (OPA) and *N*-Isobutyryl-L-Cysteine (IBLC) stock solutions at a concentration of 37.23 mM and 52.29 mM respectively, were made by dissolving the chemicals in methanol. The solutions were stored in aliquots at -20°C. For the derivatization of the lactamide enantiomers, the following solutions were prepared in HPLC vials as follows: MilliQ water for washing the needle, 1 ml of 133 mM sodium borate buffer, 1 ml 3% acetic acid, 1 ml of the OPA/IBLC methanol stock solution was freshly prepared by addition into sodium borate buffer and 3x1 ml vials with samples (D-lactamide, L-Lactamide and D,L-Lactamide). The molar ratio of the analyte to the OPA/IBLC solution was 1:25:25 (Brucher *et al.*, 2010). The standard used was an amine.

3.7.1.2 Instrumentation

Analyses of samples from the derivatization reaction were conducted by a LaChrome L-7400 HPLC system (Merck, Hitachi, Germany), equipped with a UV/VIS detector and an Autosampler. The HPLC was also equipped with a robotic Autosampler, a binary pump delivery system, a column thermostat and a fluorescent light detector.

3.7.1.3. Derivatization of lactamide enantiomers with OPA and IBLC

The derivatization of lactamide with OPA/IBLC was performed robotically by programming the auto sampler of the LaChrome L-7400 HPLC system as shown in Table 3.4. Following derivatization, the samples were injected onto a reverse phase C18 column (150mmx4mm), at 25°C, with detection at a wavelength of 340 nm. An isocratic solvent system of 40 mM, sodium phosphate buffer (pH 6.5)/methanol (65:35) was used (flow rate of 1 ml/min). The mobile phase was filtered through a 0.22 µm filter (Express® PES membrane, Millipore,

Corp.) and degassed prior to use (Brucher *et al.*, 2010). The Programming instructions for the Autosampler are given in Appendix D.

3.7.2 Direct methods

3.7.2.1 Separation of lactamide enantiomers on chiral stationary phases (CSPs)

The lactamide enantiomers were sent to Macherey Nagel (Karlsruhe, Germany), specialists in chromatography and manufacturers of chiral HPLC and GC columns and chiral TLC plates. Chiral columns with different stationary phases were tested, to determine the most suitable for the separation of lactamide enantiomers. Different mobile phases were also tested, (Table 3.1).

Table 3.1: Separation methodologies applied by Macherey Nagel to resolve lactamide enantiomers.

Chiral stationary phase	Mobile phase	Flow rate	Temperature	Wavelength
Nucleosil 100-5	0.2mM $CuS0_4$ Isg	04ml/min	30°C	210nm
Nucleosil 100-5	0.5mM $CuS0_4$	04ml/min	22°C	210nm
Nucleocel Alpha RP-S	Acetonitrile/ Water	0.5ml/min	20°C	210nm
Nucleocel Alpha RP-S	Methanol	0.5ml//min	20°C	210nm

The injection volume for all the analyses was 5.0µl

3.7.2.2. Separation of lactamide enantiomers by chiral TLC

Samples (2 µl, 25 mM) of the lactamide enantiomers, dissolved in methanol, were spotted on a TLC glass plate impregnated with a chiral selector and copper (II) ions. The mobile phase used was a mixture of methanol/water/acetonitrile (50:50:200, v/v/v) as described in the Macherey Nagel manual. The tank was saturated for 10 min with the mobile phase prior to the loaded CHIRALPLATE® being placed in it. After drying with a hand dryer, spots on the plate were visualized using a variety of reagents: Ninhydrin, Ehrlich's reagent and hydroxylamine-iron (III) chloride reagent. The control was a chiral amino acid, proline.

Chapter 4: Production, Purification and Immobilization of the *Geobacillus pallidus* RAPc8 amidase

4.1 Introduction

Processes involved in the production of heterologous protein depend on their ability to produce high cell biomass as well as high levels of the target protein, in order to be economically sustainable. In recent years, the ability to attain high biomass and protein yields have been made possible by the application of a combination of molecular and bioprocessing techniques (Kilikian *et al.*, 2000). The molecular strategies involve, as an example, the use of strong promoters for the expression of foreign protein, such as the *lac* promoter which allow the expression of foreign genes in host organisms such as *Escherichia coli*. Bioprocessing strategies involve the manipulation of cell induction time and inducer concentration, as examples (Kilikian *et al.*, 2000; Olaofe, 2009). Depending on the industry or process for which the enzyme is used, the downstream processes also contribute significantly to the costs of protein production and preparation. Some of these downstream activities include the purification of the enzyme and immobilization. Whether these steps are incorporated into the process depends on the property requirements of the biocatalyst, which is dependent on the biotransformation reaction the biocatalyst is expected to perform. For example, in producing low volume, high value products such as enzymes used for therapeutics in the pharmaceutical industry, the purification techniques applied should allow for a biocatalyst of high purity to be obtained. When the target products are high volume and low value, such as the bulk commodity enzymes, the purity of the enzymes does not need to be very high (Zhang, 2010).

For an enzyme to be considered for any industrial application, one of the criteria is that it should be able to be produced in large enough quantities to meet the commercial needs. The biocatalyst should also exhibit high stability properties. However, most mesophilic enzymes are heat labile, a problem that has been overcome, in some cases, by using enzymes from extremophiles (Lee *et al.*, 1997; Karan *et al.*, 2012). Nature offers a variety of enzymes which are able to catalyze almost all reactions known to man, but only a few of these enzymes have been used successfully in industrial processes. This drawback is largely due to the fact that the native strains in which these enzymes are found are difficult to cultivate in the laboratory and those that are successfully grown in the laboratory produce small quantities of enzymes that are insufficient for use at industrial scale (Johannes *et al.*, 2006). Recently, recombinant

technology has been developed that allows for production of large quantities of such enzymes via a host-vector expression system. *Escherichia coli* BL21 is a common expression system for heterologous proteins because it has been widely studied and it is easy to genetically modify and cultivate to high cell densities (Kayser *et al.*, 2005). This *E. coli* strain is particularly suitable for protein expression because of its defective *lon* and *ompT* proteases, which prevents the production of proteases which may inactivate the target enzyme synthesized from the vector (Paliy & Gunasekera, 2007).

The *G. pallidus* RAPc8 amidase, which is the subject of this study, was produced by over-expressing it in *E. coli* BL21. The gene for this amidase was cloned from a thermostable *G. pallidus* RAPc8 strain into *E. coli*, by our collaborators (Pereira *et al.*, 2005). Over-expression of the enzyme is stimulated by addition of an inducer, isopropyl-B-D-thiogalactopyranoside (IPTG) which regulates transcription of the protein (Kilikian *et al.*, 2000).

4.2 Results

The recombinant *E. coli* BL21 strain carrying the *G. pallidus* RAPc8 amidase gene was cultivated in a 110 BIOFLO bioreactor, in two different media. The complex Luria Broth medium and the defined glucose based medium were used for the growth of the *E. coli* BL21 strain and biomass obtained from each was compared. This would in turn confirm the previous studies conducted, that the defined medium gives a higher biomass yield of 9.40 g/l than the complex medium which gave a biomass yield of 1.80 g/l (Olaofe, 2009). The medium that gave the largest biomass yield was used for further studies in this project, as high biomass yield often correlates to high enzyme yield.

The *G. pallidus* RAPc8 amidase was extracted from the *E. coli* cells by ultrasonification. The crude extract was subsequently subjected to different purification methods, in the following order: the first purification step was by heat shock at 75°C for 45min, this is an important step because most of the *E. coli* BL21 proteins are denatured at this temperature, while the amidase is not, (the producing strain is a thermophilic strain) (Makhongela *et al.*, 2007). The next purification step was ammonium sulphate precipitation of the amidase, through mixing the amidase with an ammonium sulphate salt. The third purification method was by hydrophobic interaction chromatography (HIC) and the last purification step of the amidase

was by ion exchange chromatography (IEX). The purification step that gave a purified enzyme of an acceptable standard for this study was used for further studies.

The purified *G. pallidus* RAPc8 amidase was immobilized onto Eupergit C beads, and cross-linked with 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDAC), as recommended by Makhongela *et al.*, (2007). The immobilized amidase was applied in non-aqueous organic synthesis for the synthesis of enantiopure amides (Chapter 5).

4.2.1 Recombinant *Escherichia coli* BL21 fermentation in a BIOFLO bioreactor

E. coli is the most commonly used bacterial strain for the expression and production of heterologous proteins. To improve the efficiency of recombinant protein bioprocesses, it is essential that high cell concentrations are achieved which, in turn, lead to high protein yields. A variety of techniques have been tested by industrial microbiologists, to formulate a method that will allow for the production of high cell density of *E. coli* cells, namely: manipulation of the medium composition; manipulation of the physical conditions such as temperature, oxygen, mixing and testing of different types of fermentors such as batch, and fed-batch reactors (Aristidou *et al.*, 1999; Shiloach & Fass, 2005).

In order to develop the growth technique that gives a high cell biomass, previous studies were conducted that compared the yield of the cell biomass by growing the *E. coli* BL21 in 5L shake flasks as well as a 5L batch fermentor. The yields from the two studies were compared and it was discovered that growing the cells in a fermentor gave a higher biomass yield of 1.70 g/l as compred to growing the cells in shake flasks which gave a biomass yield of 1.02 g/l (Olaofe, 2009). This is because in a bioreactor, the physical conditions are controlled, for example the pH of the medium is kept constant by automatic addition of sodium hydroxide or phosphoric acid in LB medium and 25% (v/v) ammonia solution in defined medium, as required. In a flask, pH of the medium is not controlled. The operating conditions of the reactor were set at the highest values possible, but minimising foaming: aeration of 3 vvm and 500 rpm agitation, as suggested by Makhongela *et al.* (2007) and Olaofe (2009). Growing the cells in the bioreactor gave a high cell biomass, however, the volumetric productivity of the protein was very low. Therefore, in this study, further investigations were conducted to determine a method that will allow for high cell growth. Two types of growth media were tested to determine which one would give high biomass.

The two media that were tested for the growth of *E. coli* BL21 to a high cell concentration and high enzyme concentration, were complex Luria Broth and a glucose based defined medium. The difference between the two media is that the defined medium is supplemented with glucose as a carbon source, while the complex medium is not. From the previous study, it was determined that the defined medium gave a higher cell biomass of 9.40 g/l as compared to the complex medium with a biomass yield of 1.80 g/l (Olaofe, 2009).

In this study, a comparison of the two media was done in order to confirm the results of the previous study, that the defined medium was the most suitable for achieveing high cell concentrations of *E. coli* BL21 as well as high yields of the *G. pallidus* RAPc8 amidase. The amidase gene was cloned into an isopropyl-β-D-thiogalactopyranoside (IPTG)- induced plasmid, hence for expression of the amidase gene, IPTG was used as the inducer. For induction, IPTG was added to the growing culture at a final concentration of 0.4 mM, at the time when the growing culture reached an optical density (OD) of 0.4, which is at the beginning of the exponential phase. The culture reached the 0.4 OD after 60 min of growth in the complex LB medium, and after 5 h of growth in defined medium. These conditions were used based on the previous studies conducted to determine the most appropriate time for inducing cells to achieve high protein expression (Olaofe, 2009). Studies to determine the effect of the concentration of the inducer and of the time of induction on the culture and its ability to express the protein were conducted previously in our laboratory (Olaofe, 2009). From these studies, the most suitable IPTG concentration, to achieve maximum expression was determined to be 0.4 mM and the best time to induce was at the beginning of the exponential phase. After induction, cultures were allowed to grow for a further 5 h in the LB medium and 10 h in case of the defined medium.

4.2.2 Comparison of *E. coli* BL21 growth and biomass yield in complex and defined media

The effects of the complex and defined media were determined by growing *E. coli* BL21 cells in each one of them. The cell biomass was measured in terms of optical density (OD) at A660 nm. The optical densities of the cells were plotted against time in a graph. The difference in biomass obtained from the complex and defined media is observed in the growth curves of the *E. coli* BL21 strain as shown in Figure 4.1. Growth was started at an OD of 0.1 in both media. The lag phase in the complex medium lasted for 1 h and for 5 h in defined medium.

The highest biomass yield was obtained at OD values of 2 and 10, represented by 1.5 g/l and 5 g/l in complex and defined medium respectively, which is an increase of approximately 3 fold. This study confirms that the defined medium gave a higher cell biomass compared to the complex medium. Luria broth does not accommodate high density cultures, and for it to be able to do so, the concentration of the medium composites should be increased, with addition of some phosphorus, magnesium, sulphur and some trace elements (Shiloach & Fass, 2005). Previous studies have revealed that in certain cases, this medium is not able to support high cell densities due to the fast depletion of the nutrients and fast production of acetate (Eiteman & Alteman, 2004). However, there are cases were the complex medium has given a higher biomass yield as compared to the defined medium (Basar *et al.*, 2010).

In the glucose-supplemented defined medium, the rate at which cells grow is very low in the initial phase, as shown by the long lag phase of the growth cycle. Also, the concentrations of the minerals can be manipulated by increasing or reducing them, to allow for maximum cell production without any inhibition from by-product formation. The slow growth of the cells allow for the carbon source to be assimmilated into the cells for protein production, therefore less carbon will be available for acetate formation (Eiteman & Alteman, 2004; Aristidou *et al.*, 1999;Yee & Blanch, 1992). The defined medium was therefore selected as the better growth medium because of the maximum high biomass achieved during growth, considered to be associated with high volumetric productivity of protein (Shiloach & Fass, 2006).

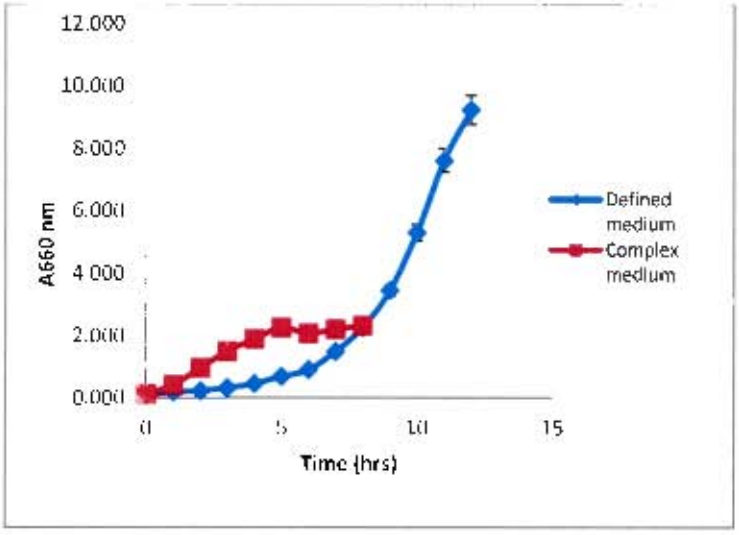

Figure 4.1: Comparison of the growth profiles of *E. coli* BL21 strain grown in defined medium (blue) and complex medium (red), in a 5 L BIOFLO fermentor.

4.2.3 Recovery and purification of *Geobacillus pallidus* RAPc8 amidase

The crude amidase extracted by sonification and centrifugation of the *E. coli* BL21 cell extract grown in defined medium, (section 3.4.1.2) was purified by the following purification methods: heat treatment, ammonium sulphate precipitation, HIC and IEX. These methods were previously used as procedures for the determination of the crystal structure of the amidase (Pereira, 1998; Agakar *et al.*, 2006). In this study, these purification methods were explored for the purification of the amidase to determine the purification method best suited for the studies conducted here.

4.2.3.1 Amidase purification by heat treatment

Earlier work demonstrated that the *G. pallidus* RAPc8 amidase displayed high thermal stability after incubation at 50°C and 60°C for 50 min (Makhongela *et al.*, 2005). Based on these findings and the fact that *E. coli* BL21 proteins are heat labile, a heat treatment method was employed as the first purification step. The crude extract was subjected to heating at 75°C for 45 min. The heat treated samples were assayed for amidase activity and protein concentration, see sections 3.5.1 and 3.5.2 respectively. The heat treatment step resulted in the precipitation of some 40% of the total proteins (from 374 mg to 139 mg) and a recovered total activity of 83% (Table 4.1). This step gave a fold purification of 2.0. Qualitative analysis of the heat treated samples on the SDS-PAGE gel also revealed that most of the contaminating proteins were precipitated by the heat treatment method (Figure 4.4).

4.2.3.2 Amidase purification by ammonium sulphate precipitation

The heat treated extract was subjected to purification by ammonium sulphate precipitation at 20% saturation. Undesired proteins can be removed out of the protein solution by 'salting out', which is done by adding a salt at a certain concentration in the protein solution. The salting out happens when the protein is no longer soluble in the salt-protein solution due to an increasing salt concentration. A number of salts can be used to purify proteins by precipitation through the salting out method, however, ammonium sulphate is the most commonly used salt for this purpose. Large protein molecules are precipitated by low salt concentrations. The unwanted proteins are preciptated out of solution while the protein of interest remains in solution. A small amount of proteins were precipitated during this purification step, however, the recovered activity and purification yield after ammonium sulphate precipitation could not be determined due to the ammonia interference with the ammonia assay and SDS-PAGE. However, this purification step is essential as it removes a proportion of the unwanted proteins by precipitating them out of the solution and it is relatively inexpensive.

4.2.3.3 Amidase purification by Hydrophobic Interaction Chromatography (HIC)

The extract from the ammonium sulphate precipitation step was dialysed in a 1 M ammonium sulphate concentration, prior to being subjected to phenyl-sepharose HIC. The extract was then loaded onto a HiLoad 16/10 phenyl sepharose column equilibrated with phosphate buffer containing 1 M ammonium sulphate. The mechanism of differentiating proteins using HIC methods relies on the differences in the hydrophobicities of proteins. The HIC purification method was also applied by Cameron (2002) and Agarkar *et al.* (2007), for the purification of the native and recombinant *G. pallidus* RAPc8 amidase, which resulted in a near homogenous amidase extract. The two peaks in Figure 4.2 represent protein, detected at an absorbance of 280 nm. The protein fractions with low background or less contaminating protein were collected and assayed for amidase activity and protein concentration. A recovered activity of 76% and a purification yield of 6 were obtained (Table 4.1).

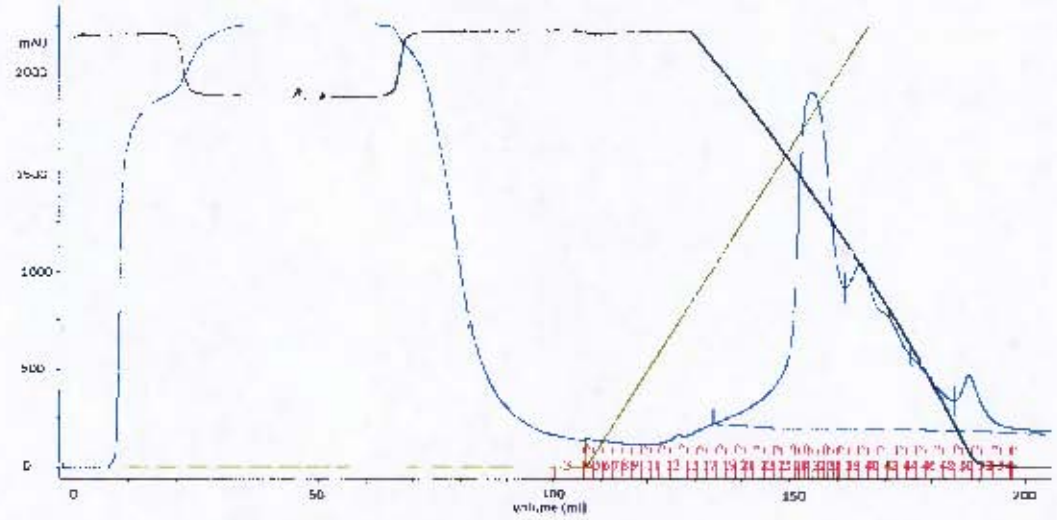

Figure 4.2: A hydrophobic interaction chromatography (HIC) chromatogram of the ammonium sulphate purified *G. pallidus* RAPc8 amidase using a Hi-Load 16/10 Phenyl-Sepharose column.

4.2.3.4 Amidase purification by Ion Exchange (IEX) Chromatography

To attain a further degree of purification of the *G. pallidus* RAPc8 amidase enzyme, the anion exchange purification method was used as the final polishing step. The HIC-treated protein solution was loaded onto a HiPrep 16/10 Q-Sepharose column equilibrated with low salt buffer. The bound proteins were eluted with an increasing sodium chloride concentration buffer. The IEX purification method was also used by Cameron (2005) and Agarkar *et al.* (2007), to achieve a highly purified amidase enzyme. The peak shown in Figure 4.3 represents the protein as detected by the UV detector at an absorbance of 280nm. The recoverd activity and purification fold obtained from this purification step were 46% and 11 respectively (Table 4.1).

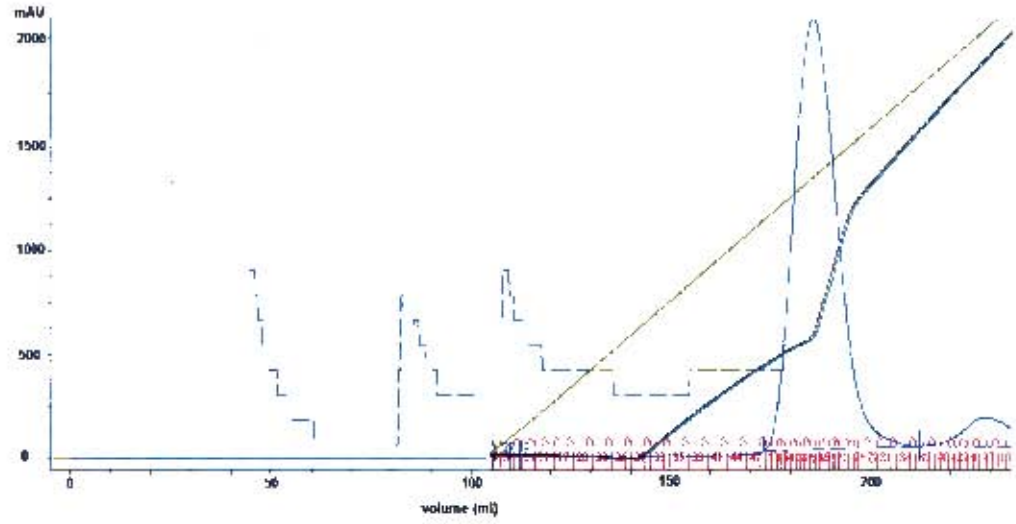

Figure 4.3: Ion exchange chromatography (IEX) chromatogram of the *G. pallidus* RAPc8 amidase extract previously purified by HIC, using a HiPrep 16/10 Q-Sepharose column.

4.2.3.5 Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE)

Samples were collected at each purification step described above, and these were then loaded onto a SDS-PAGE gel, to confirm the efficiency of the purification of the amidase. As shown in Figure 4.4, the amidase subunit is represented by the 35kDa band on the gel. This confirms the work reported by Cameron (2001) and Kimani *et al.* (2007), that the amidase has a single subunit with molecular weight of 35kDa (Makhogela *et al.*, 2007). The crystal structure of the amidase was determined by Kimani *et al.* (2007), and the *G. pallidus* RAPc8 amidase is reported to exist as a homo-hexamer. The SDS-PAGE gel confirmed that the heat treatment step resulted in the removal of most of the unwanted proteins, where approximately 40% of the total protein was removed.

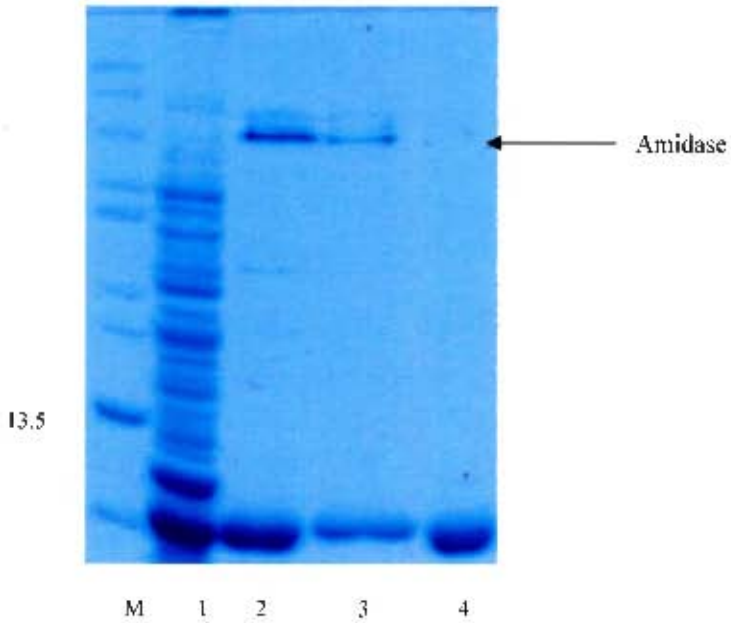

Figure 4.4: SDS-PAGE analysis of the *G. pallidus* RAPc8 amidase crude extract (lane 1), heat treated supernatant (lane 2), pooled from fractions from HIC (lane 3) and pooled from fractions from IEX (lane 4).

4.2.3.6 Purification table for the purification of *Geobacillus pallidus* RAPc8 amidase

The purification table (Table 4.1) gives a summary of the efficiencies of each of the individual purification methods in the purification of the *G. pallidus* RAPc8 amidase protein. The summary shows that the heat treatment method was very effective at removing the *E. coli* contaminating proteins. This is represented by removal of 40% of the proteins and a recovered activity of 80%. The chromatographic methods gave a final product of highly pure enzyme, as shown by the higher total activity and single band, which represents the *G. pallidus* RAPc8 subunit, however the recovered enzyme activity was low as a significant prorportion of enzyme was lost with the succession of the purification steps.

Table 4.1: Purification table for the purification of the Geobacillus pallidus RAPc8 amidase.

Purification step	Total Protein (mg)	Total activity Units	Specific Activity (Units/mg)	Yield (%)	Purification (Fold)
Crude Extract	374	20895	56	100	1
Heat Treatment	139	17397	126	83	2
Phenyl-Sepharose	47	15983	342	76	6
Q-Sepharose	16	9549	591	46	11

4.2.3.7 Covalent immobilization of *Geobacillus pallidus* RAPc8 amidase on Eupergit C ® beads

The heat purified amidase was immobilized onto Eupergit C beads and subsequently cross-linked using ethylenediaminetetraacetic acid (EDAC) according to the method developed by Makhongela *et al.* (2007). The immobilization of the amidase onto the Eupergit C beads occurs through covalent interaction of the epoxy groups on the surface of the beads with the amino acid residues on the surface of the enzyme (Katchalski-Katzir & Kraemar, 1999). The cross-linking step is important in obtaining an immobilized biocatalyst with a high activity and stability, possibly because the cross-linker establishes a multi-point covalent enzyme-enzyme attachment (Fernandez-Lafuente *et al.,* 1999). The immobilization procedure was carried out at 25°C, with shaking, for 72 h. 0.5% (w/v) EDAC was added to the immobilized enzyme for 3h and the beads were then filtered and washed to determine the amount of protein bound on the beads (Table 4.2). This method was selected for the immobilization of the amidase as previous studies revealed that Eupergit C beads, together with crosslinking, resulted in a high protein binding yield and recovered activity (Makhongela *et al.*, 2007) where the binding yield, which represents the amount of protein bound onto the support, obtained was 59.6% and the recovered activity, which is the specific activity of the immobilized enzyme, was 78.9% (Makhongela *et al.*, 2007). The binding yield and recovered activity obtained in this study were 56% and 54% respectively. The binding yield is comparable as to that obtained in previous studies (Makhongela *et al.*, 2007). However, the recovered activity is lower by a magnitude of about 1.5. The reduction in recovered activity as compared to the value obtained in previous studies could be a contribution of the inhibitory effect of the cross-linker, EDAC (Makhongela *et al.*, 2007).

Table 4.2: The activity of the free G. pallidus RAPc8 amidase before immobilization and that of the filtrates.

Free amidase			Filtrates/washes		
Activity (U)	Protein concentration (mg)	Specific activity (U/mg)	Activity (U)	Protein concentration (mg)	Specific activity (U/mg)
14720	210	70	2912	92	32

4.3 Discussion and Conclusion

This chapter describes the methods used for the production and preparation of the *G. pallidus* RAPc8 amidase, by purification and immobilization. The method used for the production of the amidase was by growing the recombinant *E. coli* BL21 cells in an automated BIOFLO 110 series fermentor which allowed for the control and maintenance of the suitable growth conditions. A higher maximum biomass yield was obtained by growing cells in the bioreactor than growing in shake flasks. This is because in shake flasks, critical growth conditions such as oxygen and pH cannot be regulated (Makhongela *et al.*, 2007; Olaofe, 2009).

Further studies were conducted to determine the effects of the growth media on the biomass yield, as well as the protein yield. Two media were compared: a complex medium (LB) and a defined medium (glucose-supplemented), to determine their effects on the growth of the *E. coli* BL21 cells. The maximum biomass values were determined through optical density at A660 nm. The cells grown in defined medium gave a higher maximum biomass value compared to that obtained by growing the cells in complex medium, Figure 4.1. In defined medium, a dry biomass weight of 5 g/l was obtained which is 3 fold higher than that obtained in complex medium, of 1.5 g/l. This is because defined media contains a carbon source in high concentrations which acts as a source of energy for the synthesis of protein inside the cell (Aristidou *et al.*, 1999).

Defined medium on the other hand has glucose which is a carbon and energy source, which can support high cell growth and the minerals present in it are vital for protein production. One would argue that if the concentration of the complex medium was increased it would be able to support high cell densities. However, studies conducted by other groups have shown that increasing the concentration leads to cell inhibition, and hence a decrease in protein production (Shojaosadati *et al.*, 2008). The ability of the *E. coli* cells to grow well in a bioreactor and the high enzyme activity obtained make this enzyme a potentially useful biocatalyst for an industrial scale biocatalytic process because scaling-up from the 5 L laboratory bioreactor to industrial scale bioreactor would be possible based on the methods which have been established at lab scale.

In order to obtain an enzyme of a desired purity, the crude extract of *G. pallidus* RAPc8 amidase obtained by sonification and centrifugation of the *E. coli* BL21 cells was subjected

to different purification methods. A four step procedure was undertaken, which involved the purification of the amidase by heat treatment purification, ammonium sulphate precipitation, phenyl-Sepharose (HIC) and Q-Sepharose (IEX) chromatography. From previous studies that involved the resolving of the amidase structure, the above mentioned purification methods were employed to obtain an amidase enzyme of a high purity (Agarkar *et al.*, 2006). In this study, the same procedure was followed for the purification of the *G. pallidus* RAPc8 amidase, to determine the most suitable method for this study. The first purification step, which was the heat treatment took advantage of the thermostable properties of the amidase as described in a previous study (Makhongela *et al.*, 2007) and the fact that the *E. coli* proteins are from a mesophilic strain, making them heat labile. The heat treatment step resulted in the removal of 40% of unwanted proteins and a recovered activity of 80%.

The next purification step involved the precipitation of the heat treated amidase extract in ammonium sulphate salt. However, qualitative and quantitative analysis of the ammonium sulphate precipitated extract could not be determined due to the interference of the ammonia with the ammonia assay and the SDS-PAGE. Salt precipitation is important because it removes some of the contaminating proteins that the other previous purification steps employed could not remove, which could have resulted from the fact that those steps could not differentiate between the types of proteins due to similarities in some of their characteristics. The ammonium sulphate amidase extract was loaded onto a phenyl-sepharose HIC column as the next purification step. This step resulted in a purification yield of 6 and recovered activity of 76%, therefore resulting in a near homogenous amidase extract. The last purification step was loading the amidase extract onto a HiLoad column for ion exchange chromatography. The purification yield obtained through this step was 11 and the recovered protein yield was 46%.

After qualitative and quantitative analysis of extracts from each of the purification steps, the heat treatment method appeared to be the most suitable method for the purpose of this study. This method presents a cheap method and also because the resulting amidase was of a purity high enough for the rest of the study and it resulted in a removal of 40% of contaminating proteins. Compared to the chromatographic methods, the heat treatment is also suitable for industrial scale applications as it does not require any special, costly equipment. At an industrial scale where the volumes of the protein to be purified are very high, the

chromatographic methods would be very costly and therefore make the process not economically sustainable. Also, a lot of biocatalyst is lost during chromatographic purification of the enzyme, with recovered activity of 46% in IEX. The recovered activity of the amidase from the IEX extract would not be enough to reach the required volumetric productivities. However, if the heat treated amidase extract does not meet the requirements of a particular process, one of the chromatography methods might be used in conjunction with the heat treatment to obtain a purity level sufficient for the synthesis of speciality compounds, at much lower costs.

Enzyme immobilization is a useful method to enhance the stability and sometimes the selectivity of an enzyme. Immobilization also allows for the easy recovery of the enzyme from a biocatalytic reaction mixture, which ultimately makes biocatalysts recyclable. Eupergit C is the most commonly used support matrix for the immobilization of biocatalysts used for industrial applications (Boller *et al.*, 2002). Eupergit C beads were previously shown to be a suitable immobilization support for *G. pallidus* RAPc8 amidase compared to other methods that were tested (Makhongela *et al.*, 2007). Cross-linking of the immobilized enzyme with EDAC was a crucial step as it improved the enzyme binding yield. The amidase is a hexamer, and therefore cross-linking may assisted in binding the individual subunits together thereby preserving activity. In previous studies, the recovered activity of the immobilized amidase was obtained at 78% (Makhongela *et al.*, 2007), in this study only 54% of recovered activity was obtained. This could be due to the fact that the cross-linking agent has resulted in the aggregation of too many enzyme molecules per area of the beads, therefore resulting in the hindrance of the enzyme active site making it difficult for substrate to reach the active site.

The results reported in this chapter resulted in the production of an active and stable immobilized amidase biocatalyst. The *G. pallidus* RAPc8 amidase was shown to have 54% activity of the purified unimmobilized form (U/mg). The immobilized amidase biocatalyst was subsequently used in amide synthesis in non-aqueous media, as described in the next chapter.

Chapter 5: Amide synthesis in non-aqueous media by *G. pallidus* RAP c8 amidase

5.1 Introduction

The traditional method of synthesizing organic and inorganic compounds by chemical processes puts a lot of pressure on the environment in terms of pollution as well as the depletion of natural resources. Bioprocesses are fast gaining ground as alternative means of synthesizing the compounds by biotransformations through the use of enzymes. The increasing environmental awareness as well as pressure from governments are major contributors to the shift towards environmentally benign processes (Mallakpour & Yousefian, 2007).

Biocatalysis presents a useful alternative, to the traditional chemical synthesis method for the synthesis of optically pure compounds, used in most industrial processes such as in pharmaceuticals for the synthesis of drugs. One of the major challenges experienced in chemical processes is their lack of selectivity and substrate solubilities, which consequently lead to diminished product yields. The use of biocatalysts in processes in combination to applying medium engineering helps overcome the challenges experienced in aqueous media synthesis as mentioned above. Also, the discovery by Klibanov, (1989), that enzymes remain active when introduced to non-aqueous media, has broadened the applications of biocatalysts and the synthesis of new compounds. This discovery has led to increased research both in academic and industrial laboratories, as demonstrated by the large number of publications that have been published in this field since then (Carrea & Riva, 2000; Baker *et al.*, 2005). This interest in using non-aqueous media as reaction media for biotransformations is fuelled by the many advantages that solvents have over non-aqueous media such as the shift in the thermodynamic equilibrium.

Alternative, 'new generation' non-aqueous media have been identified such as room temperature ionic liquids (RTILs) which have been successfully used for organic biotransformations. The RTILs have been identified as better alternatives due to their environmentally benign properties (Wimmer & Zarevucka, 2010; Nara *et al.*, 2002; Ventura

et al., 2012). This chapter investigates the effects of organic solvents and room temperature ionic liquids as reaction media have on the product yield of acetamide and lactamide.

In this study, a comparative study of acetamide synthesis by the *G. pallidus* RAPc8 amidase, was conducted using both organic solvents and ionic liquids and the results were compared. The organic solvents used as the reaction media were: acetonitrile, toluene, tetrahydrofuran and *n*-heptane. The ionic liquids used as reaction media were: 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF_6], 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4] and 1-octyl-3-methylimidazolium hexafluorophosphate [omim][PF_6]. The reaction for the synthesis of acetamide from acetic acid and ammonium carbamate by the thermostable *G. pallidus* RAPc8 amidase was applied as the model reaction. From the organic solvent reactions, the solvent that was most suitable to support the synthesis of acetamide, determined by high yields of product, would be used for further studies. The ionic liquid that gave the highest product yield was also determined. This would then be applied in future studies to determine the effect of solvent on the enantioselectivity of the *G. pallidus* RAPc8 amidase.

Lactamide synthesis was studied in the organic solvent reaction medium using a racemic mixture of D,L-lactic acid. To determine the enantiomeric excess (ee) of the products of the *G. pallidus* RAP c8 amidase, chiral separation techniques were applied to determine the (ee) once the reaction was complete.

5.2 Results

5.2.1 Effect of water content on water activity in acetonitrile and [bmim][PF_6]

The effect of water content on the water activity of the solvent systems was studied by adding differing amounts of water % (v/v) to the solvents, acetonitrile and [bmim][PF_6]. In each of the systems, water content was varied between 0 and 10%. A mass of 20 mg (2.36 U/mg) of the immobilized *G. pallidus* RAPc8 amidase biocatalyst was added to both the acetonitrile and the [bmim][PF_6] solvents and were incubated at 50°C for 24 h, to allow the water activity to equalise between the amidase biocatalyst and the solvent (Bell *et al.*, 1995). The amidase used in this study was lyophilized prior to use and the assumption were made that the biocatalyst did not contain any water. If the biocatalyst mass is kept constant and the different

phases in the system are equilibrated before taking the readings, then each phase would have the same water activity. Equilibration of the different phases in the solvent is vital for consistency during the taking of the water activity values. The water activity values are taken with a NOVASINA hygrometer equipped with a humidity-temperature sensor.

The water activity versus water content % (v/v) profile for the polar organic solvent acetonitrile and the hydrophobic ionic liquid [bmim][PF_6] systems containing *G. pallidus* RAPc8 amidase, are shown in Figure 5.1. As the water added to the system increased, the a_w values in both systems increased, but with [bmim][PF_6] having higher values than acetonitrile. At water content of 8% (v/v), the water activity value of acetonitrile was 0.68, while that of [bmim][PF_6] was 0.80. Acetonitrile has a logP value of -0.33; this makes it hydrophilic and therefore water miscible. [Bmim][PF_6] on the other hand, has a hydrophobic anion (PF_6), which gives the ionic liquid non-water-miscible properties (Kragl *et al.*, 2002). In a water miscible solvent, the water mixes with the solvent, therefore leaving little or no amounts of free water, which is termed as water activity. In hydrophobic solvents, the water does not mix with the solvent; therefore, the free water in the system is abundant. The results obtained confirmed that water miscible solvents have lower water activity values compared to water immiscible solvents, whose water activity values are always higher. Thus, the [bmim][PF_6], being water-immiscible, gave higher a_w values than the water miscible acetonitrile. The a_w values compatible with the *G. pallidus* RAPc8 amidase enzyme could therefore be determined by monitoring the synthesis of acetamide from acetic acid and ammonium carbamate, at varying water concentrations, and extrapolating results from Figure 5.1.

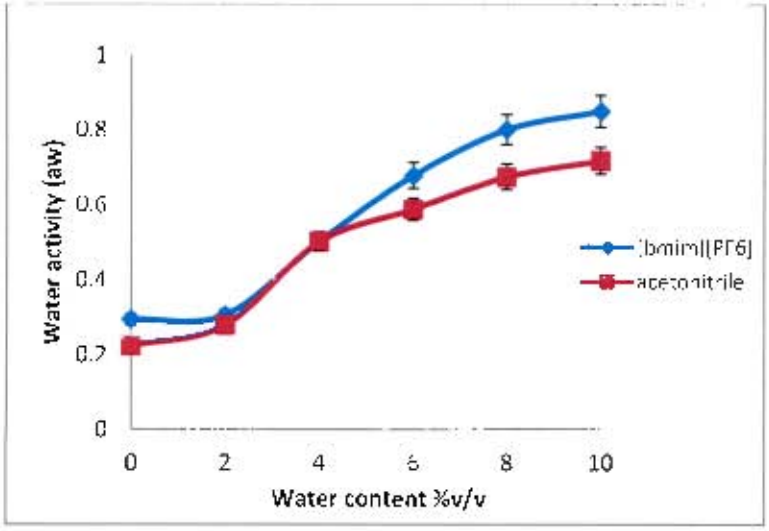

Figure 5.1: Variation in water activity (a_w) of the G. pallidus RAPc8 amidase reaction system, with increasing water concentration, in acetonitrile (red squares) and 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF6] (blue diamonds). Conditions: 1 ml solvent or RTIL, 20 mg *G. pallidus* RAPc8 amidase, maintained at 50°C for 24 h.

5.2.2 Acetamide synthesis in organic solvents

Preliminary results obtained in our laboratory showed that the *G. pallidus* RAPc8 amidase was able to catalyze the synthesis of amides in low water reaction systems (0.006%) (Glowacka, 2007, unpublished results). These findings led us to further investigate the effect of solvent and the water content on the activity of the amidase biocatalyst. Table 5.1, lists the organic solvents used as reaction media in the synthesis of acetamide and their varying range of logP values. The polarity of the solvents decreases with increasing logP values. The logarithm of partition coefficient (logP) of a solvent is a valuable parameter used to describe the polarity of a particular solvent and hence the effect it would have on the enzyme activity. The general consensus is that high enzymatic activity is achieved in solvents with logP values higher than 4.0 or hydrophobic solvents (Salleh *et al.*, 2002). In this study, solvents with varying logP values were studied to determine the most suitable for the *G. pallidus* RAP c8 amidase.

To determine the optimum water content and activity, as well as the most suitable solvent for the synthesis of acetamide by *G. pallidus* RAPc8 amidase, varying water content % v/v was added to each of the solvents as in section 5.2.1. The substrates, acetic acid and ammonium carbamate as well as 20 mg (2.36 U/mg) of the biocatalyst, were added to the reaction reaction systems and incubated in an orbital shaker at 50°C for 24 h, with shaking at 200 rpm.

At 50°C, the *G. pallidus* RAPc8 amidase operates at an optimum activity, temperatures lower than that will result in a low reaction rate and product yield. However, increasing the temperature above 50°C, will lead the rapid loss in enzymatic activity (Makhongela *et al.*, 2007).

Table 5.1: Organic solvents and their logP values.

Solvent	logP
Acetonitrile	-0.33
Tetrahydrofuran	0.49
Toluene	2.5
n-heptane	4

LogP: logarithm of partition coeffient of a particular solvent between water and 1-octanol (Salleh *et al.*, 2002)

The graph in Figure 5.2 shows a profile of the acetamide yield (represented by the HPLC peak areas) versus the water content % (v/v) in acetonitrile, tetrahydrofuran, toluene and heptane. In the different reaction systems, the highest acetamide yield was achieved at a water content of 8% (v/v) in acetonitrile, followed by 4-6% (v/v) in tetrahydrofuran, 4% (v/v) in toluene and 0.1% (v/v) in heptane. The highest acetamide yield was observed in acetonitrile at 90%, followed by 41% in tetrahydrofuran, 29% in toluene and 11% in heptane. The trend onserved here is that, with decreasing solvent polarity (increase in logP values) (Table 5.1), the product yield also decreases.

From the results obtained in this study, it can be observed that in solvents that are more polar, a higher water content is required to obtain the optimum product yield, whereas in non-polar solvents, a lower water content is required (Bornscheuer & Kazlauskas, 2006). In a similar study, amidase Penicillin G acylase was used for the synthesis of an amide in toluene. The product yield obtained was 97% for over 30 h with controlled water activity. This shows that the accumulation of water can impact negatively on the product yield. In this study, controlling the water content by absorbing it kept water accumulation in the system at zero, thereby maintaining the reaction equilibrium (Ebert *et al.*, 1998). This could be an explanation for the low product yields in the non-polar solvents, toluene and heptane, since the acetamide synthesis reaction produces water as a by-prodct. From Figure 5.2, it is shown that, at high water contents in non-polar solvents, the product yield achieved is low. It is the

accumulation of water in the system that may have resulted in the low product yield, due to the reversal of the reaction equilibrium from synthesis to hydrolysis. In future, a water control mechanism should be put in place to maintain the water content in the reaction system, such as the addition of molecular sieves or the the addition of salt soluitons of known a_w, such as the $CuSO_4.5H_2O/CuSO_4.3H2O$ pair, with a a_w of 0.32 at 25°C (Bornscheuer & Kazlauskas, 2006).

From this study, the highest product yield was achieved in acetonitrile as the reaction medium. Therefore, for further studies, acetonitrile was used as the reaction milieu. The water activity value of *G. pallidus* RAPc8 amidase in acteonitrile can be determined by extrapolation from Figure 5.1. The optimum 8% water content correlates to a water activity value of 0.65.

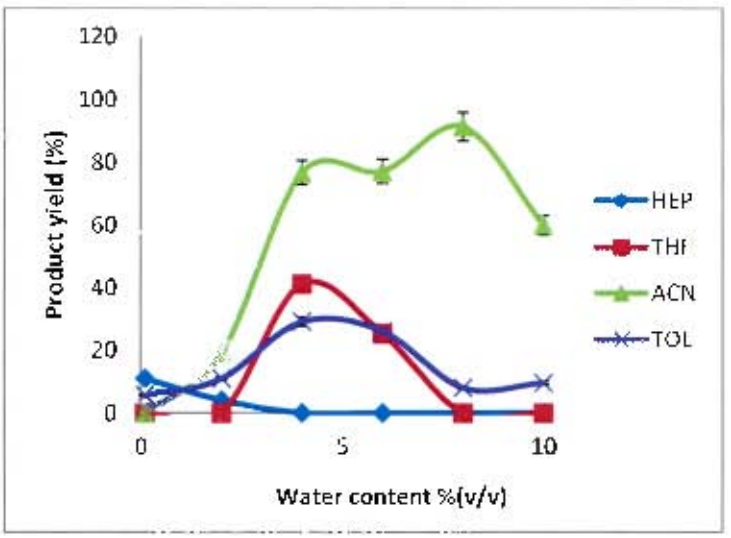

Figure 5.2: The correlation between the water content in the reaction system (% v/v) and the acetamide yield produced by *G. pallidus* RAPc8 amidase in: (ACN:Acetonitrile), (HEP:Heptane), (THF:Tetrahydrofuran) and (TOL:Toluene). Conditions: Temp: 50°C; Time: 24 h at 200rpm.

5.2.3 Acetamide synthesis in room temperature ionic liquids

Three room temperature ionic liquids were also studied as reaction media for acetamide synthesis and these ionic liquids and their characteristics are listed in Table 5.2. In this study, the effect of different ionic liquids as well as the effect of the water content, on the product yield, were investigated. The effects of water content were determined by varying the water content in the ionic liquids used. The water concentrations were varied between 0.1 and 10%. Ionic liquids are polar solvents, and the differentiating characteristics between the three

solvents are the anions used and the length of the carbon chain. The BF_4^- anion gives the ionic liquid water miscible properties while the PF_6^- gives the ionic liquid water-immiscible properties.

Table 5.2: Names, stucture and water miscibility of ionic liquids used in acetamide synthesis.

Name	Abbreviation	Structure	Miscibility with H20
1-Butyl-3-methylimidazolium tetrafluoroborate	[bmim][BF4]		Yes
1-Butyl-3-methylimidazolium hexafluorophosphate	[bmim][PF6]		No
1-methyl-3-octylimidazolium hexafluorophosphate	[omim][PF6]		No

Table adapted from; De Diego *et al.* (2009)

The graph in Figure 5.3 shows that the highest product yields obtained in [bmim][PF_6] and [bmim][BF_4] were at a water content of 8 and 10% v/v respectively. Above 10% (v/v), the product yield decreases, due to a saturation of water in the reaction system. The highest yield of 95%, was detected in [bmim][BF_4] and a 51% yield was observed in [bmim][PF_6]. No product was detected in [omim][PF_6]. The synthesis results obtained in ionic liquid media were quite different from those obtained in the application of Penicillin amidase, where no product was obtained when [bmim][BF_4] was used (Zhang *et al.*, 2006).

Both the [bmim][BF_4] and [bmim][PF_6] ionic liquids are highly polar, which explains the high water content required to obtain the highest product yield. The difference in the water content required to reach the optimum water activity in the two ionic liquids is due to the difference in the anions of the two ionic liquids. The [BF_4] anion makes the ionic liquid water miscible while the [PF_6] makes the ionic liquid water immiscible, therefore making the [bmim][BF_4] reaction mixture require more water to reach the optimum water activity value.

The absence of product in [omim][PF_6] for *G. pallidus* amidase could be explained by the fact that the substrates used for the synthesis of acetamide (acetic acid and ammonium carbamate), are more soluble in water miscible solvents than in water-immiscible solvents. Hence, similarly, the low yield detected in heptane. Also [omim][PF_6] is very viscous compared to the other two ionic liquids used in this study, because of the longer alkyl chain

of the cation. Also, cations with longer alkyl chain have been shown to decrease enzyme activity by obstruction of the its non-polar active site (Ventura *et al.*, 2012). Therefore, the absence of product in this ionic liquid could be a result of inadequate mass transfer in the reaction mixture, or a result of reduced enzyme acitivity due to the long alkyl chain. The anion could not have played a significant role because some product was detected in [bmim][PF_6]. Further, the stability studies in [omim][PF_6] confirmed that the lack of product in [omim][PF_6] was not a consequence of the loss of activity of the amidase in this ionic liquid, because the deactivation profile of the amidase showed a similar trend to that of the other ionic liquids (Figure 5.6).

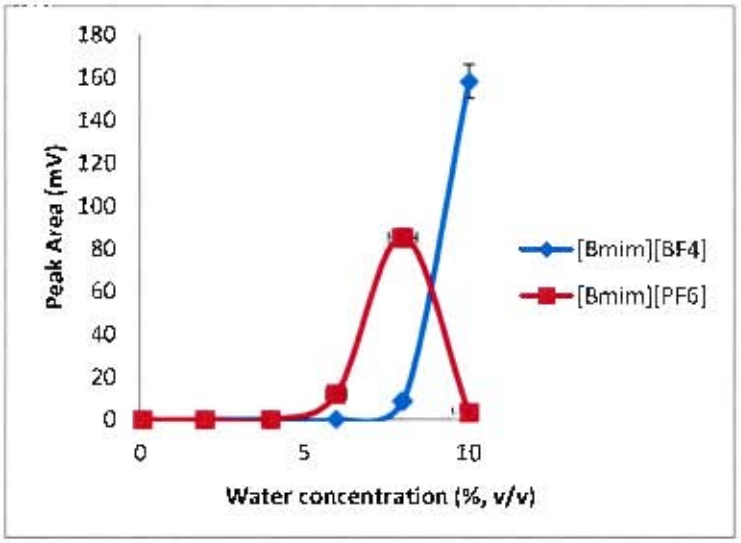

Figure 5. 3: The correlation between water content (%, v/v) and acetamide yield (represented by peak area, mV) in the ionic liquids, [bmim][BF4] and [bmim][PF6]. Conditions: Temp: 50°C; Time: 24 h; 200 rpm.

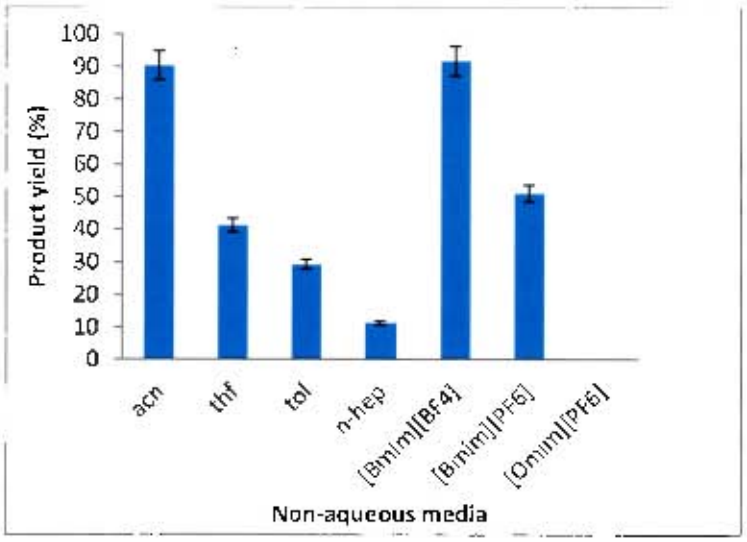

Figure 5.4: Evaluation of the effects of various non-aqueous media in acetamide synthesis; acetonitrile (acn), tetrahydrofuran (thf), toluene (tol), 1-butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4], 1-butyl-3-methylimidazolium hexafluorophosphate [bmim][PF_6] and 1-octyl-3-methylimidazolium hexafluorophosphate [omim][PF_6], on acetamide synthesis. Conditions: Temp: 50°C; Time: 24 h; 200 rpm.

5.2.4 Comparison between organic solvents and ionic liquids as reaction media

Synthesis of acetamide was carried out in two types of solvents: organic solvents and room temperature ionic liquids. The motivation behind the investigation of the two types of solvents was to determine which type would be most suitable for the synthesis of acetamide by *G. pallidus* RAPc8 amidase, in terms of keeping the enzyme stable for extended time periods and high product yield. The results obtained from section 5.2.1, reveal that, the highest product yield of 90% was achieved in the polar solvent, acetonitrile, while a low yield of 11% was observed in the non-polar solvents. In section 5.2.2, the synthesis of acetamide was carried in three ionic liquids: [bmim][BF_4], [bmim][PF_6] and [omim][PF_6]. The highest product yield of 95% was observed in [bmim][BF_4], and a lower yield of 51% in [bmim][PF_6]. No product was obtained in [omim][PF_6]. [Bmim][BF_4] is hydrophilic, while [bmim][PF_6] is hydrophobic. The same trend observed in the organic solvents is observed with the ionic liquids as reaction media; the more hydrophilic solvent gave a higher acetamide yield, while the hydrophobic solvent gave a lower yield. However, the difference between the organic solvents and ionic liquids is that, even though the product yield in hydrophobic ionic liquids is lower than that obtained in the hydrophilic ionic liquids; it is still higher at 51%, compared to that of its counterpart organic solvents, at 41, 29 and 11%. The higher product yield in hydrophobic ionic liquids may be due to the fact that, even-though the

ionic liquids are water-immiscible, they are still polar in nature as they are composed of mainly cations and anions (Kragl *et al.*, 2002).

5.2.5 Stability studies of the *G. pallidus* RAPc8 amidase in organic solvents and ionic liquids

The influence of non-aqueous media on the stability of the amidase biocatalyst was studied by incubating the enzyme in organic solvents and ionic liquids in the absence of substrate. The mixtures were incubated in an incubator equipped with a shaker at 50°C and agitation at 200 rpm. The graphs in Figures 5.5 and 5.6 represent the deactivation profiles of the amidase biocatalyst in organic solvents and ionic liquids respectively. The deactivation profiles in the two figures show a similar trend with rapid loss of enzymatic activity in the first few hours. The biocatalyst retained between 30 and 40% of its activity after incubating for 96 h at 50°C.

5.2.5.1 Deactivation profile *G. pallidus* RAPc8 amidase in organic solvents

The deactivation profile in Figure 5.5, shows that incubation in organic solvents resulted in a decrease of activity after 3 hr of incubation by 40-50%, reaching an average residual activity of 53% in all three organic solvents. After 3h, the activity decreases gradually in all three solvents to a value of 40% after 96 h of incubation. All three solvents, acetonitrile, tetrahydrofuran and toluene have the same effect on the activity of *G. pallidus* RAPc8 amidase. However, from the deactivation profile it can be observed that acetonitrile always has a higher activity value as compared to the other two solvents, by a difference in activity of 5%. This could mean that the enzyme retains activity for longer in polar solvent than in non-polar solvent, which could be a possible explanation of the higher product yield in acetonitrile.

5.2.5.2 Deactivation profile *G. pallidus* RAPc8 amidase in ionic liquids

The deactivation profile for the *G. pallidus* RAPc8 incubated in ionic liquids for 96 h is shown in Figure 5.6. The trend observed in this graph is similar to that of the organic solvents. After 3 h of incubation, the amidase lost activity by 40-50%, reaching an average residual activity value of 55%. After 96 h of incubation, the residual activity of the amidase was reduces to 40%. The activity of the *G. pallidus* RAPc8 amidase diminished in a similar trend in all three ionic liquids: [bmim][BF_4], [bmim][PF_6] and [omim][PF_6]. However, another trend is observed in the ionic liquid deactivation profile, where the residual activity

of the amidase in [bmim][PF_6] and [omim][PF_6] is always higher than that in [bmim][BF_4], by a difference in resudual activity of 10%. This is the opposite of the trend observed in the organic solvent deactivation profile where the residual activity is higher in hydrophilic solvent; in ionic liquids, the residual is higher in the hydrophobic ionic liquids, [bmim][PF_6] and [omim][PF_6]. Even though the residual activity is higher in the hydrophobic ionic liquids, the acetamide yield is high in the hydrophilic ionic liquids, [bmim][BF_4], with no product observed in [omim][PF_6]. This could be due to the fact that in the water-immiscible ionic liquids, the mass transfer and also due to the solvents not being able to solvate the substrates as a consequence of lack of water mixed with the solvent.

The stability in both organic solvents and ionic liquids of the *G. pallidus* RAc8 amidase showed a similar trend to that reported for stabilization studies of Penicillin G acylase (PGA) in [bmim][BF_4], [bmim][PF_6] and toluene (Basso *et al.*, 2005). However, PGA experienced a more gradual loss of activity in [omim][PF_6] than *G. pallidus* RAPc8 amidase. These stability studies show the effects of storing the biocatalyst in the respective solvents at 50°C, but do not represent the way the amidase would behave in a batch reaction synthesis in the presence of the substrates. Studies performed on the stability of the *Candida antarctica* lipase B showed that in the absence of substrate, the lipase lost activity more rapidly, and similar profiles as those in Figures 5.5 and 5.6 were obtained. However, when the lipase was incubated in the presence of substrates, the stability of the enzyme was enhanced by up to 7500 times in [bmim][PF_6] (Lozano *et al.*, 2001). Therefore, for future research, in order to determine the dynamics of amidase stability in non-aqueous organic synthesis, the stability studies should be conducted in the presence of substrate.

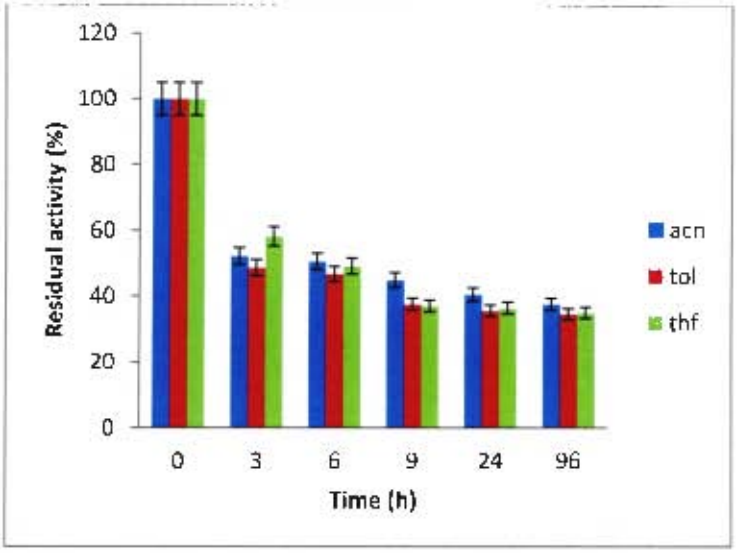

Figure 5.5: Evaluation of amidase stability (in terms of activity change over time) in various organic solvents: acetonitrile (acn;blue), toluene (tol;red) and tetrahydrofuran (thf; green). Conditions: Vol: 1 ml; Temp: 50°C; Time: 0-96 h; 200 rpm.

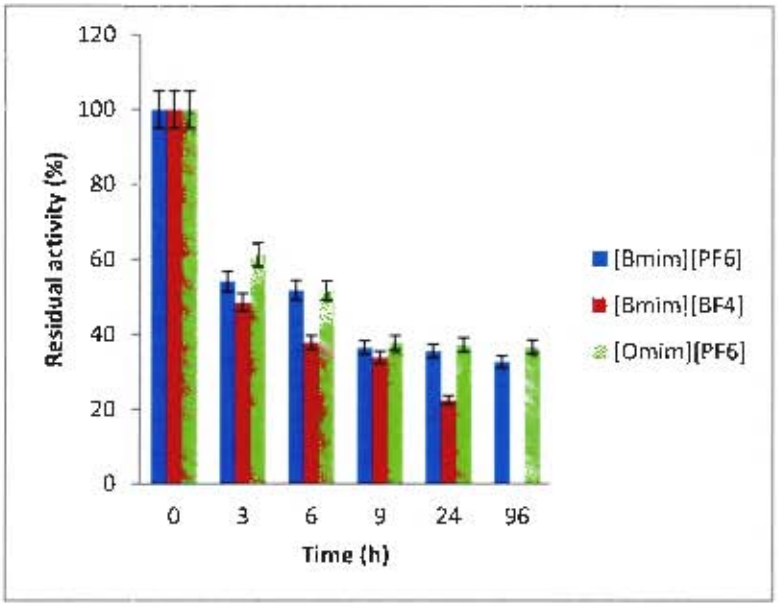

Figure 5.6: Evaluation of amidase stability (in terms of acitivity over time) in various room temperature ionic liquids: [bmim][BF_4] (red), [bmim][PF_6] (blue) and in [omim][PF_6] (green). Conditions: Vol: 1 ml; Temp: 50°C; Time: 0-96 h; 200 rpm.

5.2.6 Acetamide synthesis optimization

5.2.6.1 Effects of substrate concentration on product yield and volumetric productivity in batch synthesis of acetamide

The effect of substrate concentration on the *G. pallidus* RAPc8 amidase catalyzed synthesis of acetamide from acetic acid and ammonia was investigated at varying concentrations of acetic acid (25 mM-150 mM) while keeping the ammonium carbamate concentration and biocatalyst mass constant at 25 mM and 20 mg respectively. The effect of substrate concentration was investigated by measuring the product yield, as determined by the peak areas (mV) (HPLC chromatogram, results not shown) and volumetric productivity, which is defined as the product concentration per cubic volume of the bioreactor per unit time.

From Figure 5.7, it can be seen that as substrate concentration increased, the product yield declined slightly. The highest yield was 24%, at substrate concentration of 25 mM and it decreased to 19% at a concentration of 150 mM, the product yield declined by 5%. For the synthesis of acetamide, acetic acid is the acyl donor and the ammonium carbamate is the nucleophile. This reaction is reversible, and therefore it would be expected that an increase in the acyl donor concentration would result in higher product yields, by shifting the reaction equilibrium towards synthesis. However, as observed in Figure 5.7, this was not the case, since increasing the acyl donor concentration resulted in a lower product yield. The relationship between the declining product yield and increasing substrate concentration could be related to a decrease in enzyme activity due to substrate inhibition of the enzyme, as observed in similar studies (Scherbakova *et al.*, 2004; Habulin & Knez, 2008). Alternatively, the reduction in product yield could be attributed to by the effect of the ammonium carbamate salt on the water activity of the reaction. However, it is not known whether the reason for the decline in product yield was a result of substrate inhibition of acetic acid on the *G. pallidus* RAPc8 amidase. Further studies should be conducted to study the substrate inhibition on *G. pallidus* RAPc8 amidase. In addition, to confirm whether the change in product yield is a result of the change in water activity on adding ammonium carbamate, further studies monitoring the water activity in the reactions should be conducted.

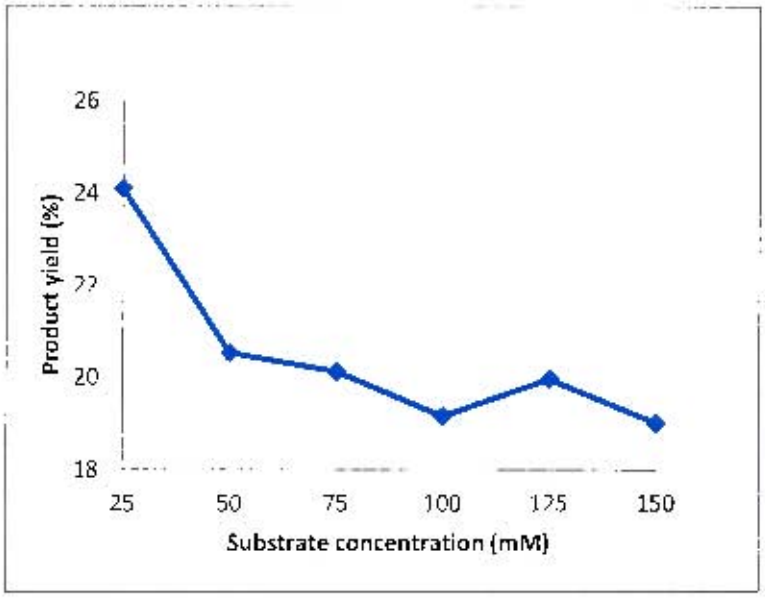

Figure 5.7: Effect of increasing substrate concentration on the product yield (%). The biocatalyst mass was kept constant. Product yield was calculated as a ratio of the initial substrate concentration. Conditions: Vol: 1 ml; Temp: 50°C; Time: 24 h; 200 rpm; 20 mg of biocatalyst; 25 mM ammonium carbamate.

An investigation was carried out to determine the relationship between substrate concentration and volumetric productivity. The volumetric productivity is defined as the concentration of product formed per unit volume and time. The volumetric productivity is calculated using the following equation:

$$Qp = \frac{P}{V.t} \qquad 5.1$$

Where P is the product quantity (mg), V is the reactor volume and t is the time taken for the reaction to reach completion.

The graph in Figure 5.8 shows an increase in the volumetric productivity with increasing substrate concentration. At the highest substrate concentration of 150 mM, the highest volumetric productivity of 74 mg/L.h was obtained, and the lowest of 15 mg/L.h was obtained at a substrate concentration of 25 mM. Thus, for this reaction, for high volumetric productivity to be achieved, the substrate concentration needs to be high. Volumetric productivity is a better way to measure the efficiency of a bioprocess reaction because it gives the product yield in terms of time and reactor volume; therefore it makes it easier to

predict the amount of product to expect in a reactor of a certain volume. This type of information is useful for the development of processes and in determining the time period it would take to synthesize a certain quantity of the target product.

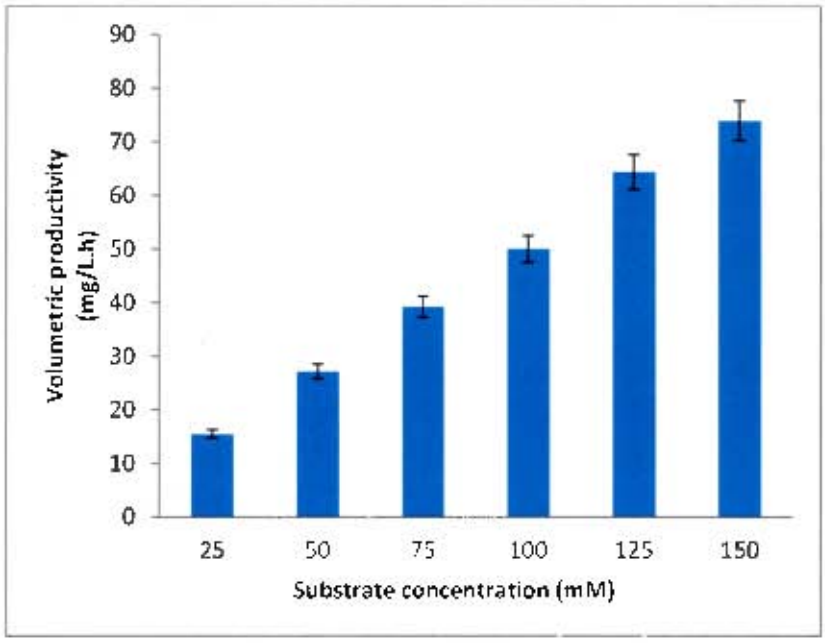

Figure 5.8: effects of increasing substrate concentration on the volumetric productivity (mg/L.h). The volumetric productivity was calculated as a change in product mass (mg) per litre and time (hr). Conditions: Vol: 1 ml; Temp: 50°C; Time: 24 h; 200 rpm; 20 mg of biocatalyst; 25 mM ammonium carbamate.

5.2.6.2 Effect of substrate ratio on the acetamide synthesis reaction

The molar ratio of acetic acid to ammonium carbamate in the acetamide synthesis reaction is a very important parameter. An acyl-enzyme intermediate is formed by the acyl donor and the enzyme. The nucleophilic attack by ammonium carbamate will result in the formation of acetamide, however, if the nucleophile concentration is low, water may be the preferred nucleophile, which will lead to the formation of a hydrolysis product (Habulin & Knez, 2008). If the ratio is not optimized, it may result in the acetic acid being unavailable for the reaction due to salt formation. In the acetamide synthesis reaction, acetic acid acts as an acyl donor and ammonia as a nucleophile, which reacts with the acetic acid to form acetamide and water. Investigations on the effects of acetic acid concentration of the product yield were conducted previously, (Section 5.2.6.1). This present section reports the ratio effect of ammonia concentration to acetic acid on the product yield. A high concentration of ammonia can decrease the acetamide synthesis reaction rate binding with the acetic acid, making it

unavailable for the synthesis reaction. A low ammonia concentration can also affect the reaction as there would be insufficient ammonia to react with the acetic acid. Therefore, it was important that the optimal molar ratio between ammonia carbamate and acetic acid was determined, to achieve the highest possible product yield.

To determine the effect of molar ratio of ammonium carbamate/acetic acid on the volumetric productivity, different ratios of the two substrates (0.5 to 4), were used; acetic acid concentration was kept constant at 150 mM and ammonium carbamate concentration was varied between 75 mM and 600 mM. As evident from Figure 5.9, there was a substantial decrease in volumetric productivity with increasing ammonium carbamate concentration. The highest productivity of 23 mg/L.h was obtained at a substrate ratio of 0.5. As the molar ratio was increased from 0.5 to 1, the volumetric productivity decreased from 23 mg/L.h to 7 mg/L.h, giving a decline in volumetric productivity by 70%. At molar ratios of 2, 3 and 4, the volumetric productivity values are at a constant value of 2 mg/L.h. Ammonium carbamate produces two moles of ammonia which react with acetic acid; therefore, an increase in ammonium carbamate concentration could lead to formation of the ammonium carboxylate salt, which is insoluble, reducing the concentration of acetic acid available for acetamide synthesis, which could be an explanation for the reduction in volumetric productivity with increasing molar ratio. Also, the reduction in volumetric productivity with increasing ammonium carbamate could be attributed to by the change in water activity of the solution, as the ammonium mops up free water. Changes in water activity will have an effect on the activity of the enzyme. For further experimentation, a molar ratio of 0.5 of the substrates was used.

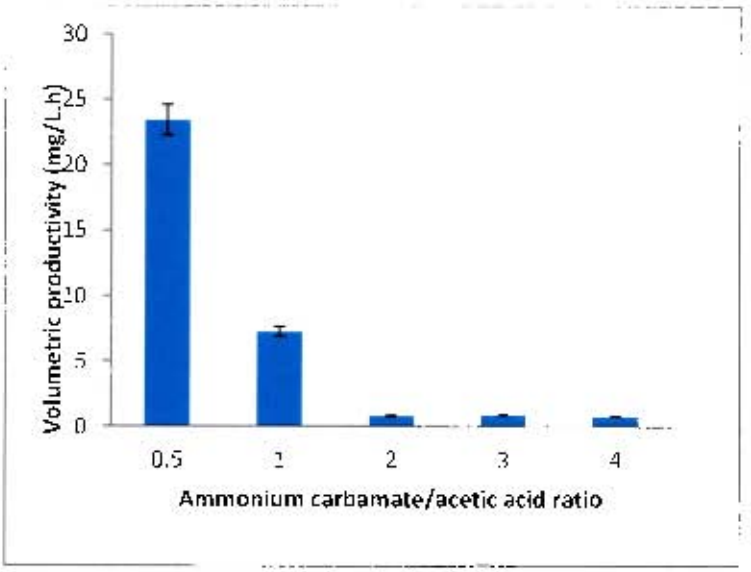

Figure 5.9: Effect of the molar ratio of the substrates: acetic acid/ammonium carbamate om the volumetric productivity of the reaction. Conditions: Vol: 1 ml; Temp: 50°C; Time: 24 h; 200 rpm; 150 mM of acetic acid; 20 mg of biocatalyst.

5.2.6.3 Effect of the amount of immobilized *G. pallidus* RAPc8 amidase biocatalyst used on acetamide yield and volumetric productivity

In an ideal situation, increasing the concentration of the biocatalyst in the reaction mixture would be expected to result in increased product synthesis. However, this is not always the case, due to other reaction dynamics that may result from the increasing concentration of the biocatalyst, such as saturation, which may lead to mass transfer limitations and may have a negative effect on the product yield. In this study, the influence of the biocatalyst mass on the product yield and volumetric productivity on the synthesis of acetamide was investigated. Differing amounts of the *G. pallidus* RAPc8 amidase biocatalyst mass (between 20 mg and 500 mg), were added to 1 ml reaction mixtures consisting of acetic acid and ammonium carbamate in acetonitrile. The reactions were performed at 50°C and 200 rpm. The optimal acetic acid and ammonia concentrations were used (obtained in sections 5.2.6.1 and 5.2.6.2, of 150 mM and 75 mM respectively). The organic solvent used as the reaction medium, was acetonitrile, the one in which the highest acetamide product yield was obtained (section 5.2.1).

From the results, it was clear that an increase in biocatalyst mass resulted in a decrease in product yield as seen in Figure 5.10. The highest product yield of 12% was obtained at a biocatalyst mass of 20 mg. The low product yield may be due to an insufficient availability of

biocatalyst for the amount of substrate present in the reaction, Some of the substrates may bind to other sites on the enzyme, inhibiting it and preventing the enzyme from binding substrate on the active site. However, further studies are required to confirm as to whether the low product yield is a consequence of the substrate inhibitory properties, since the substrates concentrations have been increased to 150 mM and 75 mM of acetic acid and ammonium carbamate, respectively. However, increasing the biocatalyst mass to 500 mg, resulted in a decline in product yield to a low value of 4%. This could be due to an increase in solid mass into the reaction mixture since the *G. pallidus* RAPc8 amidase is immobilized onto Eupergit C® beads. The more solid is introduced, the lower the mass transfer rates in the reaction system, which may hamper the mixing of the enzyme with the substrates, leading to lower reaction rates. The lower product yield could also be a consequence of the immobilization support taking up the water from the system produced by the synthesis reaction, thereby increasing the water around the biocatalyst and therfore affecting its optimum water activity. Therefore, for further studies, this mass was used for the amide synthesis reactions.

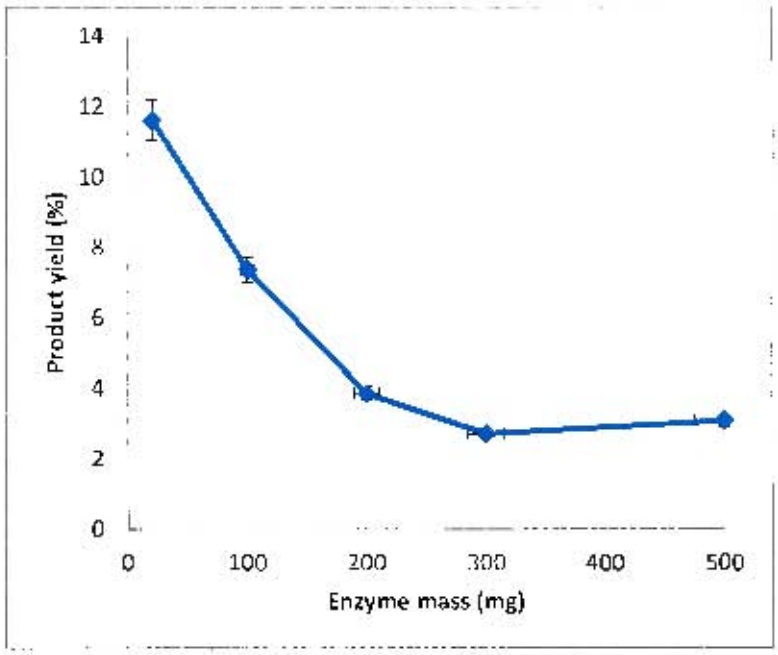

Figure 5.10: Effect of biocatalyst mass on the product yield (%). Conditions: Vol: 1 ml; Temp: 50°C; Time: 24 h; 200 rpm; 150 mM acetic acid; acetic acid/ammonium carbamate ration of 0.5.

An investigation was also conducted to determine the effect of biocatalyst concentration on the volumetric productivity of the acetamide synthesis reaction. The graph of Figure 5.11 shows the same trend to that in Figure 5.10. Increasing the biocatalyst mass resulted in a decrease in the volumetric productivity. The decrease in both volumetric productivity and product yield could be a consequence of the increase of the solid phase in the reaction mixture. The reaction mixture for acetamide synthesis was heterogenous because of the different phases of the reactants, biocatalyst and the reaction medium. The amidase used was immobilized onto a solid support, Eupergit C, which makes it insoluble in the organic solvent reaction mixture. An increase in the biocatalyst mass introduced more solids to the reaction system, making the mixture gelatinous. As a consequence of this, the mass transfer of substrates in the heterogenous mixture presumably decreased, as diffusion and mixing of substrates was hampered (Fernandez-Perez & Otero, 2001; Habulin & Knez, 2008). For future studies, an enzyme mass lower than 20 mg should be investigated, to determine its effects on both the product yield and the volumetric productivity.

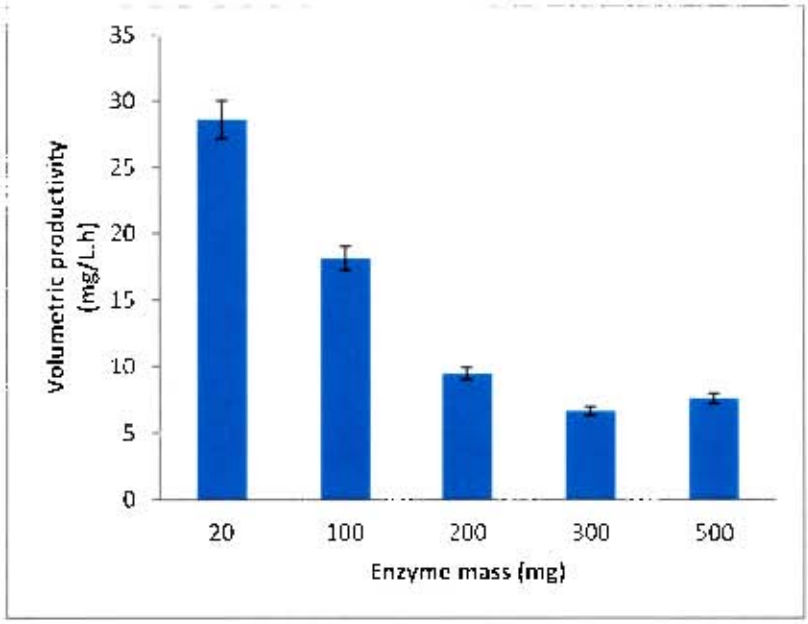

Figure 5.11: Effects of biocatalyst mass on the volumetric productivity (mg/L.h). Conditions: Vol: 1 ml; Temp: 50°C; Time: 24 h; 200 rpm; biocatalyst mass (20-500 mg); 150 mM acetic acid; acetic acid/ammonium carbamate ratio of 0.5.

5.2.7 Effects of different solvents on the enantioselectivity of *G. pallidus* RAPc8 amidase

Previous studies in the field of non-aqueous media organic synthesis have revealed that the type of solvent used may have an effect on the selectivity of the enzyme used. With increasing need for the synthesis of optically pure compounds, it is important to determine what effect the solvent used will have on the enzyme selectivity (Habulin & Knez, 2008). This study investigated the effects of the organic solvent acetonitrile and ionic liquid [bmim][BF_4], on the enantioselectivity of the *G. pallidus* RAPc8 amidase. Previous studies of the hydrolysis of D-lactamide and L-lactamide enantiomers revealed that the *G. pallidus* RAPc8 amidase was active towards the D- enantiomer, while no activity was observed on the L-enantiomer (Makhongela *et al.*, 2007).

For lactamide synthesis, the optimal reaction conditions determined in the previous sections (5.2.6.1 and 5.2.6.2), were used, such as 150 mM acyl donor, 75 mM of ammonium carbamate and 20 mg of the biocatalyst, with a water content of 8% v/v. To determine the lactamide yield, the HPLC method discussed in section 3.6.2.1, was used. A lactamide standard was used for the extrapolation process. The yield of lactamide obtained from the synthesis in acetonitrile was 57%, as shown in the Figure 5.12. However, no product was observed in [bmim][BF_4], and the substrate concentration, lactic acid was 100%. This

lactamide yield of 57% is low compared to the 97% yield of acetamide in acetonitrile obtained in section 5.2.2. The reason for the lower yield of lactamide under the same reaction conditions for acetamide synthesis could be due to the fact that the *G. pallidus* RAPc8 amidase is only active towards the D-enantiomer of lactic acid and not towads the L-enantiomer, as it was the case in the hydrolysis reaction of D,L-lactamide (Makhongela *et al.*, 2007). This could be explained by the fact that the *G. pallidus* RAPc8 amidase is active towards the D-enantiomer of lactic acid and no activity towards the L-enantiomer. The 50% yield could be due to the fact that the amidase was able to utlise 50% of the substrate, form the 100% racemic mixture. To determine whether the D- or L-lactic acid enantiomer was the preferred substrate, an investigation into the method that could be used to identify the enantiomer of the final product was conducted, see section 5.8.

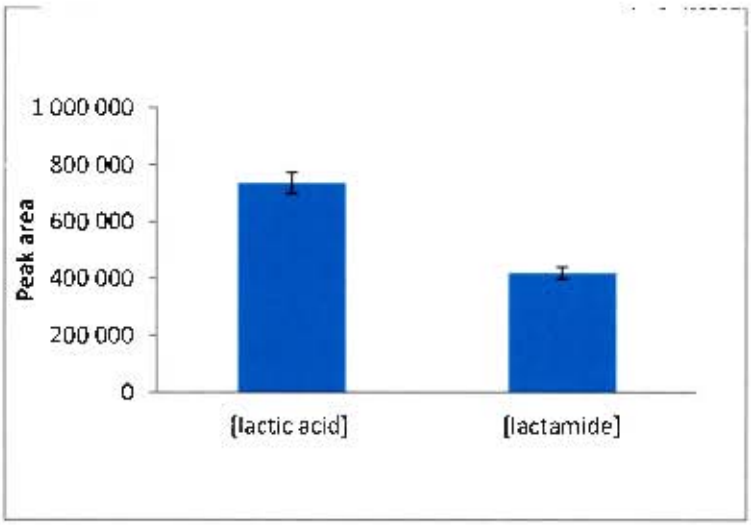

Figure 5.12: Lactamide synthesis from a racemic mixture of 150 mM of D,L-lactic acid and 75 mM of ammonium carbamate, under the following conditions: Vol: 1 ml; Temp: 50°C; Time: 24 h; 200 rpm. The peak area represents the product yield.

5.3. Chiral separation of lactamide enantiomers

5.3.1. Indirect Methods

5.3.1.1 Derivatization of lactamide enantiomers with OPA and IBLC

The method used in this study, to derivatize lactamide enantiomers using *o*-phthaldialdehyde (OPA) and *N*-Isobutyryl-L-cysteine (IBLC), was developed by our collaborators in Germany at the Karlsruhe Institute of Technology, for the enantioseparation of amines. The

derivatization reaction was carried out robotically in an Autosampler on the HPLC, due to the fact that the diastereomers formed are not stable and in this method, standing time was reduced and the enantiomers are injected into the column immediately after derivatization. The robotic Autosampler was programmed as shown in Appendix D. The lactamide enantiomers were added at a molar ratio of 1:25:25 to the OPA and IBLC respectively as prescribed in the method used in the German laboratory (Brucher *et al.*, 2010).

The agents, OPA and IBLC were selected as the chiral derivatizing agents based on literature documenting the successful separation of enantiomers of amino acids and aliphatic amines, with the OPA/IBLC combination. Amino acids and amines, like amides, have amino groups in their structures, and the assumption was that if the reaction worked with the amino acids and aliphatic amines, then there was a possibility that it would work with lactamide. The amino group in both amino acids and amines is involved in the nucleophilic reaction with the OPA. The lone pair of electrons on the nitrogen is involved in the bond formation between the nitrogen and the carbons on OPA.

The reagent OPA, in combination with IBLC (with reduced sulfhydryl groups), reacts with primary amines to form fluorescent moieties. The samples were analyzed at a wavelength of 340nm, which is the wavelength at which the OPA/IBLC complexes fluoresce. However, no peaks were visualized on the detector as the samples were ran through the UV/VIS analyzer attached onto the HPLC. Clearly the reaction was not successful, probably due to the fact that unlike amines, amides are weak bases. In amides, there is a carbonyl functional group, and the -OH group in the carboxyl group of carboxylic acids is replaced by an amino group in amides. The lone pair of electrons on the amino group is responsible for the basic character of amines. In amides, this lone pair of electrons is withdrawn by the carbonyl group, delocalizing it by resonance, the electrons form a partial double bond with the carbonyl carbon, and results in a negative charge on the oxygen. Hence, the lone pair of electrons is not available for protonation reactions, and as a result, the basic nature of amides is diminished and the amide group is a poor nucleophile (Zumdahl & Zumdahl, 2000).

Another option to consider in seeking to derivatize amides would be to use derivatization reagents that have been used for chiral carboxylic acids, since acids are even weaker bases than amides. The common procedure for labelling chiral carboxylic acids is by tagging them

with a chiral primary amines in the presence of activation reagents such as 1-ethyl-3(-3-dimethylaminopropyl) carbodiimide (EDC) and 2,2'-dipyridyl disulfide (DPDS)/ triphenylphosphine (TPP). A variety of organic reactions suitable for other compounds with the carbonyl functional group such as aldehydes and ketones have been identified. These involve amine- and hydrazine- reagents which produce the corresponding nitriles and hydrazones. (Toyo'oka, 2002). Therefore, for future studies, it is advisable that for derivatization purposes, the chiral amines are investigated as potential derivatization agents for lactamide enantiomers.

5.3.2. Direct methods

5.3.2.1. Separation of lactamide enantiomers on Chiral Stationary Phases (CSPs)

Finding a chiral column with a suitable chiral stationary phase for a specific separation is challenging because not all columns are suitable for separation of different types of compounds. Chiral columns are also quite costly, and therefore the best solution to identifying a column suitable for lactamide enantiomers was to send the lactamide enantiomers to Macherey Nagel, Karlsruhe, Germany. The figures below, (5.13, 5.14, 5.15 and 5.16) show the results obtained from the analyses performed on a variety of chiral columns and conditions at Macherey Nagel, Germany, using lactamide standards.

The separation of lactamide enantiomers was investigated by using two types of chiral columns and varying conditions. In Figures 5.13 and 5.14, the results obtained were analyzed using a Nucleosil 100-5 column, whose silica base is coated with sulphonic acid, which is a strong acidic cation exchanger. The mobile phase was 0.2 mM $CuSO_4$ solution and the column temperature started at 30°C ($CuSO_4$ acts as a ligand exchanger). The flow rate and injected volumes were set at 0.4 ml/min and 5 µl respectively. The second analysis was done using the same column, but the $CuSO_4$ mobile solution was at a concentration of 0.5 mM and the initial temperature was 22°C. The flow rate and injected volume were kept constant. However, the separation of the lactamide enantiomers was not successful as only one peak was visible on the HPLC spectra, instead of two, representing the two chiral structures. The peaks shown on the chromatograms (Figures 5.13 and 5.14), had a similar retention of 6.3 min, and only one peak was visible, showing that the separation was not successful. Thus, no resolution was observed. The use of the mobile phase containing copper was to take advantage of the ligand exchange process, where the analytes would be expected to form

diastereomeric copper complexes with the copper present in the mobile phase. The diastereomers would then subsequently bind onto the stationary phase on the column. The enantiomers would then be retained on the column for different amounts of time, since the retention time depends largely on the strength and stability of the interaction of enantiomer with the column stationary phase. This method has been documented to have been successful with amino acids (Zaher *et al.*, 2008; Corradini *et al.*, 2005; Natalini *et al.*, 2005; Lu *et al.*, 2002). Clearly, it was not successful with the amide, lactamide.

Since no separation was achieved using the Nucleosil 100-5 column, a second column was used in attempt to separate the lactamide enantiomers, which was a Nucleocel Alpha RP-S column (Figures 5.15 and 5.16). This column has silica gel as the base, which is then coated with amylose-tris-3,5-(dimethylphenyl carbamate).The amylose units have a propeller-like shape and they are believed to form helical shapes. The separation of enantiomers on these columns depends on attractive interactions such as polar and π-π interactions between the analytes and the amylase functional groups on the chiral stationary phase. For the separation of lactamide enantiomers, different mobile systems were tried, as an optimization step, these were: methanol and acetonitrile:water, (40:60% v/v). The HPLC was set a wavelength of 210 nm with a flow rate of 5 µl. However, none of the methods were successful in the separation of the lactamide enantiomers as only one peak was visible on the HPLC chromatogram, at retention value of 5.5 min.

Other studies that involve the separation of chiral amides have been successfully carried out on columns containing silica immobilized with macrocyclic antibodies. Three types of antibodies have been used, these are: vancomycin, rifamycin and teicoplanin. A carboxylic group and a secondary amine group attached to vancomycin are the groups involved in ionic interactions with the enantiomers. The vancomycin column has proved successful in separating neutral amides but it is less effective for acidic molecules (Gubitz & Schmid, 2001). Therefore, since lactamide is both a weak base and not an acid, it might be effectively separated on this column. Thus, for future studies, these types of columns should be investigated in the separation of the lactamide enantiomers.

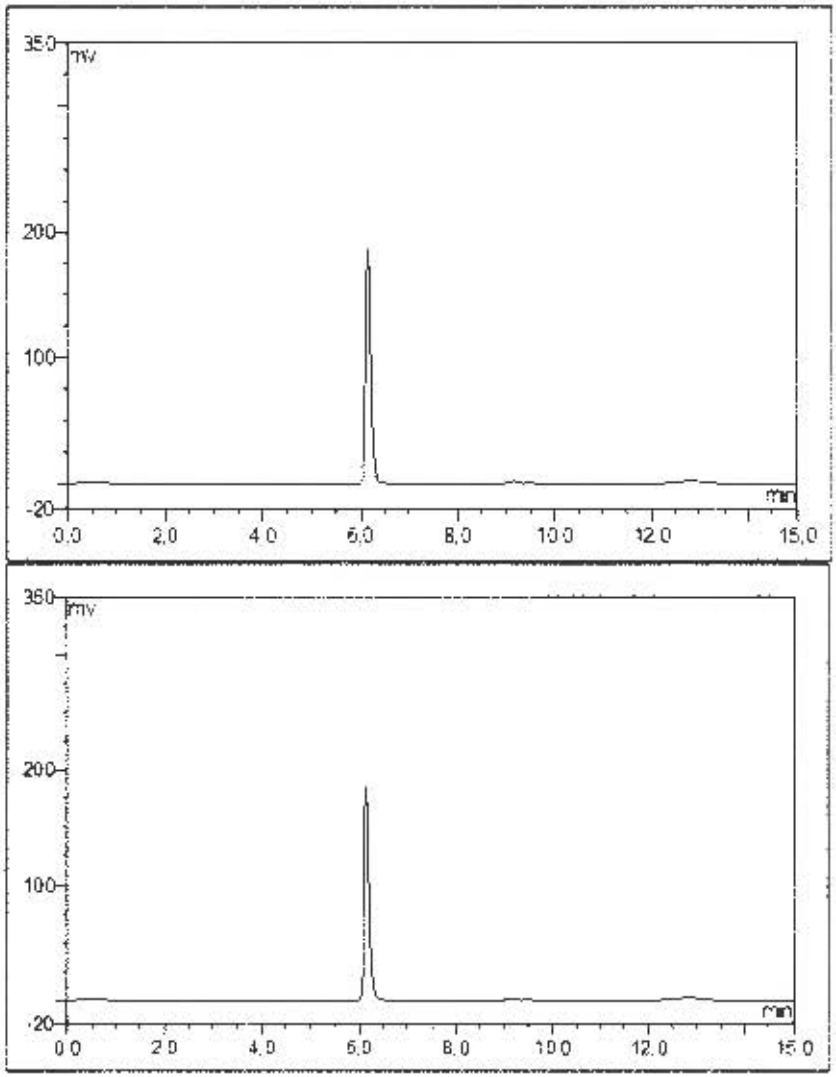

Figure 5.13: HPLC chromatogram showing the elution of D- and L-lactamide enantiomers (top and bottom respectively). Conditions: Column: Nucelosil 100-5; flow rate: 0.4 ml/min; Temp: 30°C; Wavelength (λ): 210; Injected volumes: 5 µl; Eluent: Acetonitrile (with 0.2 mM CuSO4 lsg).

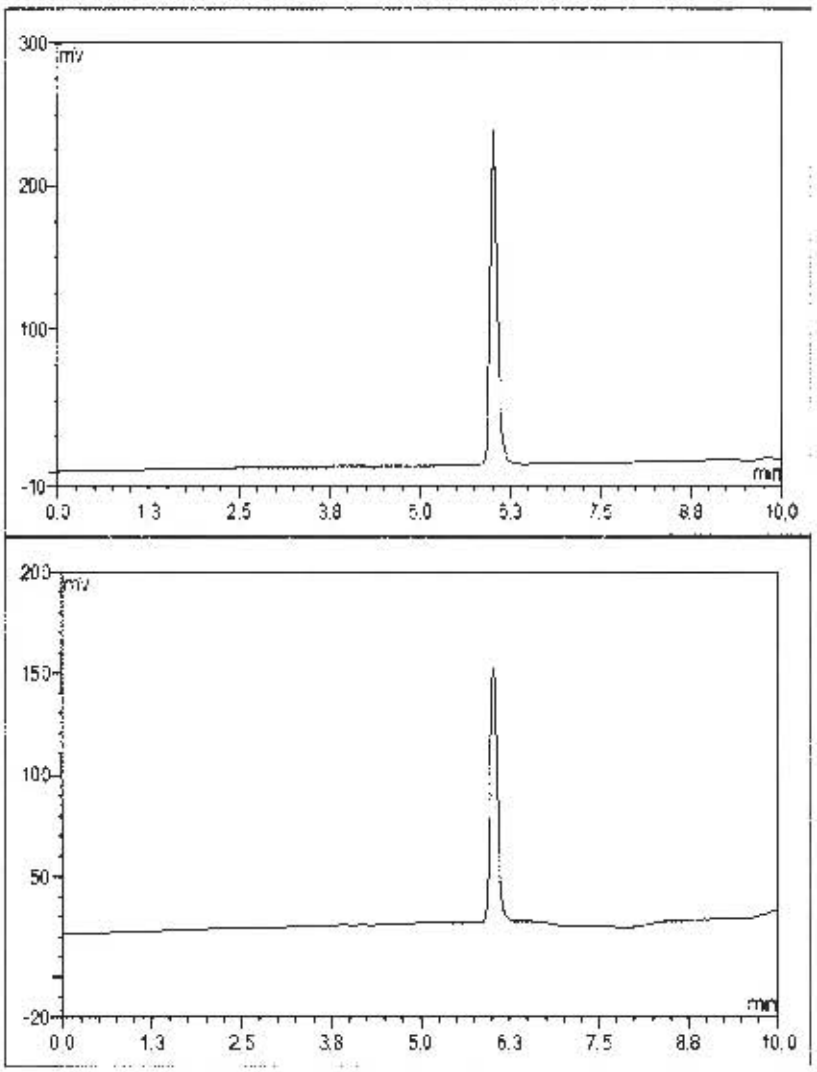

Figure 5.14: HPLC chromatogram showing the elution of D and L-lactamide enantiomers (top and bottom respectively). Conditions: Column: Nucleosil 100-5; flow rate: 0.4 ml/min; Temp: (22oC); Wavelength (λ): 210 nm; Injected volume: 5 µl; Eluent: (with 0.5 mM CuSO4 Isg).

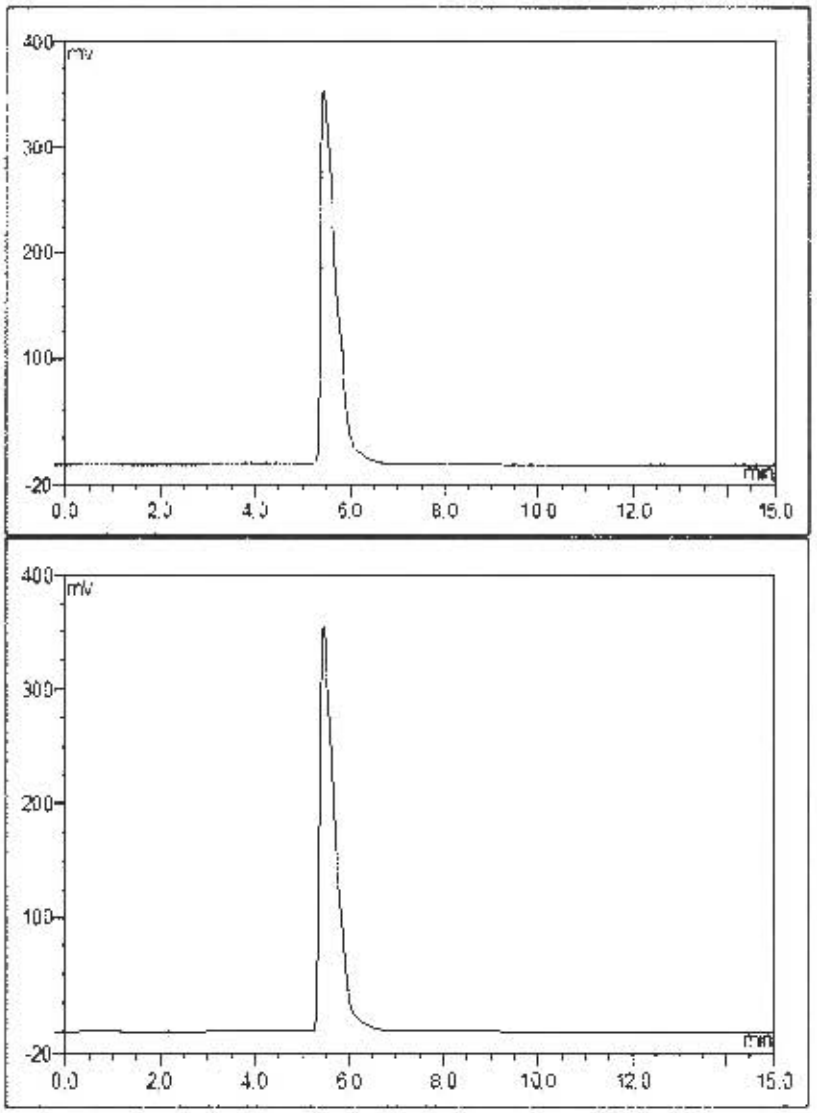

Figure 5.15: HPLC chromatogram showing the elution of D- and L-lactamide enantiomers (top and bottom respectively). Conditions: Column: Nucleosel Alpha RP-S; flow rate: 0.5 ml/min; Temp: (20°C); Wavelength (λ): 210 nm; Injected volume: 5 µl; Eluent: Acetonitrile/Water (40:60, v/v).

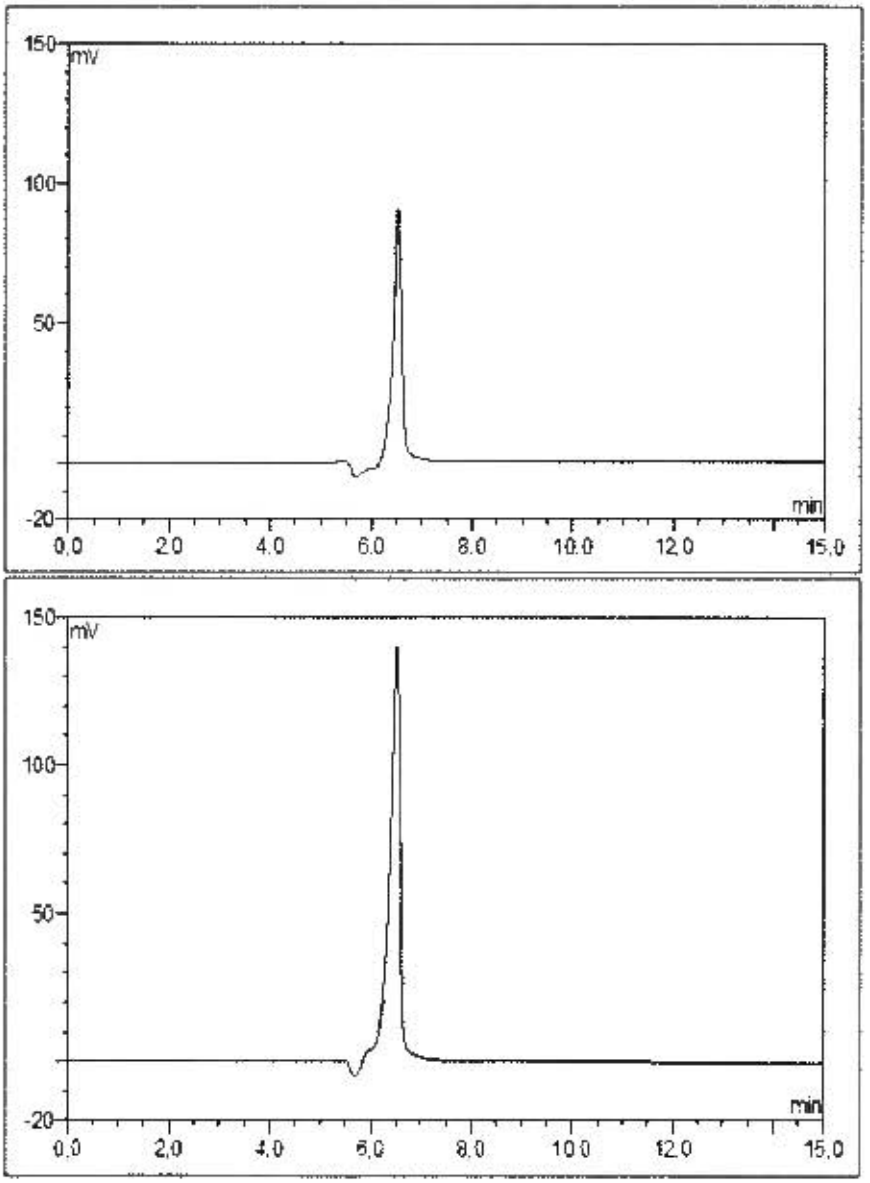

Figure 5.16: HPLC chromatogram showing the elution of D- and L-lactamide enantiomers (top and bottom respectively). Conditions: Column: Nucleosel Alpha RP-S; flow rate: 0.5 ml/min; Temp: (20°C); Wavelength (λ): 210 nm; Injected volume: 5 µl; Eluent: Methanol.

5.3.2.2. Separation of lactamide enantiomers by chiral thin layer chromatography (TLC)

An alternative chromatography technique was applied in effort to separate the lactamide enantiomers, namely by direct application to a chiral stationary phase. In this case, a chiral TLC plate was used for the separation of the enantiomers; the reverse phase (RP) silica gel coated plate was impregnated with a chiral selector and copper (II) ions. The separation of chiral compounds is based on ligand exchange, where the chiral compounds to be separated from diastereomic chelate complexes with the chiral selector and the copper.

Chromatographic separation depends on the strength of the complexes formed with the different enantiomers. A variety of different detection reagents were used to visualize the spots on the TLC plate, such as Ehrlich's reagent and hydroxylamine-iron (III) chloride reagent. The amino acid, proline was used as a positive control.

The separation was not successful, for the same reasons, namely of the chemical inertness of the lactamide due to the displacement of the lone pair of electrons on the amino group, owing to resonance. The control, proline, was visible on the TLC plate after applying the amino acid detection reagents (Ninhydrin and Erlich's) but not with hydroxylamine-iron (III) chloride reagent, which is used for amide detection (Figure 5.17).

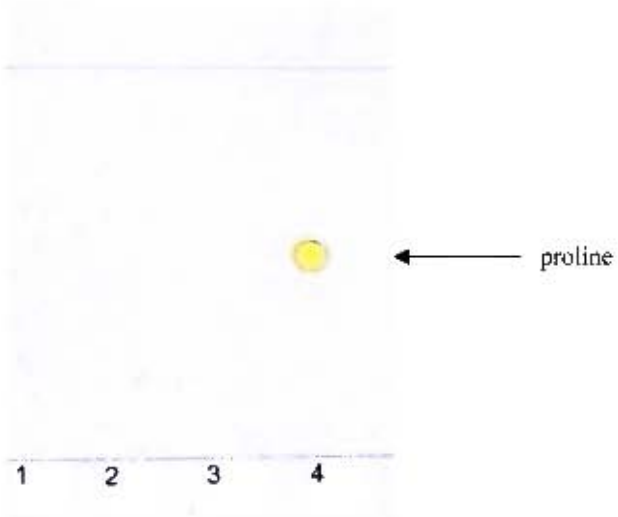

Figure 5.17: A chiral TLC plate showing the separation of lactamide enantiomers. 1: D,L-Lactamide, 2: D-Lactamide, 3: L-Lactamide, 4: Proline (control). The spots were visualized with Erlich's reagent.

5.4 Discussion and Conclusion

The work described in this chapter investigated the conditions required for the batch synthesis of acetamide using the immobilized *G. pallidus* RAPc8 amidase as a biocatalyst in non-aqueous media, using organic solvents and ionic liquids as reaction media. It was important that a suitable solvent system was selected that would be able to support the acetamide synthesis reaction. It was also necessary to study the different effects of the solvents on the biocatalyst activity. Thus, in this study, a variety of non-aqueous media were tested for the *G. pallidus* RAPc8 amidase synthesis reaction. In reactions that involve synthesis in low water systems, it is necessary that the optimal water activity of the biocatalyst is known, because an excess of water in the system could result in the reaction equilibrium being shifted from synthesis towards hydrolysis and too little water may impact negatively on the biocatalyst activity. Therefore, studies were undertaken to determine the optimum water content and water activity for the *G. pallidus* RAPc8 amidase in the solvents and the ionic liquids. The optimum water content required to obtain the highest product yield in the organic solvents: acetonitrile, tetrahydrofuran, toluene and *n*-heptane were 8%, 6%, 4% and 0.1% respectively. The water content required to reach the optimum water activity of the *G. pallidus* RAPc8 amidase biocatalyst increased with increasing solvent polarity. This is due to the fact that highly polar solvents absorb water while solvents of a lower polarity absorb little or no water. Therefore, in order to reach a certain water activity value, more water would be required in polar solvents, hence the high water content of acetonitrile required for the amidase enzyme. The product yields obtained at the optimal water content were 90%, 41%, 29% and 11% in acetonitrile, tetrahydrofuran, toluene and *n*-heptane. Therefore, for further studies, acetonitrile was the organic solvent selected for acetamide synthesis.

The water content optimization studies were also performed for the acetamide synthesis reaction carried out in ionic liquids. The ionic liquids [bmim][BF_4], [bmim][PF_6] and [omim][PF_6] were tested. The optimum water content at which the highest product yield was obtained in each of the ionic liquids were 8% and 6% in [bmim][BF_4] and [bmim][PF_6] respectively. No product was detected in [omim][PF_6]. The difference in the optimum water content for the two ionic liquids could be a result of the different anions in their molecular structures. The anions contribute to the physical and chemical characteristics of the ionic liquids. The BF_4^- anion gives the ionic liquid water miscible properties, while the ionic liquids with the PF_6^- anion are water immiscible. However, the water content is high for both

the water miscible and the non-miscible ionic liquids because ionic liquids are highly polar in nature.

Studies to determine the effect of various solvents on the stability of the *G. pallidus* RAPc8 amidase were also conducted. The enzyme retained almost equal activity in organic solvents and ionic liquids. In the studies involving organic solvents, the loss in enzymatic activity was the same for all the solvents studied. The residual activity after incubation of the amidase in acetonitrile, tetrahydrofuran and toluene was approximately 40% for all the solvents. The same effect was observed for stability studies involving ionic liquids. The residual activity of the amidase biocatalyst after 96 h of incubation was also approximately 50% for all the ionic liquids. No difference was observed in the enzyme stability in ionic liquids having different anions: [bmim][BF_4] and [bmim][PF_6]. The alkyl chain length of the cations did not have an impact on the stability of the enzyme but significantly affected the enzyme activity, as shown in the activity obtained in the ionic liquids; [bmim][PF_6] and [omim][PF_6] (Ventura *et al.*, 2012). From the results obtained in this study, it can be concluded that the absence of product in the presence of [omim][PF_6] is not due to the loss of activity of the amidase. The possible explanations could be that because this ionic liquid has a long alkyl chain, it makes the ionic liquid viscous. The high viscosity could be hampering the substrates to dissolve in the reaction mixture and also leads to mass transfer limitations.

The availability of a substrate in a reaction plays an important yield in terms of the product yield and the volumetric productivity that could be obtained, and these parameters are important in the designing a bioreactor process for a particular reaction. Hence, studies were conducted to evaluate the effects of substrate concentration and the molar ratios of the substrates on overall yields of the reaction. An increase in volumetric productivity was achieved by increasing the acetic acid concentration, while keeping that of the ammonium carbamate constant. The highest volumetric productivity achieved was 74 mg/L.h at a substrate concentration of 150 mM. By increasing the substrate concentration, the volumetric productivity was increased by substantial amounts from the lowest of 15 mg/L.h to 75 mg/L.h, which is a 5-fold increase. However, with increasing substrate concentration, the product yield showed a decline, by 5%. This could be a result of the substrate inhibition phenomena on the biocatalyst, as reported for Penicillin acylase (Shcherbakova *et al.*, 2004). The volumetric productivity is, however, a better parameter to use to determine the efficiency of a process, as it measures the product quantity per unit volume and time in the bioreactor,

while the product yield only gives a ratio of the product formed from the substrate added into the reactor. The product yield does not give an indication as to the performance of the bioreactor.

To further optimize the reaction, the effect of the immobilized biocatalyst mass on the overall performance of the reaction was evaluated at varying biocatalyst mass, used in the synthesis of acetamide. With increasing biocatalyst mass from 20 mg to 500 mg, there was a decline in the product yield. The highest product yield was obtained when 20 mg of the biocatalyst was used. The volumetric productivity also declined with increasing biocatalyst mass in the reaction mixture, the highest value was obtained at 20 mg of the biocatalyst. This study revealed that an increase in biocatalyst did not necessarily contribute to an increase in product yield or to the volumetric productivity. The decrease in the product yield and volumetric productivity with increasing biocatalyst mass could be a result of the limitation in mass transfer due to the saturation of the reaction mixture by the solid immobilized biocatalyst. However, due to the high cost nature of enzymes, the requirement of low biocatalyst mass for the process means that if this process were to be scaled up, then the enzyme production would not contribute largely to the cost of the process, therefore making it economically sustainable.

In order to determine the effect of solvent on the enantioselectivity of the *G. pallidus* RAP c8 amidase, studies were undertaken to synthesize lactamide from a racemic mixture of D,L-lactic acid and ammonium carbamate. Based on the previous studies that showed that the *G. pallidus* RAPc8 amidase is only active on the D-enantiomer of lactamide due to its inherent stereoselectivity, this study was done to determine whether the same principle would apply in the synthesis reaction. Lactamide was synthesized in acetonitrile as it gave the highest product yield in the synthesis of acetamide. About 50% product yield was obtained from the synthesis reaction, involving lactic acid and ammonium carbamate as substrates. The 57% product yield could be due to the fact that the amidase is only active on the D-enantiomer of lactic acid and it is not active towards the L-enantiomer, which means only half of the substrate was utilized. To determine whether the low product yield is a result of the enantioselectivity of the amidase, chiral separation techniques can be used to separate the isomer products in the reaction mixture and determine which of the two enantiomers of lactamide is high in quantity compared to the other.

The techniques investigated for the separation of the lactamide enantiomers in the reaction mixture to determine the enantiomeric excess of the amidase employed both direct and indirect methods. The indirect methods involved the derivatization of pure lactamide enantiomers to OPA and IBLC. However, this method was unsuccessful because the derivatization reagents used did not have functional groups complimentary to the carbonyl functional group on lactamide. Different chiral columns were tested to determine one that would be able to separate the lactamide enantiomers. This part of the study was done by Macherey Nagel, Germany. The results obtained did not show a differentiation in the lactamide enantiomers by the chiral columns. The last attempt was by separating the lactamide enantiomers on a chiral TLC plate. This method also proved unfruitful. Therefore, the enantiomeric excess of the *G. pallidus* RAPc8 amidase could not be determined because a suitable method for the separation of lactamide enantiomers could not be established. However, the vancomycin column has been tested successfully for the separation of neutral amides. This column could be successful in the separation of lactamide enantiomers since lactamide is not an acid.

Chapter 6: General discussion and conclusions

6.1 Introduction

The primary aim of this study was to investigate the application of the thermophilic *Geobacillus pallidus* RAPc8 amidase in non-aqueous organic synthesis, for the synthesis of enantiopure amides. This research was pursued following the growing trend in the biotechnology sector of shifting towards bioprocesses for the synthesis of important compounds (as opposed to the traditional chemical synthesis methods). Biocatalysis-based processes are environmentally benign and operate under mild conditions. For a biocatalytic process to be economically viable and sustainable, the techniques used for the biocatalyst preparation should preferably not contribute significantly in terms of costs to the processes. Most importantly, the biocatalyst should be produced in large quantities, it should be stable enough to allow completion of the reaction and should preferably be recyclable. The *G. pallidus* RAPc8 amidase functions optimally at high temperatures (50°C) as it was isolated from a thermostable *Geobacillus* strain (Pereira *et al.*, 1998). This is an advantage as it allows for the amidase to be purified at high temperatures, where most of the *E.coli* BL21 proteins are denatured. During this process, most proteins are removed. Also, the ability of the *G. pallidus* RAPc8 amidase to be stable at high temperatures makes it suitable for industrial use because, in industrial processes, operating conditions, especially the temperatures, may be high, in order to achieve sufficiently high reaction rates and substrate solubility. The thermostability properties of the amidase may save costs in a bioprocess as the amidase may be recycled.

6.2 Production, purification and immobilization of the *G. pallidus* RAPc8 amidase

One of the most crucial steps in the development of a biocatalysis reaction is the efficient production and preparation of the biocatalyst of interest. Chapter 4 describes the expression, production, purification and immobilization of the *G. pallidus* RAPc8 amidase, in preparation for the synthesis of enantiopure amides, acetamide and lactamide in non-aqueous media. In preparing a biocatalyst for a particular bioprocess, the medium used in the production of the host cells should be one that allows for the high cell growth results in a high biomass yield, and a high volumetric enzyme activity. The *G. pallidus* RAPc8 amidase gene was isolated, cloned and expressed in *E. coli* BL21 by Pereira *et al.* (1998). The *E. coli* BL21 cells were

grown in two different media: complex medium and glucose-based defined medium. This study confirmed the results obtained by Olaofe, (2009), which showed that a higher biomass yield of 9.40 g/l was obtained through growing the cells in defined medium. In this study, a biomass yield of 5 g/l was obtained when the cells were grown in defined medium, which is 2.5 fold higher than the biomass yield obtained by growing the cells in complex medium. Complex medium was not able to support growth over an extended time period, due to the production of acetate which is a growth inhibitor (Olaofe, 2009). The defined medium, conversely, could support cell growth for longer time periods, but with lower growth rates. To increase the biomass yield, the cells were grown in a 5L BIOFLO 110 series bioreactor, where the growth parameters such as pH, temperature and oxygen transfer could be adjusted in order to obtain a high cell biomass yield. This study was performed to emphasize the importance of selecting the correct medium for the production of recombinant or native cells, in order to produce large quantities of protein, which helps reduce the process costs.

The volumetric production of proteins from recombinant cells also depends on the induction method used for the expression of the amidase gene in the *E.coli* BL21 cells, 0.4 mM of IPTG is generally required. Studies have shown that the concentrations of the inducer, as well as the timing of induction, are important factors in obtaining high enzyme yields. To obtain high specific activity yields of the *G. pallidus* RAPc8 amidase, the optimum IPTG concentration which resulted in a high enzyme specific activity yield was found to be 0.4 mM. Also, inducing the cells at the start of the log phase proved to be significant in reaching the high specific activity yield (Olaofe, 2009). Due to the high cost of this inducer, it is advisable that in future, and for industrial applications, alternative methods of inducing the enzyme should be explored, as a way of reducing the costs of enzyme production.

The growth of the *E. coli* BL21 cells on defined was successful as a higher biomass and enzyme yields were obtained. Similar studies conducted have also shown that generally, *E. coli* cells give high biomass and enzyme yields in defined medium supplemented with glucose as compared to growth in complex medium (Shiloach & Fass, 2005). However, there are studies where the complex medium has given a higher maximum biomass yield as compared to defined medium (Basar *et al.*, 2010). For higher cell biomass to be achieved in complex medium, salts such as carbon, nitrogen, magnesium, phosphate, iron, manganese and some growth factors are required, which are present in the defined medium (Shiloach &

Fass, 2005). The results obtained in this study (Figure 4.1), coupled with those obtained from previous studies (Olaofe, 2009) confirm that the glucose defined medium is the most suitable to grow a recombinant strain of *E. coli*.

The crude *G. pallidus* RAPc8 amidase extract produced by the recombinant *E. coli* BL21 strain was purified by using different purification techniques in a four step procedure. Previously, the amidase was purified by heat treatment (Makhongela *et al.*, 2007). In this study, the amidase was subjected to heat purification and other forms of purification such as: ammonium sulphate precipitation, Hydrophobic Interaction Chromatography (HIC) and Ion-Exchange Chromatography (IEX), as previously done by Agarkar *et al.*, (2006). This was done in order to determine the most suitable method for purifying the amidase.

The efficiency of each method was determined by calculating the purification folds and specific activities of each method, as shown in the purification table in Chapter 4. SDS-PAGE was also used as an additional method of determining the efficiency of the methods. From the results obtained, the heat treatment method proved to be efficient in purifying the amidase to a purity level suitable for the laboratory based work. The heat treatment removed almost 40% of the contaminating protein as shown on the SDS-PAGE gel. The 40% removal of contaminating proteins was sufficient for the intended purpose, of synthesizing acetamide in non-aqueous media. Therefore, the heat treatment is a cheap and efficient way of purifying the *G. pallidus* RAPc8 amidase, at laboratory scale. The other methods of purification, even though they purify the amidase to near homogeneity, are however costly and about 50-60% of the enzyme yield is lost during the process.

Other important requirements of a biocatalyst used at industrial scale are that it should be thermostable and also be recyclable. The *G. pallidus* RAPc8 amidase was previously immobilized onto Eupergit C beads followed by cross-linking with EDAC. This step of immobilizing the amidase resulted in an enhancement of the thermostability of the amidase (Makhongela *et al.*, 2007). In this study, the immobilization of the *G. pallidus* RAPc8 amidase in preparation for the non-aqueous synthesis process resulted in protein binding yields and recovered activities of 56 and 54% respectively. These values are lower than those obtained in the previous study done by Makhongela *et al.* (2007), but, they were sufficient to use in biocatalytic reactions at laboratory scale for preliminary studies. The immobilization step, allowed for the production of a viable industrial enzyme. The lower enzymatic activity

obtained after immobilization, could be a result of the aggregation of a large number of enzyme molecules on a single Eupergit bead, which could impede the access of the substrate to the enzyme active site. The large number of oxirane groups on the surface of the Eupergit beads increases the surface area of the beads, therefore allowing more enzyme molecules to bind. Therefore, for future studied, the starting protein solution should be diluted with the respective buffer, to prevent over-crowding of enzyme molecules on the beads, or alternatively, more washing steps should be incorporated to wash off the excess enzyme (Katchalski-Katzir & Kraemer, 1999; Boller *et al.*, 2002).

6.3 Amide synthesis in non-aqueous media by *G. pallidus* RAPc8 amidase

The main objective of this study was to synthesize enantiopure amides, using non-aqueous media. Amides are essential compounds as they are used as intermediates in a number of fields such as the pharmaceutical and food industries. Currently, amides are synthesized using conventional methods of chemical synthesis. However, only a few methods for the synthesis of amides in low water systems using biocatalysts have been developed at laboratory scale. Most of these methods involve the use of lipase as the catalyst, but none have reported on the use of amidases. Hence this study focused on synthesizing amides using a thermophilic *G. pallidus* RAPc8 amidase, since amidases are also excellent acyl transferases, like lipases. In aqueous medium, hydrolases such as lipases and amidases catalyse the hydrolysis of compounds such as esters and amides, but when placed in non-aqueous media such as organic solvents, the reaction equilibria are shifted towards synthesis. For example, the *G. pallidus* RAPc8 amidase in buffer catalyzes the hydrolysis of amides to their corresponding carboxylic acids and ammonia. Preliminary studies revealed that the *G. pallidus* RAPc8 amidase can also synthesize amides in organic solvents, with little water present in the reaction mixture.

6.3.1 Selection of the most suitable organic solvent and ionic liquid

The most suitable solvent for the synthesis of acetamide by *G. pallidus* RAPc8 amidase was determined by testing the synthesis of acetamide in a variety of organic solvents and ionic liquids. The organic solvents used were: acetonitrile, tetrahydrofuran, toluene and *n*-heptane, and the ionic liquids investigated were: 1-Butyl-3-methylimidazolium tetrafluoroborate [bmim][BF_4], 1-Butyl-3-methylimidazolium hexaflurorphosphate [bmim][PF_6] and 1-Octyl-3-methyl hexafluorophosphate [omim][PF_6]. The most suitable solvent and ionic liquid, and

optimal water content, were determined by synthesizing acetamide in the solvents at varying water concentrations (between 0.1 and 10%). The highest yields of acetamide were obtained in acetonitrile and [bmim][BF_4], at yields of 90% and 95% respectively. The optimal water content for this reaction in both solvents was 8% (v/v). To determine the actual water activity during the synthesis of acetamide by *G. pallidus* RAPc8, water activity measurements must be taken prior to the start of the reaction and again once the reaction has been completed. From this study, these preliminary results show that the amidase exhibits a high activity in polar solvents. In a similar study, an amidase from Rhodococcus erythropolis AJ270 exhibited enhanced activity of 1.5-fold in the ionic liquid [bmim][PF_6] as compared to that in water (Xue *et al.*, 2011). Most studies report the opposite of this, stating that hydrolases exhibit higher activities in non-polar solvents. However, there are a few studies that have shown that some enzymes like the *G. pallidus* RAPc8 amidase, actually prefer polar solvents to the non-polar, such as Xanthine oxidase, Porcine pancreatic lipase and subtilisin, which retained activity when suspended in water-miscible solvents (Diaz-Garcia & Valencia-Gonzalez, 1995). The results obtained in these studies correspond to the results obtained in this study, which show that *G. pallidus* Rapc8 amidase prefers polar solvents to non-polar solvents. Other studies involving the synthesis of amides in non-polar solvents such as toluene, also had optimal water activities between 8 and 10%, v/v (Ebert *et al.*, 1998).

6.3.2 Stability studies of *G. pallidus* RAPc8 amidase in organic solvents and ionic liquids

The stability of the amidase in the different molecular solvents and ionic liquids was investigated to determine whether the stability resulted in the low yields obtained in some solvents. The enzyme samples incubated in each of the molecular solvents and ionic liquids displayed a similar trend, where 40% of the activity was retained after 96 h of incubation. This leads to the conclusion that the difference in the product yields obtained from the individual molecular solvents and ionic liquids was a result of the different properties of the solvents and their abilities to dissolve the substrates, and not due to the fact that other solvents inhibited the enzyme activity to a greater extent than others. For example, no product was obtained when the ionic liquid [omim][PF_6] was used as the reaction medium, however, product formation was observed in [bmim][PF_6]. The difference between [omim][PF_6] and [bmim][PF_6] is the length of the alkyl chain in the cation. [Omim][PF_6] has a longer carbon chain, which increases its viscosity. In a similar study, stability studies of the enzyme Penicillin acylase were conducted in water miscible and water immiscible ionic liquids:

[bmim][PF_6] and [bmim][BF_4] respectively. The enzyme exhibited a high relative activity of 88 in [bmim][PF_6] and no activity in [bmim][BF_4] (Zhang *et al.*, 2006). These results are contradictory to the results obtained in this study, which showed an equal decline in activity in both [bmim][PF_6] and [bmim][BF_4].

Therefore, from this observation it can be concluded that the lack of product in the [omim][PF_6] reaction mixture is due to the substrates not being able to dissolve in the ionic liquid and low mass transfer rates are minimized. The study also shows that the reaction time should be kept a minimum to prevent rapid loss of the biocatalyst activity which happens within 24 h. With [bmim][BF_4]as the ionic liquid of choice. Since the results presented in this chapter are preliminary, further studies should be conducted to confirm these observations. The reactions can be optimized to allow for high product yields in a short space of time, therefore allowing for the enzyme to be re-used in more than one reaction cycle.

The stability experiments in this study were conducted in the absence of substrate. The conditions reported here do not mimic those in the synthesis reaction during the synthesis of acetamide. In a study where the stability of α-Chymotrypsin was determined in both the presence and absence of substrate, revealed that in the absence of substrate, the activity of the enzyme is rapidly diminished, whilst the activity of the enzyme in the presence of the substrate declines at a slower rate. For example, in the ionic liquid [bmim][PF_6], the relative activity in the absence of the substrate is 15%, compared to the relative activity of 180% in the presence of substrate (Lozano *et al.*, 2006). Therefore, for future studies, a comparison of the stability of the *G. pallidus* RAPc8 amidase should be conducted in the absence and presence of the substrate.

6.3.3 Substrate optimization studies in the synthesis of acetamide by *G. pallidus* Rapc8 amidase

Further studies were conducted to optimize the acetamide synthesis reaction in molecular solvents by determining the effect of substrate concentration, substrate ratio and biocatalyst mass on the volumetric productivity of the enzyme. In the substrate concentration studies, the concentration of the acetic acid was varied between 25 mM and 100 mM. The effect of the concentration was determined in terms of product yield and also as volumetric productivity. The volumetric productivity is a better way of determining the dynamics of a biocatalytic

process, because it indicates the progress of the reaction per unit volume and time. The volumetric productivity makes it easier to effectively determine the amount of product to expect in a reactor of a certain volume, within a certain time period. Increasing the acetic acid concentration led to a decrease in acetamide yield by 5%. On the contrary, the volumetric productivity increased from 15 mg/L.h to 75 mg/L.h, by increasing the substrate concentration from 25 mM to 75 mM.

With the substrate ratio studies, the ratio of the two substrates added at the beginning of the synthesis reaction (acetic acid and ammonium carbamate) were varied to determine the ratio effects on the product yield and volumetric productivity. The results showed that the ratio that gave the highest product yield was 0.5. Ammonium carbamate releases two moles of ammonia, and therefore if the ammonium concentration is too high, it could lead to the formation of the ammonium carboxylate salt, which uses up the acetic acid making it unavailable for the synthesis reaction. The effect of the enzyme mass on the reaction was determined by varying the mass of the biocatalyst between 20 mg and 500 mg, in 1ml reactions. With an increasing biocatalyst mass there was a decrease in the product yield and volumetric productivity. This confirms that an increase in the biocatalyst does not always lead to a higher product yield, possibly because the increase in the biocatalyst mass, which introduced more solids to the reaction system, makes the mixture gelatinous. Also it is important to bear in mid that in non-aqueous media, enzymes are insoluble, and therefore suspended in the reaction mixture. The ability of the enzyme to perform a reaction depends on the dispersion of the biocatalyst, which is hampered in a system saturated with the biocatalyst (Noritomi *et al.*, 2009). As a consequence of this, the mass transfer of substrates in the heterogenous mixture decreased, as diffusion and mixing of substrates was hampered (Fernandez-Perez & Otero, 2001; Habulin & Knez, 2008).

6.4 Lactamide synthesis in non-aqueous media by *G. pallidus* RAPc8 amidase

Non-aqueous media such as organic solvents and ionic liquids can have an effect on the enantioselectivity of the biocatalyst of interest. Enzymes naturally possess some stereoselectivity towards certain compounds that exist as enantiomers; previous studies have shown that when applied in organic solvents, the selectivity properties are affected, in most cases they are enhanced, making the biocatalyst highly selective. The selectivity of enzymes is one of the sought after properties in industrial processes because they eliminate the

formation of by-products, thereby making the processes highly productive and also minimize pollution in the environment. In the hydrolysis reaction of lactamide to lactic acid and ammonia, the *G. pallidus* RAPc8 amidase had been shown to be selective towards the D-lactamide enantiomer and not towards the L-enantiomer of lactamide (Makhongela *et al.*, 2007). This study was done to investigate whether the enantioselectivity properties of the *G. pallidus* RAPc8 amidase would still be observed in the synthesis of lactamide in non-aqueous medium. The model reaction used to carry out this study was the synthesis of lactamide from a racemic mixture of D,L-lactic acid, and ammonium carbamate. A 57% yield of lactamide from the preliminary experiments was obtained from the synthesis reaction mixture. However, the HPLC method used here could not differentiate between the D- and L-enantiomers of lactamide; therefore, the 50% product yield could not be assigned to the formation of the D- or L-enantiomer of lactamide. However, based on the enantioselective properties of the *G. pallidus* RAPc8 amidase towards the D- and L-enantiomers of lactamide during a hydrolytic reaction (Makhongela *et al.*, 2007), the 50% lactamide yield could be attributed to the enantioselectivity of the amidase. To confirm the yield of 50%, further studies should be undertaken to re-do these experiments, as the results presented here are preliminary and were only tried once.

6.5 Chiral separation of lactamide enantiomers

The final section of this study investigated the different techniques of separating the chiral enantiomeric compounds of lactamide. Previous studies on the selectivity of the amidase enzyme revealed that the enzyme preferred the D-enantiomer to the L-enantiomer counterpart of the lactamide substrate (Makhongela *et al.*, 2007). Therefore, the aim of this study was to determine which of the two enantiomers was in excess after a synthesis reaction leading to the formation of lactamide, from D,L-lactic acid and ammonium carbamate. Determining whether the amidase is enantioselective is important as this will have an impact on the choice of raw material used. If the amidase is enantioselective, then there may be no need to refine and purify the starting material, which would help reduce the costs of amide production.

Methods to separate the lactamide enantiomers were investigated to select one that would be suitable to separate the lactamide enantiomers to determine the enantiomeric excess of the *G. pallidus* RAPc8 amidase. Three techniques were tested in attempt to separate the enantiomers of lactamide, two of the methods were direct and one was an indirect method. The direct

methods involved the use of a variety of commercially available chiral columns and chiral TLC, while the indirect method involved the application of a chiral derivatizing agent OPA, in conjunction with IBLC. None of these methods were successfully applied in the separation of the lactamide enantiomers. Finding a technique or method that works in the separation of a particular chiral compound is not easy as there is no universal method that applies to all compounds. This is due to the fact that, the chiral separation mechanism involves the interaction of the functional groups on the compounds to be separated and those on the chiral separator. Therefore, factors such as functional group the pH of mobile phases, operation temperatures, and the type of mobile phase also contribute substantially to the development of separation methods.

The separation of the lactamide enantiomers was also attempted by using direct methods such as chiral HPLC and TLC. The lactamide enantiomers were sent to Machery Nagel, Germany, so that different chiral columns could be tested for the separation of the enantiomers. The results from Macherey Nagel did not result in the separation of the enantiomers. Applying the enantiomers to chiral TLC was also unfruitful in separating the two enantiomers.

In summary, the significance of this work was to show that the *G. pallidus* RAPc8 amidase can be applied in the synthesis of enantiopure amides at industrial scale, replacing the traditional chemical ways of synthesis. This was shown by the high acetamide yields obtained of 90% in acetonitrile and 97% in [bmim[BF_4]. This study also showed that the *G. pallidus* RAPc8 amidase can retain activity in non-aqueous media to allow for the target product to be obtained. Due to the advantages associated with synthesis in low water systems, it can be concluded that, optimizing the synthesis reaction of acetamide by *G. pallidus* RAPc8 amidase could cut costs significantly, making this process competitive with the chemical synthesis processes. The non-aqueous processes is advantageous compared to the chemical processes because of enhanced productivities through eliminating by-product formation and also because it complies with the new trends concerning environmental conservation, in terms of pollution and the use of renewable natural resources. However, much research still has to be done to optimize the synthesis reactions, in order to make this process more economically viable than the processes used at present.

6.6 Recommendations

Some of the results obtained in this study are preliminary results and therefore further work may be conducted to optimize conditions and to improve on the results obtained here. Based on the results obtained, the following recommendations can be made:

- The IPTG used as an inducer for the expression of the amidase in the recombinant *E. coli* BL21 cells is very costly and can have some toxic effects on the cells. Using IPTG at laboratory scale is practical, but for scaling up of the process to industrial level, the IPTG is not a practical inducer to use. Therefore, different methods of inducing the cells should be investigated.

- The ultrasound cell disruption method of sonification used to extract the enzyme from cells is suitable for laboratory scale work. However, at industrial scale, this method would not be suitable. This calls for the development of other methods that can be applicable at large scale, for the release of the amidase from the *E. coli* cells.

- The development of a continuous method for the synthesis of enantiopure amides using other types of reactors such as the packed-bed reactor, for the synthesis of amides in non-aqueous media should be pursued. The use of a packed-bed reactor could lead to improved volumetric productivities as compared to those obtained in batch synthesis reactions. Packed-bed reactors will also allow for the replenishing of the substrates for the synthesis reaction, which will make the processes more economically viable. The continuous synthesis method also allows for better control of the thermodynamic water in the reaction system (Slotema *et al.*, 2002).

- In this study, the effects of the type of solvent used as well as other factors such as substrate concentration and biocatalyst mass were determined in terms of the product yield. However, more accurate methods of determining the effects could be described in terms of enzymatic reaction rates and also through the use of the Michaelis Menten parameters such as K_m and V_{max}.

- Maintaining the water concentration in the reaction system is critical in order to achieve a high product yield. If water is allowed to accumulate in the reaction mixture, the thermodynamic equilibrium will be shifted towards hydrolysis, resulting in the hydrolysis of the product. Therefore, methods of controlling the water content in the reaction mixture should be investigated such as the addition of molecular sieves or through pervaporation.

- To be able to determine the enantiomeric excess of chiral amides synthesized by the amidase, a technique that is suitable for the separation of the compounds should be determined. This will help in determining the amount of enantiomeric product produced, and hence the enantioselectivity of the reverse reaction, which is a useful parameter to know in chiral synthesis.

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Appendices

Appendix A: Culture medium and reagents

A1. Luria Broth medium

Add 10.0 g/l of Tryptone, 10.0 g/l sodium chloride (NaCl) and 5.0 g/l yeast extract, to 1000ml deionized water. Autoclave at 121°C for 20min. Add filter-sterilized ampicillin or carbenicillin to a final concentration of 100 µg/l.

A2. Luria Bertani Agar

Add 10.0 g/l of Tryptone, 10.0 g/l sodium chloride (NaCl), 5.0 g/l yeast extract and 15.0 g/l of agar to 1000 ml deionized water. Autoclave at 121°C for 20 min and cool down to room temperature. Add filter-sterilized ampicillin or carbenicillin to a final concentration of 100 µg/l. Pour the medium into sterile petri-dishes under sterile conditions.

A3. Defined glucose-supplemented medium

Medium composition	Per litre (l^{-1})
Glucose	25.0 g
KH_2PO_4	13.3 g
$(NH_4)SO_4$	4.0 g
$MgSO_4.7H_2O$	1.2 g
Citric acid	1.7 g
EDTA	8.4 mg
$CoCl_2.6H_2O$	2.5 mg
$MnCl_2.4H_2O$	15.0 mg
$CuCl_2.2H_2O$	1.5 mg
H_3BO_3	3.0 mg
$Na_2MoO_4.2H_2O$	2.5 mg
$Zn(CH_3COO)_2.2H_2O$	13.0 mg
$FeCl_3$	110 mg

A4. Reagent A for ammonia assay

Add 5.55 g (0.59 M) phenol and 1mM sodium nitroprusside to 100 ml of distilled, deionized water. Stir and cover the bottle with foil. Store at 4°C.

A5. Reagent B for ammonia assay

Add 8.0 g (2.0 M) sodium hydroxide and 0.11 M sodium hypochlorite to 100 ml of distilled, deionized water. Store at 4°C for a maximum of 2 weeks.

A6. $FeCl_3$ reagent for the acyl transferase activity

Using distilled, deionized water, dilute 32% HCl (13 M) to a final concentration of 0.65 M. Add $FeCl_3$ to a final concentration of 356 M. Stir well and store at room temperature.

Appendix B: Buffers and standards

B1. 50 mM potassium phosphate buffer

To make 0.1 M K_2HPO4 stock solution: Add 17.4 g of K_2HPO4 in 1000 ml deionized, distilled water. Stir well.

To make 0.1 M KH_2PO4 stock solution: Add 13. 6g of KH_2PO4 in 1000 ml deionized, distilled water. Stir well.

To acquire the desired pH of the buffer solution, add the K_2HPO4 and KH_2PO4 stock solutions in 100 ml distilled deionized water, as follows:

Desired pH	K_2HPO4 volume to be added (ml)	KH_2PO4 volume to be added (ml)

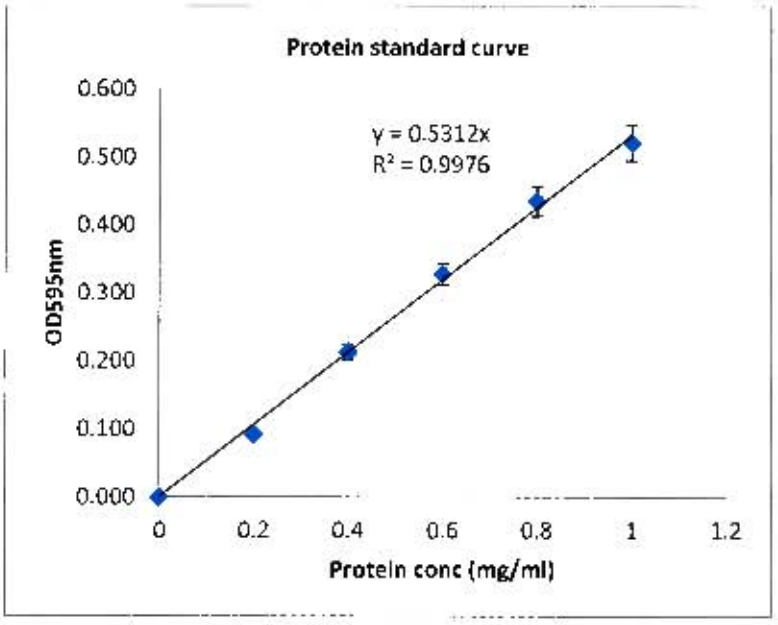
5.7	6.5	93.5
6.0	12.3	87.7
6.5	31.5	68.5
7.0	61.0	39.0
7.5	84.0	16.0
8.0	94.7	5.3

B2. Bovine serum albumin (BSA) standard

To prepare a 1mg/ml stock solution of BSA, add 10 mg of BSA to 100 ml of deionized, distilled water. The BSA standards are prepared from the BSA stock solution as follows:

BSA standard concentration (mg/ml)	Volume of BSA stock to be added (ml)	Volume of water to be added (mg/ml)
0	0	10.0
0.2	2.0	8.0
0.4	4.0	6.0
0.6	6.0	4.0
0.8	8.0	2.0
1.0	10.0	0

Add 1ml of Bradford's reagent to 1ml cuvettes, then add 20 µl of the BSA standard to each cuvette and mix well. Leave at room temperature for 5 min. Measure the absorbance at 595 nm, using a spectrophotometer.

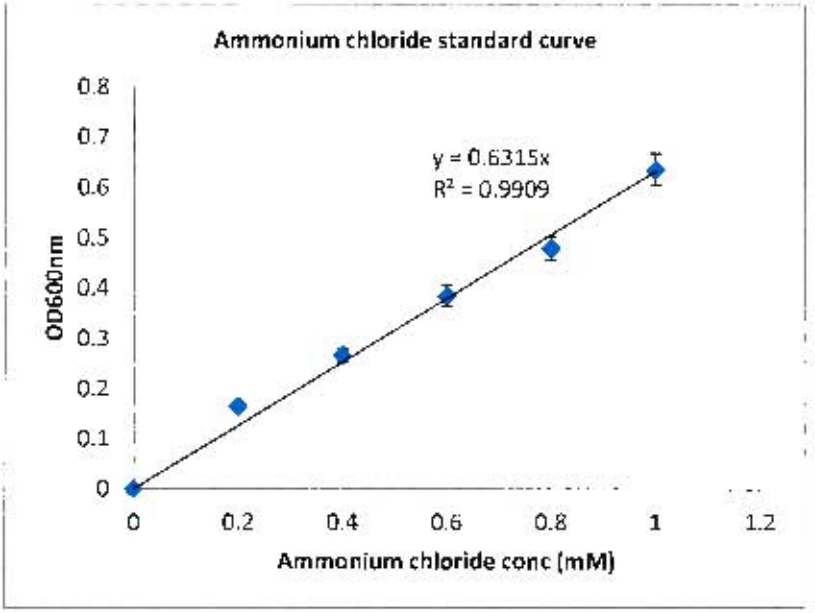


B3. Ammonium chloride standard

Prepare a stock 2.0 M stock solution of ammonium chloride (NH_4Cl) by dissolving 0.10698 g in 1000 ml distilled, deionized water. Prepare the various concentrations of NH_4Cl using the stock solution as follows:

NH4Cl standard conc (mM)	Volume of 2.0M stock to be added (ml)	Volume of water to be added (ml)
0	0	10.0
0.2	1.0	9.0
0.4	2.0	8.0
0.6	3.0	7.0
0.8	4.0	6.0
1.0	5.0	5.0

Add 350 µl of Reagent A of the ammonia assay to 1 ml cuvette. Add 100 µl of the standards to the cuvette, followed by 350 µl of Reagent B of the ammonia assay to each cuvette. Mix well. Leave at room temperature for 15 min. Measure the absorbance values at 600 nm using a spectrophotometer.

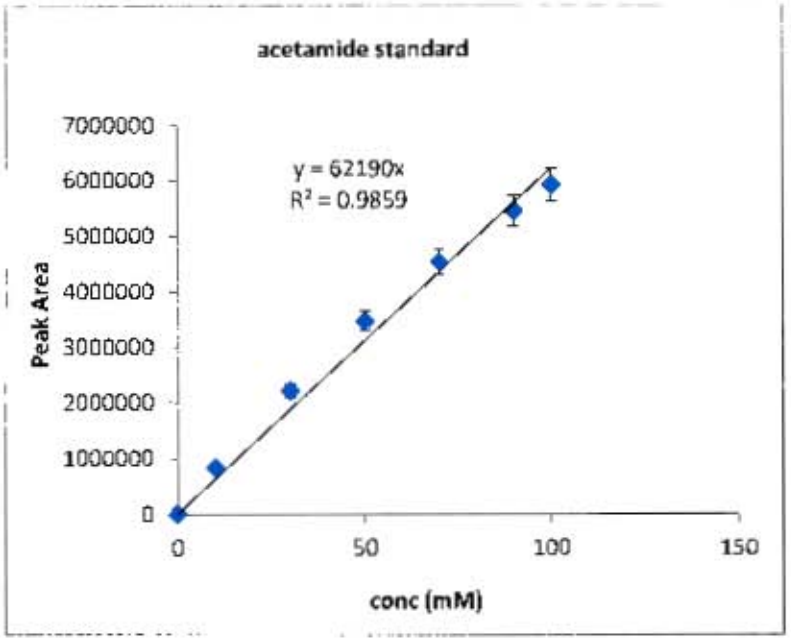


B4. Acetamide standard

Prepare a 1.0 M stock solution of acetamide by adding 0.5907 g of acetamide to 10 ml of distilled, deionized water. Mix well. Prepare the standard solutions using the stock solution as follows:

Add the standard solutions to an HPLC vial, measure the peak areas at a wavelength of 210 nm.

Acetamide concentration (mM)	Volume of acetamide added (µl)	Volume of mobile buffer added (µl)
10	10	990
20	20	980
30	30	970
40	40	960
50	50	950
60	60	940
70	70	930
80	80	920
90	90	910
100	100	900

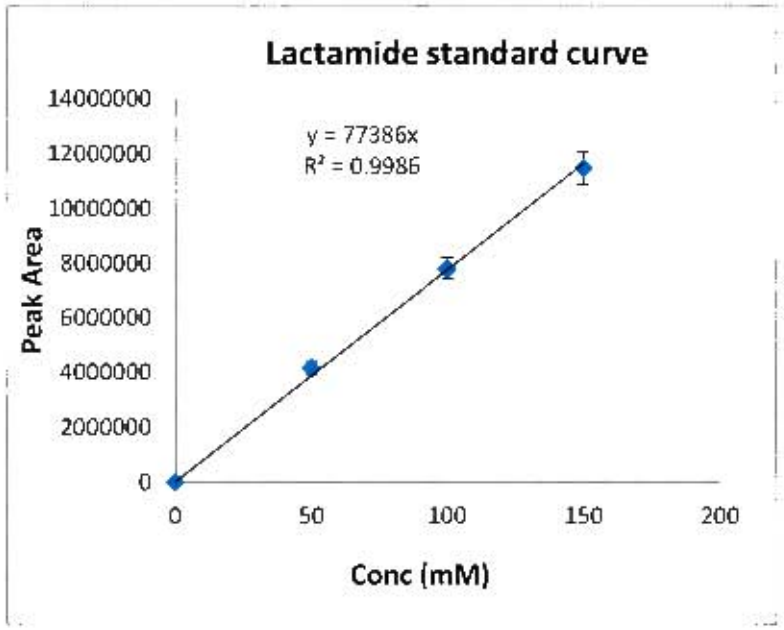


B5. Lactamide standard

Prepare a 1.0 M stock solution of lactamide by adding 0.8909 g of lactamide to 10 ml of distilled, deionized water. Mix well. Prepare the standard solutions using the stock solution as follows:

Add the standard solutions to an HPLC vial, measure the peak areas at a wavelength of 210 nm.

Lactamide concentration (mM)	Volume of lactamide added (µl)	Volume of mobile buffer added (µl)
10	10	990
20	20	980
30	30	970
40	40	960
50	50	950
60	60	940
70	70	930
80	80	920
90	90	910
100	100	900


Lactamide standard curve
y = 77386x
R² = 0.9986
Peak Area
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
0
50
100
150
200
Conc (mM)

Appendix C: SDS-PAGE

C1: Reagents

Reagents	Composition
SDS-PAGE running buffer (10x)	0.25M Tris HCl 2M Glycine 1% (w/v) SDS pH 8.3
Resolving gel buffer (1M, pH 8.8), 500ml	60.6g Tris 7.3ml HCl
Stacking gel buffer (1M, pH 6.8), 500ml	60.6g Tris 41.0ml HCl
30% Acrylamide stock, 250ml	75g Acrylamide 2g Bis-Acrylamide
Dissociation buffer, 50ml	5g SDS 5.0ml Mercaptoethanol 7.5ml Glycerol 2.5ml 0.2% Bromophenol blue 6.3ml Tris-HCl (1M, pH 6.8) 28.7ml Distilled water
SDS-staining solution	0.2% (w/v) Coomassie Blue R250 40% (v/v) Methanol 10% (v/v) Acetic acid
SDS-destaining solution	40% (v/v) Methanol 10% 9v/v) Acetic acid

Appendix D: Derivatization by OPA and IBLC

D1. HPLC derivatization commands

STEP	COMMAND	REAGENT
1	Draw 2.0µl from vial 99, maximum speed	Borate Buffer
2	Draw 3.0µl from vial 98, max. speed	OPA/IBL solution
3	Mix 5.0µl in air, max speed 2x	
4	Needle wash in vial 100, 1x	MilliQ water
5	Draw 0.5µl from sample, max. speed	Sample
6	Mix 5.5µl in air, max. speed, 2x	
7	Needle wash in vial 100, 1x	MilliQ water
8	Wait 2 minutes	
9	Draw 2.0µl from vial 97, max. speed	3% Acetic acid
10	Mix 7.5µl in air, max. speed, 6x	
11	Needle wash in vial 100, 1x	MilliQ water
12	Inject	