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NATHIELE CONTRERA GIMENES

# PURIFICAÇÃO E CARACTERIZAÇÃO DE PROTEASE DE *ASPERGILLUS TERREUS* VSP-22

CAMPINAS, SP.

2018

NATHIELE CONTRERA GIMENES

# PURIFICAÇÃO E CARACTERIZAÇÃO DE PROTEASE DE *ASPERGILLUS TERREUS* VSP-22

*Dissertação apresentada à Faculdade de Engenharia Química da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Engenharia Química.*

*Orientador:* Prof. Dr. Elias Basile Tambourgi

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELA ALUNA NATHIELE CONTRERA GIMENES, E ORIENTADA PELO PROF. DR. ELIAS BASILE TAMBOURGI.

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*"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota."*

Madre Teresa de Calcutá

# Resumo

A indústria catalítica possui as enzimas proteolíticas como uma das mais importantes classes de produtos do mercado global, uma vez que representam mais de 65% do mercado total de enzimas. Estas exibem importantes funções no processo metabólico das células, além de serem investigadas, principalmente as enzimas extracelulares, para aplicações industriais, como a de alimentos e em aplicações detergentes. Contudo, os métodos de separação e purificação envolvem inúmeras etapas, demandando um longo tempo de execução e tornando-os onerosos, uma vez que podem atingir 70-90% do custo total da produção. Assim, novas estratégias econômicas e eficientes para os *downstream processes* devem ser desenvolvidas para atender as exigências da indústria. Dessa forma, o objetivo do trabalho foi investigar a purificação e a caracterização bioquímica da protease produzida por *Aspergillus terreus* VSP-22. As técnicas de precipitação foram realizadas a 20, 40, 60 e 80% de saturação. A precipitação com etanol alcançou uma atividade específica de 174,19 $U.mL^{-1}$ e um fator de purificação de 15,05 vezes, a 80% de concentração, porém a recuperação atingiu 41,05% nesta fração. A precipitação com sulfato de amônio, a 60% de saturação, mostrou 46,45% para recuperação e 2,07 vezes para o fator de purificação. Um planejamento fracionário ($2^4$) para extração em sistema bifásico aquoso foi realizado, a fim de avaliar o efeito da massa molar de polietileno glicol (PEG), as concentrações de PEG e sulfato de amônio, volume da amostra e pH. O melhor fator de purificação (4,65 vezes) foi obtido com 18% (w/w) de PEG 2000 (g/mol), 18% (w/w) de sulfato de amônio a pH 9,8 e 0,5 mL de volume da amostra, o qual mostrou uma recuperação de 59,03%. A metodologia de superfície de resposta foi realizada para examinar os efeitos das concentrações de PEG e sulfato de amônio, e observou-se que o maior fator de purificação (5,16) e recuperação (70,82%) foram no ponto central. O sistema bifásico aquoso otimizado teve sua escala aumentada 8 e 64 vezes, os quais evidenciaram conformidade com as atividade específica (109,71 e 109,87 $U.mg^{-1}$) e recuperação (69,40 e 70,60%, respectivamente). A caracterização bioquímica encontrou um pH ótimo de 9,0 e temperatura de 50 °C para extrato enzimático, já para protease purificada, um ótimo de pH 9,8 e 40 °C. Os parâmetros cinéticos evidenciaram a alta afinidade da protease purificada para azocaseína (Vmax = 24,4499 $U.mL^{-1}$ e Km = 0,0318 mM). Portanto, além das técnicas de purificação terem sido eficientes, a protease demonstrou ter propriedades interessantes, sugerindo seu potencial em aplicações industriais.



Palavras-chave: *Aspergillus terreus*, protease, recuperação, fator de purificação, sistema bifásico aquoso.

# Abstract

Enzyme industry has proteases as one of the most important classes of global market products, account for more than 65% of the total enzyme market. Peptidases display essential role in metabolic process of cells, in addition have been investigated, mainly extracellular peptidases, to applications on industries as food and detergent applications. However, the bottleneck of production is found on downstream processes, which involve innumerous steps, are costly and time-consuming and can attain 70-90% of total production costs. Thus, new cost-effective and efficient strategies for downstream processes have to be improved to attend industries where peptidases are requested. In this way, the aim of the present work was to investigate the purification and biochemical characterization of protease produced from *Aspergillus terreus* VSP-22. The techniques of precipitation were performed at 20, 40, 60 and 80% of saturation. Ethanol precipitation achieved a specific activity of 174.19 $U.mL^{-1}$ and a purification factor of 15.05-fold at 80% concentration, however recovery attained 41.05% in this fraction. Ammonium sulphate precipitation at 60% saturation showed 46.45% on recovery and 2.07-fold on purification factor. The $2^4$ factorial design of aqueous two-phase system (ATPS) was performed to evaluate the effect of polyethylene glycol (PEG) molar mass, PEG and ammonium sulphate concentrations, sample volume, and pH. The best purification factor (4.65-fold) was obtained with 18% (w/w) PEG 2000 (g/mol), 18% (w/w) ammonium sulphate at pH 9.8 and 0.5 mL of sample volume, showing a recovery of 59.03%. Response Surface Methodology was conducted in order to examine PEG and ammonium sulphate concentrations effects, and it was observed that a higher purification factor (5.16-fold) and recovery (70.82%) in the center point. The optimized ATPS was scaled up 8 and 64 fold and their behaviour were in agreement with specific activity (109.71 and 109.87 $U.mg^{-1}$) and recovery (69.40 and 70.60%, respectively). The biochemical characterization found an optimum pH of 9.0 and temperature of 50 °C for enzyme extract, whereas for purified protease an optimum of 9.8 pH and 40 °C. Kinetic parameters evidenced the high affinity of purified protease for azocasein ($V_{max} = 24.4499$ $U.mL^{-1}$ and $K_m = 0.0318$ mM). In this way, the purification techniques were efficient and protease has proven to have interesting properties, suggesting its potential in industrial application.



Keywords: *Aspergillus terreus*, protease, recovery, purification factor, aqueous two-phase system.

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# Lista de Tabelas

# Lista de Abreviaturas e Siglas

| | |
|---|---|
| ANOVA | Analysis of Variance |
| ATPS | Aqueous-Two Phase System |
| Bmim | 1-Butyl-3-methylimidazolium |
| $C_{(NH4)2SO4}$ | Ammonium Sulphate Concentration (% w/w) |
| $C_{PEG}$ | PEG Concentration (% w/w) |
| DFP | Diisopropyl Fluophosphate |
| EDTA | Ethylene Diamine Tetraacetic Acid |
| $F_{cal}$ | Fisher's Calculated Value |
| $F_{tab}$ | Fisher's Tabulated Value |
| FDA | Food and Drug Administration |
| GRAS | Generally Regarded as Safe |
| GSE | Glutamate-Specific Endopeptidases |
| IL | Ionic liquids |
| $K_m$ | Michaelis Constant |
| Log $k_a$ | Protease Partition Coefficient |
| Log $k_p$ | Partition Coefficient of Proteins |
| $M_{PEG}$ | PEG Molar Mass (g/mol) |
| $n$ | Extraction Yield by ATPS (%) |
| NaPA | Sodium Polyacrylate |
| NC-IUBMB | Nomenclature Committee of the International Union of Biochemistry and Molecular Biology |
| [P6,6,6,14]Dec | Trihexyltetradecylphosphonium Decanoate |
| PA | Protease Activity ($U.mL^{-1}$) |

| | |
|---|---|
| PAA | Poly(acrylic acid) |
| PEG | Polyethylene glycol |
| PF | Purification Factor |
| PMSF | Phenylmethane Sulfonylfluoride |
| R | Recovery (%) |
| $R^2$ | Coefficient of Determination |
| $R^2_{adj}$ | Adjusted Coefficient of Determination |
| RSM | Response Surface Methodology |
| SA | Specific activity ($U.mg^{-1}$) |
| SBA | Sistema Bifásico Aquoso |
| SmF | Submerged Fermentation |
| SSF | Solid–State Fermentation |
| TP | Total Protein ($mg.mL^{-1}$) |
| Tris | Tris(hydroxymethyl)aminomethane |
| $V_{max}$ | Maximum Rate |
| Y | Yield (%) |

# Sumário

# Capítulo 1 – Introdução

## 1.1. Organização Estrutural da Dissertação

Um modelo alternativo de dissertação é apresentado neste trabalho e, dessa forma, faz-se necessária a criação de um tópico esclarecedor da disposição dos assuntos. A dissertação está organizada em capítulos e, primeiramente, são apresentadas uma introdução geral e os objetivos. Posteriormente, os artigos referentes à revisão bibliográfica e purificação por precipitação e extração em sistemas bifásicos aquosos são retratados e discutidos. Por fim, as conclusões e sugestões para trabalhos futuros são expostos.

O Capítulo 1 contempla uma introdução geral sobre o tema proposto do trabalho, em que é apresentada uma visão geral de enzimas no mercado mundial enfatizando o uso de proteases, quanto ao seu modo de ação e aplicações. Além disso, são abordados os métodos de produção e as estratégias de recuperação e purificação de peptidases, como precipitação e sistemas bifásicos aquosos, utilizados na pesquisa.

O Capítulo 2 apresenta o objetivo geral do trabalho, assim como os específicos.

O Capítulo 3 expõe a revisão bibliográfica em forma de artigo de revisão, o qual é intitulado "*An Overview of Proteases: Production, Downstream Processes and Applications on Industry*". O artigo aborda as características de proteases em relação à sua ação, funções, classificações, aplicações, além de descrever as diferentes fontes em que enzimas proteolíticas podem ser encontradas. Os modos de produção por fermentação submersa e fermentação em estado sólido também são descritas. O enfoque principal do artigo refere-se as metodologias de separação e purificação, também nomeadas *downstream processes*, utilizadas para proteases, destacando a precipitação por etanol e sulfato de amônio, além dos sistemas bifásicos aquosos.

O Capítulo 4 apresenta o artigo experimental intitulado "*Precipitation and Aqueous Two-Phase System Extraction of Protease from Aspergillus terreus VSP-22*", em que a investigação acerca da purificação por etanol e sulfato de amônio são realizadas, além da extração em sistemas bifásicos aquosos (PEG/$(NH_4)_2SO_4$) por meio de planejamentos experimentais. A caracterização bioquímica também foi avaliada por meio de testes como efeito de pH, temperatura e presença de íons metálicos, teste de estabilidade para pH e temperatura, além da avaliação dos parâmetros cinéticos.

O Capítulo 5 contempla as principais conclusões e considerações finais desta dissertação e, o Capítulo 6 descreve algumas propostas para trabalhos futuros.

## 1.2. Enzimas Proteolíticas e suas Perspectivas no Cenário Mundial

As enzimas têm revolucionado a indústria biotecnológica uma vez que são biodegradáveis e apresentam eficiência catalítica. Estas substituem agentes químicos por tecnologias limpas e economicamente viáveis, tornando os processos industriais mais ecológicos e econômicos. A utilização de enzimas no mercado global tende a aumentar cada vez mais, já que foi previsto um crescimento anual de 4,7% para 2016-2021, um aumento de $5 bilhões em 2016 para $6,3 bilhões em 2021, no qual as aplicações na indústria alimentícia e de ração se destacam (1). As proteases são uma das classes mais importantes na indústria catalítica, uma vez que representam mais de 65% do mercado total de enzimas (2).

Enzimas proteolíticas são proteínas capazes de quebrar ligações peptídicas, as quais podem ser encontradas em todos os organismos, dentre microrganismos, plantas e animais (3). Estas enzimas exercem relações fisiológicas e, também, bioquímicas envolvidas nas funções celulares e em organismos, agindo na regulação do destino, posição e ação da maioria das proteínas, modulação de interações entre proteínas, na propagação da informação celular, além da criação de novas biomoléculas, incluindo produção, tradução e amplificação de sinais moleculares (4).

Em aplicações biotecnológicas, os seres vivos capazes de produzir uma quantidade expressiva de protease extracelular, não somente como uma propriedade inerente, podem ser explorados para atividade comercial, uma vez que possuem facilidade na recuperação e purificação e, podem apresentar diversas aplicações (5). Dessa forma, as proteases de origem microbiana, provenientes de bactérias, leveduras e fungos filamentosos, apresentam destaque na indústria, uma vez que as enzimas são secretadas para o meio de cultivo.

As peptidases têm recebido atenção nas áreas de química de proteínas e engenharia de enzimas e, também, possuem diversas aplicações práticas, como agentes de limpeza, em detergentes, indústria alimentícia, de ração e química, processamento de couro, recuperação da prata, aplicações médicas e de tratamento de água. Ainda, podem ser utilizadas em química fina, na síntese enzimática de peptídeos, e na indústria farmacêutica, com inibidores de peptidases (6; 7).

A produção de proteases por microrganismos é influenciada pelos componentes do meio, principalmente, fonte de carbono e nitrogênio, assim como de fatores físicos, sendo eles pH, temperatura, tamanho do inóculo, nível de oxigênio difundido no meio e tempo de incubação. Cada espécie requer uma condição específica de cultivo para que se consiga atingir

níveis elevados do produto de interesse (5). Muitos microrganismos são capazes de produzir proteases, contudo, a importância é dada àqueles com rendimentos significativos e propriedades adequadas. Além disso, as melhores condições de crescimento do microrganismo não resulta, necessariamente, em maiores produções de protease (8).

As proteases são, geralmente, produzidas pela fermentação submersa, representando cerca de 90% da produção de toda indústria catalítica (9). Todavia, caracteriza-se em um processo oneroso para enzimas de baixo custo com aplicação em massa. Assim, a fermentação em estado sólido é um método alternativo (10), o qual apresenta benefícios como simplicidade, baixo custo, altas concentrações de enzimas, altos rendimentos e possibilidade de uso de resíduos agroindustriais como substrato (11). Os fungos são representativos na fermentação em estado sólido, já que o meio assemelha-se com seu habitat natural e a condição de baixa umidade, 40-60%, é suficiente para seu crescimento (12). Além disso, proteases de origem fúngica são fáceis de recuperação, uma vez que somente é necessária a filtração para separação do micélio, reduzindo custos com a quebra celular.

Outro fator em questão, para produção de protease a nível industrial, é a necessidade de purificação das enzimas do meio de cultivo. Esta etapa do bioprocesso pode representar 70-90% do custo total da produção de um produto biológico. Os métodos de bioseparação são resultados de subsequentes etapas, as quais baseiam-se nas características da molécula de interesse como carga, tamanho, hidrofobicidade e/ou capacidade de ligar em ligantes específicos (13). As primeiras técnicas utilizadas objetivam a concentração da enzima, contudo, pode ocasionar em perdas na atividade enzimática e redução do rendimento (14). Dessa maneira, diversas metodologias de purificação têm sido descritas na busca de melhor eficiência na separação e purificação, tornando todo o processo economicamente viável e encontrando padrões de qualidade para o mercado.

Dentre os métodos que vêm sendo utilizados estão a purificação por precipitação e sistema bifásico aquoso. A purificação por precipitação é uma primeira etapa de recuperação que objetiva a captura da biomolécula, alcançando uma concentração e purificação parcial, aumentando a eficiência em etapas subsequentes (15). Esta é uma técnica comum que leva à formação de fase sólida amorfa, por meio da mudança de pH, temperatura, adição de sais, solventes orgânicos, ou, ainda, polímeros (16). Além disso, é um método atrativo em separação de enzimas extracelulares e pode ser utilizado em aplicações em que não há exigência de altos níveis de pureza.

Assim, a precipitação apresenta vantagens como simplicidade de operação, baixa quantidade de energia, equipamentos simples, bem como facilidade no aumento de escala (17). As principais perturbações nas interações proteína-solvente que afetam a solubilidade da proteína são causadas pela adição de sais e solventes orgânicos, como sulfato de amônio e etanol, respectivamente, os quais podem ser reciclados após uso. Estes levam à precipitação das proteínas/enzimas por modos diferentes. A adição de solventes orgânicos causa a precipitação por meio da redução na constante dielétrica da solução. Já a adição de sais permite a diminuição na camada de hidratação das proteínas, gerando interações hidrofóbicas entre as porções hidrofóbicas dos sais orgânicos e das biomoléculas, caracterizando o fenômeno de *salting-out* (16).

Um alternativo método que vem sendo explorado são os sistemas bifásicos aquosos (SBAs), os quais se baseiam em uma extração líquido-líquido composta principalmente por água em ambas as fases. Essa concentração aquosa, 65-90%, permite um ambiente moderado para extração de biomoléculas (18). As duas fases são formadas por uma mistura de dois polímeros solúveis em água ou por um polímero e sal específico, com concentração crítica e temperatura certas. Sob estas condições, duas fases aquosas imiscíveis coexistem (19). Esta metodologia apresenta vantagens como pequeno tempo de processo, baixo consumo de energia, compatibilidade com o meio ambiente (não-tóxico) e facilidade para aumentar a escala de extração (20). Além disso, SBAs formados por polímero/sal apresentam benefícios quando comparados com sistemas polímero/polímero como baixo custo, baixa viscosidade e rápida separação das fases (21).

A maioria dos processos de recuperação são baseados em subsequentes etapas de cromatografias, já que apresentam simplicidade e poder de resolução. Contudo, são métodos dispendiosos, que quando combinados reservam um alto fator de purificação, mas, podem acarretar em perdas de atividade e redução do rendimento (22). Logo, o desenvolvimento de estratégias envolvendo a recuperação de enzimas representa uma área de pesquisa de grande interesse, a fim de tornar os *downstream processes* mais eficientes, econômicos e viáveis para as aplicações industriais (23). Além disso, a exploração de novas fontes microbianas de enzimas proteolíticas deve ser cada vez mais incentivada, a fim de atender aos requisitos exigidos para as aplicações da indústria (24).

# Capítulo 2 – Objetivos

## 2.1. Objetivo Geral

Purificar e caracterizar protease de *Aspergillus terreus* VSP-22.

## 2.2. Objetivos Específicos

- Purificar por precipitação com etanol e sulfato de amônio;
- Extrair a protease por sistema bifásico aquoso;
- Aumentar a escala de extração por sistema bifásico aquoso;
- Caracterizar enzimaticamente a protease antes e após a purificação;
- Avaliar os parâmetros de cinética enzimática.

# Capítulo 3 – Artigo de Revisão a Publicar "An overview of Proteases: Production, Downstream Processes and Applications on Industry"

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# AN OVERVIEW OF PROTEASES: PRODUCTION, DOWNSTREAM PROCESSES AND APPLICATIONS ON INDUSTRY

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## Abstract

Peptidases are enzymes with the ability to break peptide bonds. These enzymes are ubiquitous and are present in every organism such as microorganisms, plants and animals. They are essential in metabolic process of cells and extracellular peptidases have been investigated due to their industrial applications, such as food and detergent. The aim of this review article was supply an update about proteases characteristics, their production, downstream processes and applications, discussing methodologies and tendencies that have been used by researchers. Production of proteases by microorganisms are predominant, which are greatly influenced by medium components. They have been produced by submerged and solid-state fermentations; the last achieves higher concentration of enzymes and uses agro-industrial residues as substrates. The bottleneck of production is found on downstream processes, which are costly and can attain 70-90% of total production costs. Precipitation using solvents, as ethanol and acetone, and salt, as ammonium sulphate, are being used, in addition to chromatographies and aqueous two-phase systems (ATPSs). The ATPS has been emerged to recovery target biomolecule, being able to clarify, concentrate and purify in just one operation. New cost-effective and efficient strategies for downstream processes have to be improved to attend industries where peptidases are requested. Furthermore, the search for new sources of proteases with high activity and tolerance in harsh conditions as pH, temperature and salinity have been increasingly encouraged in order to meet requirements for industry applications.

Keywords: Sources of peptidases, submerged fermentation, solid-state fermentation, precipitation techniques, aqueous-two phase system, industry applications.

## 3.1. Introduction

Enzymes have been revolutionizing the biotech industry due to their effectiveness as catalysts, being cost-effective and biodegradable in nature. The global market for industrial enzymes achieved approximately $4.6 billion in 2014 and $4.9 billion in 2015. The prediction

estimated an increase of 4.70% at compound annual growth rate for 2016-2021, nearly $5.0 billion in 2016 to $6.3 billion in 2021 (1). Peptidases compose one of the most important classes of enzymes in catalytic industry, which represent more than 65% of total industrial enzyme market (2). Proteases have been commonly applied on industries such as food, laundry and detergent processing, leather, pharmaceuticals, biosynthesis, silver recovery and bioremediation applications (3).

It is possible to detect peptidases in all organisms such as microorganisms, plants and animals. That are microorganisms able to produce these enzymes in a significant amount of extracellular proteases that can be exploited for business activity (4). For biotechnological industries, extracellular enzymes have higher attention due to innumerous applications, facilities on downstream processes and moderately nonspecific for substrates. However, the recovery of target enzyme is complicated and costly because there is low concentration of biomolecule of interest between other proteins contaminants, in addition to the similarity of physical properties into proteins in same solution (5).

The downstream processes are the bottleneck of the enzyme production cost to be applied on industries, which can reach 70-90% of the total cost (6). The separation techniques used in the early stages purpose a concentration and increase on recovery yield, utilizing common methodologies as precipitation and chromatography. In order to improve the purity of the biomolecule of interest additional steps are required. Aqueous two-phase system is an alternative liquid-liquid extraction that has been emerging as it decreases the stages of purification and increases enzyme recovery (7).

Microbial proteases are predominant in industry, which some degree of purity is required to be applied on enzyme sale. Therefore, the search for economic and effective downstream processes are important to minimize separation costs and making process feasible (6). Furthermore, detecting proteolytic enzymes with high activity highlighting to pH and thermo tolerance attend to many industrial requirements. Thus, the aim of the present work was to provide an overview of characteristics of proteases, with respect to their function, classification, and several sources, in addition to tendencies in their production and especially on recuperation and purification methodologies.

## 3.2. Proteases

### 3.2.1. Definition and Functions

Proteolytic enzymes, peptidases, proteinases or proteases are defined as proteins with capacity to break peptide bonds. These terms have slightly different meanings, however they are used with the same intentions and are synonymous. Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) recommends to utilize the term peptidase (8). Furthermore, the committee defines proteinases as hydrolases that act on a peptide bond. In Enzyme Nomenclature, hydrolases are classified in class 3 and peptidases subclass 3.4. Most of all proteinases are hydrolases, although exist some distinct catalytic mechanisms (9).

The simplest structure of proteolytic enzyme has two subdomains separated by the active site. Within this site are the residues accountable for catalysis, dyad or triad catalytic. A peptidase specificity is evaluated by behavior in front of its substrate and inhibitors, which distinct one homologous peptidase from another (10). In general, a reaction of hydrolysis is based as an acid-base reaction. Therefore, a peptide bond presents in the active site suffers a nucleophilic attack, leading to the formation of an acyl intermediate. These temporary complex breaks down and a proton is transferred to residue of a base on the protease. This results in hydrolysis of peptide bond with a water molecule (9).

In biology, proteases are ubiquitous and have physiological and biochemical relationships involved in cells and organisms functions, as adaptation, nutrition, growth, regulation, sporulation/germination, disease and death (11). In particular, peptidases regulate destination, position and action of majority of proteins, modulating interactions of protein-protein, inflecting in process of cellular information, creating new biomolecules, including generate, transduce and amplify molecular signals (12). Furthermore, modification in proteolytic system leads to pathological conditions, such as tumor invasion, neurodegenerative illness and inflammatory diseases, in addition to pathogens and parasites that utilize proteolytic enzymes to invade their hosts (9).

### 3.2.2. Classifications

Proteases are classified based on their specificity, catalytic type and homology. In terms of specificity, proteolytic enzymes are divided in two groups: exopeptidases, that act cleaving one or few free amino acids from N- or C- terminus, and endopeptidases, that attack internal peptide bonds. Thereby, exopeptidases act at or near the end of polypeptide chains,

being categorized as amino- or carboxypeptidases. Exopeptidases that release one N-terminus amino acid are named aminopeptidases (3.4.11). If they release a dipeptide or tripeptide are named dipeptidylpeptidase (3.4.14) and tripeptidylpeptidase (3.4.14), respectively. Carboxypeptidases (3.4.16, 3.4.17 and 3.4.18) liberate a single amino acid or a dipeptide, the last one designated peptidyl-dipeptidase (3.4.15) (9; 8). Moreover, carboxypeptidases are differentiated due to the presence of some amino acids substituents at the active site, forming 3 groups: serine-, metallo- and cysteine carboxypeptidases (11).

In regard to endopeptidases, these enzymes can be grouped by size of peptides that undergoes cleavage. They are not easily categorized as exopeptidases. Some enzymes attack peptides and proteins of any size, named endopeptidases, and others act only in short peptides, which are oligopeptidases. There is also a group known as omega-peptidases; enzymes that act on peptide bonds with amino acids modified or without presence of alpha carbon (9). In addition, classification of endopeptidases is based on their catalytic mechanism of action, which are composed by 6 types: serine, aspartic, cysteine, metallo, glutamic acid and threonine endopeptidases, that is explained below (13).

Table 3-1: Subgroups of protease categorized by catalytic types and their characteristics.

| **Catalytic Type** | **pH optimum** | **Representative Enzymes** | **Examples of Inhibitors** |
|---|---|---|---|
| Cysteine | Acid | Papain, Clostripain, Streptopain. | *p*-chloromercuribenzoate |
| Serine | 7.0-11.0 | Chymotrypsin, Subtilisin. | Diisopropyl fluophosphate (DFP); phenylmethane sulfonylfluoride (PMSF) |
| Aspartic | 3.0-4.0 | Pepsin, Rennin. | Pepstatin, diazoketone compounds. |
| Metallo | Neutral | Thermolysin | Ethylene diamine tetraacetic acid (EDTA), 1-10 phenanthroline. |
| Threonine | Neutral | - | - |
| Glutamic acid | Acid | - | - |

Obs: Threonine and glutamic acid types are recently subgroups; thus, does not have enough information.

In respect to classification based on catalytic type of proteases, both exopeptidases and endopeptidases are able to be categorized. In order to comprehend the mechanism of action it is relevant to determine pH of the catalytic action and inhibitors that can occasionally inactivate the reaction. Catalytic types of peptidases are distinguished by nucleophiles attack,

in reaction of hydrolysis. There are proteinases in which nucleophiles are a side-chain of amino acid. These protein nucleophiles can be a thiol of a cysteine residue, and a hydroxyl of a threonine or serine residues. Nucleophiles that are stimulated with a water molecule are termed as water nucleophiles. They are activated by side chains of amino acids, as aspartate and glutamate residues, or stimulate by metal ion bound of an amino acid side chain (9). Table 3-1 shows the classification of proteases by catalytic types and some characteristics as optimum pH, examples of enzymes and inhibitors (9; 13).

Finally, it is possible to describe classification by homology. This hierarchical category establishes unit peptidases into families, and these into clans. A unit peptidase is the domain responsible for protease activity, which contains the primary substrate binding sites and catalytic residues. A family contains a set of homologous sequences, in which analyses are based in a statistically relation of unit peptidases. A family is constructed around proteases that are well characterized biochemically and a model of these peptidases is known as holotype. Clans are a set of related structures and represent one or more families with similar evolutionary tertiary structure. Furthermore, if the characteristics of a family have connection to others, this clan is divided into subclans. If no crystal structure is clarified for any family it is possible to nominate this family into a clan, in agreement to the order of the active site residues (9; 14; 8; 15).

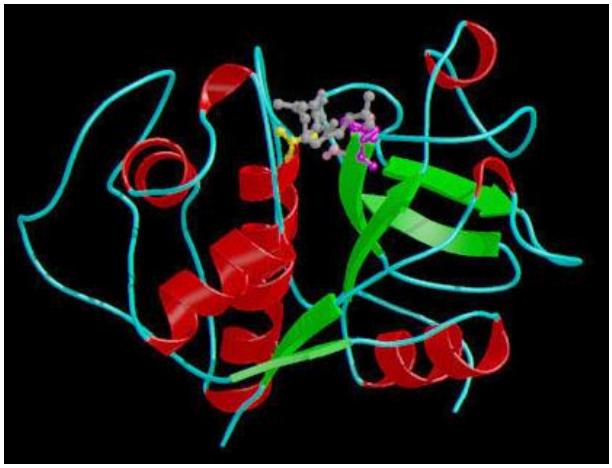
### 3.2.3. MEROPS Database

There are many databases and websites on the internet about peptidases. One relevant tool is called MEROPS, a database of proteolytic enzymes and their respective inhibitors and substrates (14), which provide their hierarchical classification and nomenclature (16). It was created in 1996 following rating system by Rawlings and Barrett (17) and is based on homology, exploring sequences alignments. It supplied secondary and tertiary structures, besides phylogenetic trees. Use of MEROPS database includes restore information about family and clan to know peptidase and inhibitor, in addition to determination of their respective sequences in order to detect clan, family, and peptidase/inhibitor specie. This tool can be applied on several applications of genome analyses, evolution studies, peptidase specificity, predictions, drug target analyses and cleavage identification (10).

Rawlings and colleagues have published updates (16; 10; 14) and scientific findings about substrate cleavage collection on the MEROPS database (18). Researchers have been using this website as support to their studies, as Franta et al. that sequenced maggot tissues of

larvae and used MEROPS to assist clusters of peptidases (19). Morya and coworkers analyzed catalytically active site residues from distinct *Aspergillus* species (20). In addition, Mai et al. analyzed relation between sequences, structures and functions of proteases, kinases and phosphatases using Enzymes Commission and MEROPS classifications. They visualized and clustered these enzymes and observed consistent information with each one (21). Figure 3-1 was collected at MEROPS database and illustrates a 3D structure of a well-known peptidase from papaya, named papain.

Figure 3-1: Structure of papain from *Carica papaya* (3.4.22.2). The image is derived from MEROPS database. Helices are shown as red coils and beta strands as green arrows. The active site residues are shown in ball- and stick- representation: Cys158 in yellow, His292 in purple and Asn308 in pink.



## 3.3. Sources of Peptidases

### 3.3.1. Microbial Peptidases

Sources of microbial proteases from bacteria, yeasts and filamentous fungi produced by fermentation represent around 40% of the total worldwide enzyme sales, due to the fact that peptidases are secreted into the medium culture (22). *Bacillus* species are the most representative microbial strains used in protease production, highlighting to neutral and alkaline serine proteases (11). Several researchers have described newly proteinases from sundry places. Rehman and colleagues (23) isolated a potent bacterium strain from hydrothermal vents, identified *Bacillus subtilis* KT004404, and characterized as promiscuous protease in use of leather and textile industries. Luo et al. (24) isolated a strain of *B. subtilis* N7 from cucumber rhizosphere and new alkaline antifungal protease was purified and partially characterized.

Moreover, Uttatree and Charoenpanich (25) purified protease from *B. subtilis* BUU1, from marine sediment collection, and results showed a promising candidate in

biotechnological applications. Although there are a lot of studies about *Bacillus* sp., there are other bacteria species that are also significant. Raj and coworkers (26) isolated proteolytic bacterium from dairy effluent sludge, identified *Pseudomonas aeruginosa* and its protease has fribrinolytic activity, with potential in detergent and pharmaceutical industries. Al-Askar et al. (27) noticed antifungal activity on *Streptomyces griseorubens* E44G strain, which is against of the agent of root rot disease in corn, and studies led to the identification of an alkaline protease.

In reference to proteases from yeasts, investigations have been searching new catalytic properties for applications on industry. Li and colleagues (28) reported the first production of cell-bound acid protease from marine yeast *Metschnikowia reukaufii* W6b, which shows similar conditions of production when compared with terrestrial yeasts and presents high skimmed milk coagulability, that has potential in cheese, food and fermentation industries. Viana et al. (4) evaluated microbial composition of cow raw milk, which 17.60% were yeasts, and *Candida buinensis* showed highest proteolytic activity, in addition to members of genera *Brettanomyces* and *Dekkera*. Factorial design was made and inexpensive medium proposed as environmentally friendly agent, further to facilitates scale-up on industry.

In respect with fungal proteases, simple recuperation is observed due to the use of filtration to separate the mycelium. Compared to bacteria this is advantageous, since bacteria require more cost intensive procedures for cells separation (29). Concerning to proteinases sources from fungi, *Aspergillus* species are a dominant producer, especially for food applications (11). Eugster et al. (30) evaluated prolyl endopeptidases from two *Aspergillus oryzae* strains, which had potential application on food industry for enzyme therapy in patients with intolerance to gluten. Abidi and coworkers (31) reported a novel alkaline protease from *Aspergillus niger*, that was purified, characterized and checked for antioxidant activity.

Futhermore, Novelli and colleagues (32) performed a comparative investigation on proteolytic production of diverse fungi strains, using agro-industrial residues. The majority reached high protease activity, highlighting to *Aspergillus oryzae*, *Penicillium roquefortii* and *Aspergillus flavipes*, which could be interesting for biotechnological industries. Omrane et al. (33) isolated a fungus producing protease from alkaline wastewater of chemical industries, identified *Trametes cingulata* CTM10101 that could be utilized in detergent formulations and synthesis of peptides. Anitha and Palanivelu (34) analyzed an extracellular keratinolytic protease from *Aspergillus parasiticus* detected in poultry soil, with applications on environmental biotechnology and pharmaceutical industries.

### 3.3.2. Plant and Animal Sources of Peptidases

The main highlights of plant proteolytic enzymes produced commercially are bromelain and papain from family of pineapple Bromeliaceae and *Carica papaya*, respectively (11). Bromelain is noteworthy in view of a large production of pineapple in Brazil, and pineapple stems, barks and leaves are able to be reusable as a source of enzyme. Further, bromelain has extended applications on pharmaceutical and food industries (35). On the other hand, papain is extracted from papaya latex and ripe fruits, having a variety of food applications involving juice and beer clarification, cheese production and meat tenderization. Both enzymes are cysteine proteases and some studies compare and analyze them for similar and newly purposes (36).

Recently, novel plant sources have been investigated in order to explore other candidates for protease production. Sun and coworkers (37) evaluated nearly ninety plant resources and highest proteolytic enzymes were from pineapple, fig and papaya, plants already commercialized for this proposal. Nevertheless, five plant extracts being kiwifruit, broccoli, ginger, leek and red pepper showed considerable protease activity, indicating the potential resources for protease production on industries.

Animal sources of peptidases are present in tissues, making the process more laborious to clarify. Moreover, the origin of raw material can bring pathogens that are potential contaminants, mainly if destination of bioproduct is for therapeutic application (38). However, applications utilizing animal proteases have been used for a long time, like a chymosin extracted from fourth stomach of unweaned calves and lambs, that are used in cheese manufacture (39). It may be noticed that researchers have described new proteases from other animal sources. Dadshahi et al. (40) extracted and purified a thermostable protease from a shrimp, *Penaeus vannamei*, from wastes of Persian Gulf, which demonstrated high stability against high temperatures and pH range of 7–9. Zaqueo and colleagues (41) isolated the first serine protease from *Bothrops brazili* venom, that was purified and characterized, which showed good fibrinolytic activity.

### 3.3.3. Proteases from Extremophiles

The microorganisms capable of surviving in extreme habitats, for instance, high or low temperature, pH and/or high concentration of salt, are called extremophiles. They present adaptations that allow their growth and emerge enzymes, which are interesting for biotechnological purposes. Extremozymes are useful for the industry due to their ability to

support conditions, that mesophiles enzymes are unable to. In this way, studies concerning the exploration of microorganisms from extreme habitats have been encouraged, which might result in new discoveries of biomolecules produced under harsh conditions.

*3.3.3.1. Thermophiles Proteases*

The first report of extremophiles was in hot springs, boosting investigations in hot and extreme environments on the Earth, including deep-sea, hydrothermal vents, volcanic islands and geothermal heated lakes (42). Microorganisms that withstand higher temperatures are known as thermophiles, and the ones that grow around 80 °C are named hyperthermophiles (43). Thermostable proteases can improve reaction rates, enhance the solubility of nongaseous reactants and decrease the contamination from mesophiles, what are interesting in terms of industry. For example, a novel thermostable protease from *Caldicoprobacter guelmensis* D2C22$^{T}$ was isolated from hydrothermal hot spring, purified and characterized as a serine alkaline protease, which showed optimum proteolytic activity at 70 °C and pH 10.0. Furthermore, half-life time at 80 and 90 °C was 180 and 60 minutes, respectively (44).

*3.3.3.2. Psychrophilic Proteases*

Cold habitats exhibit a wide biodiversity on the Earth biosphere and investigations from the Artic to the Antarctic continent have been successful. Psychrophiles, which are microorganisms that have optimum temperature growth of 15 °C or lower (45), have been found in permanently cold environments, like in deep sea, snow, permafrost, sea ice, glaciers, also in cold-water lakes, cold soils, cold deserts and caves. Psychrophilic microorganisms have adaptations in their structure, which allow metabolic activities enabling these organisms to survive and thrive near freezing point of water. There are also facultative psychrophiles, or psychrotolerants, which support a relatively broad temperature ranges and tend to inhabit environments with thermal fluctuations (46).

Lario and colleagues (47) checked production, purification and performed a characterization with a protease from the marine Antarctic yeast, *Rhodotorula mucilaginosa* L7. Although the enzyme is from a cold-adapted yeast, purified protease evidenced optimal catalytic activity at 50 °C and pH 5.0, further the stability in presence of high concentration of salt. Similar behavior was observed in bacterial isolates, genera *Pseudomonas* and *Flavobacterium*, collected near Uruguayan Antarctic Base on King George Island. The growth and protease production occurred at low temperature, 4 and 18 °C, whereas stability tests

demonstrated peptidases stable on 4 °C and 30 °C, being the last with slightly better activity (48).

*3.3.3.3. Alkaphiles and Acidophiles Proteases*

Organisms capable of tolerating high values of pH are named alkali-tolerant, which are able to grow near neutrality to pH 9.0, and alkaliphiles, which grow at pH higher than 9.0. The cytoplasmic pH of alkaliphiles is lower when compared with external pH, and this difference is reverse of chemiosmotically productive proton motive force. On the other hand, acid environments are often associated with volcanic activities and microorganisms found in these places are known as acidophiles, extreme and moderate acidophiles have optimum pH below 3.0 and around 3.0-5.0, respectively (43).

Psychrophilic bacteria from King George Island soil from Antarctica were evaluated and phylogenetic analyses detected two species with proteolytic activity. The best activity of proteases were at pH 7.0, at 27 °C, from *Sporosarcina aquimarina*, and pH 9.0 at 37 °C, from *Algoriphagus antarcticus*, indicating the first report of proteases in both species, particularly alkaline proteases (49). Natural environments, mainly that suffer action of industries, have been investigated for commercially valuable products. Anandharaj and coworkers (50) sought a potential candidate for protease production and isolated from tannery wastes a novel peptidase from *Bacillus alkalitelluris* TW13, which showed an optimum growth at 40 °C and pH 8.0. Furthermore, the purified protease showed proteolytic activity in a wide range of alkaline pH's, 8.0 to 11.0, and maximum relative activity and stability at pH 10.0.

*3.3.3.4. Halophiles Proteases*

On hypersaline habitats, it is possible to detect halophiles and haloalkaliphiles, microorganisms that can tolerate higher NaCl concentrations as well as salt that is necessary for function and stability of these enzymes. Proteases from marine sources, mainly to halophilic and haloalkaliphilic microorganisms, seem to have important applications on detergent, dehairing, deskinning, further to food industries as fish sauces and marinades (51). Two thermostable alkaline proteases isolated from haloalkaliphilic bacteria were detected on salt enriched soil in Okha, Coastal Gujarat, India. The study of protease production showed 15 and 20% of NaCl concentration (w/v), *Oceanobacillus iheyensis* and *Haloalkaliphilic bacterium*, respectively, in addition to alkaline pH at 11.0 (52). Moreover, a haloalkaliphilic bacterium, *Oceanobacillus* sp., also isolated from Coastal Gujarat, India, was investigated to grow in the presence of solvents and the tolerance of protease was analyzed against these solvents. In

organic solvents, peptidase showed optimum activity at 2 M of salt and was active at pH 8.0-11.0, with optimum at pH 10.0. The study was significant due to limited information of haloalkaliphilic bacterium and it enzymes under non-aqueous conditions (53).

*3.3.3.5. Genetic Modulation as a Tool*

Researchers have also been exploring microorganisms with significant characteristics to create genetically modified microorganisms, in order to improve growth rate and production of the target biomolecule, accelerating the process and enabling an industrial application. Studies involving molecular and genetic of *Aspergillus* species were industrially significant for microbial protease production. Therefore, the first recombinant heterologous product that got approval from Food and Drug Administration (FDA) was the calf chymosin used in cheese making produced by *Aspergillus* species (11). Strategies involving *Escherichia coli* and *Pichia pastoris* are typical examples used as bacterium and yeast hosts, respectively, and examples were described below.

Alias and coworkers (54) isolated PI12 gene, which encoded a cold-adapted serine protease. Full-length cDNA was amplified and cloned into *Pichia pastoris* expression vector, pPIC9, which had a methanol-alcohol oxidase as promoter. The enzyme was successfully expressed and secreted, which achieved 28.3 $U.mL^{-1}$ of protease after 72 hours of induction time with 0.50% of methanol inducer. Similarly, a psychrophilic bacterium, *Planococcus* sp., was isolated from deep-sea mud and its protease gene, *cpls8*, cloned and expressed in *E. coli*. Heterologous peptidase exhibited optimal activity at pH 10.0 with increasing activity at 5 and 35 °C (55). In both cases, proteolytic activity of newly proteases seemed to be attractive and cost-effective for biotechnological processes.

## 3.4. Production of Proteases by Submerged and Solid-State Fermentations

Production of extracellular proteases in microorganisms is strongly affected by medium components. Each strain contains its particular conditions to reach a maximum enzyme production. Critical factors established between physiology of microorganism and physicochemical factors, determine the appropriate model of production, such as temperature, pH, aeration, water activity, moisture, nature of substrate and bed properties (56). There are innumerous protease-producing microorganisms; however, attention is restricted for those with significance yield on suitable properties. Nevertheless, the best conditions for microorganism growth do not necessarily outcome in higher protease production (57).

Proteases are usually produced by submerged fermentation (SmF), being nearly 90% of production of all industrial enzymes (58). The technique applies liquid substrates as molasses and broths, and bacteria are suited due to their greater requirement of water to growth. As advantages, SmF displays ease in process control and recovery of extracellular enzymes (59). Nonetheless, bioproducts are diluted and enzymes extracts can be less stable, when compared to solid-state fermentation. This leads to an expensive enzyme production that has low-cost bulk application (60).

Thus, solid-state fermentation (SSF) is an alternative method, which exhibit benefits when compared to the submerged fermentation, that includes simplicity, low cost, higher concentration of enzymes and yields, product stability, lower catabolic repression, lower request on sterility as a result of low water activity utilized, in addition to use a widely agro-industrial residues as substrates (61; 56). Common substrates used are wheat bran, rice and rice straw, hay, fruit and vegetable waste, paper pulp and bagasse (62; 59). Disadvantages of SSF includes insufficient employment of the nutrients due to heat transfer in substrates and low quantity of oxygen. On the other hand, challenges with scale-up, purification of bioproduct and biomass estimation are the bottleneck of the technique that braves researchers to find solutions (56).

The physiology of solid medium brings up differences in SmF and SSF enzymes production. Some enzymes from SSF show molecular weight, kinetic parameters and optimal conditions distinct to the ones obtained in SmF. Enzymes that are intracellular in SmF can be extracellular in SSF and, some strains that are good producers in on type of fermentation are not so good on another (63). Therefore, the metabolic diversity on these techniques has direct impact on productivity of enzymes.

Fungi are the representative microorganism for solid-state fermentation, due to the resemblance between the medium and their natural habitat. Further, lower moisture condition is needed when compared with bacteria, 40-60% moisture could be enough for fungi (56). Some species of genera *Aspergillus*, *Penicillium* and *Rhizopus* are notably suitable for production of proteases, once are generally regarded as safe (GRAS) (61). Highlighting to *Aspergillus* species, they are often utilized for industrial production of enzymes, which growth can occur on inexpensive media, in addition to secrete large amounts of enzymes (64).

Table 3-2: Protease production by submerged and solid-state fermentations.

| Microorganism | Substrate | | Productivity of Protease | | References |
|---|---|---|---|---|---|
| | SmF | SSF | SmF | SSF | |
| *Bacillus subtilis* IH-72 | Soybean meal | Wheat bran | 5.86 ± 0.23 $U.mL^{-1}$<br>11.74 ± 0.47 $U.mL^{-1}$ (mutant) | 89.15 $U.g^{-1}$ (mutant) | (65) |
| *Bacillus* sp. BBXS-2 | - | Wheat straw | - | 12.20 $U.g^{-1}$ | (60) |
| *Bacillus* sp. JB-99 | - | Wheat bran | - | 7.22 $U.g^{-1}$ | (66) |
| *Aspergillus oryzae* LBA 01 | - | Wheat bran | - | 29.27 $U.g^{-1}$ | (22) |
| *Aspergillus oryzae* NRRL 2220 | Tomato pomace | Tomato pomace | 2.25 $U.g^{-1}$ | 18.11 $U.g^{-1}$ | (58) |
| *Aspergillus oryzae* (Ozykat-1) | - | Mixture rice-wheat bran | - | 1.20 $U.g^{-1}$ | (61) |
| *Aspergillus terreus* IMI 282743 | Pre filtered palm oil mill efluent | - | 129 $U.mL^{-1}$ | - | (67) |
| *Synergistes* sp. DQ640074 | Tannery solid waste | Tannery solid waste | 400-420 $U.mL^{-1}$ | 745-755 $U.g^{-1}$ | (68) |
| *Myceliophthora* sp. | Casein | Wheat bran, casein | 0.38 $U.mL^{-1}$ | 1.78 $U.mL^{-1}$ | (69) |
| *Vibrio alginolyticus* | Nutrient Broth medium | Mixture rice-wheat bran | 4.65 $U.mL^{-1}$ | 0.02 (650nm) | (70) |

In fact, many bacteria of genus *Bacillus* also exhibit great protease production in SSF and have been used as model for industry investigations. Mukhtar and Haq (65) analyzed different nutrient sources for SmF and SSF utilizing a wild strain and mutant from *Bacillus subtilis* IH-72. The best results took the substrate choice, then optimization of medium was

performed and productivity was 5.86 ± 0.23 $U.mL^{-1}$. However, mutant displayed superior biosynthesis of protease. Table 3-2 shows the protease production by submerged and solid-state fermentations from fungi and bacteria.

Many sources of substrates have been investigated in order to find the ideal condition for protease production and wheat bran has been reported as a great carbon source. The highest yield is achieved because desired sources of carbohydrates, proteins and minerals required for microorganism growth and production of protease are presented (65). The addition of another carbohydrate source should result in an increase on production, as halo-tolerant bacterium, *Vibrio alginolyticus*, that triggered better growth on wheat and rice bran medium in same proportions when compared with production of each source isolated (70). Wheat bran medium of *Aspergillus oryzae* (Ozykat-1) was supplemented with rice bran and improvement was obtained in ratio of 0.33. Whereas higher rice bran proportions cause a decrease in activity, suggesting the repression of protease production can occur in the presence of large amounts of carbon source (61). Catabolic repression was also observed in investigation of protease production from *Roseobacter* sp., when the medium was supplemented with readily available carbon sources as glucose, galactose, maltose, sucrose and lactose (71).

Concerning to protein sources, microorganisms requires complex nitrogen sources for protease production, such as casein, yeast extract, peptone and beef extract (72; 28; 73). *Micrococcus aloeverae* AE-6 MCC $2184^T$ and *Micrococcus yunnanensis* DSM $21948^T$ were induced for maximum protease production in casein medium (73). *Rhodotorula mucilaginosa* showed peptone as highest dependent variable for this purpose (74). In general, a combination of carbon and nitrogen sources shows stimulatory and inhibitory effects on protease production, depending on the species of the genus or strains of the same specie.

## 3.5. Purification of Proteases

The isolation and purification of enzymes from a fermented medium can account 70-90% of the total production costs, which usually involve several techniques according to their size, charge, hydrophobicity or capability to bind determined compounds (6). In spite of innumerous purification steps on downstream processes, considerable losses occur due to autolysis. Proteases suffer auto- and heterolytic fragmentation that is caused by the high activity of proteases, pH inactivation or complexation with endogenous inhibitors (75).

Protease purification has been commonly executed by the precipitation with organic solvents or salts and sequential chromatographic techniques. However, the last one is very

specific, expensive and exhibit difficult to scale-up, that result on enhance of final product cost (6). Even if the purification factor increases, the chromatography techniques propitiate losses on yield, what is possible to be noticed in many publications (76-79). Depending on application where certain degree of purity is needed, costs can be minimized using other separation and purification methodologies (6). A substitute technique that has been emerging is known as aqueous two-phase system, a liquid-liquid extraction that is composed in majority for water what favors extraction of biomolecules of interesting. Accordingly, precipitations and aqueous two-phase systems are discussed in following sections.

### 3.5.1. Precipitation Technique

Precipitation is the most common operation in downstream processes, which estimates that more than 80% of all recuperation and purification methodologies utilize this technique. The precipitation procedure describes a formation of an amorphous solid phase. Modifying the properties of aqueous solution induces the solid phase formation by changing pH or temperature, or adding salts, organic solvents or polymers. In this way, the procedure is usually operated without specific control of supersaturation and nucleation rate (80). Techniques of precipitation carry out two essentially actions: concentrate and purify biomolecule of interest. Firstly, the volume are reducing for next stages, and then, certain degree of purification is reached (6).

This method is generally applied at the beginning of recuperation and purification proceedings, and it is able to be used as an unique operation. Furthermore, it is useful in separation of extracellular enzymes and in applications that do not require a high level of purity (6; 5). Precipitation has advantages as it is a simple technique, which needs low energy, simple equipment requirements, easy to scale-up and materials utilized can be recycled, as ethanol and ammonium sulfate (5). The mainly perturbation of protein-solvent interactions that affected protein solubility is the addition of salts and organic solvents. For this reason, these two precipitants are discussed in this review article.

#### *3.5.1.1. Addition of Organic Solvents*

Precipitation by adding organic solvents is caused by a decrease in dieletric constant of solution. This reduction triggers an increase on electrostatic interaction of opposite charged regions of proteins, which allow an aggregation and, subsequently, precipitation. On the other hand, hydrophobic regions of protein are exposed by addition of these organic solvents, due to the water surrounding the biomolecule is displaced favoring aggregation (80). The precipitated

formed by addition of organic solvents has more stability when immersed in soluble material. These solvents do not denature biological products, as enzymes. Irreversible denaturation may occur if interactions of solvent and internal hydrophobic regions lead a disruption of secondary forms, α-helix and β-sheets. This situation is controlled by temperature near zero degree, when flexibility of biomolecule is reduced and loss of activity is minimized, in view of low penetration capacity of solvent (5). Some studies adopted 4 °C as work temperature (72; 81; 82).

Methanol, ethanol, isopropanol and acetone are relevant precipitants, highlighting the second one, which exhibits a greater solubility and satisfactory hydrophilic character to decrease denaturation. Further, ethanol is a cheap resource widely produced in world, mainly in Brazil (6). Ethanol precipitation is an attractive technique because it has good physicochemical properties as well as complete miscibility in water, high volatility, low cost and low toxicity (82), however it is flammable. Recovery of ethanol precipitants is facilitated by flash evaporation, whereas ammonium sulfate and acids precipitants need dialyses to adjust the ionic strength (75).

There are many publications analyzing organic solvents to purify peptidases. Biaggio and colleagues (83) investigated ethanol precipitation for serine peptidase from *Aspergillus terreus* and at 70% of ethanol concentration they reached 90.40% of recovery and 2.50-fold for purification factor, indicating better results when compared with subsequent chromatography techniques employed. Soares et al. (5) purified bromelain from pineapple wastes using 30-70% fraction of ethanol concentration that achieved good results for activity yield (98.60%) and purification factor (2.27-fold). Sun and coworkers (82) performed an ethanol precipitation for alkaline serine protease from tomato *Lycopersicum esculentum* that attained 31.60% of recovery and 9.80-fold for purification factor at 33% concentration.

Murthy and colleagues (84) investigated visceral proteases from little tuna (*Euthynnus affinis*), catla (*Catla catla*) and tilapia (*Oreochromis mossambicus*) using acetone, ethanol and ammonium sulphate fractional precipitations. Acetone precipitated fraction showed better recovery for all fish enzymes, highlighting to catla protease that reached 31.18% of recovery and 15.95-fold for purification factor. Sequentially, ethanol precipitated fraction from catla attained good recovery (21.47%) as well as purification factor (11.40-fold). Thus, cold acetone was efficiently used as precipitant agent when compared with ethanol and salt.

Geethanjali and Subash (85) checked extraction of proteases from the viscera of *Labeo rohita* by ethanol, acetone and ammonium sulphate precipitation. Acetone as precipitant also demonstrated the best recovery results (54%), followed by 20-40% ammonium sulphate fraction (18.70%). These indicate that ice-cold acetone precipitation was most effective in view of its low dieletric constant and boiling point; due to solubility of proteins in solvents depend on these characteristics, in addition to dipole movement. Karbalaei-Heidari et al. (72) purified a novel protease from *Halobacillus karajensis* MA-2 using acetone precipitation, which provides 83% of activity yield and 1.70-fold for purification factor at 80% concentration.

*3.5.1.2. Addition of Salts*

Salt when added to a solution it dissociates and ions interact, establishing electrostatic interactions in charged residues on the protein surface. Nonpolar interactions probably result between hydrophobic portions of organic salt and hydrophobic residues of biomolecule. At high concentration of salts, ions sequestrate water molecules that surrounding protein in hydration layer and more residues of protein are exposed, lowering its solubility and precipitating it (80). This mechanism is called precipitation by salting-out phenomenon. Ammonium sulphate is a common salt used in salt precipitation and is usually applied at purification of enzymes due to its simplicity, does not generate heating of the solution what happens with organic solvents, in addition to high solubility of the salt and non-denaturation. Nevertheless, $(NH_4)_2SO_4$ is corrosive and disposal should be monitored. Salt precipitation has also to adjust the temperature in order to avoid activity losses (76; 40; 78).

Some researchers have been studying ammonium sulphate precipitation in first step of peptidases purification. Hussain and colleagues (79) purified the enzyme from *Aspergillus terreus* at 90% saturation, where 71.70% of recovery and 1.09-fold for purification factor were achieved. Huang et al. (78) detected 5.52-fold for purification factor and 47.23% of recovery for an acid protease from *Aspergillus hennebergii* HX08, and then had verified the presence of two proteolytic enzymes. Uttatree and Charoenpanich (25) precipitated a protease from *Bacillus subtilis* BUU1 at 80-90% of saturation which attained 7.51-fold on purification factor. In addition, Rehman and coworkers (23) purified metalloproteases from *Bacillus subtilis* KT004404 where 95.78% of recovery yield and 2.62-fold for purification factor were achieved at 60% saturation of $(NH_4)_2SO_4$. All data related in section 5.1 are with respect to precipitation technique, not taking into account purity gains or recovery losses on subsequent recuperation and purification methodologies.

Abidi et al. (76) founded 89.30% of enzyme yield and 1.22-fold for purification factor at 80% saturation for a serine protease from *Penicillium italicum*. Dadshahi and colleagues (40) purified protease from *Penaeus vannamei* which they attained 83.91% of recovery yield and 2.30-fold for purification factor at 80% saturation. Abidi et al. (31) precipitated an alkaline protease from *Aspergillus niger* that reached at 80% saturation a recovery of 77.30% and 2.66-fold for purification factor. Temiz and coworkers (77) performed protease precipitation from *Engraulis encrasicholus* digestive tract and detected 57.61% of activity yield and 1.66-fold for purification factor at 40% of saturation. It is noticed that each peptidase has its own characteristics due to the variety of proteolytic enzymes sources and, consequently, each one provides its precipitation profile.

### 3.5.2. Aqueous Two-Phase Systems

*3.5.2.1. Definition and Operation of ATPS*

Aqueous two-phase system is a liquid-liquid extraction method that has been emerging in separation of several biological compounds as proteins, genetic material, bio-nanoparticles, organelles, even viruses and whole cells (86; 87). In addition, to remove inorganic molecules (88; 89). This technique replaces the use of organic solvents in traditional liquid-liquid extraction (90), which provides preservation of biomolecules, mainly 3D structure of proteins avoiding their denaturation and inactivation. Therefore, ATPS has proposed as an attractive tool for recuperation that integrates the clarification, concentration and purification of the target molecule in one unique operation (91).

ATPS is formed by adding two or more hydrophilic polymers, or polymer and a salt, under critical concentrations and temperature, implying in two immiscible aqueous phase (89). A partial immiscibility of the components allows the formation of the TOP phase (top phase), formed by more hydrophobic polymer, and the BOTTOM phase (lower phase), that is rich in salt or a second more hydrophilic polymer (92). The biological properties of target biomolecules can be preserved due to the high water content (65-90% w/w) that reduces the risks of denaturation and losses on activity and other active biomolecules. Moreover, polymers used in ATPS provide a stabilizing effect that benefits biologically macromolecules (93).

Furthermore, aqueous biphasic system exhibits economic advantages concerning the recovery, polymer reuse, ease of use, low cost, short time for phase separation and low energy consumption, when compared with traditional chromatographic techniques (94; 95). Further, ATPS has low interfacial tension, nonflammable, biocompatible environment (96) and

is easy to scale-up, showing good resolution, in addition to high recovery (97; 98). Several publications have been partitioned enzymes by aqueous two-phase system as proteases from *Lentinus citrinus* DPUA 1535 by polyethylene glycol (PEG)/phosphate ATPS (99), bromelain using PEG/$(NH_4)_2SO_4$ system (35), collagenase from *Penicillium aurantiogriseum* URM4622 by PEG/phosphate (100) and cysteine protease from Asian pear fruit using PEG/$MgSO_4$ (75).

Nevertheless, aqueous two-phase system presents two main drawbacks concerning the difficulty of isolating the extracted molecule from the polymer phase by back extraction and the employment of salts, essentially ammonium sulfate and phosphates, that make critical to scale-up the process due to requirements of disposal and corrosion considerations. The last could interfere on algal growth and generate anaerobic conditions if salts were discharged into water. Thus, a treatment of water is needed and, consequently, the increase on downstream processes cost (91). In order to enhance these conditions, biodegradable or recyclable components have been proposed, as citrate salts (101; 102).

The characteristics of the protein of interest as structure, hydrophobicity and molecular mass affect its partitioning, and according to increases on biomolecule size, its preference to one of the phase tends to intensify (98). Parameters related to system formation also influence partitioning of target molecule as well as types of salt and polymer, the polymer molar mass and its concentration, salt ionic strength, and pH (103). Critical values of salts and polymers concentrations determine the position on binodal curves (95). Consequently, the balance of enthalpic and entropic forces provide the mechanism of phase separation in ATPS (96). The equilibrium in the system is determined by interaction of biomolecule between phase-forming components, such as hydrogen bond, charge interaction, Van der Waals force, hydrophobic interaction and steric effect (104).

The extraction of biomolecules can be explained by the theory of volume exclusion that evidences an exclusion of high molecular weight molecules, such as proteins, due to increases in the molar mass of the polymer (105). The partition coefficient of biomolecules also decreases when polymer molar mass increases, showing less hydroxyl groups at same polymer concentrations, that result the increases on hydrophobicity in this phase (106). Thus, when PEG molar mass decreases is can be observed an increase in partition coefficient and the migration of enzyme to PEG-rich phase (98).

Salting out effect also can be observed in extraction by ATPS. The higher concentration of salt leads a decrease in protein solubility in the salt-rich bottom phase, resulting

in migration of protein, and hence partition, directly to polymer-rich top phase (86). In fact, occur an interaction of salt ions with the oppositely charged groups of protein, creating a double layer of ionic group. Then, hydrophobic zones of protein are gradually been exposed due to hydration effect of salt molecules surrounding the biomolecule (98).

Aqueous two-phase system has been applied for partitioning and recovery several biological macromolecules since Albertsson demonstrated the first studies involving ATPS in late of 1950s and early 1960s, where it showed a potential polymer-polymer system for a primary recovery of biomolecules (107). In order to summarize the use of ATPS in recent years a table (Table 3-3) was performed, bringing aqueous two-phase system extractions of peptidases during the last 10 years.

*3.5.2.2. Polymer-Salt System*

The polyethylene glycol is one of the most common polymer used in phase forming because it high stability and low cost. PEG solubilization occurs due to hydrogen-bonding mechanism, where water molecules are bonded with most ether oxygen sites along the polyethylene oxide chain (108). Proteolytic enzymes have been partitioned preferentially in the polymer phase, mainly those with hydrophobic characteristics (96). Peptidases extracted from the viscera of farmed giant catfish *Pangasianodon gigas* by PEG/sodium citrate (109), recombinant bromelain from *Escherichia coli* BL21-A1 using PEG/potassium phosphate (110), papain from papaya latex utilizing PEG/sodium sulphate (111), aspartic peptidases from *Aspergillus awamori* NRRL 3112 using PEG/potassium phosphate (112), protease from stomach of *Thunnus alalunga* by PEG/magnesium sulphate (113) showed partition on upper phase.

Most polymer-salt ATPS consist in PEG and salts highlighting to phosphate, sulphate or carbonate of ammonium, potassium and sodium; whereas biodegradable non-toxic salts have been proposed as citrate and tartrate (86). Wu and colleagues (7) successfully demonstrated a potential one-step purification of bromelain using PEG 4000/phosphate system, utilizing 17% (w/w) PEG 4000 and 14% (w/w) phosphate salt at pH 8.0; achieving 16.30-fold for purification factor and 55.60% of activity yield. Lario et al. (114) showed a viable and economic extraction for an extracellular protease from *Rhodotorula mucilaginosa* L7 comprising 13% (w/w) PEG 6000 and 10% (w/w) sodium tartrate at 30 °C, resulting in a purification factor of 2.51-fold and a protease yield of 81.09%. Ketnawa and coworkers (115)

displayed an extraction of bromelain by 15% (w/w) PEG 2000 and 14% (w/w) MgSO4, which showed the highest enzyme recovery of 113.54% and purification factor of 2.23-fold.

Addition of salt ions influence the protein partition due to electrostatic interactions between biomolecules and components of the system. In this way, an increase in salt concentration allows a repulsive effect of charged proteins, which they would prefer a PEG-enriched phase (116). These salts can weaken or strengthen the interactions between ionized groups with opposite net charge of proteins, affecting the partitioning. The nature of anions and cations determines the efficiency of the salt in following order: $SO_4^{-2} > HPO_4^{-2} > CH3COO^- > Cl^-$ for anions and $NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{+2} > Ca^{+2}$ for cations (106).

There are many publications related to the investigation of polymer molar mass and salts in purpose to extract proteases from diverse sources (93; 96). Senphan and Benjakul (117) demonstrated a combined strategy of three-phase partitioning followed with ATPS, which investigated PEG 1000 and $(NH_4)_2SO_4$, $MgSO_4$, $K_2HPO_4$ and sodium citrate for purification of protease from hepatopancreas of Pacific white shrimp. The best condition was 15% (w/w) PEG 1000 and 25% (w/w) $MgSO_4$ that attained 8.60-fold for purification factor and 65.50% of activity recovery yield into PEG-enriched phase. Chaiwut et al. (118) performed diversified ATPS using PEG 4000, 6000, 8000 and Na-citrate, $MgSO_4$, $K_2HPO_4$ and $(NH_4)_2SO_4$ salts in order to isolate a peptidase from the latex of *Calotropis procera*. The enzyme had highest recovery in PEG-rich phase using 12% (w/w) PEG 4000, 17% (w/w) $MgSO_4$ and 6% (w/w) NaCl at pH 7.0. The salts showed a comparable accordance with lyotropic series, where magnesium sulphate presented greater salting-out ability compared to the others.

*3.5.2.3. Polymer-Polymer System and Alternative ATPS*

Aqueous polymer-salt systems present advantages when compared to polymer-polymer systems as low viscosity, higher density, low interfacial tension between two phases, low cost of the salt, in addition to great selectivity (88; 98). The prospect on use of polymer-polymer ATPS has improved due to replacement of dextran for cheaper polymers. PEG/sodium polyacrylate (NaPA) system has been prepared with inert and relatively cheaper components that provide low viscosity and rapid phase separation (105). Barros and coworkers (92) evaluated the partition of proteases from *Penicillium restrictum* from Brazilian Savanna using PEG/NaPA and detected a highest partition coefficient in 20% (w/w) NaPA, 4% (w/w) PEG 2000 and 45% (w/w) of fermented broth; which led to 1.98-fold for purification factor and 37.73 for partition coefficient. Novaes et al. (119) investigated an alternative method to extract

bromelain from pineapple peel waste composed by PEG/poly(acrylic acid)/saline solution ($Na_2SO_4$) that could be appropriate for pharmaceutical or cosmetic formulations. The highlighted system comprising for 8% (w/w) PEG 2000, 8% (w/w) PAA 15000, 6% (w/w) of salt and at 30 °C reached a yield of 335.27% with 25.78-fold for purification factor.

Aqueous two-phase micellar system formed by surfactants has been investigated the partition of peptidases. Spir and colleagues (120) evaluated the extraction of bromelain peel waste for cosmetic formulations using Triton X-114 and McIlvaine buffer, which bromelain was partitioned into the micelle-rich bottom phase and showed a high purification factor. Amid and coworkers (121) purified a protease from kesinai plant, *Streblus asper*, leaves by non-ionic surfactants: Pluronic series and Triton X-114. The studied demonstrated that enzymes preferentially partitioned into the bottom surfactant-rich phase, where micelles were formed. In addition, the influence of salts was determined ($K_2SO_4$, $KH_2PO_4$, KCl and $KNO_3$) where improvement on protein solubility in bottom phase and an increase in hydrophobic interaction between two phases were detected.

Ionic liquids (IL) based ATPSs also have been explored to purify proteolytic enzymes as papain and bromelain. Bai and colleagues (122) evaluated the effect of [1-Butyl-3-methylimidazolium]X (X=Cl, Br) and $K_2HPO_4$ concentration on formation of ionic liquid/salt system. The optimum condition to extract papain to the IL-rich phase was 1.40 g [Bmim]Br and 1.40 g $K_2HPO_4$, which efficiency reached 98.33%. Vicente et al. (123) proposed an aqueous two-phase micellar system with ionic liquid, which acted as co-surfactants, to recovery bromelain from pineapple stem. The system comprising for Triton X-114, [P6,6,6,14]Dec and McIlvaine buffer at pH 7.0 was most selective, where enzyme partitioned preferentially toward the micelle-poor phase attaining 90% of recovery.

Table 3-3: Aqueous two-phase system extraction of peptidases.

| Production System | Enzyme | ATPS | Activity Yield (%) | PF[a] | Partition | Reference |
|---|---|---|---|---|---|---|
| *Streptomyces* sp. DPUA1576 | Fibrinolytic protease | 15% PEG 3350 - 12% phosphate | 155.00 | 1.51 | PEG-rich top phase | (116) |
| *Aspergillus tamarii* URM4634 | Protease | 24% PEG 8000 - 20% sodium citrate | 55.80 | 3.95 | PEG-rich top phase | (86) |
| *Calotropis procera* latex | Protease | 18% PEG 1000 - 14% $MgSO_4$ | 74.60 | 4.08 | PEG-rich top phase | (96) |
| *Penicillium candidum* (PCA 1/TT031) | Protease | 9% PEG 8000 - 15.90% Na-citrate - 5.20% NaCl | 93.00 | 6.80 | Salt-rich bottom phase | (97) |
| *Mucor subtilissimus* UCP 1262 | Fibrinolytic protease | 30% PEG 6000 - 13.20% $Na_2SO_4$ | 102.00 | 30.00 | Salt-rich bottom phase | (124) |
| *Serratia marcescens* P3 | Keratinolytic protease | 25% PEG 4000 - 25% sodium citrate and 25% PEG 4000 - 25% $(NH_4)_2SO_4$ | 68.00 | 2.70 and 2.60 | PEG-rich top phase | (93) |
| *Carica papaya* latex | Papain | 11.69% PEG 8000 - 9.52% sodium citrate - 0.10% alginate | 72.00 | 2.40 | PEG-rich top phase | (91) |
| Hepatopancreas of Pacific white shrimp | Protease | 15% PEG 1000 - 25% $MgSO_4$ | 65.50 | 8.60 | PEG-rich top phase | (117) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Pineapple stems, barks and leaves | Bromelain | 10.86% PEG 4000 - 36.21% $(NH_4)_2SO_4$ | 66.38 | 11.80 | PEG-rich upper phase | (35) |
| Asian pear fruit (*Pyrus pyrifolia*) | Cysteine protease | 15% PEG 1000 - 20% $MgSO_4$ | 91.83 | 3.90 | Salt-rich bottom phase | (75) |
| Pineapple peel Nang Lae cultivar | Bromelain | 15% PEG 2000 - 14% $MgSO_4$ | 113.54 | 2.23 | PEG-rich top phase | (115) |
| *Ananas comosus* pulp | Bromelain | 17% PEG 4000 - 14% phosphate | 55.60 | 16.3 | PEG-rich top phase | (7) |
| *Bacillus licheniformis* (ATCC 21424) | Alkaline protease | 25% PEG 10000 - 10% NaCl | - | - | PEG-rich top phase | (125) |
| Giant catfish viscera (*Pangasianodon gigas)* | Peptidases | 15% PEG 2000 - 15% sodium citrate | 273.00 | 12.00 | PEG-rich top phase | (109) |
| Mango (*Mangifera indica* cv. Chokanan) | Serine protease | 28.50% PEG 6000 - 28.50% $KH_2PO_4$ - 5% NaCl | 89.00 | 12.51 | PEG-rich top phase | (126) |
| *Bacillus subtilis* BP-36 | Thermophilic Alkaline Protease | 22% PEG 10000 - 18% $Na_3$citrate | 39.70 | 4.80 | PEG-rich top phase | (127) |
| Toxin from *Bothrops alternatus* venom | Protease | 12.20% PEG 3350 - 11.82% $KH_2PO_4$ | 82.00 | 1.20 | Salt-rich bottom phase | (128) |
| *Escherichia coli* BL21-A1 | Recombinant bromelain | 13% PEG 6000 - 11% $KH_2PO_4$ | 16.39 | 5.35 | PEG-rich top phase | (110) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Rhodotorula mucilaginosa* L7 | Protease | 13% PEG 6000 - 10% sodium tartrate | 81.09 | 2.51 | PEG-rich top phase | (114) |
| *Bacillus licheniformis* NCIM 2042 | Protease | 15.93% PEG 4000 - 22.36% $KH_2PO_4$ - 1.08 mM NaCl | 89.75 | 2.20 | PEG-rich top phase | (104) |
| *Lentinus citrinus* DPUA 1535 | Proteases | 17.50% PEG 6000 - 25% $KH_2PO_4$ | 151.00 | 1.10 | PEG-rich top phase | (99) |
| Catfish viscera (*Pangasianodon gigas*) | Alkaline protease | 15% PEG 2000 - 15% sodium citrate + 10% sodium citrate | 365.53 | 11.60 | PEG-rich top phase | (129) |
| *Aspergillus awamori* NRRL 3112 | Aspartic peptidases | 18.60 g PEG 1450 - 49 g $KH_2PO_4$ | 68.00 | 2.30 | PEG-rich top phase | (112) |
| *Calotropis procera* latex | Peptidase | 12% PEG 4000 - 17% $MgSO_4$ - 6% NaCl | - | 107 | PEG-rich top phase | (118) |
| Stomach of albacore tuna (*Thunnus alalunga*) | Protease | 25% PEG1000 - 20% $MgSO_4$ and 15% PEG2000 - 15% $MgSO_4$ | 85.70 and 89.10 | 7.20 and 2.40 | PEG-rich top phase | (113) |
| Farmed giant catfish viscera | Alkaline Proteases | 40% EOPO 3900 - 10% $MgSO_4$ - 17%NaCl | 77.98 | 21.50 | EOPO-rich top phase | (130) |
| *Penicillium aurantiogriseum* | Collagenase | 20% PEG 550 - 17.50% phosphate $NaH_2PO_4$ | 242.00 | 23.50 | Salt-rich bottom phase | (131) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Penicillium aurantiogriseum* URM4622 | Collagenase | 17.50% PEG 1500 - 15% $NaH_2PO_4$ | 61.68 | 5.23 | PEG-rich top phase | (100) |
| *Penicillium* sp. UCP 1286 | Collagenase | 15% PEG 3350 - 12.50% phosphate salt | 81.47 | 25.23 | Salt-rich bottom phase | (132) |
| Pineapple peel waste (*Ananas comosus*) | Bromelain | 8% PEG 2000 - 8% PAA 15000 - 6% $Na_2SO_4$ | 335.27 | 25.78 | PEG-rich top phase | (119) |
| kesinai plant (*Streblus asper*) leaves | Protease | 31% Pluronic L61 - 0.30 % KNO3 | 92.00 | 10.30 | Surfactant-rich bottom phase | (121) |
| *Penicillium restrictum* | Protease | 20% NaPA - 4% PEG 2000 | 37.73 | 1.98 | NaPA-rich bottom phase | (92) |
| Pineapple (*Ananas comosus*) Peel Waste | Bromelain | 3% Triton X-114 - 0.50 M KI - McIlvaine buffer | - | - | Micelle-rich bottom phase | (120) |
| Pineapple stem residues | Bromelain | 10% Triton X-114 - 0.30% [P6,6,6,14]Dec - McIlvaine buffer | 90.00 | - | Micelle-poor upper phase | (123) |
| Papaya | Papain | 1.40 g [Bmim]Br - 1.40 g $K_2HPO_4$ | 98.33 | - | IL-rich upper phase | (122) |
| Pineapple (*Ananas comosus*) | Bromelain | IL3 - $KH_2PO_4$ | 87.00 | 78.93 | - | (133) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Mango (*Mangifera Indica Cv. Chokanan*) | Serine Protease | 16% 2-propanaol - 19% $K_2HPO_4$ - 5% NaCl | 96.70 | 11.60 | Propanol-rich top phase | (134) |

[a] purification factor.

## 3.6. Applications

The use of enzymes brings ecological benefits for industry, due to they are environmental friendly agent. Proteolytic enzymes are known to constitute the majority of worldwide enzyme sale. They have been widely used in diverse industries, especially on food processing as dairy, cheese, brewing and bakery; as well as laundry applications such as washing detergent and textile, silk, tanning, and leather industries. In addition to pharmaceuticals, biosynthesis, silver recovery and bioremediation industries (3; 135; 51). A practical classification of proteolytic enzymes can be performed as acidic, alkaline and neutral proteases, according to the pH range they are active at; and as aspartic, serine, cysteine, glutamic, threonine and metalloproteases regarding the functional group found in their active area (136).

High-alkaline proteases represent nearly 40% of the total worldwide enzyme sale (44). These enzymes are characterized in a pH range 7.0 to 14.0, however they are commonly active between pH 9.0-11.0 (64). Moreover, alkaline proteases find a wide employment in leather, paper, pulp, laundry detergent, textile, food and pharmaceuticals industries (137). Even alkaline proteases have a number of fields, they are highlighted in detergent applications. The protease stability and activity under harsh operational conditions, such as pH and temperatures, are attractive, further in the presence of ionic detergent, surfactants and peroxide agent, due to the presence of high quantities of chelating agents in detergents that behave as water softeners and support stain removal. In this purpose, alkaline proteases are appropriate to meet requirements of detergent industry as an additive (138; 136).

Aspartic proteases, also known as acid proteases, are active under acidic conditions in a pH 3.0-5.0. These enzymes with high activity and stability at acid pH are required on important industrial processes as leather and pharmaceutical applications with cosmetic products (139). Further, in food applications proteases have capacity to coagulate casein in cheese making, improve flavor in other foods and cleave proteins from turbidity complex in juices and wine (140). There are many studies that have been described protease as aspartic enzymes from *Aspergillus foetidus* (139), *Stenocarpella maydis* (141), *Rhizopus oryzae* (142), *Withania coagulans* fruit (143) and a wine yeast *Metschnikowia pulcherrima* IWBT Y1123 (144).

Fungal aspartic proteases have been purified and characterized as rennin-like and pepsin-like enzymes, which exhibit one or two conserved aspartic acid residues at the active

site and contain molecular weights of 30 to 45 kDa (142). Proteases have the ability of clotting milk and chymosin-like proteases substitute milk-clotting enzymes in cheese manufacturing. The chymosin-like proteases are more suitable, characterizing high milk-clotting activity (MCA) and low ratio between MCA and proteolytic activity (145). The first recombinant chymosin was introduced in 1988 into cheese industry and gradually increased to commercial levels (140).

Metalloproteases are characterized by the requirement of divalent metal ions for catalytic activity, mainly in the presence of $Mg^{+2}$, $Mn^{+2}$, $Ca^{+2}$, $Zn^{+2}$ (146), which establish a stabilizing function in ternary structures and prevent them from autolysis (139). Most of metalloproteases show optimum pH near neutrality (146). Metal-dependent proteases have been related to several applications as beer brewing from *Bacillus amyloliquefaciens* SYB-001 (147), clotting milk in cheese manufacturing from *Termitomyces clypeatus* MTCC 5091 (145), as well as dehairing and removing cloth stains from *Bacillus alkalitelluris* TWI3 (50) and *Bacillus subtilis* KT004404 (23).

Concerning to serine proteases, these proteolytic enzymes exhibit disulphide bonds, which are required to maintain the activity and stability of the enzyme. There are many publications that related serine proteases, in view of their inhibition against PMSF (phenylmethylsulfonyl fluoride) and usages in wash performance and clot removal applications from *Bacillus pumilus* MCAS8 (138), food systems as antioxidative properties from *Penicillium italicum* (76) and humoral defense mechanism triggering in *Plutella xylostella* (148). Currently, many studies have been described proteases with serine and alkaline properties (44; 33; 149; 82; 150) due to stability in pH ranges, tolerance with severe conditions, as well as PMSF inhibition, which suggest the majority use of serine alkaline proteases in detergent formulations.

Regarding the cysteine proteases, most of them show optimum acid pH and catalytic residue Cys is used as a nucleophile in reaction (151). Whereas cysteine cathepsins, known as papain-like peptidases, are present in humans and approach of pathological conditions (152); industrial applications focus on plant cysteine proteases. Plant cysteine proteases are generally used in food applications in meat tenderization, cheese making, brewing, beer clarification, bread manufacture and flavor improvement (75). They are derived from extensively plant as papaya (papain) (36), pineapple (bromelain) (153), pear (75), kiwifruit (actinidine), fig (ficin) and ginger (zingibain) (37). They have high proteolytic activity; however, the limitation is due to their non-uniform or over activity on meat texture. Other

approaches have been described in the literature as cysteine protease from *Zingiber montanum* rhizome and its potential for therapeutic and food industry due to antioxidant activity (154); and enzyme from *Cissus quadrangularis* L that showed antibacterial property (155).

The glutamic protease family was recently reclassified in the MEROPS database. The reports are related to glutamate-specific endopeptidases (GSE) that have been applied in the peptide synthesis due to their high catalytic efficiency with Glu residues and wide pH, temperature and solvent adaptability. The catalytic dyad, consisting in glutamine and glutamate residues, stimulates the nucleophilic water and stabilizes the tetrahedral intermediate on the hydrolytic reaction (156). The bottleneck of application is the difficult on large scale production in the organic synthesis (157). The GSE have been identified from a variety of microorganisms, including *Bacillus licheniformis*, *Pichia pastoris* (157); in addition to widely fungi species as *Aspergillus* sp., *Fusarium graminearum*, *Phanerochaete chrysosporium* (156), and *Thermoactinomyces* sp. (158).

## 3.7. Conclusion and Perspectives

Peptidases are ubiquitous and exhibit not only important function in cellular metabolic processes, but also application in several sectors of industries (70). The article evidenced that proteases have many classifications and data that are summarized in databases as MEROPS, which assist researchers worldwide. The global market of peptidases tends to enhance increasingly due to these enzymes can be used in many industries, mainly on food and detergent applications. In addition, superior conditions of proteases production and downstream processes have to be investigated in order to detect improvement for industry applications.

Extracellular proteolytic enzymes have been investigated in order to find better circumstances to produce the enzymes with high proteolytic activity and recovery yield. Two methods of fermentation have been utilized, submerged and solid-state fermentations. The last one is cheaper and it favors extracellular peptidases production directly into medium. However, the highest cost of production is in the respect with downstream processes, methodologies that are used to separate and purify biomolecules of interest as precipitation, aqueous two-phase system and chromatographies. This is the bottleneck for application on industries and hard work has been executed in order to find more appropriate conditions to improve industrial market for peptidases.

There are many proteases that establish high activity and attend to many industrial requirements; due to their tolerance in pH, temperature and salinity. The search for new

proteases from microbial sources has to be encouraged in order to meet industry requirements (76). The advancement of new strategies that are efficient, scalable and cost-effective exhibit an attractive area in order to enhance downstream processes and, consequently, utilization on the industries (105).

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# Capítulo 4 – Artigo a Publicar "Precipitation and Aqueous Two-Phase System Extraction of Protease from *Aspergillus terreus* VSP-22"

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# PRECIPITATION AND AQUEOUS TWO-PHASE SYSTEM EXTRACTION OF PROTEASE FROM *ASPERGILLUS TERREUS* VSP-22

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## Abstract

Enzyme industry has proteases as one of the most important classes of global market products, in which downstream processes are the bottleneck of production, being costly and time-consuming. The aim of the present work was to investigate the purification, aqueous-two phase system extraction, and a partial characterization of a protease produced from *Aspergillus terreus* VSP-22, using efficient and cost-effective methodologies. The techniques of precipitation were performed at 20, 40, 60 and 80% of saturation. Ethanol precipitation achieved a specific activity of 174.19 $U.mg^{-1}$ and a purification factor of 15.05-fold at 80% concentration, however recovery attained 41.05% in this fraction. Ammonium sulphate precipitation at 60% saturation showed 46.45% on recovery and 2.07-fold on purification factor. The $2^4$ factorial design of ATPS was performed to evaluate the effect of PEG molar mass, PEG and ammonium sulphate concentrations, sample volume, and pH. The best purification factor (4.65-fold) was obtained with 18% (w/w) PEG 2000 (g/mol), 18% (w/w) ammonium sulphate at pH 9.8 and 0.5 mL of sample volume, showing a recovery of 59.03%. Response Surface Methodology was conducted in order to examine PEG and ammonium sulphate concentrations effects, and it was observed that a higher purification factor (5.16-fold) and recovery (70.82%) in the center point. The optimized ATPS was scaled up 8 and 64 fold and their behaviour were in agreement with specific activity (109.71 and 109.87 $U.mg^{-1}$) and recovery (69.40 and 70.60%, respectively). The biochemical characterization found an optimum pH of 9.0 and temperature of 50 °C for enzyme extract, whereas for purified protease an optimum of 9.8 pH and 40 °C. Kinetic parameters evidenced the high affinity of purified protease for azocasein ($V_{max} = 24.4499$ $U.mL^{-1}$ and $K_m = 0.0318$ mM). Protease has proven to have interesting properties, suggesting its potential in industrial application.

Keywords: *Aspergillus terreus*, protease, recovery, purification factor, aqueous two-phase system.

## 4.1. Introduction

The enzyme industry has advanced due to the replacement of chemicals by cleaner and cost-effective technology, becoming ecological and economical alternative for many industrial processes. Industrial enzymes in global market was predicted to increase $5.0 billion in 2016 to $6.3 billion in 2021, which food and animal feed applications are noteworthy (1). Proteases (EC 3.4.1.1-24) is one of the most important classes of enzymes and account for 65% of total industrial enzyme market (2). Proteolytic enzymes are proteins capable to break peptide bonds and are present in all organisms, such as microorganisms, plants and animals. For biotechnological purposes, extracellular proteases have higher attention because of innumerable applications and facilities on recuperation and purification (3), mainly microbial proteases. These enzymes can be applied in different industries as food, dairy, brewing, bakery, pharmaceuticals, leather, textile, silk, detergents, biosynthesis, bioremediation, highlighting to laundry, and detergents applications (4; 5).

A large-scale production of interesting biomolecules has been possible due to advances in biotechnology. Solid-state fermentation (SSF) is an alternative method (6), which exhibits benefits when compared with submerged fermentation, that includes simplicity, low cost, high concentration of enzymes, high yields; in addition, one can use a wide range of agro-industrial residues as substrates (7). Fungi are representative microorganism of growth and production of enzymes in solid-state fermentation because the medium resembles to their natural habitat and a lower moisture condition is required (40-60%) compared to bacteria (8). Furthermore, fungal proteases are generally extracellular and secreted into fermentation medium what facilitate the downstream processes (9).

The downstream processes of proteins can represent 70-90% of total production cost and are time-consuming, as a result of subsequent methods based on their charge, size, hydrophobicity and/or capability to bind specific ligands (10). Traditional methods of chromatography are gradually being replaced, even if they are very specific and high purity is achieved, disadvantages are noticed as low yields, difficult to scale-up and, consequently, the increase of the final product cost (11; 12). In order to reduce these costs, it is important to discover economic and effective methods. Precipitation is a usual procedure applied to the first steps of downstream processes. It concentrates and purifies the target biomolecule, attaining

some degree of purification. The modification in properties of the aqueous solution as pH or temperature, or adding salts, organic solvents or polymers, cause a solid phase formation leading to precipitation of proteins (13). This method presents advantages as simple equipment requirements, low energy, easy to scale-up and precipitants are able to be recycled (10; 12).

Another technique that has been highlighted is aqueous two-phase system (ATPS), a liquid-liquid extraction method that is formed under distinct thermodynamic conditions from the mixture of two or more polymers, or a polymer and a salt (14). Polymer-salts ATPSs have some benefits compared to polymer-polymer systems as low cost, low viscosity and rapid phase separation (15). Many molecules can be partitioned into some of the phases such as biological macromolecules, small organic molecules, inorganic ions and inorganic colloidal particles, viruses and cells (16). There are some advantages that this technique provides as short processing time, ease of operation, ease to scale-up, low cost, low power consumption, high resolution and biocompatible environment in view of high water content (65-90% w/w) (17; 18).

The development of efficient, cost-effective and scalable strategies involving recovery of enzymes with high yields represent an interesting area to improve downstream processes and possible applications to the industry (19). Moreover, the exploration for new microbial sources of enzymes has to be uninterrupted, in order to attend the industry requirements to be employed on technological applications (20). In the present study, the purposes were investigate the purification of protease from *Aspergillus terreus* VSP-22 by ethanol and ammonium sulphate precipitations and extraction by ATPS designs (PEG/ammonium sulphate). As well as characterizing biochemically the proteolytic enzyme.

## 4.2. Materials and Methods

### 4.2.1. Materials

Bradford reagent was purchased from Bio-Rad (Sao Paulo, Brazil). Azocasein was provided from Sigma-Aldrich (Sao Paulo, Brazil). Ethanol used in precipitation step was obtained in a gas station. All other chemicals were of analytical grades.

### 4.2.2. Microorganism

*Aspergillus terreus* VSP-22 was supplied by Department of Biochemical-Pharmaceutical Technology, Faculty of Pharmaceutical Sciences (USP). The microorganism was inoculated in Potato Dextrose Agar (potato 200 $g.L^{-1}$, glucose 20 $g.L^{-1}$ and agar 20 $g.L^{-1}$)

and incubated at room temperature for 120 hours. It was maintained at 4 °C and sub-cultured fortnightly.

### 4.2.3. Protease Production and Enzyme Extract

Enzyme production was performed by SSF. Previously, a pre-inoculum of *Aspergillus terreus* VSP-22 was fermented in 100 mL of czapek medium (sucrose 30 $g.L^{-1}$, sodium nitrate 2 $g.L^{-1}$, dipotassium phosphate 1 $g.L^{-1}$, magnesium sulphate 0.5 $g.L^{-1}$, potassium chloride 0.5 $g.L^{-1}$ and ferrous sulphate 0.010 $g.L^{-1}$) for 48 h, 100 rpm at room temperature. Then, a strainer was used to separate cells from the medium. Cells ressuspended into 100 mL of pure water were distributed into each Erlenmeyer flaks (10 mL) to SSF. Solid state fermentation was composed by 10 grams of rice bran and moisture was adjusted to 50% (w/v). Time incubation was 72 h at room temperature.

After incubation, the crude enzymatic extract was obtained adding 50 mL of distilled water to each flask. The culture medium was mixed manually with glass rod as support and flasks were stirring for 1 hour. Thus, enzyme extracts were collected by filtration utilizing a strainer (21), centrifuged at 8,000 x g during10 min and analyses were accomplished.

### 4.2.4. Protein and Protease Activity Assay

The total protein was determined by Bradford method (22), using bovine serum albumin as standard. The proteolytic activity was assayed by the azocasein method (23). Thereby, 125 µL of azocasein 1%, solubilized in 4% ethanol (v/v) and 100 mM phosphate buffer, pH 7.0; reacted with 125 µL of enzyme extract at 37 °C for 15 min. The reaction stopped by addition of 750 µL of trichloroacetic acid 5% (w/v) and samples were centrifuged at 8,000 x g for 5 min at room temperature. One unit of activity was defined as the amount of enzyme required to produce an increase in optical density of one unit at 440 nm within 1 hour. Each experiment was performed in triplicate.

### 4.2.5. Ethanol and Ammonium Sulphate Precipitation

Protease precipitations were performed in accordance with Englard and Seifter (24) methodology. Ethanol 98% (w/w), previously cooled to 0 °C, was added dropwise until desired concentration reached in enzyme extract. The solution was stirred for 20 min and centrifuged at 8,000 x g, for 20 min, at 4 °C. The resulting pellet was suspended in 100 mM phosphate buffer, pH 7.0. Correspondingly, ammonium sulphate precipitation was performed by addition of solid $(NH_4)_2SO_4$ in enzyme extract and stirred for 30 min, and then centrifuged for 30 min

at the same conditions previously described. All precipitations and analyses were performed at least in triplicate.

### 4.2.6. Experimental Design of Aqueous Two-Phase Systems

*4.2.6.1. Protease extraction using $2^4$ Factorial Design*

The ATPSs were prepared in graduated test tubes by mixing appropriate quantities of stock solutions of polyethylene glycol (PEG) of different molecular weight 50% (w/w), ammonium sulphate solid or 40% (w/w), enzyme extract and 100 mM Tris-HCl buffer. Systems weighed a total mass of 5.0 g. After preparation, tubes were stirred for 1 minute in a vortex, centrifuged at 7,000 x g for 10 min. Then, phase volumes were measured and samples of both phases were collected for subsequent assays. Blanks of each system were prepared to minimize interactions of PEG, salt and Tris-HCl buffer in analyses (25). As soon distilled water was added instead of enzyme extract.

Table 4-1: Factor levels of $2^4$ factorial design of ATPS.

| **Variable** | **Level** | | |
|---|---|---|---|
| | **Low (-1)** | **Central (0)** | **High (+1)** |
| $M_{PEG}$ (g/mol)[a] | 2000 | 4000 | 6000 |
| $C_{PEG}$ (% w/w)[b] | 14 | 16 | 18 |
| $C_{(NH4)2SO4}$ (% w/w)[c] | 14 | 16 | 18 |
| pH | 8.2 | 9.0 | 9.8 |
| Sample Volume (mL) | 0.5 | 0.75 | 1.0 |

[a] PEG molar mass; [b] PEG concentration; [c] Ammonium sulphate concentration.

Firstly, to investigate the influence of PEG molar mass, PEG concentration, $(NH_4)_2SO_4$ concentration, pH and sample volume, a $2^4$ factorial design with four replicates in the center point was carried out using Statistica 10.0 software. Table 4-1 shows the factorial experimental design used for the extraction of protease from *Aspergillus terreus* VSP-22 by PEG/ammonium sulphate ATPS.

The experiments were conducted in random order. Statistica 10.0 was used for graphical analyses of the data and only factors with significance ($p \leq 0.05$) were considered. The optimization was evaluated by desirability function combined with response surface methodology (RSM).

*4.2.6.2. Optimization by Central Composite Design*

Based on the results of factorial design, a $2^2$ central composite design was performed with PEG and $(NH_4)_2SO_4$ concentrations as variables, using four replicates in the center point to allow the estimation of pure experimental error. The values of PEG molas mass, pH and sample volume were fixed in 2000 (g/mol), 9.0 and 0.5 mL, respectively. Table 4-2 shows the central composite experimental design used for the extraction of protease.

Table 4-2: Factor levels of $2^2$ central composite design of ATPS.

| Variable | Level | | | | |
|---|---|---|---|---|---|
| | **Low (-1.41)** | **Low (-1)** | **Central (0)** | **High (+1)** | **High (+1.41)** |
| $C_{PEG}$ (% w/w)[a] | 14.757 | 16 | 19 | 22 | 23.243 |
| $C_{(NH4)2SO4}$ (% w/w)[b] | 14.757 | 16 | 19 | 22 | 23.243 |

[a] PEG concentration; [b] Ammonium sulphate concentration

The experimental data were submitted to adjustment by coefficient of determination and analyses of ANOVA and Fisher-based test. Then, a desirability optimization methodology was conducted to define the optimized level with each variable, where selecting a set of input conditions (independent variables) provides the most desirable output values (responses) (26).

*4.2.6.3. Validation and Scale-up*

The optimized model using PEG molar mass 2000 (g/mol), 19% (w/w) of PEG concentration, 19% (w/w) of $(NH_4)_2SO_4$ concentration, 100 mM Tris-HCl buffer, pH 9.0, and 0.5 mL of sample volume was performed in triplicate in order to validate the significant results obtained with this system. Characteristics of optimized system were chosen to increase the laboratory scale of the ATPS. Firstly, it was performed a system with 40.0 g total mass, and whole variable were scaled up in same proportions using 4.0 mL of enzyme extract. Accordingly, a second scale-up was carried out in same conditions, adopting a total mass of 320.0 g and 32 mL of enzyme extract. Both were accomplished in triplicate and had a blank.

Thereafter, protease was purified in subsequent steps by ammonium sulphate precipitation followed by optimized ATPS. Salt precipitation was performed at 60% saturation with 30 mL of enzyme extract and the precipitated pellet was dissolved in a small volume of 100 mM Tris-HCl buffer, pH 9.0. Then, an aqueous two-phase system was assembled for 40.0 g total mass in its optimized condition. The upper phase was collected and characterization assays performed. These procedures were performed at least in triplicate.

### 4.2.7. Equations of Response Variables

The analyses of results were measured by specific activity, purification factor, recovery and yield. Further, for ATPS systems, partition coefficient of proteases and proteins were calculated.

Specific activity ($U.mg^{-1}$) was determined as the ratio of proteolytic activity (A), $U.mL^{-1}$, to total protein concentration (C), $mg.mL^{-1}$, in sample (Equation 1):

$$SA = \frac{A}{C} \tag{1}$$

Purification factor was calculated as the ratio of specific activity in final/top phase ($SA_T$) to specific activity in the enzyme extract ($SA_{EX}$) (Equation 2):

$$PF = \frac{[SA]_T}{[SA]_{EX}} \tag{2}$$

Recovery was estimated as the ratio of proteolytic activity of recovered proteases ($A_R$) to protease activity in enzyme extract ($A_{EX}$) (Equation 3):

$$R\ (\%) = \frac{A_R}{A_{EX}}\ x\ 100 \tag{3}$$

Yield was defined as the ratio of total protein concentration of recovered proteins ($C_R$) to total protein in the enzyme extract ($C_{Ex}$) (Equation 4):

$$Y\ (\%) = \frac{C_R}{C_{EX}}\ x\ 100 \tag{4}$$

The extraction yield for ATPS was determined by the following Equation 5, in which $V_x$ is the volume of the phase where proteins are extracted (top or bottom), $C_x$ is the protein concentration in phase where proteins are extracted. Further, $V_{TO}$ and $C_{TO}$ are the total volume and total protein concentration of the system, respectively:

$$n\ (\%) = \frac{100}{\left(\frac{V_x\ x\ C_x}{V_{TO}\ x\ C_{TO}}\right)} \tag{5}$$

The protease partition coefficient was measured as the ratio of protease activity in PEG-rich top phase ($A_T$) to bottom phase ($A_B$) (Equation 6):

$$Log\ k_a = \frac{A_T}{A_B} \tag{6}$$

Partition coefficient of proteins was estimated as the ratio of protein concentration in top phase ($C_T$) to bottom phase ($C_B$) (Equation 7):

$$Log\ k_p = \frac{C_T}{C_B} \quad (7)$$

**4.2.8. Biochemical Characterization**

The biochemical characterization was performed using enzyme extract and purified protease, according to each assay. Protease was purified by ammonium sulphate precipitation at 60% saturation, followed by optimized ATPS. Graphs were plotted using Microcall Origin 8.0 (Origin LAB, USA).

*4.2.8.1. Effect of pH and Stability on Proteolytic Activity*

In order to define the optimum pH of enzyme extract and purified protease, the proteolytic activity was assayed by using citrate-phosphate (pH 3.4; 4.2; 5.0; 5.8), sodium phosphate (pH 5.8; 6.6; 7.4), Tris-HCl (pH 7.4; 8.2; 9.0), carbonate-bicarbonate (9.8; 10.6), phosphate-NaOH (pH 11.0) and KCl-NaOH (pH 12.0; 13.0) buffers at 100 mM of different pH values containing 1% of azocasein. The enzyme was incubated in these solutions and proteolytic activity was determined similarly as described in section 2.3, and defined in percentage of relative activity. For pH stability study, purified protease was submitted in same buffers 1:1 (v/v) at 40 °C for 6 h, and samples were collected at the beginning and after 20, 40, 60, 120, 180, 240, 300, 360 min. Then, enzyme assay under standard conditions was performed and residual proteolytic activity was expressed as percentage of the initial activity adopted as 100% (27).

*4.2.8.2. Effect of Temperature and Stability on Proteolytic Activity*

The optimum temperature was determined by conducting enzyme extract and purified protease to proteolytic activity at several temperatures (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 e 80 °C) utilizing azocasein 1% in 100 mM Tris-HCl, at pH 9.0. The protease activity was expressed in percentage of relative activity. The thermo stability was performed incubating purified protease in 100 mM Tris-HCl buffer, pH 9.0, 1:1 (v/v) at different temperatures from 1-80 °C for 6 h. Aliquots were withdrawn at intervals of 0, 20, 40, 60, 120, 180, 240, 300 and 360 min and then residual proteolytic activity was carried out under standard conditions (28).

*4.2.8.3. Effect of Metal Ions*

On purpose to characterize the purified protease in the presence of chemical agents, identifying potential inhibitors or activators, divalent cationic metal ions were tested: $MgCl_2$, $CaCl_2$, $MnCl_2$, $CuCl_2$, $CoCl_2$, and $NiCl_2$ at 5 mM and 10 mM concentrations. They were incubated in substrate, azocasein 1%, containing 100 mM Tris-HCl buffer, pH 9.0. The effect

on the enzyme activity was measured using standard assay protocol at 40 °C and expressed in percentage of relative activity (29). Relative activity was expressed in percentages of activity detected with respect to the maximum protease activity.

### 4.2.9. Kinetic Parameters

Purified protease was assayed in different azocasein concentrations (0.003-0.62%) in 100 mM Tris-HCl buffer, pH 9. Reactions were incubated at 40 °C during 1 minute. The Michaelis constant ($K_m$) and the maximum rate ($V_{max}$) were determined by Lineweaver-Burk plot.

## 4.3. Results and Discussion

### 4.3.1. Ethanol and Ammonium Sulphate Precipitations

The initial purification procedure accomplished in this work were ethanol and ammonium sulphate precipitations. As can be seen, the majority of proteases were recovered in 60 and 80% of concentrations in ethanol precipitation, 48.83 and 41.05%, respectively (Table 4-3). However, specific activity and, subsequently, purification factor were superior in the last step, 174.19 $U.mg^{-1}$ and 15.05-fold, respectively. Further, total proteins tended to precipitate at first stage - 20%. This study is consistent with others, which precipitated tomato proteases in a 0-33% fraction of ethanol volume and reached 31.60% of activity recovery and 9.80-fold of purification factor (30). A cysteine protease from pear fruit *Pyrus pyrifolia* was purified in 60 and 80% of ethanol, whereas 60% saturation presented better results with 1.78-fold for purification factor and 55.60% on recovery (31). These comparisons evidence that specific activity and purification factor attained here were higher and technique was efficient.

Table 4-3: Protease purification by ethanol precipitation.

| **Ethanol Concentration (%)** | **Specific Activity ($U.mg^{-1}$)** | **Purification Factor** | **Recovery (%)** | **Yield (%)** |
|---|---|---|---|---|
| **0** | 11.58 ± 1.47 | - | - | - |
| **20** | 3.19 ± 0.07 | 0.28 | 13.79 | 50.01 |
| **40** | 12.87 ± 0.46 | 1.11 | 13.76 | 12.38 |
| **60** | 63.99 ± 12.72 | 5.53 | 48.83 | 8.83 |
| **80** | 174.19 ± 40.67 | 15.05 | 41.05 | 2.73 |

For ammonium sulphate precipitation, proteolytic enzymes were substantially precipitated at 60% of saturation (46.45%). At this stage, specific activity and purification factor

were higher, which showed 13.13 U.mg$^{-1}$ and 2.07-fold, correspondently. Nevertheless, 22.38% of contaminating proteins were precipitated at these conditions (Table 4-4). These findings are comparable to protease purification studies from *Bacillus subtilis* KT004404, which found out 2.62-fold and 19.22 U.mg$^{-1}$ for purification factor and specific activity, respectively, at 60% concentration of ammonium sulphate (32). Proteases from fish viscera of catla also precipitated at 60% saturation and specific activity achieved 8.32 U.mg$^{-1}$ (33).

Table 4-4: Protease purification by ammonium sulfate precipitation.

| **$(NH_4)_2SO_4$ Saturation (%)** | **Specific Activity (U.mg$^{-1}$)** | **Purification Factor** | **Recovery (%)** | **Yield (%)** |
|---|---|---|---|---|
| **0** | 6.33 ± 1.66 | - | - | - |
| **20** | 1.19 ± 0.90 | 0.19 | 5.60 | 29.70 |
| **40** | 10.32 ± 2.77 | 1.63 | 28.52 | 17.48 |
| **60** | 13.13 ± 3.82 | 2.07 | 46.45 | 22.38 |
| **80** | 6.23 ± 1.70 | 0.98 | 19.18 | 19.49 |

Both precipitation techniques provided significant results, highlighting to 80 and 60% saturation of ethanol and ammonium sulphate, respectively. The advantages of ethanol as precipitant are total miscibility in water, high volatility and low toxicity (31). However, ammonium sulphate precipitation does not heating the solution and non-denaturing proteins, in addition to high solubility of the salt that makes recovery of precipitants easily.

### 4.3.2. Aqueous Two-Phase Systems Designs

*4.3.2.1. Evaluation of factors on protease extraction using $2^4$ Factorial Design*

The partitioning of protease from *Aspergillus terreus* VSP-22 in PEG/ammonium sulfate aqueous two-phase system was investigated at room temperature in 100 mM Tris-HCl buffer. Optimum pH of enzyme extract was performed before to know which buffer it would be used. Protease partitioned preferentially to the PEG-enriched phase, where log $k_a$ is positive. The enzyme preferentially partitioned top phase due to a salting out effect, in view of the lower protein solubility in salt-rich bottom phase, resulting in a partitioning towards polymer-rich top phase (34). Or its hydrophobicity, on account of predominance of PEG, demonstrating the higher affinity of protease for a less polar environment (12). Similar partition behaviors were reported from *Serratia marcescens* P3 keratinolytic protease using PEG-salts ATPSs (11) and *Penicillium fellutanum* protease adopting PEG/NaPA/NaCl ATPS (19).

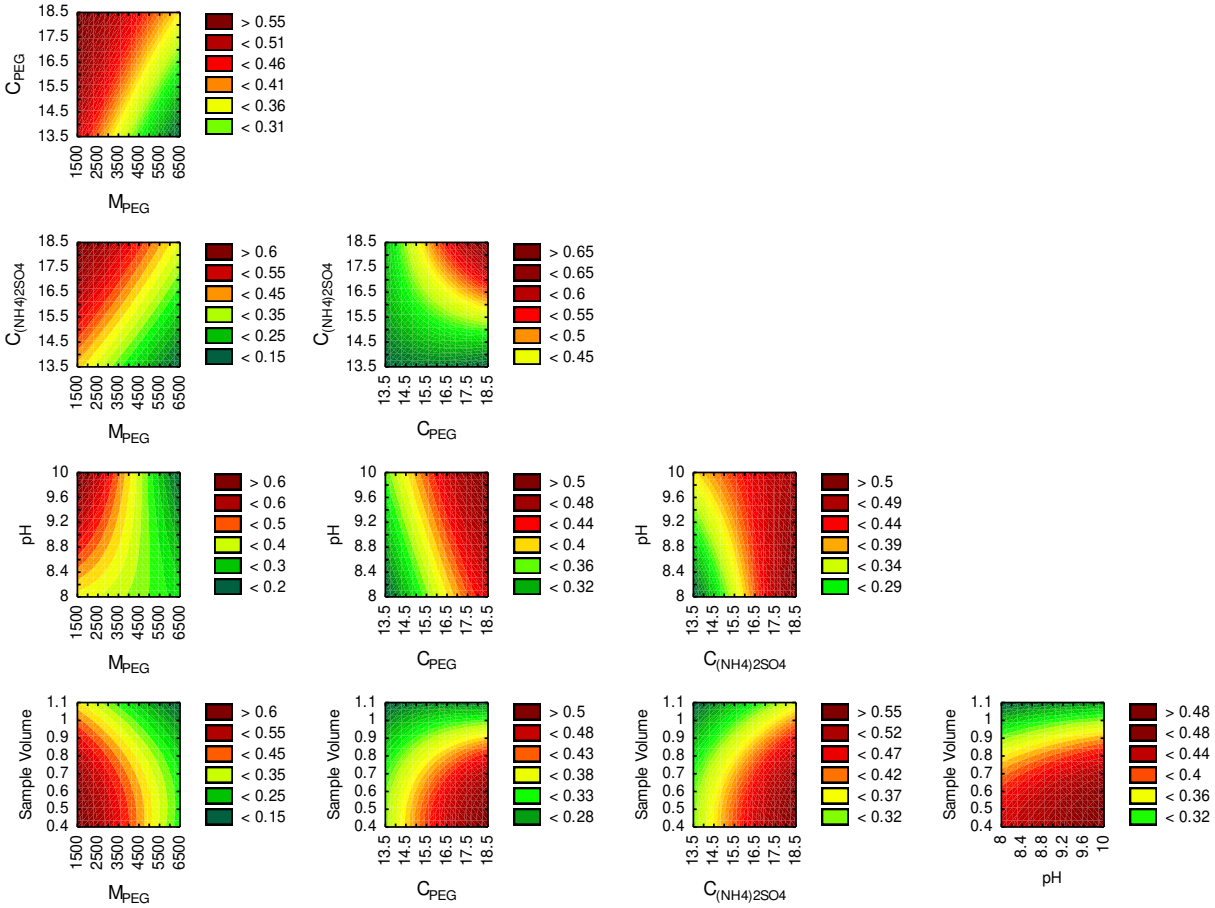
Statistical analyses from pure error at 95% of confidence level revealed the effects of independent variables on responses (supplementary file). For log $k_a$, the positive effect of salt concentration and negative effect of PEG molar mass demonstrated significance for design that were able to be observed in 7, 15 and 13 runs with 0.86, 0.77 and 0.73, respectively (supplementary file). Log $k_a$ ranged from 0.86 to 0.06 when the PEG molar mass increased from 2000 to 6000 g/mol. These results revealed that larger polymer size displays a repulsive effect on partition of protease, conducting to a reduction in the coefficient partition. In agreement of the theory of volume exclusion, increases in PEG molar mass contributes to exclusion of high molecular weight molecules, such some proteins (19). This similar behavior was reported for protease recovery from *Aspergillus tamarii* URM4634 with PEG/citrate, in that a decrease in $M_{PEG}$ from 8000 to 400 g/mol leads to a k increases from 1.18 to 2.61 (34). Furthermore, the decrease in $M_{PEG}$ and an increase of $C_{(NH4)2SO4}$ from 14 to 18%, suggesting the simultaneous occurrence of exclusion volume and salting-out effects (15).

For specific activity, only the sample volume exhibited significant negative effect, where decrease in the sample volume represents a higher response on the dependent variable. The better results showed 87.36 and 85.80 $U.mg^{-1}$ for 15 and 9 runs, containing the minimum of sample volume. For purification factor, sample volume and interaction between PEG and salt were significant, as negative and positive effect, respectively. The same 15 and 9 systems presented the higher purification factors, 4.65 and 4.09-fold, correspondently, and in both cases PEG molar mass 2000 (g/mol) were utilized. These purification factors are consistent with other works in the literature that reported purification of proteins from biological extracts, as in the case of a fibrinolytic protease from *Streptomyces* sp. DPUA1576 (1.51-fold) utilizing a PEG/phosphate (15) and a bromelain from pineapple peel (2.23-fold) applying PEG/magnesium sulphate system (35).

As can be noticed, recovery response presented sample volume and salt concentration as positive effects and, PEG molar mass as negative. The highlighted values were 88.75 and 86.62% in 13 and 7 runs, respectively, that were composed for maximum sample volume and salt concentration, further the presence of PEG molar mass 2000 (g/mol) in accordance of analysis. The great values founded for recovery indicate that PEG is an inert polymer which does not react with proteases, instead of its interaction observed with total proteins in the systems. Further, these results are comparable with other studies as recovery of phytase (115.24%) from *Absidia blakesleeana* URM5604 using PEG/citrate ATPS (36) and protease recovery (76.60%) from *Calotropis procera* latex employing PEG/magnesium

sulphate (37). As biotechnological purification processes a final activity yield of 80% are able to be considered applicable (38), which means protease from *Aspergillus terreus* VSP-22 is a potential application for industry.

Figure 4-1: Desirability surface of $2^4$ factorial design of ATPS of protease from *Aspergillus terreus* VSP-22.




The increase on sample volume, which had positive effect on recovery, tends to increase protease recovery in upper phase. However, a higher volume used in the system leads to decrease on log $k_p$, specific activity and purification factor, because its negative effect on these responses. In this way, a decision about quantity of sample volume was suitable for next design, which depends on protease applicability. The curvature method is not appropriate for optimization in this study, because each one of response variable reveals an opposite direction, as in recovery. Hence, a desirability tool had to be used as a combination of dependent variable, which combines every variable into one response. The desirability function jointly of response surface methodology (RSM) establish the desirability optimization methodology (DOM) (23). Figure 4-1 illustrates the findings of the desirability surface of $2^4$ factorial design.

Inspection on Figure 4-1 indicates that pH does not affect the system, because its relationship between other variables had not significant responses and was generally linear. Similar behavior was observed in partitioning of protease from *Aspergillus tamarii* URM4634 using PEG/citrate ATPS, which pH was not statistically significant (34). For sample volume, minimum values showed better responses for all independent variables compared. About PEG molar mass, it also displayed good responses in minimum for PEG 2000 (g/mol). It is evidenced that PEG and salt concentrations demonstrated better regions on maximum values on graphs. This indicates $C_{PEG}$ x $C_{(NH4)2SO4}$ graph had major impact with isolated responses and these concentrations could be modified and investigated in order to find an optimized system.

These imply the new variable values for a central composite design which PEG molar mass, sample volume e pH were fixed in their optimized responses: PEG 2000 (g/mol), 0.5 mL and optimum pH 9.0, respectively. The pH was established as the optimum pH of enzyme extract of protease, previously determined. Ammonium sulphate and polyethylene glycol concentrations were independent variables and had their values defined as shown on Table 4-2.

In this work, it was suitable to carry out factorial design following by a central composite design. The common procedure is stepwise refinement, in order to find out the better conditions before define levels for central composite design, and it was not performed in this study due to the solubility of ammonium sulphate. Exceeded increases in salt concentrations could affect the system, shifting binodal curves. These means, that the salt used in aqueous two-phase system has a limit of solubilization in pure water, and when salt is in presence of buffer and PEG the solubility is changed as well.

*4.3.2.2. Optimization by Central Composite Design*

In this way, in order to investigate in detail the effect of PEG and $(NH_4)_2SO_4$ concentrations on partitioning of protease in ATPS, a central composite design was performed. Highlighted values were in center points, which showed a mean of 116.08 $U.mg^{-1}$ for specific activity, a purification factor of 4.88-fold, recovery of 67.87% and 1.09 for log $k_a$. The partitioning can be influenced by presence of salts that weakens or strengthens interactions, or can interact with ionized groups with net charge opposed of proteins (16). Purification factor and log $k_a$ were higher than those obtained at the beginning of this work, where a preliminary factorial design was used. Purification factor raised from 4.65 to 5.16-fold and log $k_a$ increased from 0.86 to 1.19.

The results of recovery were submitted to the analysis of variance (ANOVA) and revealed that the regression was significant and lack of fit was not statistically significant. The model adjustment was measured by the coefficient of determination, $R^2$ and $R^2_{adj}$, and according to Equation 8, recovery followed a second polynomial model, excluding the linear concentration of salt. ANOVA demonstrated that coefficient of determination ($R^2 = 0.7236$) was satisfactory, which mean 72.36% of the total variation in recovery were explained by adjusted model. Further, the Fisher-based test suggested the significance of Equation 8 ($F_{4,7} = 4.5811 > F_{tab}$) and agreement of the lack of fit ($F_{cal} < F_{tab}$) between predicted model and the experimental values. The response surface methodology for recovery on adjusted model is shown on Figure 4-2.

$$R = - 41.3830 + 12.1074\,(x) - 0.3898\,(x^2) - 0.0508\,(y^2) + 0.0972\,(xy) \quad (8)$$

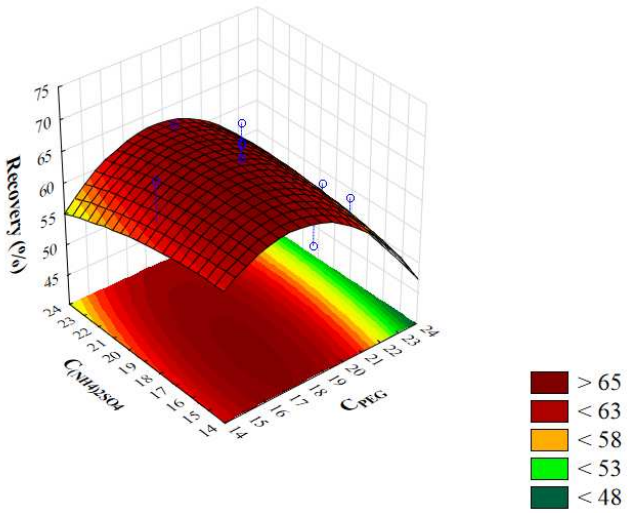
Table 4-5: Analysis of variance (ANOVA) of protease recovery in central composite design.

| Factor | SS[a] | df[b] | MS[c] | F-value | p-value |
|---|---|---|---|---|---|
| **$C_{PEG}$ (L)** | 53.2286 | 1 | 53.2286 | 10.6845 | 0.046822 |
| **$C_{PEG}$ (Q)** | 118.7546 | 1 | 118.7546 | 23.8375 | 0.016428 |
| **$C_{(NH4)2SO4}$ (Q)** | 126.8813 | 1 | 126.8813 | 25.4688 | 0.015005 |
| **$C_{(NH4)2SO4}$ x $C_{PEG}$** | 21.5631 | 1 | 21.5631 | 4.3283 | 0.128945 |
| **Lack of Fit** | 91.8304 | 4 | 22.9576 | 4.6083 | 0.119922 |
| **Pure Error** | 14.9455 | 3 | 4.9818 | | |
| **Total SS** | 386.2923 | 11 | | | |

[a] sum of square; [b] degree of freedom; [c] mean of square.

In order to find the best concentrations of PEG and ammonium sulphate a desirability optimization methodology was performed. The investigation on recovery response had aimed to increase percentages of this variable and continue with good purification factors. The desirability analysis of the global system was done through the significance and weights of responses, utilizing the coefficient of determination ($R^2$). Based on standard magnitude it was possible to obtain 19.95% (w/w) of $C_{PEG}$ and 19.95% (w/w) of $C_{(NH4)2SO4}$. The reduction in PEG concentration provides a decrease in the excluded volume effect, leading proteins to be soluble in the PEG-rich top phase (23). Thus, conditions of the optimized system were achieved as PEG molar mass 2000 (g/mol), 19.95% (w/w) of $C_{PEG}$, 19.95% (w/w) of $C_{(NH4)2SO4}$, 0.5 mL of sample volume and pH 9.0.

Figure 4-2: Three-dimensional contour plot showing the interactive effects of concentrations of PEG ($C_{PEG}$) and ammonium sulphate ($C_{(NH4)2SO4}$) on the activity recovery on adjusted model.




*4.3.2.3. Validation and Scale-up*

The results confirmed a similarity between predict and experimental data. The model was validated using PEG molar mass 2000 (g/mol), 19.95% (w/w) of $C_{PEG}$, 19.95% (w/w) of $C_{(NH4)2SO4}$, 0.5 mL of sample volume and 100 mM Tris-HCl buffer, pH 9.0. Purification factor defined the validation of the model with its 5.23-fold in experimental values and 4.79-fold in predicted (Table 4-6). It was possible to increase purification factor by 1.12 times in optimal conditions of 5.0 g system when compared to the best value obtained in $2^4$ factorial design (4.65-fold).

Table 4-6: Validation of the optimized condition and observed values on scale-up of ATPS.

| **Total mass of the system (g)** | **Results** | | | | | |
|---|---|---|---|---|---|---|
| | **SA**[a] | **PF**[b] | **R**[c] | ***n***[d] | **Log $k_a$** | **Log $k_p$** |
| **Adjusted Model** | 111.83 | 4.79 | 63.49 | 57.09 | 1.30 | 2.96 |
| **5** | 97.17 ± 8.00 | 5.23 | 60.21 | 40.38 | 1.06 | 2.70 |
| **40** | 109.71 ± 7.17 | 5.00 | 69.40 | 52.54 | 1.22 | 2.78 |
| **320** | 109.87 ± 18.41 | 5.41 | 70.60 | 50.20 | 1.36 | 2.78 |

[a] Specific Activity ($U.mg^{-1}$); [b] Purification factor; [c] Recovery (%); [d] Yield of extraction (%).

Scaling up the system demonstrated the proximity of experimental to predicted responses. In respect with 40.0 g system, it had better results in almost of all variables when compared with 5.0 g standard system, which showed a specific activity of 109.71 $U.mg^{-1}$, recovery of 69.40%, yield of 52.54% and 1.22 of log $k_a$. When 40.0 g system was compared with adjusted model, it indicated that purification factor (5.00-fold) and recovery had greater

results. Concerning to 320.0 g system, it displayed the superior results when compared with 5.0 g and 40.0 g systems and also with adjusted model, highlighting to a purification factor of 5.21-fold, recovery of 70.60% and log $k_a$ of 1.36.

This appears to be the first work where ATPSs were selected to recover a protease from *Aspergillus terreus*. Furthermore, results of optimization and scale-up indicate that these approaches were successfully conducted with PEG/ammonium sulphate system.

As a concluding remark, protease was carried out in subsequent steps using 60% saturation of $(NH_4)_2SO_4$ precipitation followed by optimized aqueous two-phase system. Both techniques were simple, low cost and reproducible that made possible to improve specific activity and purification factor when compared to the methodologies employed singly. The use of $(NH_4)_2SO_4$ salt on integration of these techniques facilitated the experiments runs. Table 4-7 summarizes purification techniques used in this study. Recovery had decreased after two procedures, what could be explained due to increased protease loss with additional step of purification. However, purification factor increases 2.92 times from ammonium sulphate precipitation to salt precipitation followed by optimized ATPS. Further, specific activity raised 1.53 times from optimized ATPS to ammonium sulphate precipitation conjugated with ATPS.

Table 4-7: Summary of the purification steps of protease from *Aspergillus terreus* VSP-22.

| Purification Method | PA[a] | TP[b] | SA[c] | PF[d] | R[e] |
|---|---|---|---|---|---|
| **Enzyme Extract** | 14.10 ± 0.02 | 0.58 ± 0.11 | - | - | - |
| **Ethanol Precipitation (80%)** | 4.73 ± 1.00 | 0.07 ± 0.03 | 174.19 ± 40.67 | 15.05 | 41.05 |
| **$(NH_4)_2SO_4$ Precipitation (60%)** | 1.33 ± 0.19 | 0,10 ± 0.02 | 13.13 ± 3.82 | 2.07 | 46.45 |
| **Optimized ATPS** | 6.89 ± 0.29 | 0.07 ± 0.005 | 97.17 ± 8.00 | 5.23 | 60.21 |
| **$(NH_4)_2SO_4$ Precipitation (60%) and Optimized ATPS** | 7.82 ± 0.005 | 0.05 ± 0,02 | 149.23 | 6.06 | 39.39 |

[a] Protease Activity ($U.mL^{-1}$); [b] Total Protein ($mg.mL^{-1}$); [c] Specific Activity ($U.mg^{-1}$); [d] Purification factor; [e] Recovery (%).

Recuperation and purification of proteases are usually performed in several stages, where commonly expensive and time-consuming techniques are used. Purifying protease from *Bacillus subtilis* KT004404 by ammonium sulphate precipitation followed by chromatography step reached a purification factor of 7.53-fold and recovery of 25.26% (32). Moreover, purification of two acid proteases from *Aspergillus hennebergii* HX08 by ammonium sulphate

precipitation and two chromatography stages attained 94.90 and 103.67 $U.mL^{-1}$ of specific activity of each enzyme (39). An alkaline protease from *Aspergillus terreus* was purified by ammonium sulphate precipitation accompanied for chromatography technique, which achieved 7.50-fold in purification factor and 23% on recovery (40). In addition, a serine peptidase from *Aspergillus terreus* was precipitated by ethanol and submitted to two chromatography steps that showed only 15.50% on recovery and 1.20-fold on purification factor (41). Therefore, it should be concluded that protease from *Aspergillus terreus* VSP-22 was successfully purified by two steps: ammonium sulphate precipitation and optimized ATPS, obtaining satisfactory activity recoveries and purification factors with simpler and cheaper methodologies.

### 4.3.3. Protease Characterization

*4.3.3.1. Effect of pH and Stability on Proteolytic Activity*

The optimum pH (5.0-13.0) of enzyme extract and purified enzyme was determined at 37 and 40 °C, respectively. The enzyme from crude enzymatic extract showed activity over a broad range pH and its maximum value was at pH 9.0. As can be noticed, the protease was stable when incubated from acid to alkali solutions, showing activity above 80% in the majority of them. The relative activity of purified protease demonstrated a maximum pH at 9.8, while the all other pH's were active above 80% (Fig. 4-3A). Further, the purified enzyme exhibited highlighted activities with 94, 96, 97, 95 and 99% from pH 6.6 to 10.6. These indicate a high activity from a range of pH (6.6-10.6) which suggests the fact that enzyme is an alkaline protease (Fig. 4-3B). These results are consistent with researches of proteases, which founded optimum pH in the range of 9.0-11.0 from *Aspergillus flavus* and pH 8.0 from *Aspergillus terreus* (42). The serine protease from *Bacillus subtilis* DR8806 was also active at a wide range of pH, which had pH 8.0 as the maximum value (4).

Protease stability at different pH values (3.4-13.0) was performed at 40 °C after 20, 40, 60, 120, 180, 240, 300 and 360 minutes. As shown in Figure 4-4A, the protease was stable in a pH range 3.4 to 5.8 after 60 min of incubation, retaining about 72, 104, 93 and 96% of residual activity. In a different buffer, pH 5.8 in sodium phosphate, preservers 46% of activity after 60 minutes, and pH 6.6 around 30% in same conditions. Moreover, after 360 min of incubation the protease was retained 62, 66 and 60% in a pH range 4.2, 5.0 and 5.8, respectively (supplementary file); all of them composed with citrate-phosphate buffer. Proteases in alkali pH did not demonstrate stability what is possible to infer that enzymes in their maximum activity had consumed the entire azocasein substrate and started enzyme autolysis, occurring auto- and heterolytic fragmentation of these enzymes (31).

Figure 4-3: Optimum pH of enzyme extract (A) and protease previously purified (B). In figure A, pH 5.8 brings citrate-phosphate and pH 7.4 evidences sodium phosphate buffers, both had superior values when compared with same pH in different buffer. In figure B, pH 5.8 and 7.4 illustrated sodium phosphate and Tris-HCl buffers, respectively. Relative activity was expressed in percentages of activity detected with respect to the maximum protease activity.

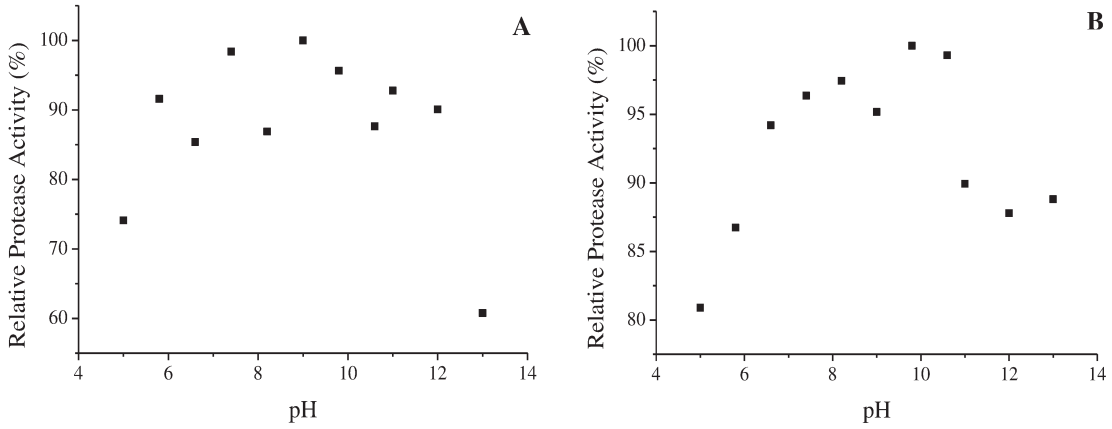
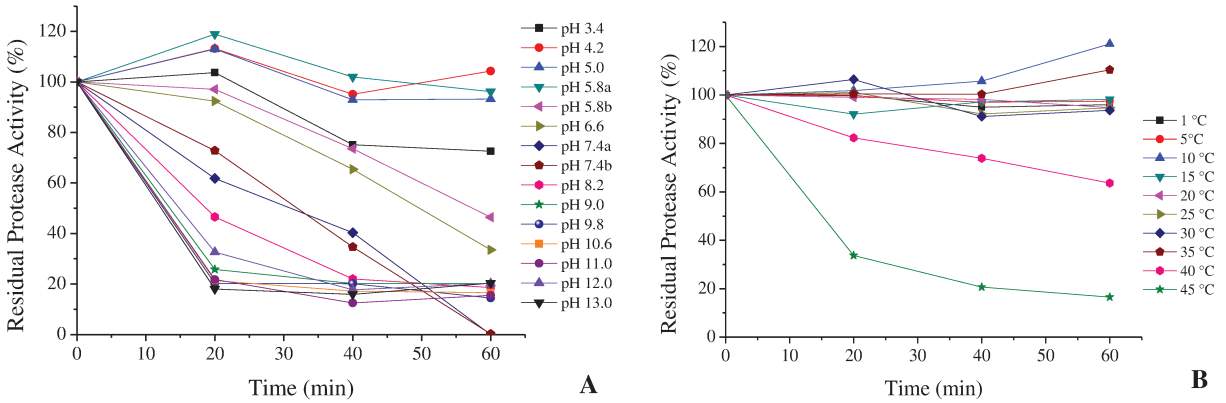



Figure 4-4: PH (A) and thermo (B) stability of purified protease from *Aspergillus terreus* VSP-22. In figure A, 5.8a e 5.8b define the activity of citrate-phosphate and sodium phosphate buffers, respectively. 7.4a and 7.4b are the activities of sodium phosphate and Tris-HCl buffer, correspondently. Residual activity was defined as the percentages of activity compared with standard assay of proteolytic activity.



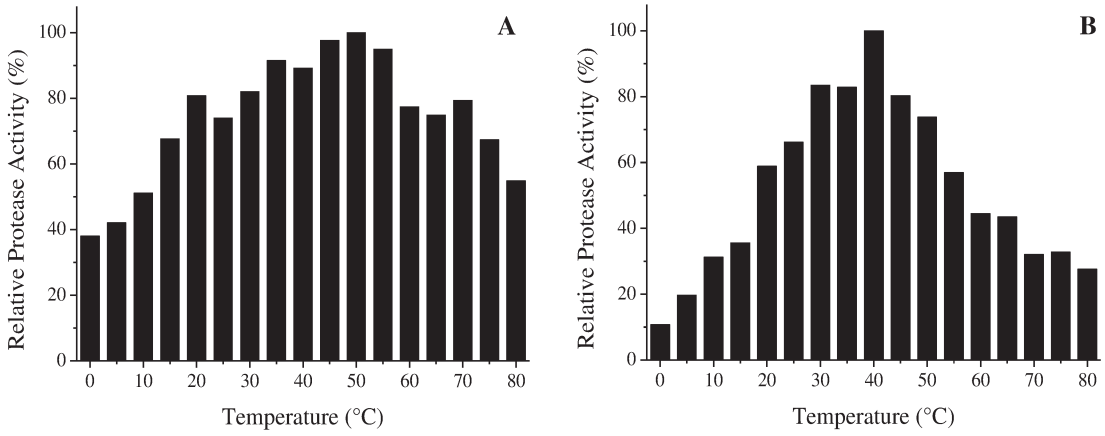

This study differs from researches that has been reported similar behavior in optimum pH and its stability, such as a thermostable serine alkaline protease from *Aeribacillus pallidus* C10 that preserved its activity more than 70% at pH 6.0 to 10.5 after 2 h of incubation at room temperature and founded optimum pH at range of pH 7.0-10.0 (27). A metallo-neutral protease from *Bacillus amyloliquefaciens* SYB-001 described an optimum pH and stability at

pH 7.0 and 6.0-7.0, respectively (29). A bacterium *Salinivibrio* sp. also reached at pH 8.0 a maximum activity and showed stability in a pH range of 5.0-10.0 for 1 h (28).

*4.3.3.2. Effect of Temperature and Stability on Proteolytic Activity*

The effect of temperature on crude enzymatic extract and purified enzyme was examined at different temperatures. The protease from enzyme extract exhibited maximum activity at a temperature of 50 °C, at pH 9.0. It was ascertained that protease was active at a broad temperature range, from 35 to 55 °C, and that it had 91, 89, 97 and 94% of activity. The enzymes showed 22 and 45% activity reductions at temperatures of 60 and 80 °C, respectively (Fig. 4-5A). For purified protease, the optimum temperature was at 40 °C, at pH 9.0. The activities reductions were higher when compared with enzyme extract, obtaining 55 and 68% of reductions at 60 and 70 °C, respectively, and 27% of activity remained at 80 °C (Fig. 4-5B). Therefore, it can be noticed that purified enzyme was more susceptible at temperature than protease from enzyme extract. The optimal temperature is comparable of purified proteases reported previously from *Penicillium italicum*, which showed a maximum at 50 °C and 85% of activity at 40 °C (20). An alkaline serine protease from fungus *Myceliophthora* sp. revealed an optimum at 40-45 °C (43). Other similar findings at 50 °C were from *Aspergillus oryzae* CH93 (44), *Bacillus subtilis* BUU1 (45) and *Alkalibacillus* sp. NM-Fa4 (5).

Figure 4-5: Optimum temperature with enzyme extract (A) and protease previously purified (B). Relative activity was expressed in percentages of activity detected with respect to the maximum protease activity.




The thermal stability was determined by performing an activity assay at 1-80 °C temperature range at 20, 40, 60, 120, 180, 240, 300 and 360 minutes. The Figure 4-4B shows the stability of purified protease from *Aspergillus terreus* VSP-22 over 60 minutes. The protease retained almost of whole initial activity (90%) after 60 min of incubation at 1-35 °C, and it had

96, 97, 121, 98, 94, 94, 93 and 110%, accordingly. Activity values exceeding 100% are explained by the fact that additional surface charges, extensive ionic networks or random genetic drift might provide extra stability of proteases (32). Following 360 min of incubation, 90% of the initial activity was maintained at 5-25 °C (supplementary file). Moreover, after 20 min of incubation at 40 and 45 °C, the enzyme had started to inactive, retaining 82 and 33% of its activity, respectively. After 60 minutes of incubation, these temperatures preserved only 63 and 16% of its activity. Purified enzyme submitted at temperatures above 45 °C were promptly inactivated in first 20 minutes, suggesting this protease is not stable at high temperatures.

As soon, the protease could be a potential enzyme for mashing process of beer brewing, where subsequent stage needs the inactivation of the enzyme, which would be lost under high temperature (29). Furthermore, the protease of this work demonstrated an advantage over the majority of cold-active alkaline proteases, which does not attend industrial requirements because they are not stable at temperatures above 20 °C and present low recoveries of target product at large-scale production (27). Thus, these data suggest a promising applicability on industry from *Aspergillus terreus* VSP-22 protease.

*4.3.3.3. Effect of Metal Ions*

Chloride salts of various metal ions, 5 and 10 mM, had their effect investigated under enzymatic activities and are illustrated in Table 4-8. The relative activity enhanced when purified protease was incubated with 5 mM metallic ions containing $Mn^{+2}$ and $Ca^{+2}$, 102 and 101%, respectively. Although other metal ions generated a decrease on relative activity. Concerning to a high level of metallic ions, 10 mM, all of them caused a slight reduction on activity when compared with control and at low concentration, 5 mM. $Mg^{+2}$, $Mn^{+2}$ and $Ca^{+2}$ had their activities preserved above 90% at 10 mM. These results means that most of metal ions did not improve activity of protease from *Aspergillus terreus* VSP-22 and suggest that enzyme is not metal-dependent.

Metal ions have varying effects on the activity of each enzyme, however proteases slightly enhanced in presence of $Ca^{+2}$ and $Mn^{+2}$, which perform a stabilizing function in ternary structures of enzymes (9) and prevent them from autolysis, which postulate these proteases as candidate for detergent formulations (46). It was reported in the literature at 5 mM that $Ca^{+2}$ and $Mn^{+2}$ ions had been improved protease activity, besides $K^{+}$ (47). A metallo-neutral protease showed stimulations with $Mn^{+2}$, $Mg^{+2}$, $Ca^{+2}$ and $Li^{+2}$ in both 5 and 10 mM concentrations (29); and $Ca^{+2}$, $Mg^{+2}$ and $Mn^{+2}$ ions had also been activated the protease at 2 mM (48). Although,

$Co^{+2}$, $Cu^{+2}$ and $Ni^{+2}$ metal ions had been reported to inhibit activity of proteases (49; 40; 50; 51).

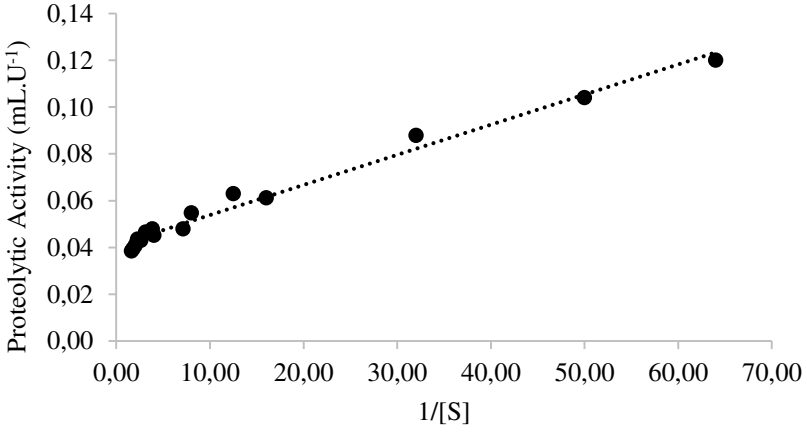
Table 4-8: Effect of metal ions on purified enzyme partitioned to the PEG-rich upper phase of PEG 2000/ammonium sulfate ATPS.

| **Metal Ion** | **Concentration** | |
|---|---|---|
| | **5.0 mM** | **10.0 mM** |
| **Control** | 100.0 | 100.0 |
| **$Mg^{+2}$** | 92.73 ± 0.0090 | 90.24 ± 0.0047 |
| **$Cu^{+2}$** | 76.09 ± 0.0042 | 73.41 ± 0.0043 |
| **$Ni^{+2}$** | 68.36 ± 0.0027 | -* |
| **$Co^{+2}$** | 74.67 ± 0.0022 | 49.66 ± 0.0085 |
| **$Mn^{+2}$** | 102.45 ± 0.0066 | 90.74 ± 0.0044 |
| **$Ca^{+2}$** | 101.61 ± 0.0057 | 93.04 ± 0.0070 |

*The effect of metal ion $Ni^{+2}$ was not possible to measure due to precipitation of azocasein in a concentrated ion solution.

### 4.3.4. Kinetic Parameters

Figure 4-6: Lineweaver-Burk plot of purified protease from *Aspergillus terreus* VSP-22.




The reaction kinetics of protease previously purified was determined from Lineweaver-Burk plot (Figure 4-6). Parameters achieved $V_{max}$ and $K_m$ ($R^2 = 0.9830$) of 24.4499 $U.mL^{-1}$ and 0.0318 mM, respectively. It was possible to compare these results with those reported in the literature in view of the azocasein uses as substrates. Thereby, proteolytic enzymes from fungi species have been described $V_{max}$ and $K_m$, such as *Aspergillus foetidus* protease ($K_m$ = 1.92 $mg.mL^{-1}$ and $V_{max}$ = 357.14 $U.mL^{-1}$) (52), *Aspergillus oryzae* acid protease ($K_m$ = 0.96 $mg.mL^{-1}$ and $V_{max}$ = 135.14 $\mu mol.min^{-1}$ $mg^{-1}$) (53) and *Aspergillus niger* LBA02 protease ($K_m$ = 0.44 $mg.mL^{-1}$ and $V_{max}$ = 344.83 $U.g^{-1}$) (54). It could be concluded that protease

described in this work had a high affinity for azocasein substrate when compared to other studies, due to the low value obtained for $K_m$.

## 4.4. Conclusion

Results obtained in this study revealed that protease from *Aspergillus terreus* VSP-22 achieved satisfactory purification factors and recoveries by ammonium sulphate (PF = 2.07-fold and R = 46.45%) and ethanol (PF = 15.05-fold and R = 41.05%) precipitations, highlighting to aqueous two-phase system. The factorial and central composite ATPS designs provided an increase in these variables, enabling to find optimized PEG/ammonium sulphate system (19.95% (w/w) of PEG 2000 (g/mol), 19.95% of ammonium sulphate (w/w) at pH 9.0) with PF = 5.23-fold and R = 60.21%. Furthermore, scale-up of this optimized ATPS indicated the system provided reproducibility on evaluated parameters, such as 40.0 (PF = 5.00-fold and R = 69.40%) and 320.0 g (PF = 5.41-fold and R = 70.60%) of total mass. In addition, the proposed strategy of ammonium sulphate precipitation followed by optimized aqueous two-phase system attained outstanding results of purification factor (6.06-fold), exhibiting low cost and simplicity for its execution, what it is highly interesting for application in downstream processes.

With respect to biochemical characterization, it provided valuable information concerning to the mechanism of catalysis reactions, which suggested that enzyme is an alkaline protease, finding an optimum pH of 9.0 for enzyme extract and 9.8 for purified protease. In evaluation of optimum temperature, enzyme showed better activity at 50 and 40 °C for enzyme extract and purified protease, respectively. Stability analyses demonstrated that proteolytic enzymes are stable in a range of pH and some temperatures (1 to 35 °C). Moreover, it was noticed that metal ions do not interfere on protease activity, indicating that enzyme is not metal-dependent. Kinetic parameters evidenced the high affinity of purified protease for azocasein, achieving $V_{max} = 24.4499$ U.mL$^{-1}$ and $K_m = 0.0318$ mM.

The findings in this work are consistent with other studies, which have been used methodologies in proteases purification and characterization. Therefore, the approach of this investigation focused in obtain an efficient, cost-effective and scalable strategy for downstream processes, which was succeed in ammonium sulphate precipitation followed by optimized aqueous two-phase system. Further, the protease characterization proved to has interesting properties, suggesting its potential application as food and detergents industries.

## 4.5. Acknowledgments

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## 4.7. Supplementary File

Table 4-9: Effect Estimates of Specific Activity in $2^4$ Factorial Design.

| Factor | Effect | Sdt. Err. Pure Err | t(3) | p | -95, % Cnf. Limt | +95, % Cnf. Limt | Coeff | Std. Err. Coeff | -95, % Cnf. Limt | +95, % Cnf. Limt |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mean/Interc.** | 55,9298 | 1,8978 | 29,4713 | 0,0001 | 49,8903 | 61,9694 | 55,9298 | 1,8978 | 49,8903 | 61,9694 |
| **(1)$M_{PEG}$** | -10,9373 | 4,2435 | -2,5774 | 0,0820 | -24,4422 | 2,5675 | -5,4687 | 2,1218 | -12,2211 | 1,2838 |
| **(2)$C_{PEG}$** | 0,6230 | 4,2435 | 0,1468 | 0,8926 | -12,8819 | 14,1278 | 0,3115 | 2,1218 | -6,4410 | 7,0639 |
| **(3)$C(NH_4)_2SO_4$** | 4,5129 | 4,2435 | 1,0635 | 0,3656 | -8,9920 | 18,0177 | 2,2564 | 2,1218 | -4,4960 | 9,0089 |
| **(4)pH** | 1,4354 | 4,2435 | 0,3382 | 0,7575 | -12,0695 | 14,9402 | 0,7177 | 2,1218 | -6,0348 | 7,4701 |
| **(5)Sample Volume** | -16,8423 | 4,2435 | -3,9689 | 0,0286 | -30,3472 | -3,3374 | -8,4212 | 2,1218 | -15,1736 | -1,6687 |
| **1 by 2** | 5,8800 | 4,2435 | 1,3856 | 0,2599 | -7,6249 | 19,3848 | 2,9400 | 2,1218 | -3,8124 | 9,6924 |
| **1 by 3** | -1,4435 | 4,2435 | -0,3402 | 0,7562 | -14,9484 | 12,0613 | -0,7218 | 2,1218 | -7,4742 | 6,0307 |
| **1 by 4** | -12,2036 | 4,2435 | -2,8758 | 0,0637 | -25,7085 | 1,3012 | -6,1018 | 2,1218 | -12,8543 | 0,6506 |
| **1 by 5** | 6,3746 | 4,2435 | 1,5022 | 0,2301 | -7,1302 | 19,8795 | 3,1873 | 2,1218 | -3,5651 | 9,9397 |
| **2 by 3** | 12,9431 | 4,2435 | 3,0501 | 0,0554 | -0,5618 | 26,4479 | 6,4715 | 2,1218 | -0,2809 | 13,2240 |
| **2 by 4** | 1,8648 | 4,2435 | 0,4395 | 0,6901 | -11,6400 | 15,3697 | 0,9324 | 2,1218 | -5,8200 | 7,6848 |
| **2 by 5** | -3,6127 | 4,2435 | -0,8513 | 0,4571 | -17,1176 | 9,8922 | -1,8063 | 2,1218 | -8,5588 | 4,9461 |
| **3 by 4** | -5,0448 | 4,2435 | -1,1888 | 0,3200 | -18,5497 | 8,4601 | -2,5224 | 2,1218 | -9,2748 | 4,2300 |
| **3 by 5** | -0,4223 | 4,2435 | -0,0995 | 0,9270 | -13,9272 | 13,0825 | -0,2112 | 2,1218 | -6,9636 | 6,5413 |
| **4 by 5** | -1,7263 | 4,2435 | -0,4068 | 0,7114 | -15,2311 | 11,7786 | -0,8631 | 2,1218 | -7,6156 | 5,8893 |

Table 4-10: Effect Estimates of Purification Factor in $2^4$ Factorial Design.

| Factor | Effect | Sdt. Err. Pure Err | t(3) | p | -95, % Cnf. Limt | +95, % Cnf. Limt | Coeff | Std. Err. Coeff | -95, % Cnf. Limt | +95, % Cnf. Limt |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mean/Interc.** | 2,9944 | 0,0905 | 33,0952 | 0,0001 | 2,7064 | 3,2823 | 2,9944 | 0,0905 | 2,7064 | 3,2823 |
| **(1)$M_{PEG}$** | -0,4619 | 0,2023 | -2,2832 | 0,1066 | -1,1058 | 0,1819 | -0,2310 | 0,1012 | -0,5529 | 0,0910 |
| **(2)$C_{PEG}$** | 0,0936 | 0,2023 | 0,4628 | 0,6750 | -0,5502 | 0,7375 | 0,0468 | 0,1012 | -0,2751 | 0,3687 |
| **(3)$C(NH_4)_2SO_4$** | 0,1585 | 0,2023 | 0,7834 | 0,4906 | -0,4854 | 0,8023 | 0,0792 | 0,1012 | -0,2427 | 0,4012 |
| **(4)pH** | -0,0756 | 0,2023 | -0,3736 | 0,7335 | -0,7194 | 0,5683 | -0,0378 | 0,1012 | -0,3597 | 0,2841 |
| **(5)Sample Volume** | -0,8929 | 0,2023 | -4,4136 | 0,0216 | -1,5368 | -0,2491 | -0,4465 | 0,1012 | -0,7684 | -0,1245 |
| **1 by 2** | 0,2198 | 0,2023 | 1,0864 | 0,3568 | -0,4241 | 0,8636 | 0,1099 | 0,1012 | -0,2120 | 0,4318 |
| **1 by 3** | -0,1955 | 0,2023 | -0,9662 | 0,4052 | -0,8393 | 0,4484 | -0,0977 | 0,1012 | -0,4197 | 0,2242 |
| **1 by 4** | -0,4173 | 0,2023 | -2,0628 | 0,1311 | -1,0612 | 0,2265 | -0,2087 | 0,1012 | -0,5306 | 0,1133 |
| **1 by 5** | 0,1934 | 0,2023 | 0,9561 | 0,4096 | -0,4504 | 0,8373 | 0,0967 | 0,1012 | -0,2252 | 0,4186 |
| **2 by 3** | 0,6939 | 0,2023 | 3,4297 | 0,0415 | 0,0500 | 1,3377 | 0,3469 | 0,1012 | 0,0250 | 0,6689 |
| **2 by 4** | 0,0469 | 0,2023 | 0,2318 | 0,8316 | -0,5970 | 0,6908 | 0,0235 | 0,1012 | -0,2985 | 0,3454 |
| **2 by 5** | -0,3025 | 0,2023 | -1,4954 | 0,2317 | -0,9464 | 0,3413 | -0,1513 | 0,1012 | -0,4732 | 0,1707 |
| **3 by 4** | -0,1105 | 0,2023 | -0,5463 | 0,6229 | -0,7544 | 0,5333 | -0,0553 | 0,1012 | -0,3772 | 0,2667 |
| **3 by 5** | -0,0960 | 0,2023 | -0,4746 | 0,6675 | -0,7399 | 0,5478 | -0,0480 | 0,1012 | -0,3699 | 0,2739 |
| **4 by 5** | -0,0630 | 0,2023 | -0,3114 | 0,7759 | -0,7068 | 0,5809 | -0,0315 | 0,1012 | -0,3534 | 0,2904 |

Table 4-11: Effect Estimates of Recovery in $2^4$ Factorial Design.

| Factor | Effect | Sdt. Err. Pure Err | t(3) | p | -95, % Cnf. Limt | +95, % Cnf. Limt | Coeff | Std. Err. Coeff | -95, % Cnf. Limt | +95, % Cnf. Limt |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mean/Interc.** | 58,0090 | 1,6571 | 35,0057 | 0,0001 | 52,7353 | 63,2827 | 58,0090 | 1,6571 | 52,7353 | 63,2827 |
| **(1)$M_{PEG}$** | -18,2582 | 3,7055 | -4,9274 | 0,0160 | -30,0506 | -6,4658 | -9,1291 | 1,8527 | -15,0253 | -3,2329 |
| **(2)$C_{PEG}$** | 1,3771 | 3,7055 | 0,3716 | 0,7349 | -10,4153 | 13,1695 | 0,6885 | 1,8527 | -5,2077 | 6,5847 |
| **(3)$C(NH_4)_2SO_4$** | 15,0668 | 3,7055 | 4,0661 | 0,0268 | 3,2744 | 26,8592 | 7,5334 | 1,8527 | 1,6372 | 13,4296 |
| **(4)pH** | 2,4777 | 3,7055 | 0,6687 | 0,5516 | -9,3147 | 14,2701 | 1,2388 | 1,8527 | -4,6574 | 7,1350 |
| **(5)Sample Volume** | 22,6565 | 3,7055 | 6,1144 | 0,0088 | 10,8641 | 34,4489 | 11,3283 | 1,8527 | 5,4321 | 17,2245 |
| **1 by 2** | 3,5548 | 3,7055 | 0,9593 | 0,4082 | -8,2376 | 15,3472 | 1,7774 | 1,8527 | -4,1188 | 7,6736 |
| **1 by 3** | 1,0287 | 3,7055 | 0,2776 | 0,7993 | -10,7638 | 12,8211 | 0,5143 | 1,8527 | -5,3819 | 6,4105 |
| **1 by 4** | -1,3338 | 3,7055 | -0,3600 | 0,7427 | -13,1262 | 10,4586 | -0,6669 | 1,8527 | -6,5631 | 5,2293 |
| **1 by 5** | -0,0963 | 3,7055 | -0,0260 | 0,9809 | -11,8887 | 11,6962 | -0,0481 | 1,8527 | -5,9443 | 5,8481 |
| **2 by 3** | 2,9895 | 3,7055 | 0,8068 | 0,4788 | -8,8029 | 14,7819 | 1,4947 | 1,8527 | -4,4015 | 7,3910 |
| **2 by 4** | -1,8982 | 3,7055 | -0,5123 | 0,6438 | -13,6906 | 9,8942 | -0,9491 | 1,8527 | -6,8453 | 4,9471 |
| **2 by 5** | -0,0922 | 3,7055 | -0,0249 | 0,9817 | -11,8846 | 11,7002 | -0,0461 | 1,8527 | -5,9423 | 5,8501 |
| **3 by 4** | -2,3169 | 3,7055 | -0,6253 | 0,5761 | -14,1093 | 9,4755 | -1,1584 | 1,8527 | -7,0546 | 4,7378 |
| **3 by 5** | 4,5227 | 3,7055 | 1,2205 | 0,3094 | -7,2697 | 16,3151 | 2,2613 | 1,8527 | -3,6349 | 8,1575 |
| **4 by 5** | 1,3590 | 3,7055 | 0,3668 | 0,7381 | -10,4334 | 13,1514 | 0,6795 | 1,8527 | -5,2167 | 6,5757 |

Table 4-12: Effect Estimates of Log $k_a$ in $2^4$ Factorial Design.

| Factor | Effect | Sdt. Err. Pure Err | t(3) | p | -95, % Cnf. Limt | +95, % Cnf. Limt | Coeff | Std. Err. Coeff | -95, % Cnf. Limt | +95, % Cnf. Limt |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mean/Interc.** | 0,4638 | 0,0200 | 23,2088 | 0,0002 | 0,4002 | 0,5274 | 0,4638 | 0,0200 | 0,4002 | 0,5274 |
| **(1)$M_{PEG}$** | -0,2607 | 0,0447 | -5,8345 | 0,0100 | -0,4030 | -0,1185 | -0,1304 | 0,0223 | -0,2015 | -0,0593 |
| **(2)$C_{PEG}$** | 0,0811 | 0,0447 | 1,8146 | 0,1672 | -0,0611 | 0,2233 | 0,0405 | 0,0223 | -0,0306 | 0,1117 |
| **(3)$C(NH_4)_2SO_4$** | 0,3187 | 0,0447 | 7,1316 | 0,0057 | 0,1765 | 0,4609 | 0,1594 | 0,0223 | 0,0882 | 0,2305 |
| **(4)pH** | 0,0327 | 0,0447 | 0,7309 | 0,5177 | -0,1096 | 0,1749 | 0,0163 | 0,0223 | -0,0548 | 0,0874 |
| **(5)Sample Volume** | 0,0324 | 0,0447 | 0,7240 | 0,5214 | -0,1099 | 0,1746 | 0,0162 | 0,0223 | -0,0549 | 0,0873 |
| **1 by 2** | 0,0349 | 0,0447 | 0,7814 | 0,4916 | -0,1073 | 0,1771 | 0,0175 | 0,0223 | -0,0536 | 0,0886 |
| **1 by 3** | 0,0056 | 0,0447 | 0,1261 | 0,9076 | -0,1366 | 0,1479 | 0,0028 | 0,0223 | -0,0683 | 0,0739 |
| **1 by 4** | -0,0234 | 0,0447 | -0,5230 | 0,6371 | -0,1656 | 0,1188 | -0,0117 | 0,0223 | -0,0828 | 0,0594 |
| **1 by 5** | -0,0019 | 0,0447 | -0,0436 | 0,9680 | -0,1442 | 0,1403 | -0,0010 | 0,0223 | -0,0721 | 0,0701 |
| **2 by 3** | 0,0453 | 0,0447 | 1,0148 | 0,3849 | -0,0969 | 0,1876 | 0,0227 | 0,0223 | -0,0484 | 0,0938 |
| **2 by 4** | -0,0475 | 0,0447 | -1,0620 | 0,3662 | -0,1897 | 0,0948 | -0,0237 | 0,0223 | -0,0948 | 0,0474 |
| **2 by 5** | 0,0078 | 0,0447 | 0,1743 | 0,8727 | -0,1344 | 0,1500 | 0,0039 | 0,0223 | -0,0672 | 0,0750 |
| **3 by 4** | -0,0615 | 0,0447 | -1,3753 | 0,2627 | -0,2037 | 0,0808 | -0,0307 | 0,0223 | -0,1018 | 0,0404 |
| **3 by 5** | -0,0100 | 0,0447 | -0,2229 | 0,8379 | -0,1522 | 0,1323 | -0,0050 | 0,0223 | -0,0761 | 0,0661 |
| **4 by 5** | -0,0157 | 0,0447 | -0,3508 | 0,7489 | -0,1579 | 0,1265 | -0,0078 | 0,0223 | -0,0789 | 0,0633 |

Table 4-13: Effect Estimates of Log $k_p$ in $2^4$ Factorial Design.

| Factor | Effect | Sdt. Err. Pure Err | t(3) | p | -95, % Cnf. Limt | +95, % Cnf. Limt | Coeff | Std. Err. Coeff | -95, % Cnf. Limt | +95, % Cnf. Limt |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mean/Interc.** | 0,2890 | 0,0333 | 8,6714 | 0,0032 | 0,1830 | 0,3951 | 0,2890 | 0,0333 | 0,1830 | 0,3951 |
| **(1)$M_{PEG}$** | -0,1207 | 0,0745 | -1,6189 | 0,2039 | -0,3579 | 0,1165 | -0,0603 | 0,0373 | -0,1789 | 0,0583 |
| **(2)$C_{PEG}$** | 0,3992 | 0,0745 | 5,3560 | 0,0127 | 0,1620 | 0,6364 | 0,1996 | 0,0373 | 0,0810 | 0,3182 |
| **(3)$C(NH_4)_2SO_4$** | 0,3647 | 0,0745 | 4,8928 | 0,0163 | 0,1275 | 0,6019 | 0,1823 | 0,0373 | 0,0637 | 0,3009 |
| **(4)pH** | 0,1621 | 0,0745 | 2,1756 | 0,1178 | -0,0750 | 0,3993 | 0,0811 | 0,0373 | -0,0375 | 0,1997 |
| **(5)Sample Volume** | -0,2729 | 0,0745 | -3,6614 | 0,0352 | -0,5101 | -0,0357 | -0,1364 | 0,0373 | -0,2550 | -0,0179 |
| **1 by 2** | -0,0182 | 0,0745 | -0,2438 | 0,8231 | -0,2554 | 0,2190 | -0,0091 | 0,0373 | -0,1277 | 0,1095 |
| **1 by 3** | -0,0352 | 0,0745 | -0,4722 | 0,6690 | -0,2724 | 0,2020 | -0,0176 | 0,0373 | -0,1362 | 0,1010 |
| **1 by 4** | -0,1980 | 0,0745 | -2,6566 | 0,0766 | -0,4352 | 0,0392 | -0,0990 | 0,0373 | -0,2176 | 0,0196 |
| **1 by 5** | -0,1182 | 0,0745 | -1,5860 | 0,2109 | -0,3554 | 0,1190 | -0,0591 | 0,0373 | -0,1777 | 0,0595 |
| **2 by 3** | 0,1532 | 0,0745 | 2,0552 | 0,1321 | -0,0840 | 0,3904 | 0,0766 | 0,0373 | -0,0420 | 0,1952 |
| **2 by 4** | 0,0514 | 0,0745 | 0,6900 | 0,5398 | -0,1858 | 0,2886 | 0,0257 | 0,0373 | -0,0929 | 0,1443 |
| **2 by 5** | -0,1443 | 0,0745 | -1,9365 | 0,1482 | -0,3815 | 0,0929 | -0,0722 | 0,0373 | -0,1908 | 0,0464 |
| **3 by 4** | -0,0720 | 0,0745 | -0,9657 | 0,4054 | -0,3092 | 0,1652 | -0,0360 | 0,0373 | -0,1546 | 0,0826 |
| **3 by 5** | -0,1015 | 0,0745 | -1,3615 | 0,2666 | -0,3387 | 0,1357 | -0,0507 | 0,0373 | -0,1693 | 0,0679 |
| **4 by 5** | 0,0214 | 0,0745 | 0,2876 | 0,7924 | -0,2158 | 0,2586 | 0,0107 | 0,0373 | -0,1079 | 0,1293 |

Table 4-14: Effect Estimates of Yield in $2^4$ Factorial Design.

| Factor | Effect | Sdt. Err. Pure Err | t(3) | p | -95, % Cnf. Limt | +95, % Cnf. Limt | Coeff | Std. Err. Coeff | -95, % Cnf. Limt | +95, % Cnf. Limt |
|---|---|---|---|---|---|---|---|---|---|---|
| **Mean/Interc.** | 47,8726 | 2,1123 | 22,6637 | 0,0002 | 41,1503 | 54,5949 | 47,8726 | 2,1123 | 41,1503 | 54,5949 |
| **(1)$M_{PEG}$** | -10,8075 | 4,7232 | -2,2882 | 0,1061 | -25,8390 | 4,2239 | -5,4038 | 2,3616 | -12,9195 | 2,1120 |
| **(2)$C_{PEG}$** | 8,7862 | 4,7232 | 1,8602 | 0,1598 | -6,2453 | 23,8176 | 4,3931 | 2,3616 | -3,1226 | 11,9088 |
| **(3)$C(NH_4)_2SO_4$** | 2,8306 | 4,7232 | 0,5993 | 0,5912 | -12,2008 | 17,8621 | 1,4153 | 2,3616 | -6,1004 | 8,9310 |
| **(4)pH** | 3,9830 | 4,7232 | 0,8433 | 0,4610 | -11,0485 | 19,0145 | 1,9915 | 2,3616 | -5,5242 | 9,5072 |
| **(5)Sample Volume** | -0,0577 | 4,7232 | -0,0122 | 0,9910 | -15,0891 | 14,9738 | -0,0288 | 2,3616 | -7,5446 | 7,4869 |
| **1 by 2** | -1,8458 | 4,7232 | -0,3908 | 0,7221 | -16,8773 | 13,1857 | -0,9229 | 2,3616 | -8,4386 | 6,5928 |
| **1 by 3** | 8,1324 | 4,7232 | 1,7218 | 0,1836 | -6,8991 | 23,1638 | 4,0662 | 2,3616 | -3,4496 | 11,5819 |
| **1 by 4** | 2,8880 | 4,7232 | 0,6114 | 0,5841 | -12,1435 | 17,9195 | 1,4440 | 2,3616 | -6,0717 | 8,9597 |
| **1 by 5** | 0,5880 | 4,7232 | 0,1245 | 0,9088 | -14,4435 | 15,6194 | 0,2940 | 2,3616 | -7,2217 | 7,8097 |
| **2 by 3** | -7,6949 | 4,7232 | -1,6292 | 0,2018 | -22,7264 | 7,3366 | -3,8474 | 2,3616 | -11,3632 | 3,6683 |
| **2 by 4** | -1,3415 | 4,7232 | -0,2840 | 0,7949 | -16,3730 | 13,6899 | -0,6708 | 2,3616 | -8,1865 | 6,8450 |
| **2 by 5** | 5,1650 | 4,7232 | 1,0935 | 0,3541 | -9,8664 | 20,1965 | 2,5825 | 2,3616 | -4,9332 | 10,0982 |
| **3 by 4** | -2,7505 | 4,7232 | -0,5823 | 0,6012 | -17,7820 | 12,2810 | -1,3753 | 2,3616 | -8,8910 | 6,1405 |
| **3 by 5** | 2,2551 | 4,7232 | 0,4774 | 0,6657 | -12,7764 | 17,2866 | 1,1276 | 2,3616 | -6,3882 | 8,6433 |
| **4 by 5** | 1,9747 | 4,7232 | 0,4181 | 0,7040 | -13,0568 | 17,0062 | 0,9874 | 2,3616 | -6,5284 | 8,5031 |

Table 4-15: Results of upper phase on $2^4$ factorial design of ATPS.

| | Actual values | | | | | Results | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Run** | **$M_{PEG}$**[a] | **$C_{PEG}$**[b] | **$C_{(NH4)2SO4}$**[c] | **pH** | **S.V.**[d] | **SA**[e] | **FP**[f] | **R**[g] | ***n***[h] | **Log $k_a$** | **Log $k_p$** |
| **1** | 2000 | 14 | 14 | 8.2 | 1.00 | 49.80 | 3.00 | 66.11 | 38.60 | 0.39 | -0.12 |
| **2** | 6000 | 14 | 14 | 8.2 | 0.50 | 57.71 | 3.36 | 27.74 | 28.07 | 0.06 | 0.06 |
| **3** | 2000 | 18 | 14 | 8.2 | 0.50 | 50.81 | 3.06 | 45.97 | 63.14 | 0.38 | 0.16 |
| **4** | 6000 | 18 | 14 | 8.2 | 1.00 | 41.09 | 2.39 | 48.26 | 40.34 | 0.24 | -0.08 |
| **5** | 2000 | 14 | 18 | 8.2 | 0.50 | 65.74 | 3.49 | 57.99 | 51.53 | 0.68 | 0.23 |
| **6** | 6000 | 14 | 18 | 8.2 | 1.00 | 47.76 | 2.41 | 64.45 | 42.87 | 0.44 | 0.04 |
| **7** | 2000 | 18 | 18 | 8.2 | 1.00 | 49.65 | 2.89 | 86.62 | 55.44 | 0.86 | 0.47 |
| **8** | 6000 | 18 | 18 | 8.2 | 0.50 | 74.50 | 4.10 | 48.55 | 42.64 | 0.61 | 1.03 |
| **9** | 2000 | 14 | 14 | 9.8 | 0.50 | 85.80 | 4.09 | 55.82 | 51.85 | 0.52 | 0.17 |
| **10** | 6000 | 14 | 14 | 9.8 | 1.00 | 43.69 | 2.63 | 51.23 | 32.14 | 0.21 | -0.11 |
| **11** | 2000 | 18 | 14 | 9.8 | 1.00 | 44.95 | 2.26 | 68.34 | 67.91 | 0.50 | 0.45 |
| **12** | 6000 | 18 | 14 | 9.8 | 050 | 50.89 | 2.96 | 31.87 | 45.18 | 0.21 | 0.44 |
| **13** | 2000 | 14 | 18 | 9.8 | 1.00 | 52.44 | 2.79 | 88.75 | 47.66 | 0.73 | 0.35 |
| **14** | 6000 | 14 | 18 | 9.8 | 0.50 | 37.36 | 2.25 | 38.00 | 50.71 | 0.42 | 0.22 |
| **15** | 2000 | 18 | 18 | 9.8 | 0.50 | 87.36 | 4.65 | 59.03 | 45.67 | 0.77 | 1.21 |
| **16** | 6000 | 18 | 18 | 9.8 | 1.00 | 46.05 | 2.44 | 72.47 | 53.37 | 0.54 | 0.34 |
| **17** | 4000 | 16 | 16 | 9.0 | 0.75 | 49.66 | 2.37 | 53.06 | 47.82 | 0.32 | 0.18 |
| **18** | 4000 | 16 | 16 | 9.0 | 0.75 | 59.33 | 2.83 | 65.71 | 52.66 | 0.50 | 0.19 |
| **19** | 4000 | 16 | 16 | 9.0 | 0.75 | 69.52 | 3.31 | 60.00 | 38.62 | 0.40 | 0.11 |
| **20** | 4000 | 16 | 16 | 9.0 | 0.75 | 54.50 | 2.60 | 70.20 | 61.25 | 0.51 | 0.45 |

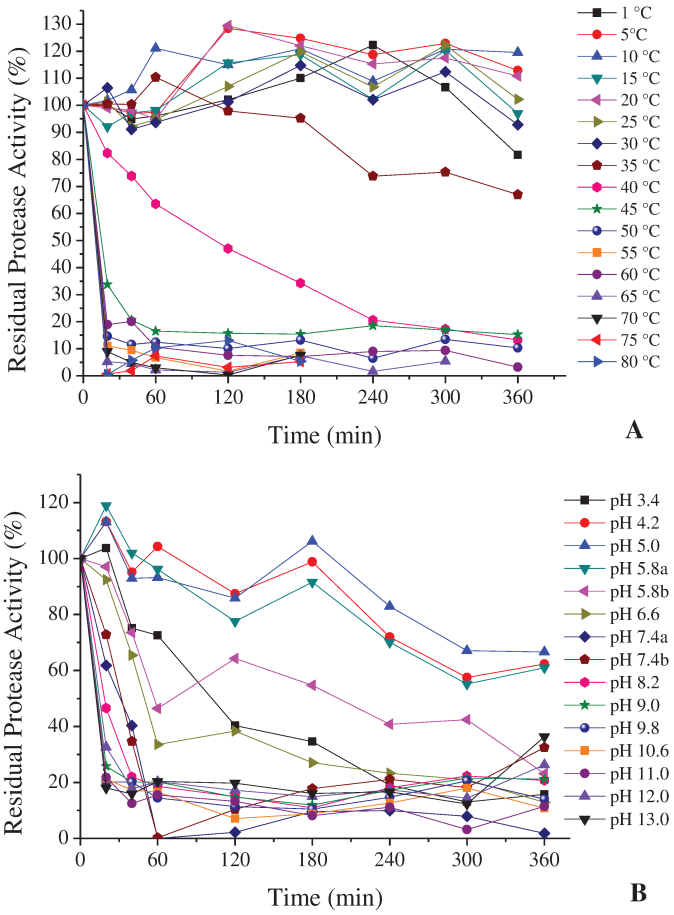
[a] PEG molar mass; [b] PEG concentration; [c] Ammonium sulphate concentration; [d] Sample volume; [e] Specific Activity ($U.mg^{-1}$); [f] Purification factor; [g] Recovery (%); [h] Yield of extraction (%).

Table 4-16: Results of upper phase on $2^2$ central composite design of ATPS.

| **Run** | Actual values | | Results | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **$C_{PEG}$**[a] | **$C_{(NH4)2SO4}$**[b] | **SA**[c] | **FP**[d] | **R**[e] | ***n***[f] | **Log $k_a$** | **Log $k_p$** |
| **1** | 16 | 16 | 71.50 | 3.46 | 56.97 | 59.24 | 0.63 | 0.52 |
| **2** | 16 | 22 | 52.58 | 2.51 | 58.22 | 64.93 | 1.62 | 1.04 |
| **3** | 22 | 16 | 94.77 | 4.52 | 59.95 | 55.64 | 0.92 | 0.78 |
| **4** | 22 | 22 | 54.69 | 2.85 | 51.91 | 69.12 | 2.10 | 2.88 |
| **5** | 14.76 | 19 | 74.28 | 3.55 | 67.73 | 55.39 | 0.77 | 0.62 |
| **6** | 23.24 | 19 | 76.35 | 3.98 | 55.49 | 55.71 | 2.05 | 2.83 |
| **7** | 19 | 14.76 | 69.89 | 3.38 | 58.92 | 73.12 | 0.84 | 0.36 |
| **8** | 19 | 23.24 | 66.93 | 3.49 | 63.73 | 58.38 | 1.34 | 2.78 |
| **9** | 19 | 19 | 112.78 | 4.74 | 67.83 | 48.62 | 1.14 | 2.66 |
| **10** | 19 | 19 | 113.42 | 4.77 | 65.41 | 49.37 | 0.91 | 2.67 |
| **11** | 19 | 19 | 122.81 | 5.16 | 70.82 | 46.62 | 1.19 | 2.63 |
| **12** | 19 | 19 | 115.30 | 4.85 | 67.44 | 47.29 | 1.12 | 2.63 |

[a] PEG 2000 concentration; [b] Ammonium sulphate concentration; [c] Specific Activity ($U.mg^{-1}$); [d] Purification factor; [e] Recovery (%); [f] Yield of extraction (%).

Figure 4-7: Thermal stability (A) and pH stability (B) of purified protease from *Aspergillus terreus* VSP-22 after 360 minutes of incubation. In Figure B, 5.8a e 5.8b define the activity of citrate-phosphate and sodium phosphate buffers, respectively. 7.4a and 7.4b are the activities of sodium phosphate and Tris-HCl buffer, correspondently.



## Capítulo 5 – Conclusão

Os resultados obtidos neste trabalho revelaram que a protease proveniente de *Aspergillus terreus* VSP-22 atingiram resultados satisfatórios para os fatores de purificação e recuperação para as precipitações com sulfato de amônio (PF = 2,07 e R = 46,45%) e etanol (PF = 15,05 e R = 41,05%), contudo, foram os sistemas bifásicos aquosos que se destacaram. O planejamento fracionário, seguido do planejamento composto central para os SBAs, proporcionaram um aumento nestas respostas, o que possibilitou o encontro de um sistema PEG/$(NH_4)_2SO_4$ otimizado. Este sistema caracterizado por 19,95% (w/w) de PEG 2000 (g/mol), 19,95% de sulfato de amônio (w/w) em pH 9.0, alcançou 5.23 vezes para o fator de purificação e 60.21% para recuperação da protease. Além disso, o aumento de escala do sistema otimizado evidenciou uma boa reprodutibilidade dos parâmetros avaliados, tanto em 40,0 (PF = 5,00 e R = 69,40%) como 320,0 g (PF = 5,41 and R = 70,60%) de massa total do sistema. A estratégia proposta em realizar a precipitação com sulfato de amônio e, em seguida, extrair a enzima utilizando o SBA otimizado atingiu excelentes resultados para o fator de purificação (6,06), mostrando simplicidade e baixo custo para sua execução, o que é muito interessante para ser aplicado nos *downstream processes*.

A caracterização bioquímica evidenciou informações importantes acerca do metabolismo das reações catalíticas, a qual sugeriu tratar-se de uma protease alcalina, já que obteve-se um ótimo de pH em 9,0, para o extrato enzimático, e 9,8, para a protease purificada. A avaliação da temperatura ótima atingiu máxima atividade proteolítica em 50 e 40 °C, para o extrato enzimático e protease purificada, respectivamente. As análises de estabilidade demonstraram que as proteases são estáveis em uma ampla gama de pH e temperaturas (1 a 35 °C). Além disso, percebeu-se que íons metálicos não interferem na atividade proteolítica. Os parâmetros cinéticos evidenciaram uma alta afinidade para a protease purificada, com $V_{máx}$ = 24.4499 U/mL e $K_m$ = 0.0318 mM.

As proteases são ubíquas e exibem importantes funções nos processos metabólicos celulares, além de poderem ser aplicadas em diversos setores da indústria. As metodologias de purificação foram investigadas, as quais representam o maior custo da produção. Dessa forma, encontrou-se uma estratégia eficiente, econômica e escalável para os *downstream processes*, utilizando a precipitação com sulfato de amônio seguida do sistema bifásico aquoso otimizado. Além disso, a caracterização da peptidase provou ter propriedades interessantes, podendo ser utilizadas em aplicações nas indústrias de alimentos e detergentes, por exemplo. Assim, a protease proveniente do fungo filamentoso *Aspergillus terreus* VSP-22 demonstrou

características desejáveis, além do procedimento de extração e purificação ter sido eficiente, simples e acessível para ser utilizado nas aplicações industriais.

## Capítulo 6 – Sugestões para Trabalhos Futuros

- Aplicação da protease em testes como coagulação do leite, amaciamento da carne, além de avaliações em compatibilidade detergente e desempenho de lavagem;
- Imobilização da protease em suportes como alginato de sódio e quitosana;
- Caracterização da enzima imobilizada e comparação com seu estado livre.

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