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NATÁLIA BITTENCOURT MELANI

PRODUÇÃO, CARACTERIZAÇÃO BIOQUÍMICA E PURIFICAÇÃO DE LIPASES DE *METSCHNIKOWIA SP*. PROSPECTADAS DO AMBIENTE ANTÁRTICO UTILIZANDO SISTEMA BIFÁSICO AQUOSO

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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

Coorientador: Prof. Dr. Edgar Silveira Campos

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Os membros acima assinaram a ata da defesa, na qual encontra-se no processo de vida acadêmica do aluno.

Epígrafe

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie

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À Deus, por nos conceder com o milagre da vida;

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Resumo

Estudos de recursos naturais do planeta tem se mostrado como o caminho para o avanço dos processos tecnológicos em detrimento aos métodos químicos. Dessa forma, a necessidade de busca por enzimas com características especiais que confeccionem a sua aplicação no ramo industrial, aliado à capacidade de sobrevivência e adaptação de seres em habitats ambientes extremos, como o continente Antártico, fazem desses microrganismos alvos de grande interesse biotecnológico. Especificamente, a extração de lipases de fungos psicrófilos e psicrotolerantes tem chamado a atenção devido a sua elevada atividade a baixas temperaturas, o que confere uma propriedade peculiar. Tecnologias alternativas estão gradualmente vindo à tona na purificação de lipases, onde sistemas bifásicos aquosos apresentam importante destaque. Neste trabalho, lipases de *Mestschnikowia australis* CRM 1589, isoladas do ambiente Antártico, produzidas por fermentação submersa, foram purificadas por sistema bifásico aquoso e caracterizadas bioquimicamente. A enzima foi eficientemente extraída com sistemas bifásicos aquosos compostos por PEG/fosfato de sódio e PEG/sulfato de amônio, com tendência a partição em fase rica em PEG. Além disso, o primeiro sistema apresenta melhores resultados de partição e o segundo, fatores de purificação mais elevados. Os principais estudos indicaram que a lipase possui características alcalinas, estabilidade em ampla faixa de temperatura e pouca inibição a íons metálicos. Essas características conferem potenciais aplicações industriais para esta lipase.

Palavras-chave: Sistema Bifásico Aquoso, Lipase, Purificação, *Metschnikowia australis*, Metodologia de Superfície de Resposta.

Abstract

Studies of planet's natural resources have been shown as the path to the advancement of technological processes and to the detriment of chemical methods. Thus, the need to search for enzymes with special characteristics that make their application in the industrial field, together with the capacity of survival and adaptation of beings in habitats extreme environments, such as the Antarctic continent, make these microorganisms targets of great biotechnological interest. Specifically, the extraction of lipases from psychrophilic and psychrotolerant fungi has attracted attention due to its high activity at low temperatures, which confers a peculiar property. Alternative technologies are gradually surfacing in the purification of lipases, where aqueous two-phase systems (ATPS) are prominent. In this work, lipases of *Mestschnikowia australis* CRM 1589., isolated from Antarctic environment, produced under submerged fermentation, were purified and characterized. The enzyme was efficiently extracted with an aqueous two-phase system composed by PEG / sodium phosphate and PEG / ammonium sulfate, with a tendency to phase partition in PEG rich. In addition, the first system has better partitioning results and the second onde showed higher purification factors. The main studies indicated that lipase has alkaline characteristics, stability in a wide range of temperature and low inhibition values to metallic ions. These characteristics confer potential industrial applications for this lipase.

Keywords: Aqueous Two-phase System, Lipase, Purification, *Metschnikowia australis*, Response Surface Methodology

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Sumário

CAPÍTULO 1 - INTRODUÇÃO

1.1 Organização Estrutural da Dissertação

O presente trabalho encontra-se disposto na forma de capítulos, nos quais:

- O Capítulo 1 refere-se a uma breve introdução sobre o ambiente antártico e seus microrganismos, bem como a importância e utilização das lipases;
- O Capítulo 2 descreve os objetivos do trabalho desenvolvido;
- O Capítulo 3 envolve uma revisão bibliográfica sobre lipases, por meio do artigo intitulado: "A 10-year overview of lipases: from production to application";
- O Capítulo 4 é composto pelo manuscrito experimental "Extraction and purification of cold-adapted lipase from *Metschnikowia australis* CRM 1589 using Aqueous Two-Phase System (ATPS) composed by PEG / Sodium Phosphate", onde descreve a extração de lipases por sistema de duas fases aquosas;
- O Capítulo 5 apresenta o manuscrito experimental initiulado "Purification of cold-adapted lipase from *Metschnikowia australis* CRM 1589 using Aqueous Two-Phase System (ATPS) composed by PEG / Ammonium Sulfate and its biochemical characterization", no qual avalia purificação da lipase de *Metschnikowia australis* CRM 1589 por sistema de duas fases aquosas do tipo polímero/sal, bem como sua caracterização bioquímica para potenciais futuras aplicações;
- O Capítulo 6 indica as principais conclusões da dissertação, a partir dos resultados encontrado ao longos dos capítulos anteriores;
- O Capítulo 7 sugere possíveis futuras abordagens para continuidade desta pesquisa;
- O Capítulo 8 indica as referências bilbiográficas utilizadas na introdução deste trabalho,
- O Capítulo 9 é composto por um apêndice no qual foi realizado testes de fermentação extrativa para avaliar a viabilidade de estudos futuros de produção e purificação desta enzima.

1.2 O Ambiente antártico e seus microrganismos

Mais de 80% do ambiente terrestre apresenta temperaturas abaixo de 5°C (CAVICCHIOLI et al., 2000), onde a maior fração de habitats frios é representada por oceanos profundos, neves e geleiras do Ártico e da Antártica, solos *permafrost*, gelo do mar e geleiras de altas montanhas. O exemplo mais significativo de um habitat terrestre frio é a Antártica, com cerca de 14 milhões de km², dos quais são quase totalmente cobertos por gelo e neve

(HOLDGATE, 1977). O isolamento a longo prazo resultante de eventos climáticas e geológicas podem estar entre os mais importantes fatores históricos que influenciam padrões biogeográficos na Antártica. Além disso, a combinação de condições como frio, seca, e alta incidência de raios UV faz com que este seja um lugar desafiador para se viver (BUZZINI; MARGESIN, 2013). A maioria das espécies de leveduras encontradas nos desertos da Antártica são dos gêneros *Cryptococcus* e *Rhodotorula*. Para o sucesso da sobrevivência neste ambiente, as adaptações das leveduras incluem: piscrofilia, alteração de esteróis, capacidade de resistir a dessecação, bem como a capacidade de aproveitar com eficiência minerais em um habitat oligotrófico (BUZZINI; MARGESIN, 2013). Devido à forte habilidade de viver em ambientes extremos, estes organismos apresentam potencialidades versáteis para a síntese de biomoléculas funcionais (CHI, 2016).

As enzimas de organismos psicrófilos são propensas a desenvolver uma estrutura mais flexível quando comparada com os seus homólogos providos de organismos mesofílicos e termofílicos (SATYANARAYANA; KUNZE, 2009), e são denominadas “frio ativas”. Lipases, amilases, glucoamilases, proteases e xilanases representam os mais famosos exemplos de enzimas “frio ativas” produzidas por leveduras adaptadas ao frio (SHIVAJI; PRASAD, 2009; JOHNSON; ECHAVARRI-ERASUN 2011; BUZZINI et al., 2012). Entre elas, as lipases fúngicas constituem um importante grupo de enzimas biotecnologicamente importantes devido à versatilidade das suas propriedades enzimáticas e especificidade de substrato, além da facilidade de produção em massa (SINGH; MUKHOPADHYAY, 2012).

1.3 Lipases

De acordo com o Comitê de Nomenclatura da União Internacional de Bioquímica e Biologia Molecular (IUBMB), lipases (ou, formalmente, triacilglicerol lipases, EC 3.1.1.3) pertencem à primeira subclasse de hidrolases, ou seja, que atuam em ligações éster (EC 3.1) e à sub-subclasse de hidrolases do éster carboxílico (EC 3.1.1), que também inclui outras lipases de carboxilesterases (EC 3.1.1.1). A diferença fundamental entre as lipases e outras carboxilesterases é a sua capacidade de hidrolisar os ésteres insolúveis em água de ácidos graxos de glicerol e de cadeia longa. Além disso, sob condições termodinamicamente favoráveis, catalisam reações de síntese, acidólise, alcoólise, e aminólise dos ésteres. Devido a suas propriedades de quimio, regio e estereosseletividade, seu potencial de aplicação é amplo (BUZZINI; MARGESIN, 2013). Ademais, lipases microbianas tem recebido muito mais atenção comparadas às mesmas enzimas providas de plantas e animais. Essa consideração parte devido a sua diversidade na atividade catalítica, alto rendimento e baixo custo de produção,

assim como a relativa facilidade de manipulação genética. Além disso, também são estáveis em solventes orgânicos, não necessitam de cofactores e possuem uma ampla especificidade de substrato (JAEGER; REETZ, 1998).

A utilização desta enzima ocorre em duas formas distintas, podendo ser empregada como catalisador biológico para a fabricação de ingredientes alimentares, bem como adotada na produção de produtos químicos finos. São comumente usadas no processamento de óleos e gorduras, couro, têxtil, detergentes e formulações desengordurantes, fabricação de papel, síntese de produtos de química fina, processamento de alimentos, formulação de cosméticos, etc (HOUDE et al., 2004). Além disso, nas últimas décadas, as lipases também tem apresentado uma série de aplicações na fabricação de produtos farmacêuticos, pesticidas, proteína de célula única, preparação de biossensor e na gestão de resíduos (SATYANARAYANA; KUNZE, 2009).

Especificamente, a extração de lipases de fungos psicrófilos e psicrotolerantes tem chamado a atenção devido a sua elevada atividade a baixas temperaturas, o que confere uma propriedade útil para muitos empregos biotecnológicos, incluindo a produção de detergentes utilizados na lavagem a frio, em empresas agrícolas, e processos de fermentação que requerem baixas temperaturas (LI et al., 2014; GERDAY et al., 2000). As principais restrições em estratégias de purificação tradicionais incluem baixos rendimentos e longos períodos de tempo. Por este motivo, tecnologias alternativas, tais como processos de membrana, sistemas de duas fases aquosas e imunopurificação estão gradualmente vindo à tona na purificação de lipases. As indústrias buscam por estratégias de purificação que são baratas, com alto rendimento, rápidas e passíveis de operações de grande escala (SAXENA et al., 2003).

Sistemas bifásicos aquosos é uma dessas potenciais alternativas (ZHOU et al., 2013; SOUZA et al., 2015). Esta tecnologia consiste em uma técnica onde a extração, concentração, e purificação parcial podem ser integradas num único passo de extração líquido-líquido. Este método oferece muitas vantagens, tais como ambiente biocompatível para as biomoléculas, menor tensão interfacial, e as possibilidades de operação contínua (NANDINI; RASTOGI, 2011). Além disso, esses sistemas têm outras vantagens, incluindo a viabilidade de extrações de grande escala, redução de volume, separação rápida com pouca desnaturação, separação seletiva e baixa tensão interfacial com rápida transferência de massa (BEŞEL, 2003). A partição de proteínas padrão depende da concentração e do peso molecular da fase de formação de polímero(s), do pH, e da concentração e do tipo do sal (RAGHAVA RAO et al., 2003; CHETHANA et al., 2007).

O presente trabalho se justifica pela atual crescente necessidade de abordagens mais seletivas e menos prejudiciais para o meio ambiente. Dentro deste contexto destaca-se a tecnologia enzimática, que é hoje um dos campos mais promissores dentro das novas tecnologias para síntese de compostos de alto valor agregado. Entre os processos de interesse, estão a reação de hidrólise, transesterifitcação e síntese de lipídios por intermédio de lipases (COSTA-SILVA, 2010). Assim, o estudo de purificação por diferentes sistemas de duas fases aquosas do tipo polímero/sal, utilizando diferentes massas moleculares de polietileno glicol (PEG) e sais de fosfato de sódio e sulfato de amônio, bem como a caracterização bioquímica de lipases pertencentes à *levedura Metschnikowia australis* CRM 1589 prospectadas do ambiente Antártico foi realizado na busca por enzimas de grande potencial biotecnológico com características especiais que permitam a sua aplicação no ramo industrial.

CAPÍTULO 2 - OBJETIVOS

2.1. Objetivo Geral

Produzir, caracterizar bioquimicamente e purificar, pela técnica de sistema bifásico aquoso, lipases de *Metschnikowia australis* CRM 1589 oriundas do Ambiente Antártico.

2.2. Objetivos Específicos

- Realizar fermentação submersa para produção lipase de *Metschnikowia australis* CRM 1589;
- Determinar atividade enzimática da lipase produzida por fermentação submersa;
- Purificar por métodos de precipitação com sulfato de amônio;
- Purificar a lipase por sistemas de duas fases aquosas compostos por PEG/sais de fosfato e PEG/sulfato de amônio;
- Produzir e purificar a lipase através de fermentação extrativa;
- Caracterizar bioquimicamente a lipase purificada;
- Determinar os parâmetros cinéticos da lipase.

CAPÍTULO 3 - MANUSCRITO DE REVISÃO BIBLIOGRÁFICA “A 10-YEAR OVERVIEW OF LIPASES: FROM PRODUCTION TO APPLICATION” SUBMETIDO AO PERIÓDICO SEPARATION AND PURIFICATION REVIEWS

A 10-year overview of lipases: From production to application

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Abstract:

Lipases (triacylglycerol hydrolases E.C. 3.1.1.3) are enzymes produced by mammals, plants, fungi, and bacteria that catalyze the hydrolysis of triacylglycerols into glycerol and fatty acids. Lipases are versatile in lipid biotechnology due to their selective properties, which have been applied in recent years in emulsifiers, pharmaceuticals, cosmetics, flavors, fragrances, the pretreatment of lipid-rich wastewater, the bioremediation of oils, and biodiesel synthesis. This review offers information collected over the last 10 years on lipase sources as well as advances in production, purification strategies, and the main applications in industry.

3.1 Introduction

Lipases (E.C. 3.1.1.3) are hydrolases capable of catalyzing the hydrolysis of triacylglycerols (TAGs) into glycerol and fatty acids (FAs). These enzymes are adapted to operate at the interfaces of biphasic systems, which is a phenomenon known as interfacial activation. The characteristic substrate is an aggregate, micelle, or monomolecular film formed by ester molecules. In an organic medium, lipases catalyze esterification, transesterification, and interesterification reactions [82]. The substrates of lipases are diversified and can be composed by neutral lipids, phospholipids and lysophospholipids, ether lipids, etc. [67].

In the presence of excess water, lipases act at the organic-aqueous interface to catalyze the hydrolysis of carboxylate ester bonds into free fatty acids (FFAs) and organic alcohols, as illustrated in Figure 3.1 [14]. Substrate specificities of lipases are classified into alcohol specificity, acylglycerol specificity, FA specificity, positional specificity, TAG specificity, and stereo and chiral specificities. These enzymes exhibit a high degree of activity and stability even in non-aqueous media, do not require cofactors, and are easily immobilized

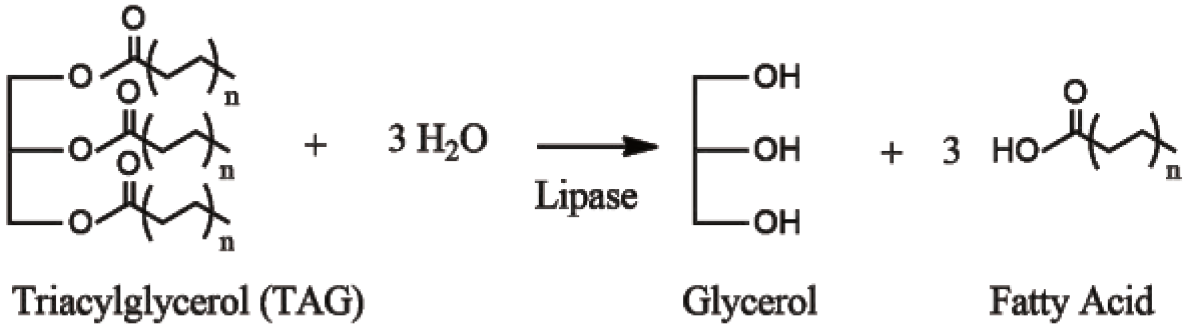

Figure 3.1 - General scheme of lipase reaction

on different matrices [61]. Most lipases of animal and microbial origin exhibit alkaline pH optima (pH 8-9). However, special conditions, such as the presence of salts, and the type of emulsifier used can shift the optimum pH to the acidic range [49].

In aqueous media, a polypeptide chain, denominated a lid, blocks the hydrophobic catalytic center. This structure has a hydrophobic internal face and a hydrophilic external face and moves away upon interaction with the substrate [9]. Some groups, such as human pancreas lipase and *Rhizopus miehei* lipase, exhibit this lid, whereas two nearly parallel amphipathic helices seem to enfold the active site of other groups, such as *Geotrichum candidum* lipase. The conformational change allows activation at an oil-water interface. However, this phenomenon does not apply to all lipases. Some lipases, such as those from *Pseudomonas aeruginosa* and *Candida antarctica* B, do not have a lid and therefore do not exhibit interfacial activation [42].

The active sites of lipases are generally characterized by the triad composed of serine, histidine, and aspartate [116], which has been confirmed by structures of the human pancreatic lipase and *Rhizomucor miehei* lipase (RML). However, the catalytic triad in *Geotrichum candidum* lipase is composed of Ser-Glu-His. In all these cases, the side chains of the active site amino acids are considered stereochemically similar to serine proteases due to their configuration [42].

Based on the positional specificity of hydrolysis on a triacylglycerol molecule, there are two groups of microbial lipases. The first is nonspecific and can cause complete hydrolysis of triacylglycerol to glycerol and free fatty acids, such as lipases from *Geotrichum candidum*, *Staphylococcus aureus*, *Corynebacterium acnes*, *Penicillium cyclopium* and *Chromobacterium viscosu*. The other hydrolyzes fatty acids esterified only in the sn-1 and sn-3 positions, which results in diacylglycerols and monoacylglycerols [49].

Lipases exhibit region-selective properties and enantioselective catalytic behavior and are therefore considered the most versatile catalysts in lipid biotechnology [51]. These enzymes can be employed in a large number industrial processes, including the production of pharmaceuticals and emulsifiers, the synthesis and degradation of engineering thermoplastics, the production of agrochemicals, cosmetics, flavors, and fragrances, the synthesis of biodiesel through the transesterification of triglycerides with short-chain alcohols, the production of concentrated fatty acids through the hydrolysis of oils and fats, etc. [82].

3.2 Source of lipases

Lipases are ubiquitous esterase enzymes produced by animals, plants and microorganisms. Table 3.1 lists some source of lipases.

Table 3.1- Source of lipases

Source	Specie	Reference
Animal	-	[115]
Animal	*Hexaplex trunculus*	[114]
Animal	*Litopenaeus vannamei*	[78]
Bacterial	*Burkholderia ubonensis*	[109]
Bacterial	*Staphylococcus warneri*	[104]
Bacterial	*Bacillus sp*	[87]
Bacterial	*Serratia marcescens*	[86]
Bacterial	*Enterococcus faecium*	[76]
Bacterial	*Acinetobacter sp.*	[37]
Fungal	*Penicillium sp.*	[108]
Fungal	*Trichoderma harzianum*	[98]
Fungal	*Rhodotorula glutinis*	[97]
Fungal	*Sporidiobolus pararoseus*	[93]
Fungal	*Candida cylindracea*	[81]
Fungal	*Talaromyces thermophilus*	[79]
Fungal	*Geotrichum candidum*	[77]
Fungal	*Debaryomyces hansenii*	[70]
Fungal	*Aureobasidium pullulans*	[55]
Fungal	*Leucosporidium scottii*	[26]
Fungal	*Botryosphaeria ribis*	[3]
Plant	*Coffea arabica*	[72]
Plant	*Avena sativa*	[44]
Plant	*Jatropha curcas*	[40]
Plant	*Cucurbita moschata*	[1]

3.2.1 Plant lipases

Plant lipases have been isolated from the leaves, oils, stems, latex, and seeds of oleaginous plants and cereals [82]. The part of the plant and the desired degree of purification determine the most appropriate method for preparing biocatalysts from plant biomass [62]. The

different uses of plant lipases are well documented, although there is a need for further studies on applications in the production of concentrated fatty acids from vegetable oils and biotransformation reactions [82]

These enzymes are especially attractive owing to their ease of purification, low cost, and diversity [25]. However, the unique substrate specificity of plant lipases for industrial applications has yet to be fully determined due to the lack of studies on the optimal production of plant lipases [40]. The direct application of crude lipase extracts from seed oils offers an advantage in the oleochemical industry due to the non-need for purification and immobilization steps, which lowers production costs. The enzymes are naturally immobilized onto solid materials from seeds, which allows easy recovery of the biocatalyst [111].

De Sousa et al. [25] characterized a new plant enzyme extracted from physic nut seeds. The vegetable enzyme extract from germinated seeds (VEEG) was able to hydrolyze different biodiesel bases (tallow, vegetable oils, and biodiesel waste), with high hydrolysis conversion (97% FFA). The lipase activities found were 111 ± 19 U/g for tributyrin, 106 ± 49 U/g for tricaprylin, and 96 ± 4U/g for olive oil, demonstrating that the VEEG had no selectivity for the chain length of the substrates tested.

Santos et al. [82] evaluated the catalytic properties of lipases from different seed plant sources, such as castor bean (*Ricinus communis*), sunflower (*Helianthus annuus*), corn (*Zea mays*), and passion fruit (*Passiflora edulis*), for application in oil hydrolysis for the production of concentrated fatty acids. The results suggested that the use of lipase from dormant castor bean seeds has potential for the hydrolysis of different vegetable oils.

Hidayat et al. [40] studied methods to enhance the indigenous lipase activity of germinated *Jatropha* seeds for the degradation of phorbol ester, which is a compound with the potential to cause high levels of tumor production. High germinated seed lipase activity was obtained when seeds were soaked in pH 6 for 12 h without aeration. Phorbol ester proved to be completely degraded during germination.

3.2.2 Animal lipases

The animal lipases used in industrial processes include pancreatic and pregastric lipases. However, only those produced from porcine pancreas (phospholipases A_2) are employed in industrial applications. These enzymes are used for the production of an emulsifier and antifungal agent in foods denominated lysolecithin [14]. Porcine pancreatic lipase is one of the most widely employed lipases in biotransformation reactions because is cheaper than other

commercial microbial and animal lipases. However, the semi-purified form of this lipase is expensive [60].

Currently, the main sources of the commercially available enzymes are bacteria, yeasts and filamentous fungi. However, porcine and human pancreases were the first sources of lipases and phospholipases used in food processing. This change can be attributed to the fact that lipases and phospholipases from natural sources do not always meet the requirements for industrial biocatalysis in terms of activity, stability, and specificity [14]. Some lipases have been targeted by pharmaceutical companies for therapeutic purposes. Table 3.2 displays some examples. Moreover, there are several drugs that have currently been approved or are in clinical trials [67].

Table 3.2 - Use of lipases in pharmaceutical preparations according to Carvalho et al. [20]

Pharmaceutical ingredient	**Common commercial lipase or species**
Crizotinib	*Candida antarctica* lipase B (CAL-B), *Rhizopus delemar*
Pregabalin and Analogues	Novozyme 435, Lipolase®, Lipozyme TL IM®
Prosimpal	*Arthrobacter (ABL)*
Naproxen	*Candida rugosa* lipase
Ketoprofen	*Aspergillus terreus*
Dihydropyridine Derivatives	*Candida antarctica* A (CAL-A, NZL-101), Porcine Pancreatic Lipase
Ketorolac	Novozyme 435
Chloramphenicol	*C. antarctica* lipase B (CAL-B, Novozyme 435)
Nebracetam	*Burkholderia cepacia* (lipase PS-D)
Aminohydroxypiperidine Derivatives	*C. antarctica* type A (lipase NZL-101), *Burkholderia cepacia* (PSL IM), *Pseudomonas fluorescens* (AK)

Most cells in the body hydrolyze TAGs using lipases to supply fatty acids for energy demands through similar pathways [60]. Lipoprotein lipase hydrolyzes circulating TAGs into free fatty acids and glycerol and is present in nearly all extrahepatic tissues. The highest activities are found in mammary glands, the heart and adipose tissue. Moreover, its tissue-specific regulation drives the flow of circulating TAGs in the body [8].

Scherer et al. [85] investigated the influence of immobilization time and the enzyme-to-support-mass ratio on the yield of immobilization and esterification activity of porcine pancreatic lipase. The greatest immobilization yield (38.2%) was obtained for pillared montmorillonite with 120 min of immobilization and an enzyme-to-support-mass ratio of 2:0.5. The highest esterification activity (1403 U/g) was achieved after 180 min of immobilization with an enzyme-to-support-mass ratio of 2:1. Gilani et al. [33] immobilized porcine pancreas lipase on mesoporous chitosan beads.

3.2.3 Bacterial lipases

Microbial lipases are mostly extracellular and their production is highly influenced by the composition of the medium. Thus, agro-industrial waste could be an option to reduce production costs as well as solve the problem of the disposal of such waste [15]. Bacterial lipases are commonly influenced by nutritional conditions, such as carbon sources, lipids, nitrogen, and inorganic salts [54].

The genera *Pseudomonas* and *Burkholderia* are the most widely used species for the production of bacterial lipases, due mainly to the high enzyme activity at a wide range of temperatures and pH values as well as high enantioselectivity [36]. *Streptomyces* have a particularly complex secondary metabolism that produces a large number of biological target compounds [6].

Bose and Keharia [15] studied the feasibility of Jatropha seedcake as a substrate for lipase production under submerged condition and found that *Pseudomonas aeruginosa* AAU2, which is a cysteine hydrolase, isolated from dumped rotting Jatropha seedcake produces an extracellular solvent-tolerant lipase with activity of 0.432 U ml^{-1}. Kanmani et al. [45] evaluated a lipase produced from *Staphylococcus pasteuri* isolated from sites contaminated with oil and grease using a medium based on coconut oil mill waste, the results of which demonstrated significant extracellular lipase activity.

Gururaj et al. [37] isolated, purified and optimized the production conditions of *Acinetobacter sp*. AU07 isolated from distillery waste using the response surface methodology. This species was found to produce a thermostable and organic solvent-tolerant lipase, with maximum production evidenced at pH 7 and 30 ºC using 2% (v/v) castor oil (inducer), 0.5% (v/v) inoculum and agitation at 150 rpm, achieving 14.5 U/mL.

3.2.4 Fungal lipases

Fungi able to produce lipases are found in several habitats, including seeds, waste vegetable oil, soil contaminated with oils as well as deteriorated food and dairy products [90]. The most cited genera for lipase production are *Aspergillus*, *Rhizopus*, *Penicillium*, *Mucor*, *Geotrichum,* and *Fusarium [23]*. Filamentous fungi are also considered the best source of extracellular lipase for large scale industrial production among all organisms used as sources of lipases [10].

Ramos et al. [77] report the production of an extracellular lipase from *Geotrichum candidum* using cotton seed oil as the inducer in a submerged fermentation system with immobilization via physical adsorption on polyhydroxybutyrate particles. Maximum lipase activity was 22.91 IU/mL after 48 h of fermentation and specific activity was 229.10 IU/mg, while the maximum immobilized protein (4.43 mg/g) and hydrolytic activity (404.4 ± 2.3 IU/g) were obtained with 75 mL of crude enzymatic extract per gram of polyhydroxybutyrate particles.

Colla et al. [23] used a 16-experiment Plackett-Burman factorial design to evaluate the production of lipases by filamentous fungi. The following factors were investigated: bran type used as the main carbon source, nitrogen source, nitrogen source concentration, inducer, inducer concentration, agitation, pH and fungal strain. *Aspergillus niger* and *Aspergillus flavus* were considered good lipase producers via submerged fermentation.

Since yeasts have the GRAS (Generally Considered as Safe) status and do not have toxins or other pathogenic substances, these microorganisms are widely used in food applications and a variety of industrial biotransformations. The major genera of lipase producers are *Candida*, *Rhodotorula*, *Yarrowia*, *Geotrichum*, and *Trichosporon* [63].

Maldonado et al. [57] investigated lipase production by *Geotrichum candidum* in shaken flasks and a bench-scale bioreactor using different inducers. The effects of temperature, initial pH, peptone, $NaNO_3$, $MgSO_4$, and soy oil concentrations were evaluated using the experimental design methodology. *G. candidum* was grown with different inducers and had no significant preferences for any particular inducer with regard to lipase production.

3.3 Production

3.3.1 Submerged fermentation

Improvements to the fermentation conditions (C/N ratio, carbon source, nitrogen source, temperature, inducers, etc.) are the key to enhancing lipase production [53]. The large-scale industrial production of enzymes mainly uses submerged fermentation (SmF) technology due to the ease in monitoring and controlling the process [89]. A quantitative comparison between solid-state fermentation (SSF) and SmF is difficult due to the different methods employed for determining lipase activity [100].

Khoramnia et al. [48] measured lipase production of an *Acinetobacter sp.* isolate in SmF and SSF. The results demonstrated that this was one of the few bacteria able to grow and produce lipase better in SSF than SmF. Narasimha et al. [65] evaluated lipase production from a bacterial strain of *Pseudomonas sp* using SmF with different carbon sources and concluded that enzyme production was maximized when olive oil was used as the carbon source in the medium.

Basheer et al. [10] analyzed a marine fungus (BTMFW032) isolated from seawater and identified as *Aspergillus awamori*, which produces an extracellular lipase. Cultivation with SmF demonstrated enzyme production after 36 hours of incubation and reached a maximum at 96 hours (495 U/ml), whereas maximum specific enzyme activity was recorded at 108 hours (1164.63 U/mg protein).

Shakila Begam et al. [86] used low-cost defatted soybean flour and non-defatted soybean flour for lipase production from *Serratia marcescens* MBB05. Greater enzyme production was obtained using defatted soybean flour in SmF. Moreover, a high increase of approximately 28 U/mL in lipase production occurred when the protease enzyme was inhibited by EDTA at the concentration of 0.2% in the crude enzyme of *S. marcescens* MBB05.

3.3.2 Solid-state fermentation

SSF is a process involving a solid matrix and is carried out in the absence or near absence of free water. Thus, the substrate needs to have moisture to support the growth and metabolism of the microorganism and the solid matrix must either be the source of nutrients or be a support containing nutrients to enable the development of the microorganism [91].

Silveira et al. [89] used palm oil industrial waste as raw material for lipase production by *Aspergillus niger* under different cultivation conditions. In SSF, maximum lipase

activity after 72 h of cultivation was 77.04 IU/g of dry substrate, whereas cultivations performed with SmF led to a peak activity of 10.46 IU/mL reached after 48 h.

Gutarra et al. [38] conducted a comparative study of *Penicillium simplicissimum* lipase production and morphology using SSF on babassu cake and SmF in a semi-synthetic medium as well as a medium based on suspended babassu cake grains. Maximum lipase production of 3.3 U/mL occurred after 120 h with SmF and maximum lipase activity of 77.3 U/g occurred after 72 h of fermentation.

Fungal species are preferably cultivated with SSF, whereas yeast and bacteria are cultivated with SmF [27]. Indeed, the high water activity required in the latter case (generally higher than 0.9) is the main factor for the preferable cultivation of most bacteria in SmF. However, there are some exceptions. Sarkar and Laha [83] found that *Aspergillus niger* lipase activity produced by SSF (4.8 IU/mL) was greater than that reported for SmF (1.46 IU/mL), demonstrating that lipase obtained by SSF was more concentrated.

Martin del Campo et al. [58] studied lipase/esterase production by the halophilic archaeon *Natronococcus sp.* TC6. When the bacterium was cultured in SSF, production reached 29.2 U L^{-1} with p-nitrophenyl laurate, which was 6.3-fold greater than the activity reached in SmF. Moreover, enzyme activity was 387.8 U L^{-1} with p-nitrophenyl butyrate in SmF, which was approximately 2.5-fold less than the activity obtained using SSF, suggesting that SSF is an excellent system for the production of esterase/lipase activity from this archaeon.

Coradi et al. [24] evaluated lipase production by *Trichoderma harzianum* in SmF and SSF using a variety of agro-industrial wastes. Considering the same volume (40 mL), total enzyme activity was 30% higher in SmF (0.5% yeast extract and 1% olive oil) than SSF (castor/cane medium). However, the cost of this carbon source with SSF production was approximately tenfold lower, which compensates greatly for the lower yield in activity.

3.3.3 Fermentation by cell immobilization

The use of the whole cells for immobilization offers an important advantage, since they can be used directly as a biocatalyst without a cell disruption step. Moreover, whole cells catalysts could be applied to the varied transformation of oil feedstock with no different requirements regarding the amount of water, free fatty acids or phospholipids in the feedstock [2]. Fungal hyphae contain many types of enzymes that constitute natural biocatalysts, since these enzymes can be immobilized by growing fungi on a solid substrate [30].

The preparation of immobilized lipase for industrial application involves complicated recovery, immobilization and purification processes. Thus, the direct use of whole-cell lipase is an attractive process, since it does not require purification or immobilization [39].The immobilization process is advantageous, as it can prevent biomass washout at high dilution rates. Therefore, the separation of biomass from the medium is favored due to the high cell concentration in the reactor [28].

Chen and Lin [22] analyzed simultaneous cell growth and immobilization of fungus cells from *Rhizopus oryzae* for application in the production of biodiesel through the methanolysis of soybean oil. The best conditions for cell growth and immobilization were determined when a circulating packed-bed bioreactor system using fibrous nonwoven fabric as the immobilization matrix was used. Andrade et al. [2] evaluated different fungal biomasses with high lipase activities immobilized on different biomass support particles. With the exception of *Penicillium citrinum* URM 4216, all fungal strains exhibited high lipase activity (20 to 50 Ug^{-1}) when immobilized *in situ* using polyurethane foam particles.

He et al. [39] studied biodiesel production catalyzed by whole-cell immobilized lipase from *Rhizopus chinensis* CCTCC. Time courses of the esterification of oleic acid with methanol catalyzed by RCL M201021 showed high catalytic ability in the transesterification of oil to produce fatty acid methyl ester (biodiesel) when used directly in the solvent-free system and organic solvents.

In a study on different types of lipase production, Ferrarezi et al. [30] found that the use of immobilized hyphae enabled a very high increase in lipase activity as well as an improvement in enzyme stability. Lipases produced through both SSF and SmF exhibited maximum lipolytic activity at 40 ºC. These lipases were from a fungus grown on loofah sponges and the immobilized hyphae exhibited 108 Ug lipolytic activity, which a profile including both alkaline and acidic activities.

3.4 Purification Strategies

3.4.1 Concentration

Conventional techniques for lipase extraction and purification include ultrafiltration, ammonium sulfate precipitation, and extraction with organic solvents, such as cold ethanol and acetone [26]. This stage is highly important, since chromatography steps are expensive and involve large volumes of water, which increases costs.

The choice of method depends mostly on the characteristics of the lipase, composition of the extract, and cost of the process. The most practical technique is known as separation by precipitation, which consists of modifying the solvent solution to alter the solubility of proteins and favor the formation of protein aggregates [16].

Ammonium sulfate is likely the most common protein precipitant used. This neutral salt is particularly popular due to its low cost, high solubility, lack of denaturing properties toward most proteins, and its stabilizing effect on many proteins [105]. To preserve the biological properties of lipase, it is important for the entire procedure to be performed at temperatures below 4 °C, which keeps the structures of the biomolecule from being significantly affected.

Ayaz et al. [6] used ammonium sulfate (80%) precipitation and gel filtration chromatography to purify an extracellular alkaline lipase obtained from *Streptomyces sp*. OC 119-7, which resulted in 5.52-fold purification, with 68.055 U/mg specific activity. Studies of this type demonstrate that concentration techniques combined with chromatographic procedures tend to generate better results.

Bose and Keharia [15] collected the supernatant containing lipase in a glass beaker. Crystalline ammonium sulfate was slowly added with continuous stirring up to 40% saturation and the solution was kept overnight at 20 ºC to enable protein precipitation. Gururaj et al. [37] investigated a thermostable, organic solvent-tolerant lipase from *Acinetobacter sp.* AU07 isolated from distillery waste. To precipitate the proteins, solid ammonium sulfate (60% saturation) was added to the culture supernatant with continuous stirring and incubated at 4 ºC for 24 h. The sample was centrifuged at the same temperature for 15 min at 12,500 × g. The resulting pellet was dissolved in 10 mM Tris-HCl buffer (pH 8.0) and dialyzed with the same buffer for 12 h at 4 ºC.

Following the same line of low temperatures, Yang et al. [109] investigated a new lipase from *Burkholderia ubonensis* SL-4. The culture supernatant was collected by centrifugation at 12,000 rpm for 15 min at 4 ºC. The clear supernatant containing extracellular lipase was regarded as the crude enzyme preparation and precipitated by the addition of ammonium sulfate (80% saturation) at 4 ºC overnight. The pellet was centrifuged at 12,000 rpm for 30 min and re-suspended in a small amount of Tris-HCl buffer (50 mM, pH 8.0) to enable protein solubilization.

The pretreatment of lipases with organic solvents prior to the esterification process results in the elimination of the time lag [4]. The addition of different organic solvents to a protein solution can also promote precipitation, as such solvents lower the dielectric constant of an aqueous solution, which enables an increase in the electrostatic attraction between bodies with opposite charges in the solution. This increase in interactions between proteins of opposite charges eventually leads to their precipitation [105].

Protein precipitation using organic solvents must be carried out at temperatures close to 0 °C to prevent protein denaturation and preserve biological properties. Patel et al. [71] investigated an extracellular solvent-stable alkaline lipase from *Pseudomonas sp.* DMVR46, which demonstrated significant stability in organic solvents, such as cyclohexane, isooctane and n-hexane, retaining more than 70% of its initial activity.

Toscano et al. [98] characterized an extracelular lipase from *Trichoderma harzianum*. Precipitation of the crude lipase was achieved by adding ethanol at an enzyme solution-to-solvent ratio of 1:3 at 4 °C for 2 h under stirring. The solution was allowed to rest overnight. The precipitate was then filtered under reduced pressure, washed with a hydroalcoholic solution at the same concentration as that of the precipitation media, and dried under reduced pressure at room temperature (16). The lipase powder obtained had an activity of 12.000 $U \cdot g^{-1}$.

Ultrafiltration constitutes another type of concentration, in which separation is determined by particle size. Ultrafiltration membranes are characterized by their molecular-weight cutoff, which is defined by the smallest molecular-weight species for which the membrane demonstrates more than 90% rejection [21]. There are different sizes of commercially available membrane pores. The technique can also be employed in the desalination of a medium. This step is generally combined with different extraction methods to obtain a better performance in the purification process.

Li et al. [52] ultrafiltered crude lipase with a 30 kDa cutoff centrifugal membrane filter (Millipore, USA). The supernatant sample was subjected to ultrafiltration concentration, ammonium sulfate precipitation (60 to 80% saturation) and phenyl sepharose chromatography in sequence, resulting in partial purification by 60.5-fold with a specific activity of 38.9 U/mg. Sarkar et al. [84] studied a new alkali-thermostable lipase from *Staphylococcus aureus* isolated from *Arachis hypogaea* rhizosphere. The supernatant was centrifuged with the PALL Microsep centrifugal device (10 kDa cut off).

3.4.2 Chromatography

Chromatography is a multistage technique for separating different, but similar solutes with high resolution and is the most widely employed separation method in biotechnology. This strategy is based on the differential distribution of solutes with biomolecules of interest in the mobile phase carried through the stationary phase. These columns, known as chromatographic matrices, are generally composed of hydrophilic materials that demonstrate no interactions with the biomolecules [95]. Ion exchange chromatography is the most frequently employed method, the main anion and cation exchangers of which are the diethylaminoethyl group and carboxymethyl, respectively [96].

Trodler et al. [102] applied an efficient one-step method for the purification of lipase B from *Candida antarctica* by ion-exchange chromatography developed through a rational design. The crude solutions were purified using cation-exchange chromatography. *C. antarctica* lipase B was bound to the chromatography column and purified with a purification factor of 2.4.

Sivaramakrishnan and Incharoensakdi [92] obtained lipase from *Bacillus sp.* isolated from oil-contaminated soil. The enzyme was purified by ammonium sulfate precipitation and ion-exchange chromatography. The precipitated ammonium sulfate fraction was applied to a phenyl sepharose CL-4B column and pre-equilibrated with 250 mM phosphate buffer (pH 7.0). The lipase was purified 5.1-fold, with a yield of 10.5%.

Hydrophobic interaction chromatography (HIC) is another often employed technique. The aim of this form of liquid chromatography is to separate and purify biomolecules through their hydrophobic interaction with hydrophobic ligands coupled to porous media. The stationary phase contains weak ligands, such as short-chain phenyl and alkyl immobilized on a hydrophilic matrix [95]. In this type of chromatography, hydrophobic groups, such as phenyl, octyl, or butyl, are attached to the stationary column [103].

Hydrophobic materials have been shown to increase the performance of lipase. It is important to note that the support has to be chosen in accordance with the properties of the reaction system [34]. Rivera-Pérez et al. [78] purified intracellular lipase from pleopods of *Litopenaeus vannamei*. The enzymes were separated by hydrophobic chromatography using a fast-protein liquid chromatography system (GradiFracSystem, Pharmacia Biotek) operating at 27 °C. The sample was loaded into a previously equilibrated phenyl-sepharose CL-4B column and washed with Tris-HCl containing ammonium sulfate at pH 8.0.

Kanmani et al. [45] used coconut oil mill waste as a substrate for *Staphylococcus pasteuri* to optimize lipase production, oil biodegradation, and enzyme purification. The protein was purified using a phenyl sepharose CL-4B column equilibrated with ten bed volumes of equilibration buffer and washed with five bed volumes of the buffer. Elution was performed using a buffer with a similar composition, followed by ion-exchange chromatography. The phenyl sepharose CL-4B column resulted in 1.98-fold purification of the enzyme and a yield of 0.63%.

Gel filtration or size-exclusion chromatography is a technique in which the column is packed with a gel filtration matrix with a defined distribution of pore sizes that enables molecules to pass through the column along different paths based on size. The difference in the pore-size distributions of different gel filtration media constitutes the difference in their fractionation range. Thus, pore size is crucial to the separation performance. The main advantages of this method include the use of mild conditions, simple operation, isocratic elution, and easy scaling up [95].

Ayaz et al. [6] determined the lipolytic activity of a lipase obtained from *Streptomyces sp*. OC 119-7 using a sephacryl S100 HR (GE Healthcare) gel filtration column (ÄKTAprime™ plus). The elution fractions with the highest lipase activity were collected and submitted to ultrafiltration. The result was a 5.52-fold purification of the extracellular lipase, with a specific activity of 68.055 U/mg. Yong et al. [110] studied an extracellular lipase from *Botryococcus sudeticus* (UTEX 2629). The dialyzed filtrate with lipase activity was purified using size-exclusion column chromatography. The pure protein was separated from the crude enzyme extract after treatment with 15 to 65% ammonium sulfate precipitation and size-exclusion column chromatography.

Affinity chromatography is an adsorptive chromatography based on interactions with immobilized ligands, such as hormones and monoclonal antibodies, which bind to complementary receptors and antigens, respectively, in a highly specific manner. Bioaffinity chromatography is advantageous due to its extremely high selectivity [95]. This method can be applied in an early stage, but requires expensive affinity matrices. Thus, it is necessary to determine inexpensive materials for the purification of enzymes, especially in industrial applications [101].

Volpato et al. [104] obtained three different lipases from the crude extract of *Staphylococcus warneri* purified by specific lipase-lipase interactions using different lipases

(TLL, RML, PFL, BTL2) covalently attached to a solid support as the adsorption matrix. The hypothesis of this method is that the immobilization of a lipase on a support with the active center exposed to the medium would enable the adsorption of other lipase molecules.

Pauwels and Van Gelder [73] reported a new purification process for lipase A (LipA), which is an endogenous enzyme secreted by *Burkholderia glumae*. This affinity purification technique combined the specific binding scaffold of a lipase-specific foldase and the resistance of LipA to chemical denaturation. Trimukhe et al. [101] conducted a study on the purification of a lipase from *Aspergillus niger* NCIM 1207, in which trimellitic anhydride (TMA)-crosslinked deacetylated chitin adsorbed lipase selectively, with approximately fivefold purification of the crude lipase and a yield of 70%.

3.4.3 Aqueous two-phase systems

An aqueous two-phase system (ATPS) is considered an ideal purification technique for the separation, concentration and extraction of biomolecules due especially to the high productivity, simplicity, short processing time, scalability, cost effectiveness, and versatility of the system [88]. Other advantages of this technique include scale-up potential, process integration capability, the use of low-toxicity-forming chemicals, and biocompatibility [12].

ATPS consists of a combination of polymers, such as dextran, polyethylene glycol, polypropylene glycol, phosphates, sulfates, low-molecular-weight alcohols (ethanol and propanol), surfactants (n-decyl tetraethylene oxide and octylphenol ethoxylate) and/or ionic liquids (1-butyl-3-methylimidazolium hexafluorophosphate and 1-ethyl-3-methylimidazolium acetate) above their critical concentrations, which enables the formation of two phases. This technique is categorized into five main groups: polymer-polymer, polymer-salt, alcohol-salt, ionic liquid-based and micellar systems [12].

Ramakrishnan et al. [76] indentified an intracellular lipase from *Enterococcus faecium* MTCC5695, which was submitted to purification and concentration using ATPS, achieving activity recovery and a purification factor after ultrafiltration of 5.99% and 82.09%, respectively. Zhou et al. [115] analyzed the purification of porcine pancreatic lipase by ATPS involving polyethylene glycol (PEG 1500) and potassium phosphate (PEG 1500/potassium phosphate: 17%/13%) at pH 7.0 and an enzyme partition coefficient of 12.7, extraction efficiency of 94.7%, and purification factor of approximately 4.

Souza et al. [94] developed an ATPS involving tetrahydrofuran + potassium phosphate buffer (pH 7) for the purification of lipases. The optimum conditions for the

purification of an extracellular lipase obtained by SmF enabled a purification factor of 103.9 ± 0.9 and enzyme recovery of 96.4 ± 1.1%. Khayati and Alizadeh [47] studied fermentation conditions for *Rhodotorula glutinis* in an ATPS involving PEG and salts. Fermentation at pH 6.6 and 24 ºC with 17.5% PEG 4000 (w/w), 12.5% oxalate potassium (w/w) and 12.5% fermentation broth (v/w) proved to be the best system, with a purification factor of 13.9 and enzyme yield of 71.2% in the upper phase. Amid et al. [1] demonstrated a novel ATPS for a lipase from *Cucurbita moschata*. The system was composed of a surfactant and xylitol. The enzyme was recovered with a purification factor of 16.4 and yield of 97.4%. In addition, more than 97% of the phase components were also recovered and recycled.

3.5 Novel Purification Strategies

3.5.1 Reverse micellar system

Reverse micellar extraction is a liquid-liquid extraction method composed of water droplets stabilized by a layer of surfactant molecules within an organic solvent. This technique offers several advantages, such as ease of scale-up, low interfacial tension, and continuous mode of operation [64]. Since the insolubility of the substrates and lipase products hinder the characterization and manipulation of these enzymes in an aqueous system, the reversed micellar system seems to be a potential solution for barriers stemming from the heterogeneity of the medium [51].

Wang et al. [106] studied a filamentous fungus, *Rhizopus chinensis* (CCTCC 2010021), that demonstrated the ability to produce mycelium-bound lipase (mb-RCL) for the synthesis of ester in non-aqueous solutions. It has been reported that the mycelial aggregation during fungal growth leads to more mb-RCL production compared to free cell growth. Gaikaiwari et al. [31] found that the aerosol OT (bis 2-ethylhexyl) sodium sulfosuccinate)-isooctane system under optimized conditions led to 15-fold purification, 80% recovery and 2.5-fold concentration of a *Pseudomonas* lipase with a process time of 45 min. Nandini and Rastogi [64] applied reverse micellar extraction using a cationic surfactant (CTAB) for the separation and primary purification of lipase. The maximum activity recovery, extraction efficiency, and purification factor were 82.72%, 40.27%, and 4.09-fold, respectively.

3.5.2 Membrane processes

Membrane processes are used in clarification, reaction, and recovery schemes for the production of particles, molecules, and emulsions. The advantages of membrane systems include selectivity, high surface area per unit of volume, and the control of the level of contact

between two phases. Such processes have been used for bioseparation since before the start of the modern membrane industry. However, new modules, membranes, and systems have been developed over the last 20 years specifically to meet the requirements of the biotechnology industry [21].

The principle of membrane separation is based on the difference between the permeability of materials and substances and the driving force for the separation is given by the difference in the pressure, concentration, and electric potential [41]. When a product is obtained by fermentation, components of this solution need to be separated and purified. In such cases, integrated membrane systems can be used for continuous production and downstream separation [34].

Bhavya et al. [13] investigated lipase extraction from *Aspergillus niger* using a liquid emulsion membrane. The mechanism of enzyme transport through this technique consisted of three steps: 1) solubilization of lipase in reverse micelles, 2) transportation of the lipase-loaded reverse micelles through the liquid membrane, and 3) the release of lipase into the internal aqueous phase. Under optimum conditions, enzyme activity recovery and purification were 78.6% and 3.14 fold, respectively.

Yujun et al. [113] built a biphasic enzymatic membrane reactor by immobilizing lipase from *Candida Rugosa* onto the dense surface of a polysulfone ultrafiltration membrane, followed by cross-linking with a glutaraldehyde solution. This process greatly improved the reaction rate per unit of membrane area and extended the life of the enzyme. The highest reaction rate reached 0.089 μmol FFA/min cm^2 when the enzyme loading density was 0.098 mg/cm.

3.5.3 Extractive fermentation

The extractive fermentation technique consists of a two-phase system in which one of the phases synthesizes an extracellular product and the other enables the product to be recovered. This method reduces the number of steps required, since the process results in a single-unit operation and achieves the process objectives of two or more different stages, thereby contributing to the optimization and cost reduction of products [12].

The use of two aqueous phases in extractive fermentation is an attractive approach for obtaining high product yields and provides the product in a cell-free phase. Using this method, both small molecular weight and large molecular weight extracellular products have been recovered [107]. To substitute an organic solvent as the second phase, polymers are added

to the broth until two separate phases form. The phases are generally biocompatible and contain 85 to 95% water. Thus, microbial cells often remain in one phase, while the product of interest is distributed across the phases [41].

Pan et al. [69] investigated extractive microbial fermentation for the production of lipase by *Serratia marcescens* ECU1010 carried out in cloud point system composed of a mixture nonionic surfactants with a 4:1 ratio of Triton X-114 to Triton X-45 in an aqueous solution. The purified lipase had a concentration factor of 4.2 and purification factor of 1.3.

Ooi et al. [68] studied *Burkholderia pseudomallei* growth and lipase production using different types of two-phase partitioning systems. An aqueous two-phase system composed of PEG 8000 and Dextran T500 provided the best conditions for extractive lipase production. A high yield of 92.1% was recorded in the single batch operation. Repeated-batch fermentation was performed with the continuous replacement of the top phase every 24 h, which resulted in an average enzyme concentration of 16.5 U/ml for seven extractive batches over the course of 168 hours.

Show et al. [88] successfully produced lipase from *Burkholderia cepacia* and purified it using thermoseparating reagents in a single step through extractive fermentation. Repeated-batch fermentation was performed with the continuous replacement of the top phase every 24 h, resulting in an average cell growth mass of 4.7 g/L for 10 extractive batches over 240 h. Operating the system under optimized conditions, a high average production of 55 U/ml was found as well as a 14-fold purification factor, cell growth mass of 4.8 g/L, and 99% yield.

3.6 Industrial Applications

Table 3.3 presents the main lipase applications on the market. Among the currently used industrial enzymes, hydrolases, including proteases and lipases, remain the dominant type, which are extensively used in the biodiesel, dairy, and chemical industries. In non-aqueous media, lipases catalyze reversed ester-forming reactions, such as esterification, interesterification, and transesterification. [116]. These enzymes are used for diverse purposes, such as flavor development in dairy products, fat hydrolysis, the transesterification of fats and oils, ester synthesis, the production of chiral organic compounds, cleaning products, and the treatment of domestic and industrial waste [80].

Microbial lipases have significant potential in the food, biomedical, detergent, and chemical industries. In the food industry, these enzymes are typically used in the production of dairy products, baked foods, and fruit juices as well as the interesterification of fats and oils to

produce modified acylglycerols [32]. This sector is a major consumer of lipases and biocatalysts in general on the global enzyme market. Most of the synthetic activity of lipase is directed toward the production of food aromas that successfully mimic more complex natural aromas [99].

Table 3.3 - Examples of industrial applications of lipases

Application	**Species**	**Reference**
Biodiesel	*Burkholderia ubonensis* SL-4	[109]
Biodiesel	*Bacillus sp.*	[92]
Biodiesel	*Jatropha curcas L.*	[25]
Biodiesel	*Rhizopus chinensis*	[39]
Biodiesel	*Rhizopus oryzae*	[5]
Biodiesel	*Pseudomonas aeruginosa* AAU2	[15]
Biodiesel	Recombinant *Candida rugosa*	[50]
Bioremediation	*Aspergillus awamori* BTMFW032	[10]
Chemical Industry	*Candida antarctica*	[43]
Chemical Industry	*Pseudomonas aeruginosa*	[35]
Detergent Industry	*Hypocrea pseudokoningii*	[74]
Detergent Industry	*Staphylococcus aureus*	[11]
Food Industry	*Candida rugosa*	[99]
Food Industry	*Candida rugosa*	[66]
Food Industry	*Candida antarctica*	[18]
Pharmaceutical and Food Industries	*Candida antarctica* ZJB09193	[56]
Pharmaceutical and Food Industries	*Thermomyces lanuginosus*	[59]
Pharmaceutical Industry	*Pseudomonas stutzeri*	[75]
Pharmaceutical Industry	*Bacillus subtilis*	[46]
Pharmaceutical Industry	*Pseudomonas fluorescence*	[7]

An important industrial application of lipases is the modification of fats and oils to produce biodiesel and other structured lipids [89]. They are able to synthesize biodiesel in the

presence of high water content, which is a beneficial strategies when waster oils are used, since they usually contain a high amount of waters [112]. However, the presence of glycerol is the main disadvantage on biodiesel production by enzymatic catalysis [19]. To solve this problem, there are studies focusing on the production of biodiesel-like biofuels that keeps glycerol as monoglycerides. Specific lipases for the *sn*-1,3-position are used to form a biofuel composed by a mix of glycerol derivatives and fatty acid methyl esters (FAME) or fatty acid ethyl esters (FAEE). Therefore, it can prevents the need to separate the glycerol from the biodiesel and enhance the yield of the process [19, 29]. Furthermore, there is also a patented product named Ecodiesel-100 with have similar properties produced from the partial 1,3-regiospecific alcoholysis using pig pancreatic lipase (PPL)[17].

Some lipases have piqued the interest of pharmaceutical companies due to their therapeutic benefits and several drugs are currently approved or are in clinical trials for the treatment of cardiovascular disease, obesity, anxiety, inflammation, and pain [67]. The organic chemical and pharmaceutical use of lipases in the preparation of optically active compounds, such as pure alcohols, amines, and carboxylic acids, demonstrate that these enzymes can be used in practical synthetic methods [116].

The growing concern with regard to waste disposal and the preservation of the environment has led to the search for bioremediation techniques to ensure sustainable development. In this context, the use of extracellular enzymes that hydrolyze ester bonds, such as lipases, esterases, and proteases, is an attractive solution. These enzymes are able to enhance the bioremediation of effluents enriched with fats, oils, and proteins that are discharged by the dairy industry, restaurants, slaughter houses, hospitals, and health resorts [10]. Furthermore, lipases are the most widely employed enzymes in mediating reactions in organic synthesis due to their wide range of substrate selectivity, stability in organic solvents, and lack of a cofactor requirement [80].

3.7 Concluding Remarks

Lipases can be produced from various types of organisms. This review shows filamentous fungi are considered the best source of extracellular lipase for large scale. Improvements to fermentation conditions are the key to enhancing lipase production. Fungal species are preferably cultivated with SSF, whereas yeast and bacteria are cultivated with SmF, however, there are some exceptions. Besides, an attractive process is the use of whole-cell lipase is because it does not require purification or immobilization.

Downstream processing is also important step to study and it is necessary to search for techniques that unite the production and separation of the product with as few steps as possible in accordance with the desired use. After a detailed investigation, the choice of method depends on the characteristics of the lipase, composition of the extract, and cost of the process. The most practical technique is known as separation by precipitation using ammonium. Among chromatographic techniques, ion exchange is the most frequently employed method, but due to hydrophobic properties of lipases, hydrophobic interaction chromatography is also widely applied. Gel filtration chromatography has simple operation and easy scaling up whereas bioaffinity chromatography is extremely high selectivity, but requires expensive matrices. Moreover, ATPS is also an important purification technique for extraction of biomolecules due especially to its high biocompatibility, simplicity, scalability and low cost. Novel purification strategies have been studied, and reversed micellar system seems to be a potential solution for barriers stemming from the heterogeneity of the medium. Furthermore, new modules, membranes, and systems have been developed specifically to meet the requirements of the biotechnology industry. In addition, another potential purification strategy is extractive fermentation, which reduces the cost of products with a single-unit operation of both enzyme production and extraction.

These enzymes can be employed in a large number industrial processes. They are commonly used in the biodiesel, dairy, and chemical industries. In the food industry, these enzymes are typically used in the production of dairy products, baked foods, and fruit juices as well as the interesterification of fats and oils to produce modified acylglycerols. In addition, some lipases have been target of pharmaceutical companies due to their therapeutic benefits. Moreover, they are able to enhance the bioremediation of effluents enriched with fats, oils. Therefore, the market for lipases is significant, but the high costs remain the main obstacle to further expansion.

3.8 References

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CAPÍTULO 4 - MANUSCRITO "EXTRACTION AND PURIFICATION OF COLD-ADAPTED LIPASE FROM *METSCHNIKOWIA AUSTRALIS* CRM 1589 USING AQUEOUS TWO-PHASE SYSTEM (ATPS) COMPOSED BY PEG / SODIUM PHOSPHATE" A SER SUBMETIDO AO PERIÓDICO "BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY"

Extraction and purification of cold-adapted lipase from *Metschnikowia australis* CRM 1589 using aqueous two-phase system (ATPS) composed by PEG / Sodium Phosphate

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Abstract: Lipases (EC 3.1.1.3.) are ubiquitous enzymes with important industrial applications. The bottleneck of downstream process is the purification step, which mostly controls the costs of the process. An effort has been allies over the years to find purification techniques with viable costs, and aqueous two-phase system has advantages such as low cost, easy scale up and preserve the characteristics of enzymes. This report shows the partitioning of cold-adapted lipase from *Metschnikowia australis* CRM 1589 using aqueous two-phase system (ATPS) by polyethylene glycol and sodium phosphate. Parameters such as molecular weight of PEG, sample volume, salt and PEG concentrations were analyzed and optimized in in two experimental design sessions using response surface methodology (RSM). The best result for log of enzymatic partition coefficient in complete factorial design 2^5 was found using a 12% PEG 2000, 12% sodium phosphate, pH 7.8 and 1 mL enzyme extract system, where log was observed an enzyme partition coefficient 7.08, yield 60.7% and purification factor 0.55. However, the subsequent experiments were carried out at pH 7.4 to preserve lipase activity. Then, a central composite planning 2^3 was performed to optimize the significant parameters of system and the best conditions for purification of lipase. The new planning presented the coefficient of determination R^2 0.97, suggesting a predictive model of response surface to the enzymatic partitioning. The best conditions were found at the central points of the planning,

containing 15% of sodium phosphate salts and 0.75 mL of enzymatic extract and obtaining enzymatic partition coefficient of 5.75 and 5.66 and purification factor 1.18 and 1.37, respectively. Therefore, lipases partitioned preferentially into the PEG rich phase and method could be applied as an auxiliary method to isolate the enzyme from the other components of the fermentation extract.

Keywords: Lipase, Aqueous Two-Phase System, Response Surface Methodology

4.1 Introduction

The major microbial sources of cold active lipases are bacteria, yeast and filamentous fungi (1). Cold-active enzymes have high flexibility, resulting in excellent benefits for applications in biotransformations including the cosmetics industry, volatile substrates, and the pharmaceutical industry, especially on production of enantiomers, lipids and sugars (2). Since these enzymes require low activation energy and have capacity to possess high activity at low and moderate temperatures, there is a high potential for development of energy saving products (3). Recently, some of the main characteristics of lipases, such as activation in organic interfaces, accommodation to a wide range of substrates and the lack of cofactors, have attracted attention to commercial interest (4).

The development of efficient purification methods is essential and will allow high yields of final purification to be obtained since crude extracts of lipase preparations usually contain other proteins such as esterases and in many cases other lipases (5). The high costs of purification associated with chromatography have become a bottleneck in the biotechnology industry, especially in the case of large-scale processes. Therefore, there is a continuous and growing demand in the development of alternative technologies to improve downstream processing (6).

Since proteins are sensitive and, consequently, may be denatured by solvent, the use of conventional organic-aqueous solvent systems in protein purification is not a commonly and viable alternative (7). In this context, aqueous two-phase system (ATPS) is an excellent alternate solution for the processing of proteins, since they contain a high-water content and allows the unit operations of concentration clarification and partial purification of the target product can be achieved in a step (8). In addition, this system is suitable for purification especially to show advantages such as: continuous mode of operations, potential for scale on an industrial scale, low chemical toxicity and high biocompatibility (7).

A relevant feature of ATPS is its ease of scale-up, since the partition enzymes and proteins in general, not dependent on the concentration and volume of the system (8). The five main groups are polymer-polymer, alcohol-salt, polymer-salt, ionic liquid-based systems and micellar systems. Among them, polymer-salt systems are used for a wide variety of biomolecules, mainly proteins and particles (9). The well-executed manipulation of the partition coefficient of the partitioning system in two phases is what causes the separation of the target proteins from the other biomolecules. This coefficient can be altered by factors such as: the

type of ions in the aqueous phase; the ionic strength of the salt; adjustment of the average molecular weight of the polymers used, or by the inclusion of an extra salt such as, for example, NaCl (7). In this work, lipases from *Metschnikowia australis* CRM 1589 isolated from Antarctic environment were isolated by extraction in an aqueous two-phase system of polymer-salt type.

4.2 Material and Methods

4.2.1 Microorganism

Metschnikowia australis 6A-1C2II was isolated from marine sediment collected at Refugio 2 intertidal zone (2.4 °C) in the Admiralty Bay region, King George Island, South Shetlands Archipelago, Maritime Antarctica (62˚ 04.341'S 58˚ 25.233'W). The yeast is deposited at Central of Microbial Resource - CRM-UNESP (Biosciences Institute, São Paulo State University - UNESP, Rio Claro, SP, Brazil) under the accession numbers CRM 1589.

4.2.2 Fermentation conditions

The culture medium for lipase production was previously optimized. *Metscnikowia australis* CRM 1589 was incubated in substrate medium containing 2 g · L^{-1} peptone; 2 g · L^{-1} sodium nitrate; 2 g · L^{-1} urea; Yeast extract 4 g · L^{-1}; olive oil (2% v / v); Tween 80 (2% v / v); glycerol (2% v / v). The pH was adjusted with 1M HCl to 6.0 and the fermentation was taken in a 250 ml erlemeyer flask at 20 °C under agitation stirred (200 rpm) for 144 hours. After fermentation, the crude extract was centrifuged at 2000 g for 15 minutes. The supernatant was collected and used as lipase extract.

4.2.3 Lipase Activity and Total Protein Determination

Lipase activity tests were performed according to the methodology described (10). 3.8 mg of p-nitrophenyl palmitate (pNPP) were dissolved in 500 μL of dimethylsulfoxide (DMSO) and diluted to 20 mL of 0.05 M sodium phosphate buffer and pH 7.0 containing 5 gL-1 of Triton X-100. Then, 100 μL of the enzyme extract and 900 μL of substrate were incubated at 37 ° C for 10 minutes. The reaction was quenched by heat shock at 96 ºC for 1 minute in a water bath, followed by the addition of 1 mL of saturated sodium tetraborate solution. Samples were quantified by spectrophotometry at 405 nm and 25 °C. Blank was prepared by replacing the enzyme extract with the same amount of distilled water. Total proteins were quantified by spectrophotometry according to the methodology described by Bradford (11).

4.2.4 Aqueous Two-Phase System (ATPS)

Lipase extraction was conducted using the aqueous two-phase system composed by polyethylene glycol (PEG)/salts. The systems, with a total mass of 5g, were prepared from stock

solutions (50% w/w) of PEG 2000, 4000 and 6000 and stock solutions of sodium phosphate monobasic and bibasic (40% w/w). The enzymatic extract partially purified by ammonium sulfate precipitation was added to the system, followed by homogenization of the same by vortexing. After separation of the phases, aliquots of the upper and lower phases were analyzed when the amount of proteins and enzymatic activity present.

4.2.5 Experimental design

Optimization of lipase extraction using ATPS was conducted by two subsequent experimental designs. First, a 2^5+4 central points complete factorial design was performed, containing 36 trials. The variables studied were: molecular weight of PEG (MWPEG), sample volume (V. sample), salt concentration ([Salt]) and PEG concentration ([PEG]). Then, an analysis of significance of the studied parameters was carrying out in relation to the partition coefficient of the enzyme (Ke) and proteins (Kp). From the results found, a central composite planning 2^3 was performed to optimize the significant parameters of system.

4.2.6 Response Surface Methodology (RSM)

Statistical and data analyzes were evaluated by *Statistisca* software (v.7) using the response surface methodology tool.

4.2.7 Analytical Methods

The enzyme partition coefficient (Ke) and the protein partition coefficient (Kp) are given by the ratio of the total protein concentration in the upper phase (up) to the lower phase (lp):

$$\log K_e = \frac{activity_{up}}{activity_{lp}} \quad [1]$$

$$\log K_p = \frac{[protein]_{up}}{[protein]_{lp}} \quad [2]$$

The specific activity (S_a) was calculated as the ratio of lipase activity to the total protein concentration of the sample:

$$S_a = \frac{C_{lipase}}{C_{protein}} \quad [3]$$

Since the purification factor (PF) given as the ratio between the initial and final specific activities:

$$PF = \frac{S_{a_{initial}}}{S_{a_{final}}} \quad [4]$$

4.3 Results and discussion

4.3.1 Aqueous two-phase system (ATPS)

The aqueous two-phase system allows to separate the target protein in one phase and contaminant protein in the other step in a single step when the process parameters are optimized. Factors such as hydrophobicity, ionic strength of the salt used, molecular weight of the enzyme influence the partitioning and separation of proteins from the system. Table 4.1 shows the tests performed in the complete experimental factorial design. The factors studied (molecular weight of PEG, sample volume, salt concentration and PEG concentration) were evaluated in order to find the best partitioning and purification conditions of the enzyme by aqueous two-phase system. Log K_e> 0 means that the protein prefers the upper (more hydrophobic phase) and log K_e <1, the protein will mainly partition to the lower phase.

The 5% significance analysis and response surface methodology for complete factorial design showed that the pH, salt concentration and sample volume were significant and therefore influenced the parameters of protein partition, enzymatic partition and yield. The best result for log of enzymatic partition coefficient was found using a 12% PEG 2000, 12% sodium phosphate, pH 7.8 and 1 mL enzyme extract system, where log was observed an enzyme partition coefficient 7.08, yield 60.7% and purification factor 0.55. When the system was maintained under the same conditions and only the pH was changed to 6.2, similar values were found for the log enzyme partition coefficient (6.86); however, the yield was 100% and the purification factor was 1.19.

This demonstrates that electrochemical interactions are important in enzymatic partitioning behavior since opposing charges, which is generated by the ionization of salts in system, attract and tend to partition in a specific phase (9). Therefore, the ionic strength of the salts used (monobasic sodium phosphate and bibasic sodium phosphate) should be taken into account for separation of the molecule of interest. Zhou, Hu (12) used an aqueous two-phase system for the purification of porcine pancreatic lipase (PPL) from crude PPL using polyethylene glycol (PEG) and potassium phosphate. The system that showed the best results for the purification of the enzyme was in PEG 1500 / potassium phosphate (17% and 13%, respectively) at pH 7.0 to 4 °C. This system obtained an enzymatic partition coefficient of 12.7, a purification factor of approximately 4 and an extraction efficiency of 94.7%.

Table 4.1 - Full factorial planning 2^5.

Run	MWPEG	[PEG] (%)	[Salt] (%)	pH	Sample Volume (mL)	Log Kp	Log Ke
1	2000	12	12	6,2	0,50	0,61	5,97
2	6000	12	12	6,2	0,50	-0,65	6,27
3	2000	18	12	6,2	0,50	0,42	0,00
4	6000	18	12	6,2	0,50	0,15	-0,59
5	2000	12	18	6,2	0,50	0,37	0,00
6	6000	12	18	6,2	0,50	0,70	6,67
7	2000	18	18	6,2	0,50	0,85	6,50
8	6000	18	18	6,2	0,50	6,41	6,63
9	2000	12	12	7,8	0,50	-0,32	0,00
10	6000	12	12	7,8	0,50	0,39	-0,56
11	2000	18	12	7,8	0,50	0,30	0,00
12	6000	18	12	7,8	0,50	0,79	0,00
13	2000	12	18	7,8	0,50	0,21	0,00
14	6000	12	18	7,8	0,50	6,37	0,00
15	2000	18	18	7,8	0,50	0,25	0,00
16	6000	18	18	7,8	0,50	1,62	0,00
17	2000	12	12	6,2	1,00	0,53	6,86
18	6000	12	12	6,2	1,00	-0,15	0,00
19	2000	18	12	6,2	1,00	0,61	5,15
20	6000	18	12	6,2	1,00	0,25	6,82
21	2000	12	18	6,2	1,00	1,93	6,31
22	6000	12	18	6,2	1,00	0,62	0,00
23	2000	18	18	6,2	1,00	0,33	6,23
24	6000	18	18	6,2	1,00	-0,44	5,39
25	2000	12	12	7,8	1,00	0,11	7,08
26	6000	12	12	7,8	1,00	-0,11	6,20
27	2000	18	12	7,8	1,00	0,16	0,00
28	6000	18	12	7,8	1,00	1,32	0,00
29	2000	12	18	7,8	1,00	0,87	5,58
30	6000	12	18	7,8	1,00	0,84	0,25
31	2000	18	18	7,8	1,00	0,48	0,40
32	6000	18	18	7,8	1,00	0,96	0,00
33 (C)	4000	15	15	7,0	0,75	0,72	6,43
34 (C)	4000	15	15	7,0	0,75	0,73	5,39
35 (C)	4000	15	15	7,0	0,75	0,88	6,49
36 (C)	4000	15	15	7,0	0,75	1,03	6,27

The meshes formed by PEG favor the phenomena of molecular exclusion, and PEG protein attraction forces favoring the partition of protein in the upper phase. This factor is reinforced by increasing the length of the polymer chain. Thus, low molecular weight PEG

polymer containing systems may interact, depending on their characteristics, such as their molecular weight and hydrophobicity (13). Alhelli, Abdul Manap (14) investigated the partition and purification of alkaline extracellular lipase from *Penicillium candidum* by ATPS in which it consisted of 13.8% (w/w) phosphate buffer, 3.3% (w/w) NaCl and 9.2% (w/w) PEG 3000 at 25°C. Under these conditions, the partition coefficient found was 4.0 the purification factor was 33.9 and the yield was 84.0%. It is important to take into account that lipases are enzymes present in several organisms, with different molecular weights and amino acid and hydrophobicity chains. Thus, the best conditions will not always lead to similar results for the same enzyme, and case-specific studies are required in order to obtain a better separation.

The pH parameter was significant for the enzymatic partition coefficient ($p \leq 0.05$). However, parallel studies have shown that this enzyme has a good pH 7.4. In order to preserve lipase activity to the maximum extent, the subsequent experiments were carried out by fixing this value. Thus, response surface graphs aided in the choice of parameters to be used for central composite planning. The different levels of PEG concentration as well as their molecular mass in the system (MWPEG) were not significant variables and did not interfere in the partition coefficient with pH 7.4 (Figures 4.1 and 4.2). However, lower levels of molecular mass and PEG concentration implied an increase in the partition coefficient. As the best enzyme partition coefficient results are at the surface end (Figure 4.3), a step of shifting in the maximum slope direction was performed through central composite planning.

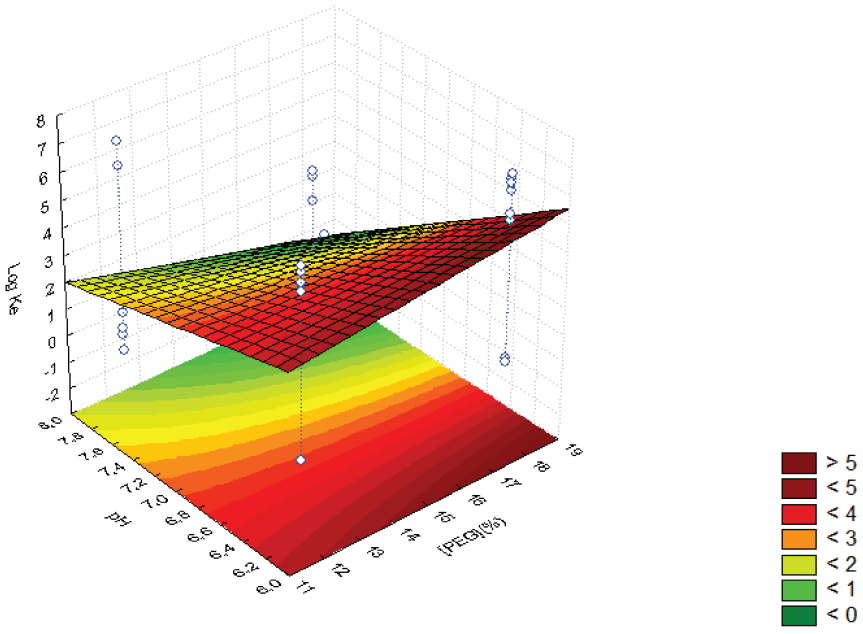

Figure 4.1 - Response surface graphic for interaction effects of PEG concentration (%) with pH on lipase enzymatic coefficient for PEG/sodium phosphate aqueous two-phase system

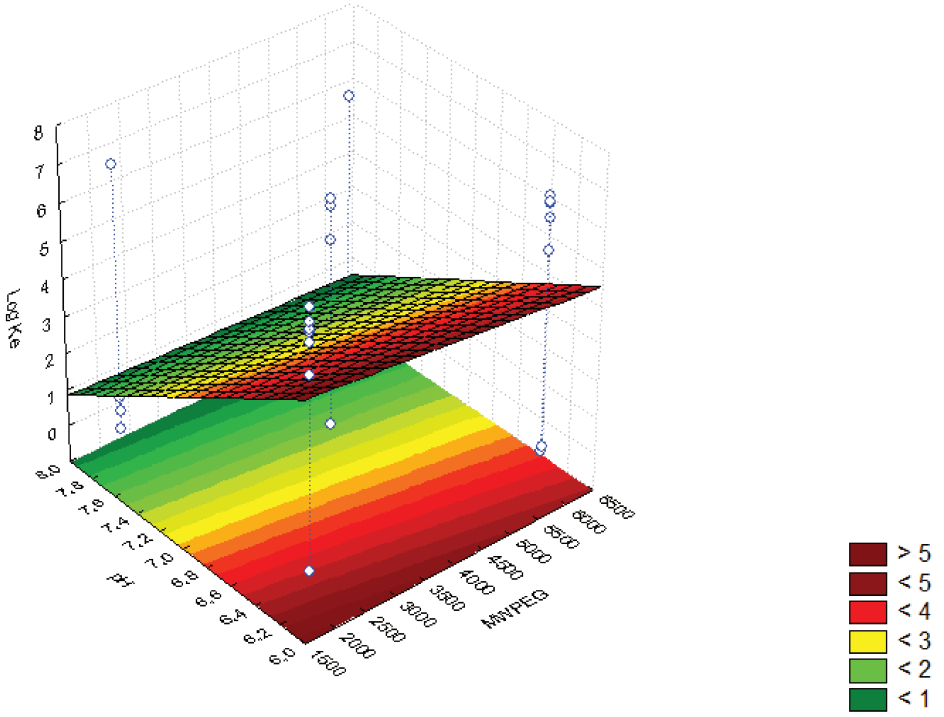

Figure 4.2 - Response surface graphic for interaction effects of the molecular mass of PEG (MMPEG) with pH on lipase enzymatic coefficient for PEG/sodium phosphate aqueous two-phase system

4.3.2 Response Surface Methodology (RSM)

From these analyzes, the composite central planning was drawn with the fixed values in 12% of PEG 2000 and pH 7.4. Thus, a central composite planning with four central points were conducted using as variables salt concentration (10.7% to 19.2%) and sample volume (0.4 to 1.10 mL) and PEG 2000 at 12% at pH 7.4. Table 4.2 presents the tests performed for central composite planning. The best conditions for purification of lipase were found at the central points of the planning (15% of sodium phosphate salts and 0.75 mL of enzymatic extract) obtaining enzymatic partition coefficient of 5.75 and 5.66 and purification factor 1.18 and 1.37, respectively. The new planning presented the coefficient of determination R^2 0.97, suggesting a predictive model of response surface were appropriate in relation to the enzymatic partitioning variable. The quadratic sample volume and salt concentration, both linear and quadratic, were significant ($p \leq 0.05$) for the enzymatic partition coefficient. Lee, Khoiroh (13) in a study of partition and purification of alkaline extracellular lipase from *Penicillium candidum* obtained results revealed that the most significant effect ($p \leq 0.05$) on the value of alkaline lipase partition coefficient was caused by the primary impact and quadratic concentration of PEG and salt.

Table 4.2 - Central composite planning 2²

Run	[Salt] (%)	Sample Volume (mL)	Log Ke	Log Kp
1	12	0,50	4,24	0,57
2	12	1,00	5,24	0,62
3	18	0,50	0,00	0,81
4	18	1,00	0,21	0,60
5	10,76	0,75	5,45	0,85
6	19,24	0,75	0,00	0,95
7	15	0,40	0,00	1,37
8	15	1,10	0,28	0,36
9 (C)	15	0,75	5,77	0,61
10 (C)	15	0,75	5,53	0,89
11 (C)	15	0,75	5,54	0,83
12 (C)	15	0,75	5,40	0,81
13 (C)	15	0,75	5,52	0,58
14 (C)	15	0,75	5,66	0,68

The response surfaces are graphical representations based on the regression model equation. These graphs allow the interactions between the tested variables to be evaluated for the purpose of determining the optimal response level (15). The contour and the three-dimensional interactions between the variables (Figures 4.4 and 4.5) showed an increase in the enzymatic partition and reduction of the protein partition coefficient in the central point region, as well as its purification factor. This can be explained by the fact that the effect of salting out entails the migration of the hydrophilic proteins to the lower phase, ignoring the meshes formed by the PEG, which controls the volume of the upper phase (14). However, high concentrations of salts do not tend to modify the surface tension of the system, implying alteration of the partition coefficient.

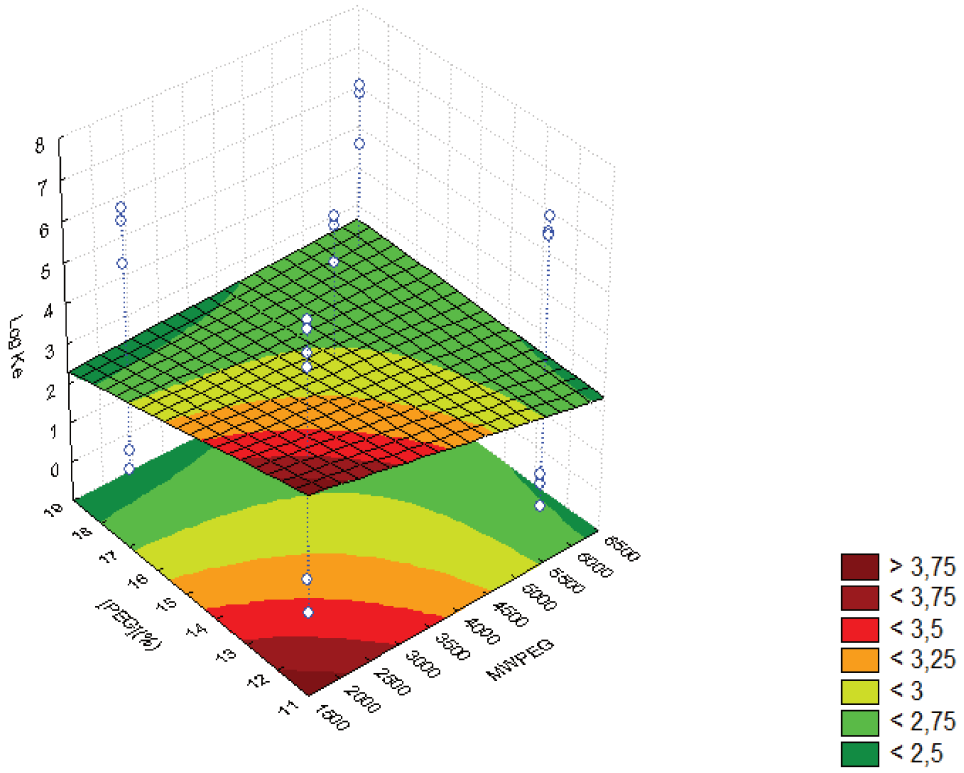

Figure 4.3 - Response surface graphic for interaction effects of molecular weight of PEG with PEG concentration (%) on lipase enzymatic coefficient for PEG/sodium phosphate aqueous two-phase system

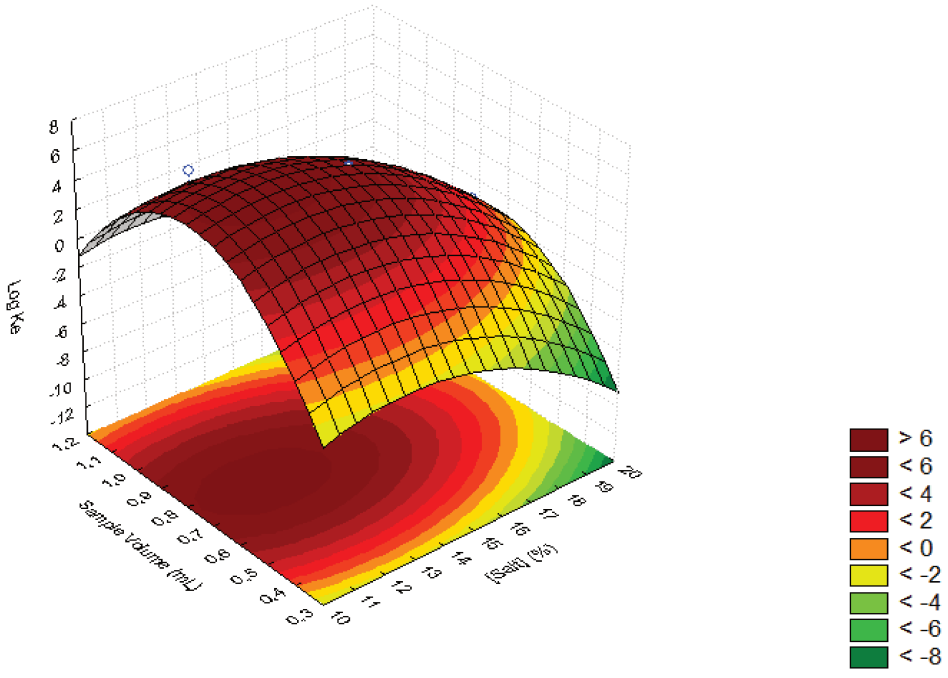

Figure 4.4 - Response surface graphic for interaction effects of sample volume with phosphate salts concentrations ([Salt]) on lipase enzymatic coefficient for PEG/sodium phosphate aqueous two-phase system

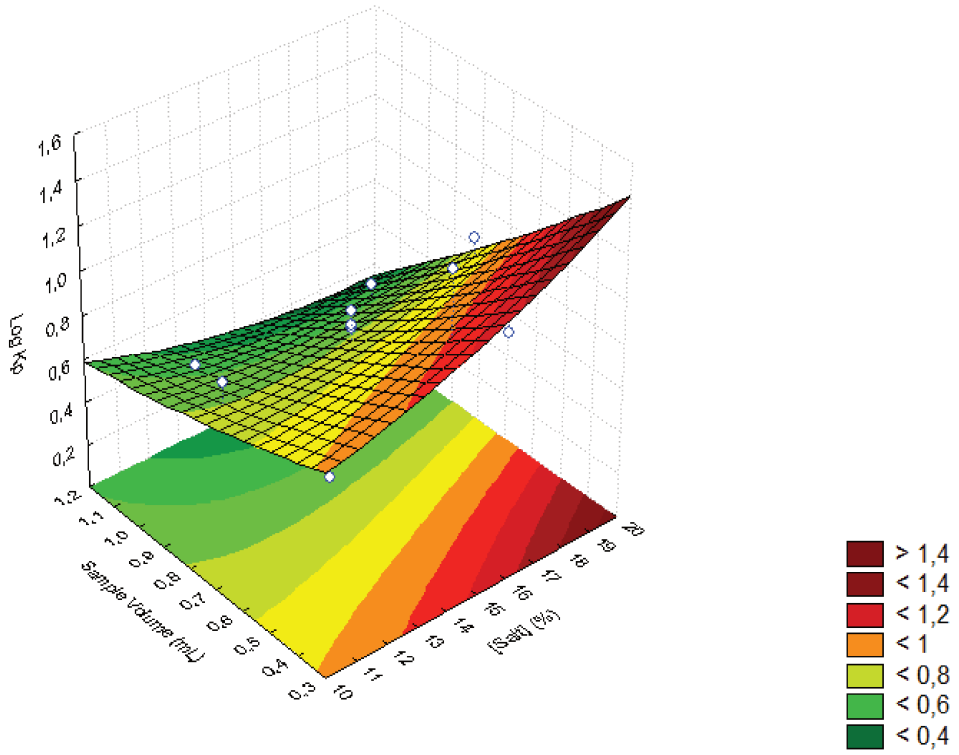

Figure 4.5 - Response surface graphic for interaction effects of sample volume with the phosphate salts concentrations ([Salt]) on protein coefficient for PEG/sodium phosphate aqueous two-phase system

4.4 Conclusion

Although purification of lipases from *Metschnikowia australis* CRM 1589 by ATPS using PEG/phosphate salts showed better enzymatic partitioning with pH 6.2, the extraction using its optimum pH was also led to the separation of biomolecule of interest of the other proteins (log Ke 5.75). Lipases partitioned preferentially into the PEG rich phase, with purification factor 1.37. This method could be applied as an auxiliary method to isolate the enzyme from the other components of the fermentation extract, since it does not modify its molecular structure and requires low cost. In addition to using existing methodologies to improve enzyme production, the use of statistical optimization of the constituents of the phases through MSR continues to be used due to its simplicity and practicality.

4.5 . Acknowlodgements

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CAPÍTULO 5 - MANUSCRITO "PURIFICATION OF COLD-ADAPTED LIPASE FROM *METSCHNIKOWIA AUSTRALIS* CRM 1589 USING AQUEOUS TWO-PHASE SYSTEM (ATPS) COMPOSED BY PEG / AMMONIUM SULFATE AND ITS BIOCHEMICAL CHARACTERIZATION" A SER SUBMETIDO AO PERIÓDIO "SEPARATION SCIENCE AND TECHNOLOGY"

Purification of cold-adapted lipase from *Metschnikowia australis* CRM 1589 using aqueous two-phase system (ATPS) composed by PEG / Ammonium Sulfate and its biochemical characterization

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Abstract: This is the first report in literature about biochemical characterization of lipases from *Metschnikowia autralis* CRM 1589. The enzyme was partially purified by ammonium sulfate precipitation 20% saturation and extracted using aqueous two-phases system (ATPS) polymer-salt composed by polyethylene glycol and ammonium sulfate. Parameters such as molecular weight of PEG, sample volume, salt and PEG concentrations were analyzed in both full factorial and star design of experiments. The lipase of *M. australis* partitioned towards PEG-rich phase. The best result of enzymatic partition coefficient was achieved using a system composed by 7.93% PEG 6000, 12% ammonium sulfate and 1 mL enzymewith log Ke = 5.89 and log Kp = -0.042, 60.7% yield and 3.36 fold-purification. The surface response methodology and ANOVA analysis were performed and coefficient of determination R^2 0.95 was found. Two bands of 97 kDa and 82 kDa approximately were observed. The enzyme showed alkaline characteristics with optimum activity at pH 7.4. Thermal stability was tested and relative activities above 80% were found at temperature range of 20-45ºC, with two peaks of optimum temperatures at 10ºC and 40ºC. Besides, no metallic ion interferences to the relative activity were observed, which is an important characteristic for potential industrial application.

Keywords: Lipase, Aqueous Two-Phase System, Response Surface Methodology

5.1 Introduction

Lipases catalyze the hydrolysis of triacylglycerols to glycerols and free fatty acids. Furthermore, they possess regio-, chemo-, and stereoselective properties, which make them one of the most important enzymes for industrial applications (1). The substrates of lipases are usually long chain triacylglycerols which are insoluble in water, however, due to their solubility in water, these enzymes can catalyze reactions in two types of systems, which are aqueous and organic medium. They can be found in animal, plant, and microbial sources. Except that only the latter can be produced at industrial levels. Therefore, microbial lipases represent the most widely used class of enzymes in biotechnological applications and organic chemistry (2). *Metschnikowia* is an important genus of the family *Metschnikowiaceae* of the order *Saccharomycetales.*

Cold-adapted enzymes have flexible structures: they show reduced activation enthalpy and negative entropy of activation compared to enzymes produced by mesophilic and thermophilic homologs. Due to these characteristics, structure, they have properties that could be useful in many biotechnological processes. The main benefits to the industries are the reduction of energy costs of the production processes carried out at moderate temperatures, since these enzymes are up to ten times more active than their mesophilic counterparts in low and moderate temperatures. Moreover, some characteristics may cause selective inactivation in the case of complex mixtures as well as temperature before the unfolding of the protein structure (3).

Although a large number of microbial lipases have been studied and evaluated for their biotechnological potential for applications in pharmaceuticals, food, textile, dairy, cosmetic and paper industry, their high costs of purifications is the bottleneck to restrict large-scale production. In this context, aqueous two-phases system is a practical and low-cost protein purification method. It is usually formed by mixing a polymer and salt or two polymers. Due to the high-water content and low interfacial tension of the systems, it is possible to preserve the biological activity and to avoid the denaturation of target compounds. In addition, the polymers used may also have a stabilizing effect (4).

Separation of target proteins is achieved by manipulating the partition coefficient by altering the average molecular weight of the polymers, the type of ions in the system, the ionic strength of the salt phase or by adding an additional salt such as NaCl. In order to optimize parameters such as partition, recovery and purification of proteins and enzymes in PEG-salt

ATPS, the of Design of Experiments (DoE) has been shown as a potential technique, obtaining promising and efficient results (5). Among them, Response Surface Methodology (RSM) is composed by a set of mathematical and statistical techniques useful for analysis and optimization of complex processes. By varying different factors simultaneously and performing a limited number of experiments, it is possible to study how the effects of these parameters can influence the responses. Therefore, this methodology is widely applied in different biotechnological processes. (6).

The characterization of enzymes is essential to direct their biotechnological application, since the several existing industrial processes require biocatalysts specific properties (7). In this paper, we report production, partial purification and characterization of lipase from *Metschnikowia australis* CRM 1589 isolated from Antarctic environment.

5.2 Materials and Methods

The microorganism *Metschnikowia australis* 6A-1C2II was collected from marine sediment at Refugio 2 intertidal zone (2.4 °C) in the Admiralty Bay region, King George Island, South Shetlands Archipelago, Maritime Antarctica (62° 04.341'S 58° 25.233'W). The yeast accession number is CRM 1589 and is storage at Central of Microbial Resource - CRM-UNESP (Biosciences Institute, São Paulo State University - UNESP, Rio Claro, SP, Brazil). *M. australi*s was incubated in substrate medium composed by 4 g· L^{-1} yeast extract; 2 g· L^{-1} sodium nitrate; 2 g· L^{-1} peptone; 2 g· L^{-1} urea; olive oil (2% v/v); Tween 80 (2% v/v); glycerol (2% v/v) and the pH was adjusted with HCl 1M to 6.0. The fermentation was performed in 250 mL Erlenmeyer flask at 20ºC under agitation shaker (200 rpm) for 144 hours. The crude extract was centrifuged at 2,000 G for 15 minutes and the supernatant was adopted as lipase extract.

5.2.1 Lipase Activity and Total Protein Determination

Lipase activity tests were determined as reported by Duarte et al. (2015), using p-nitrophenyl palmitate (pNPP) as lipase substrate. A solution composed by 3.8 mg of (pNPP) and 500 µL of dimethylsulfoxide (DMSO) was diluted to 20 mL of 0.05 M sodium phosphate buffer and pH 7.0 containing 5 g L-1 of Triton X-100. For lipase assays, 900 µL of substrate and 100 µL of the enzyme extract were incubated at 37 ºC for 10 minutes and subsequently stopped by thermal shock at 96 °C for 1 minute in a water bath. Then, 1 mL of saturated sodium tetraborate solution was added and samples were quantified by spectrophotometry at 405 nm and 25 °C. Total proteins were spectrophotometrically quantified followed by Bradford (8) method.

5.2.2 Ammonium sulfate precipitation

Precipitation by ammonium sulfate were performed according to the methodology previously described by Englard and Seifter (9). Solid ammonium sulfate was periodically added according to saturation percentage (20%, 40%, 60% and 80%) on lipase extract in cold bath at 4 ºC. The extract was mixture with magnetic agitation for 30 minutes and subsequently centrifuged at 2,000 g for 30 minutes at 4º C. The supernatant pellet was dissolved in 50 mM phosphate buffer (pH 7.0) and both suspended pellet and supernatant were used to analyze protein concentration and lipase activity.

5.2.3 Aqueous Two-Phase System (ATPS) Preparation

Aqueous Two-Phase system composed by politethyleneglicol (PEG)/ ammonium sulfate was used for lipase extraction. Stock solutions of PEG / sodium phosphate buffer at optimum enzyme pH (50% w/w) were prepared for use in the aqueous two-phase system. Relevant concentrations of PEG and salts were added to graduated test tubes and supplemented with enzyme pH optimum buffer. The system was homogenized and, finally, the partially purified extract samples were inserted, totaling 5g. The tube was sealed and then homogenized in vortex for 30 seconds. They were centrifuged at 1000g for 1 minute at 4 ºC for phase separation acceleration. Then, the volume of each phase was measured, and upper and lower samples were collected for partition analysis. The blank of each system was assembled by the addition of distilled water instead of sample.

5.2.4 Surface Response Methodology

Factors such as molecular mass of PEG, sample volume, salt concentration and PEG concentration were selected to be applied in an Experimental Design 2^4 with 4 center points in order to analyze the influence of these variables on enzyme partitioning. After identifying significant factors for extraction, a central composite planning with these parameters was performed. By using *Statistica* v. 07 software. Response surface graphs were evaluated for optimization of system parameters.

5.2.5 Analytical Methods

The enzyme partition coefficient (Ke) and the protein partition coefficient (Kp) ws calculated by the ratio of the total protein concentration in the upper phase (up) to the lower phase (lp):

$$K_e = \frac{activity_{up}}{activity_{lp}} \quad [1]$$

$$K_p = \frac{[protein]_{up}}{[protein]_{lp}} \quad [2]$$

The specific activity (S_a) was given by the ration of lipase and protein concentration:

$$S_a(U/mg) = \frac{C_{lipase}}{C_{protein}} \quad [3]$$

Purification factor (PF) was determined from initial and final specific activities:

$$PF = \frac{S_{a_{initial}}}{S_{a_{final}}} \quad [4]$$

The yield (Y%) was determined by using phase volume ratio V_R enzymatic partition coefficient (Ke):

$$Y(\%) = \frac{100}{1+\left(\frac{1}{V_R * K_e}\right)} \quad [5]$$

5.2.6 Biochemical characterization of partially purified lipase

5.2.6.1 Determination of molecular weight

The molecular weight of both purified and crude extract lipases were analyzed using dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were analyzed using a 15% polyacrylamide gel, according to according to Laemmli (10). The protein molecular markers were phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (54.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and a-lactalbumin (14.4 kDa). The gel was loaded with 5µL of samples, conduced to electrophoresis at a constant current of 150 V and stained with silver.

5.2.6.2 Effect of pH and temperature on lipase activity

The effect of pH on lipase activity was carried out by incubating reaction mixture with diferentes pH (2-11) buffers and analyzing enzyme activity. The buffers were citrate-phosphate (pH 2-8), phosphate (6-8), Tris-HCl (8-9), carbonate (9-10) and phosphate-NaOH (11). For temperature effects, the enzyme was induced to ranging temperatures (0-95ºC) and lipase assay was measured.

5.2.6.3 Effect of pH and temperature on lipase stability

The effect of pH on lipase stability was observed by incubating the enzyme for 30 minutes at its optimum temperature in the same buffers described on item 2.7.2. Then, the residual activity was measured. Lipase thermal stability was achieved by incubating the

partially purified enzyme with its optimum pH at a temperature range (0-95 ° C) for 30 minutes, and the enzymatic activity was measured.

Residual activity was calculated by the ratio between enzymatic activity at time zero (A_o) and the final activity after 30 minutesA_{30}.

$$Residual\ activity\ (\%) = \frac{A_o}{A_{30}} \times 100 \quad [6]$$

Relative activity was calculated by the ratio between the residual enzymatic activity of the point ($Residual\ activity_x$) and the highest residual activity value reached ($Residual\ activity_h$):

$$Relative\ activity\ (\%) = \frac{Residual\ activity_x}{Residual\ activity_h} \times 100 \quad [7]$$

5.2.6.4 Effect of metal ions on enzyme activity

The effects of different metal íons (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, K^{+} and Cu^{2+}) on partially purified enzymes were investigated. The salts were dissolved at 5 mM in optimal pH buffer for enzymatic activity and equal volumes of enzyme and ionic solution were incubated for 30 minutes at the optimum temperature. Then, the enzymatic activity was measured, and relative activity was calculated.

5.2.6.5 Determination of kinetic parameters

Different substrate concentrations of (pNPP) were used to determine Michaelis-Menten kinetics constants (K_m) and maximum reaction rate (V_{max}) of partially purified lipase. The final concentration ranged from 0.019 mmol L^{-1} to 0.19 mmol L^{-1}. The kinetics parameters were calculated according to Lineweaver-Burk equation plots.

5.3 Results and Discussion

5.3.1 Ammonium sulfate precipitation

The crude extract from *Metschnikowia australis* CRM 1589 was used for enzyme precipitation by ammonium sulfate. Purification parameters from crude extracts by ammonium sulfate precipitation are summarized in Table 5.1. The supernatant fraction exhibited 82.77% and 15.34 fold purification. However, Barbosa, Souza (11) reported the excessive increase of purification factor on supernatant due to interference caused by the presence of ammonium sulfate. Thus, the best condition was found at 20% saturation, achieving 3.59 lipase fold purification, 61.76% enzyme recovery and a specific activity of 12.18 U mg^{--1}. Similar results of enzyme recovery (59.5%) was found with Lipases from *Talaromyces thermophilus*

ammonium sulfate 60% saturation (12), however, *Aspergillus niger* lipase was partially purified with 90% saturation, resulting of 99% recovery and 4.29-fold (13).

Table 5.1 - Ammonium sulfate precipitation results

$(NH_4)_2SO_4$ Precipitation	Activity (UmL^{-1})	Total Protein ($mg\ mL^{-1}$)	Specific Activity ($U\ mg^{-1}$)	Enzyme Recovery (%)	Purification Fold
Crude Extract	7.12	2.10	3.39	-	-
20%	4.40	0.36	12.18	61.76	3.59
40%	2.63	1.02	2.57	36.89	0.76
60%	0.49	0.15	3.35	6.89	0.99
80%	0.40	0.16	2.47	5.57	0.73
Supernatant	5.89	0.11	52.02	82.77	15.34

5.3.2 Aqueous two-phase system (ATPS)

A full factorial design 2^4 was performed by varying the following parameters: Molecular Mass of PEG (MMPEG) 2000 to 6000; PEG concentration 10% (w/w) as lower level to 20% (w/w); ammonium sulfate salt concentration of 8% (w/w) to 16% (w/w) and sample volume of pre-purified lipase extract (0.5 to 1 mL). The system was carried out at pH 7.4. These parameters resulted in a design with 16 runs and 4 center points (Tables 5.2 and 5. 3). Enzyme partition analysis indicated the system composed by 10% of PEG 6000, 16% ammonium sulfate and 1 mL of sample offered the best lipase/protein separation, with enzyme partition in lower phase (log Ke = -0.36) and proteins on top (log Kp = 0.44). From these results, it can be explored the possibility to obtained higher enzyme purity in the top phase. However, yield activity (31.55%) as well as the purification factor (0.15) had low values. On the other hand, the system composed by 20% of PEG 6000, salt concentration at 16% and 1mL of sample resulted an activity yield 83.10%, 1.10 fold-purification and log Ke = 6.14. Moreover, system 3 (20% PEG 6000, 8% salt and 0.5 mL of pre-purified extract) showed similar values of log Ke and yield, although the purification factor of system 16 was considerably higher, indicating a better extraction. The presence of high values of log Kp suggest the need to optimize the system to find the best separation conditions. Lipase tends to partition in the PEG-rich phase. Analyzes of interactions with $p \leq 0.05$ (Figures 5.1 and 5.2) indicated that PEG concentration and the interaction between MMPEG with the salt concentration were significant for both enzymatic partition and yield parameters. From these results, it is possible to observe that an increase in MMPEG implies greater partition of lipases. Nevertheless, the extractions performed with the values of the central points did not showed satisfactory responses. Higher values of both MMPEG and salt concentration showed better values on yield parameter. In addition, sample volumes of 1mL were presented as the best quantity for extraction. Thus, the values of 1mL for

sample and PEG with molecular mass 6000 were fixed for the optimization of the system by central composite design (CCD).

Although both phases of the ATPS are rather hydrophilic, the PEG-rich phase is more hydrophobic. This favors the partition of hydrophobic proteins towards that particular phase (14). The log Kp > 0 means that the protein prefers the top (more hydrophobic phase) and for log Kp < 0 the protein will mostly partition to the bottom phase. High concentrations of PEG led to an increase in the Kp coefficient, indicating that some of the proteins migrated to the upper phase of the system. This can be explained by the increased hydrophobicity caused by the presence of PEG. This increase may lead proteins with hydrophobic characteristics to partition in the upper phase. However, purification *Rhodotorula glutinis* lipase by PEG/oxalate potassium ATPS showed Ke decreases as the PEG molecular weight increases (15). Separation of proteins can be successfully achieved by manipulating the partition coefficient, which could be added an additional salt such as NaCl (14). Alhelli, Abdul Manap (16) evaluated partitioning and purification of alkaline extracellular lipase from *Penicillium candidum* using PEG/NaCl ATPS. Their optimum aqueous two-phases system had 13.8% (w/w) phosphate buffer, 9.2% (w/w) PEG 3000 and 3.3% (w/w) NaCl and obtained a 33.9-fold purification factor, a partition coefficient of 4.0 and the yield was found to be 84.0%.

The optimization process of the significant factors is achieved by using response surface methodology (RSM) with a steepest ascent approach. The RSM technique gives the possibility to determine optimal operating conditions and significant independent factors as well as their interactions with the dependent responses in complex multivariate systems. In steepest ascent methodology, the response changes along the path of maximum increase in the predicted response. Usually, the central composite design is employed to predicting the optimal conditions of the system, obtaining the minimum or maximum of a response surface. Therefore, it can be applied PEG–salt ATPS, considering the partitioning of biomolecules and, consequently, designing complex processes that occur in the ATPS, promoting the optimization of these systems (5, 17). However, the shift towards the maximum concentration gradient for both PEG concentration and salt concentration could not be applied due to the insolubility that ATPS exhibited when high concentrations of both reagents in the same system were added.

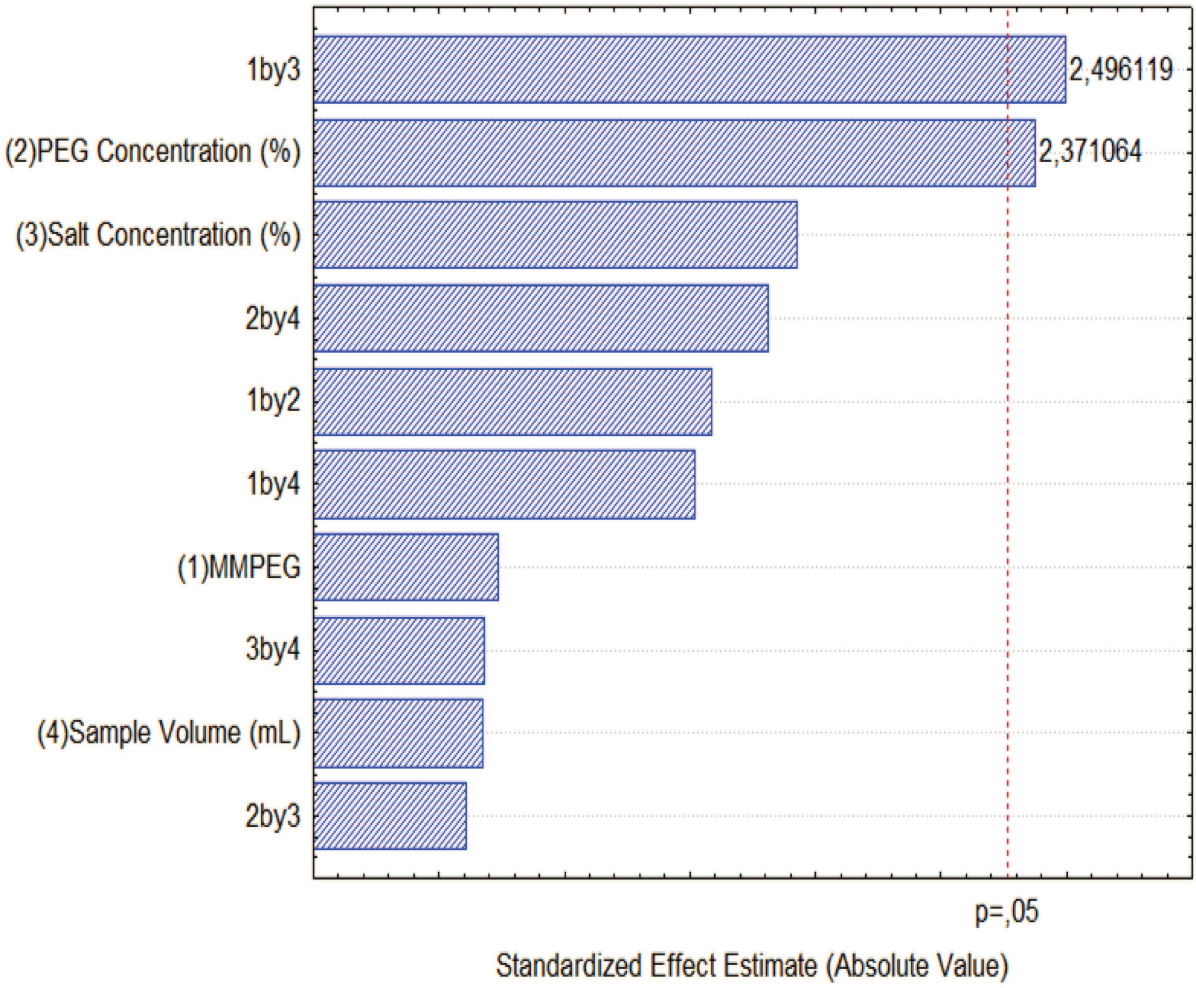


Figure 5.1 Pareto Chart of Standardized Effects ($p \leq 0.05$) with log Ke parameter as dependent variable

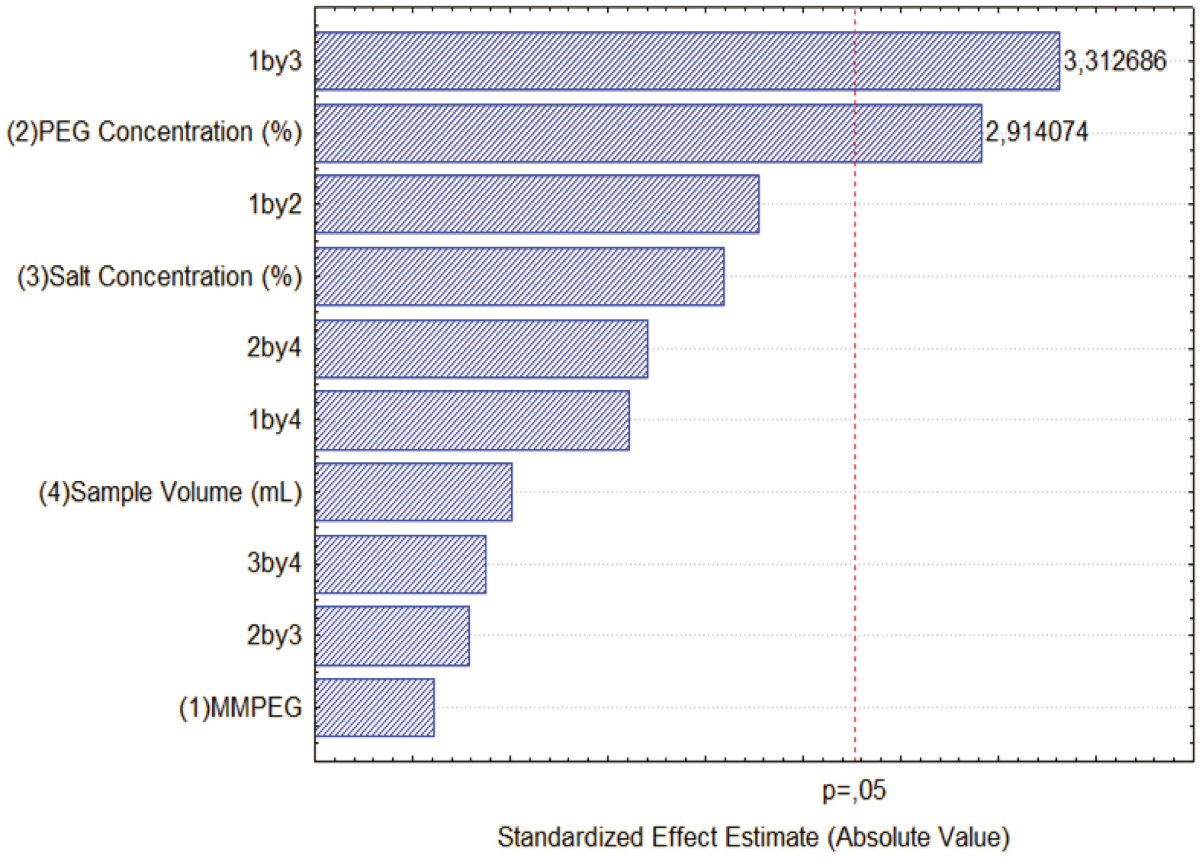


Figure 5.2 Pareto Chart of Standardized Effects ($p \leq 0.05$) with Yield parameter as dependent variable

Thus, the composite central design 2^2 was delimited from the significant factors ($p \leq 0.05$) for enzymatic partition coefficient and extraction yield found in full factorial design. The studied variables were concentration of PEG 6000 (10 to 20%) and ammonium sulfate concentration (8-16%) in the system. The star planning presented 14 runs, with 6 repetitions in the central point. From the 5g systems assembled, the activity, yield, purification factor, enzyme partition coefficient and protein values were evaluated (Table 5.4). The values found at the central point indicated good results, with yield values up to 81.06%, log Ke = 5.5 and Kp = 0.499. However, the best system for extraction of *Metschnikowia australis* CRM 1589 lipase was reached using PEG 6000 (7.93% w/w) and ammonium sulfate (12% w/w), showing approximately 68% yield activity, 3.36-fold purification, log Ke = 5.89 and log Kp = -0.042. These results indicated selectivity of protein partition in the lower phase, while the enzyme tends to stay in PEG-rich phase.

Table 5.2 - Full Factorial design 2^4 parameters

Run	MWPEG	PEG Concentration (%)	Salt Concentration (%)	Sample Volume (mL)
1	2000	10	8	0.5
2	6000	10	8	0.5
3	2000	20	8	0.5
4	6000	20	8	0.5
5	2000	10	16	0.5
6	6000	10	16	0.5
7	2000	20	16	0.5
8	6000	20	16	0.5
9	2000	10	8	1
10	6000	10	8	1
11	2000	20	8	1
12	6000	20	8	1
13	2000	10	16	1
14	6000	10	16	1
15	2000	20	16	1
16	6000	20	16	1
17 (C)	4000	15	12	0.75
18 (C)	4000	15	12	0.75
19 (C)	4000	15	12	0.75
20 (C)	4000	15	12	0.75

Response surface graphs (Figures 5.3 and 5.4), showed an increase of the ammonium sulfate concentration in system led to the increase of both the partition coefficient of the lipase to the upper phase and the yield of the extraction. However, PEG concentration did not appear to significantly interfere in ATPS ($p < 0.05$) Analysis of significance of the parameters presented linear interactions between PEG and salt concentration as well as linear PEG concentration were significant for yield while linear and quadratic salt concentrations were significant for both. The ANOVA table, based on the Fisher distribution (F test) was assembled by fixing log Ke as dependent factor (Table 5.5). The model presented $F_{calc} = 32.89$, and the Fisher distribution table with degrees of freedom 5 and 8 ($p = 0.05$) was found to be $F_{critical} = 3.68$. This means that the model is significant at $p \leq 0.05$ and does not follow Fisher's normal

distribution. Therefore, it can be applied to validate the model. The mathematical model is shown in Eq. (1), and a coefficient of determination (R^2) of 0.95 and an adjusted coefficient of determination (R^2 adjusted) 0.92 are presented in equation: $Z = 5{,}4706462106131 + {,}082755961073794 \times \text{PEG C.} - {,}21709659698156 \times \text{PEG C.} + 2{,}4562993575629 \times \text{Sal C.} - 1{,}3581757843874 \times \text{Salt C.}^2 - {,}31678616643488 \times \text{PEG C.} \times \text{Salt C.}$

Table 5.3 - Full Factorial Design 2^4 results

Run	Lipase Activity UP	Lipase Activity LP	Yield (%)	Purification Factor	Log Ke	Log Kp
1	0.00	0.00	0.03	0.00	0.00	0.38
2	0.00	0.00	0.00	0.00	0.00	0.55
3	0.35	0.00	81.93	0.40	5.54	0.66
4	0.00	0.00	0.02	0.00	0.00	1.26
5	0.00	0.00	0.00	0.00	0.00	0.87
6	0.89	0.00	68.84	3.47	5.95	1.15
7	0.00	0.00	0.00	0.00	0.00	1.48
8	0.08	0.00	78.13	0.36	4.89	0.94
9	0.00	0.00	0.00	0.00	0.00	0.88
10	0.00	0.00	0.00	0.00	0.00	0.59
11	0.01	0.00	89.00	0.01	4.05	0.80
12	0.00	0.00	0.00	0.00	0.00	0.17
13	0.00	0.00	0.00	0.00	0.00	0.84
14	0.04	0.09	31.55	0.15	-0.36	0.44
15	0.11	0.00	79.14	0.20	5.06	1.44
16	1.39	0.00	83.10	1.02	6.14	1.62
17 (C)	0.00	0.00	0.00	0.00	0.00	0.44
18 (C)	0.00	0.00	0.00	0.00	0.00	0.41
19 (C)	0.00	0.00	0.01	0.00	0.00	0.34
20 (C)	0.00	0.00	0.01	0.00	0.00	0.50

Table 5.4 - Central composite 2^2 design and results

Run	PEG C. (%)	Salt C. (%)	Activity UP	Activity LP	PF	Yield (%)	Lok Ke	Log Kp
1	10	8	0.000	0.000	0.000	0.000	0.000	0.298
2	10	16	1.027	0.000	2.659	85.738	6.011	3.930
3	20	8	0.269	0.006	1.191	74.849	1.632	0.242
4	20	16	2.378	0.000	1.350	85.914	6.376	0.328
5	7.929	12	0.792	0.000	3.361	68.052	5.899	-0.042
6	22.071	12	0.090	0.000	0.083	83.770	4.955	1.489
7	15	6.343	0.000	0.000	0.000	0.000	0.000	0.522
8	15	17.657	1.948	0.000	2.091	78.090	6.290	1.069
9 (C)	15	12	0.251	0.000	1.079	80.195	5.399	0.465
10 (C)	15	12	0.294	0.000	1.143	81.394	5.468	0.499
11 (C)	15	12	0.323	0.000	1.002	79.496	5.510	0.495
12 (C)	15	12	0.319	0.000	1.120	81.061	5.503	0.489
13 (C)	15	12	0.276	0.000	1.063	79.533	5.440	0.459
14 (C)	15	12	0.319	0.000	1.152	76.525	5.504	0.482

Table 5.5 - ANOVA results for Ke as dependent variable

Effect	Square Sum (SS)	Degrees of freedom (df)	Mean Square (MS)	$F_{calculated}$	$F_{critical}$
Regression (R)	62,69	5	12,54	32,89	3,69
Error (r)	3,05	8	0,38		
Total	65,74	13	-		

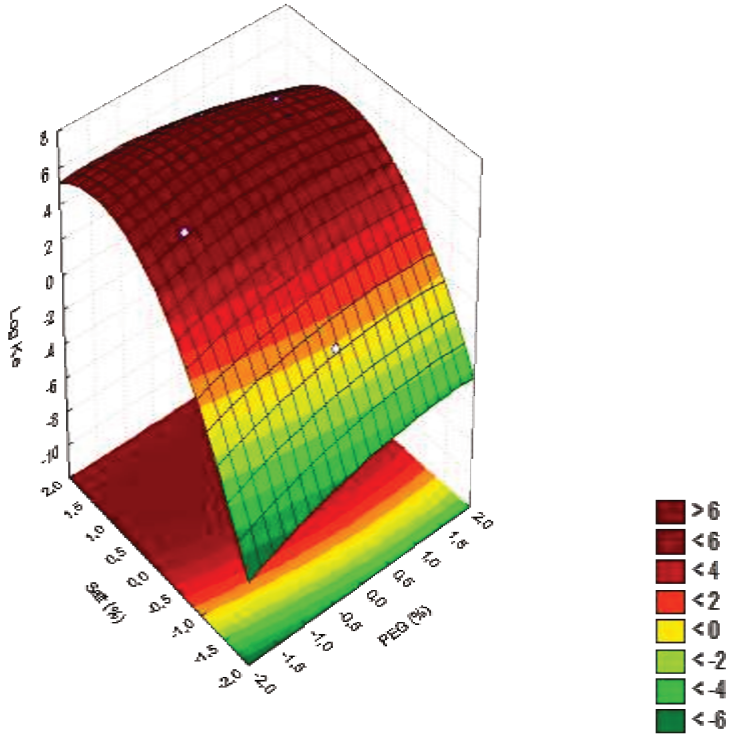


Figure 5.3 - Response surface graphic for interaction effects of PEG concentration ([PEG]) with ammonium sulfate salts concentrations ([Salt]) on enzyme partition coefficient for PEG/$(NH_4)_2SO_4$ ATPS

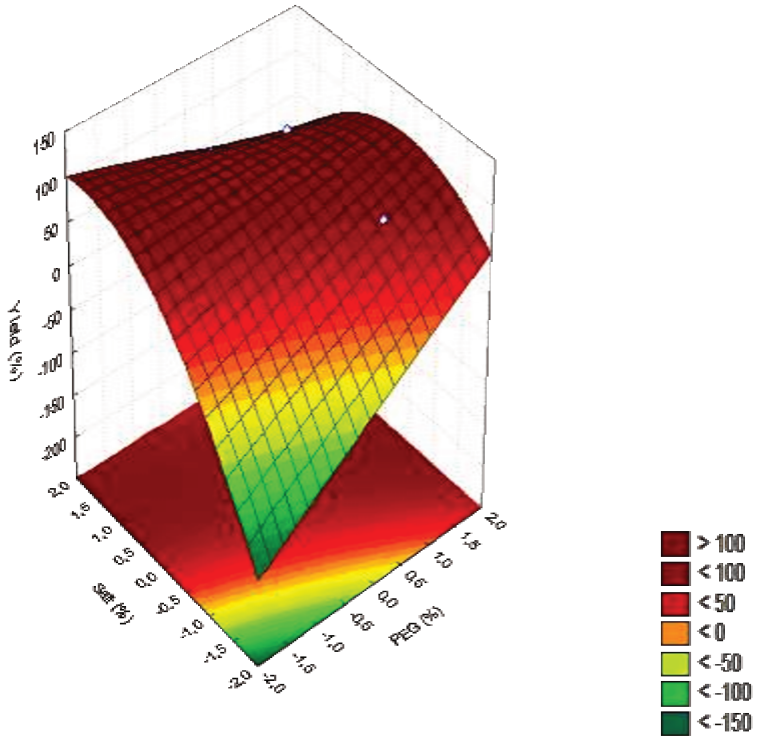


Figure 5.4 - Response surface graphic for interaction effects of PEG concentration ([PEG]) with ammonium sulfate salt concentration ([Salt]) on yield for PEG/$(NH_4)_2SO_4$ ATPS

5.3.3 SDS page

The molecular weight of target protein was determined by comparing it with a standard protein marker (Figure 5.5). This analysis indicated the enzyme were successfully separated by ATPS and lipase tends to partitioning in PEG-rich phase. Two bands were found, with approximately 97 and 82 kDa, respectively and may indicate the presence of more than one type of lipase produced by this *M. australis.* Yeast. *G. candidum* produces at least four isozymes (18). Antarctic *Pseudomonas* AMS8 protein band was observed around 65 kDa (19). The purified lipase from marine *Aspergillus awamori* exhibited molecular mass of approximately 90 kDa (20).

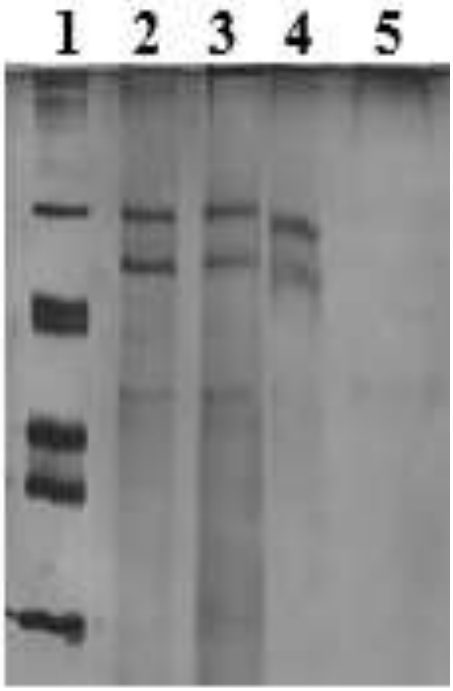


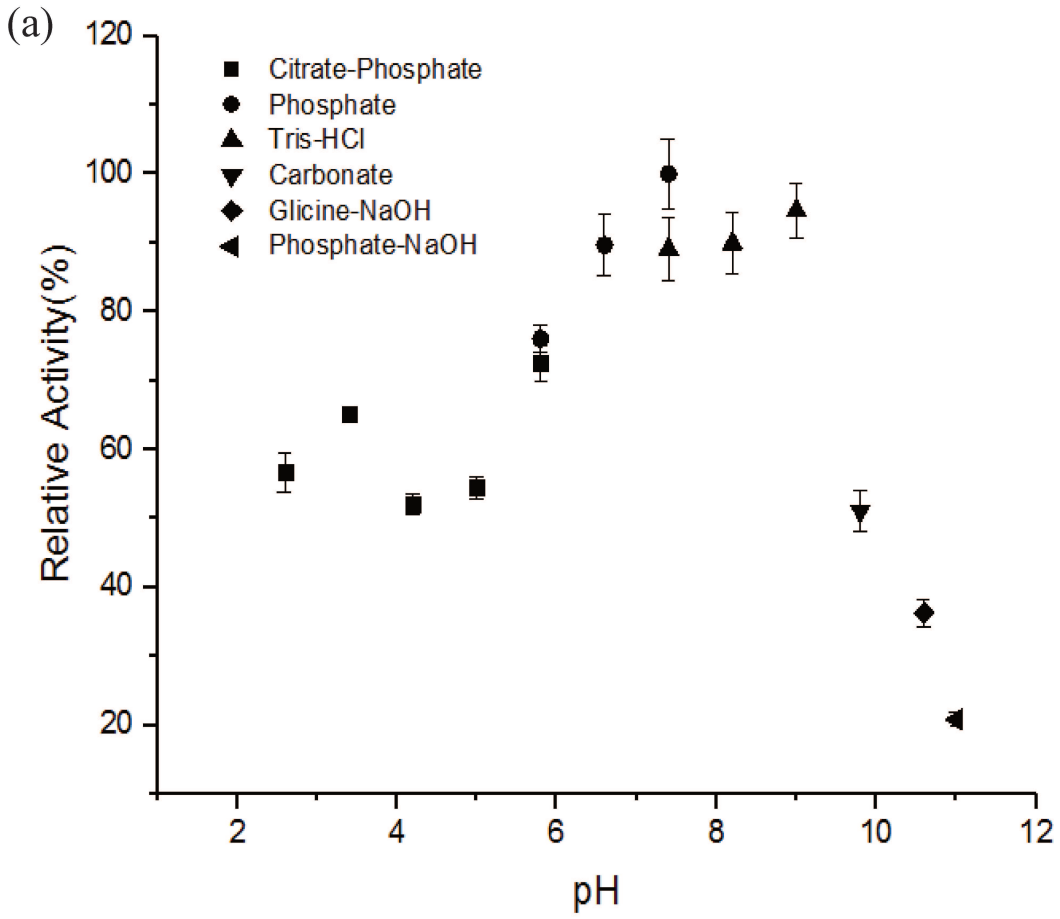
Figure 5.5 - SDS Page. Lane 1: Molecular marker; Lane 2: crude extract; Lane 3: precipitation with $(NH_4)_2SO_4$ 80% saturation; Lane: 4 PEG/$(NH_4)_2SO_4$ ATPS upper phase; Lane 5: PEG/$(NH_4)_2SO_4$ ATPS lower phase

5.3.4 Effect of pH and temperature on lipase activity and stability

Optimal pH and temperature for *M. australis* lipase activity and stability were identified (Figure 5.6). The enzyme showed good hydrolyzing activity in a pH range from 5.8 to 9.0 with relative activities of 76.1± 1.9% and 93.8 ±3.4%, respectively, however the optimum pH for its activity was pH 7.4 using 50 mM phosphate buffer. In spite of the psychrotrophic nature, this enzyme remains active between 2.0 ^{o}C and 80.0 ^{o}C and showed two main peaks temperature at 10ºC and 40ºC, which is in agreement with the two bands found in the SDS page analysis and corroborates with the possibility of isoenzymes. Okino-Delgado and Fleuri (21)

also found two optimal temperatures (20ºC and 70ºC) to lipases from orange juice byproduct, suggesting the presence of isoforms of lipases. Cold-adapted enzymes usually exhibit high catalytic activity at temperatures ranging from 0 to 30 ºC (19). *Bacillus pumilus* isolated from Antarctic soil samples produced lipases with optimum temperature and pH of 40ºC and pH 9 (22).

The enzyme showed great stability in the pH range from 7.0 to 9.8 with relative activities above 90%. For thermal stability, the best result was carried out at 10ºC. However, it is important note residual lipase activity above 80% were achieved at temperatures range from 20.0 ^{o}C to 45.0 ^{o}C, which is an advantageous characteristic for its potential application in industrial processes.



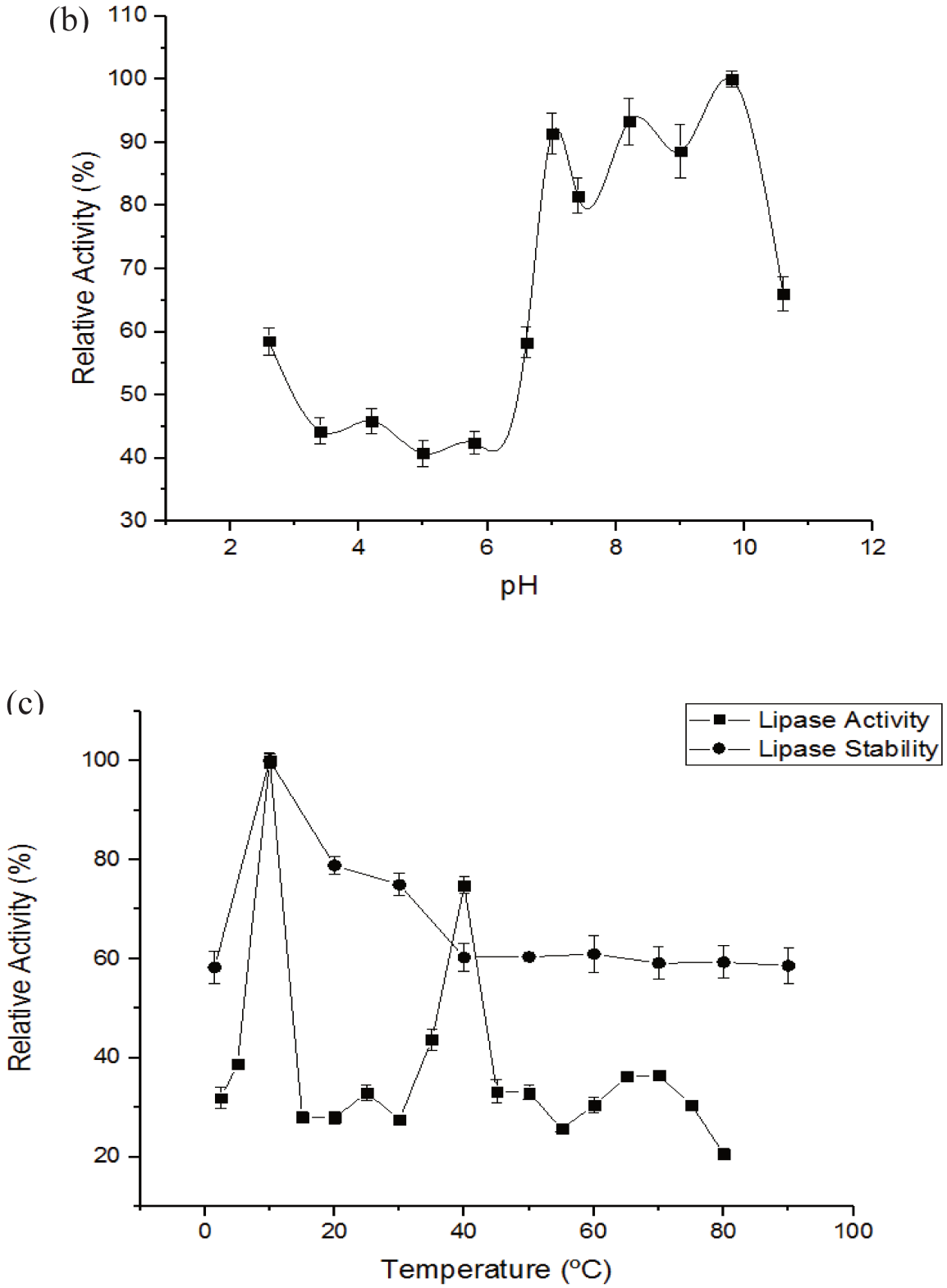


Figure 5.6 - Effect of different pH on lipase activity (a), stability (b) and thermal activity and stability (c) on partially purified lipase from *M. australis* CRM 1589.The experiments were performed in triplicate

5.3.5 Effect of metal ions

The influence of metal ions on lipase stability was evaluated by incubating equal volumes of partially purified enzyme extract and 5 mM of the salt in sodium phosphate buffer (pH 7.4) for 30 minutes at 40 °C. Lipase activity (Figure 5.7.) was increased in the presence of Zn^{2+} (114.81% and 116.22%) and Mg^{2+} (117.6 %) while decrease with K^{+} (89.49%), Mn^{2+} (92.49%) and Cu (93.16). Furthermore, Fe^{3+} (104.48%), Fe^{2+} (106.45%) Ion Ca^{2+} (106.78%) showed a slight increase in the concentration studied. Similar results were found in orange juice byproducts lipases, which recorded the increase of activity in the presence of Zn^{2+} (115%) e Mg^{2+} (122%) and decrease with Cu2+ (approximately 90%) (21). Ramakrishnan, Goveas (7) also found maximum stimulation for lipase from *Enterococcus faecium* in the case of Mg^{2+}. Compatibility with metal ions is an important character for lipase application in detergent industry (23).

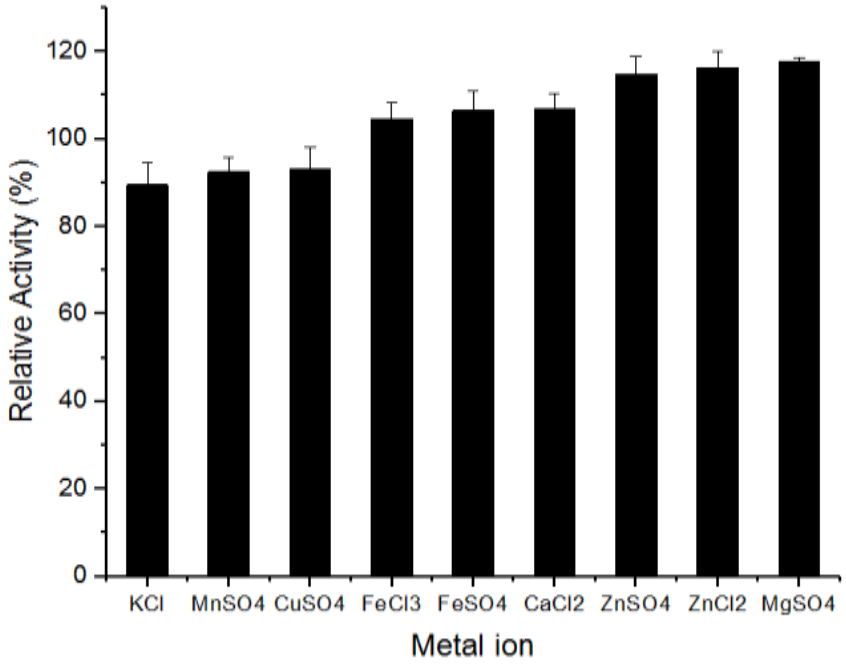


Figure 5.7 - Effect of different metallic salt on lipase stability. Equal volumes of enzyme extract and phosphate buffer (pH 7.4) with 5 mM of metal for 30 minutes at 40ºC. The experiments were performed in triplicate

5.3.6 Kinetic parameters

Kinetic studies provide information on substrate affinity to the enzyme. The concentration of substrate at which half the active sites are occupied is called K_M and this parameter gives a measure of the substrate concentration required for significant catalysis. The fastest velocity that given amount of enzyme can operate is known as Vmax i.e. this parameter

gives the maximum initial velocity when all active sites of the enzyme are occupied by substrate. (23). Applying Lineweaver-Burk plot (Figure 5.8), the values of Vmax and K_M found were 104.17 µmol $min^{-1}mg^{-1}$ and 0.052 mM, respectively. Low K_M value indicates the higher affinity of enzyme towards substrates (24). The K_M was lower and Vmax was similar to free *Botryosphaeria ribis* lipase (Km = 0.37mM; Vmax = 106.8 µmol min^{-1} mg^{-1}) (25) and lower than *G. candidum* lipase and *C. rugosa* using p-nitrophenylbutyrate (p-NPB) (26), indicating *Metschnikowia australis* CRM 1589 lipase have high affinity to p-NPP substrate.

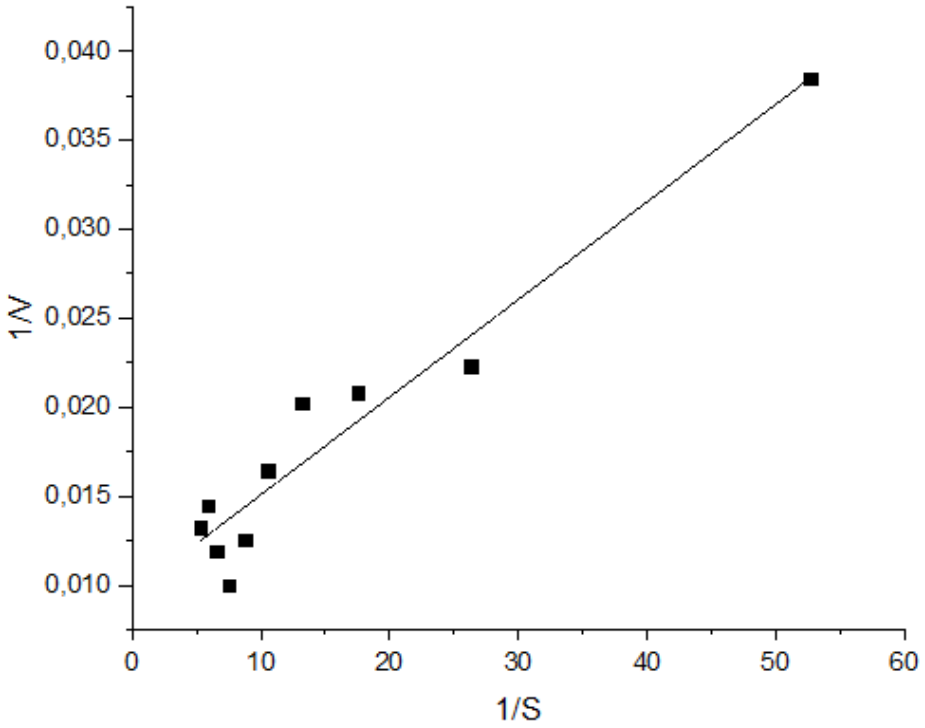


Figure 5.8 - Effect of different concentration of pNPP (0.019 mM – 0.19mM) on the activity of lipases from *M. australis* kinetic parameters were calculated based on of Michaelis-Menten plots

5.4 Conclusion

This is the first report at the present moment in literature of partially purification and biochemical characterization of lipases from *Metschnikowia australis* CRM 1589. The results indicated purification by ammonium sulfate precipitation coupled to ATPS allows the enzyme extraction from fermentation broth. Optimization by response surface methodology showed to be a useful tool to delimited contour plots and find the best conditions for extraction, with more than 80% yield, with log Ke = 5.89 , log Kp = -0.042 and 3.36 fold-purification. The SDS page technique indicated two bands of 97 and 82 kDa approximately, and two optimal

temperatures (10 and 40ºC) were registered, which may indicated this species produces isoforms of lipase. Moreover, purified lipase exhibited stability at alkaline pH and high temperatures. It was observed low inhibition against metallic ions, with potential application interest in industrial processes. However, it is important to explore new extraction approaches by changing salt, polymer or type of system in order to obtain better efficiency for purification parameters and conferring to this enzyme with interesting properties, the capability of industrial applications.

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CAPÍTULO 6 - CONCLUSÃO

A utilização de enzimas frio adaptadas provenientes de microrganismos isolados de ambientes em condições extremas apresenta grande potencial para aplicações industriais, sendo de grande importância pela sua identificação e purificação. Pela primeira vez na literatura, lipases de *Metschnikowia australis* foi purificada por sistema bifásico aquoso e caracterizada bioquimicamente.

Foi possível realizar a concentração enzimática pela técnica de precipitação por sulfato de amônio, onde os melhores resultados foram obtidos com 20% de saturação.

A extração de lipase de *M. australis* por sistema bifásico aquoso do tipo polímero-sal mostrou-se como uma alternativa viável de purificação. A utilização de PEG 2000 com fosfato de sódio e PEG 6000 com sulfato de amônio gerou coeficientes de partições enzimáticas consideráveis e fatores de purificação de aproximadamente 3 vezes. É importante ressaltar que as lipases são produzidas por diferentes fontes de organismos, e, consequentemente, apresentam características de tamanho, massa e hidrofobicidade distintas. Sendo assim, é importante a adequação do sistema para cada caso específico a fim de se obter melhores resultados de separação.

A aplicação do planejamento de experimento para otimização de extração por sistema bifásico mostrou-se como uma ferramenta de grande relevância na busca pelas melhores condições do processo. Para esta enzima, as diferentes interações que a massa molecular de PEG e força iônica do sal promoveram respostas específicas de significância estatística distintas para os dois sistemas polímero-sal estudados. A utilização da superfície de resposta permitiu delimitar os pontos ótimos para fatores de purificação e coeficientes de partições.

Durante os testes de temperatura ótima, dois picos de altas atividades foram registrados, e por meio da a técnica de SDS–Page, foi possível observar a presença de duas bandas de, aproximadamente 97 e 82 kDa, sugerindo que a levedura *M. australis* produz isoformas de lipase. No entanto, estudos de atividade lipolítica dessas regiões lipolítica (por exemplo, zimograma) são necessários para confirmação desta teoria. A enzima purificada apresentou característica alcalina e boa estabilidade em uma ampla faixa de temperatura, além da exposição de diferentes íons metálicos sobre a enzima não promover inibições significativas. Estas características conferem potencial para futuras aplicações industriais, especialmente em processos que envolvam oscilação de temperaturas.

A realização da integração dos processos de produção e purificação, por meio da fermentação extrativa, mostrou-se viável, com os melhores resultados sendo atingidos no quinto dia de fermentação. No entanto, existe a necessidade de estudos mais aprofundados sobre os parâmetros que envolvem esse processo, como tipo de sistema bifásico aquoso e os componentes empregados, bem como suas concentrações ideais para atingirem melhores resultados de produção e separação do produto de interesse de outras partículas do meio.

CAPÍTULO 7 - SUGESTÃO DE TRABALHOS FUTUROS

É importante a busca de novas alternativas de sais e até mesmo uma possível inserção de detergentes ou outros polímeros no sistema bifásico aquoso para extração da enzima. Embora os resultados encontrados sugerem a partição enzimática satisfatória, ainda é

possível encontrar melhores resultados de rendimento e fatores de purificações mais elevados, segundo a literatura, para maior sucesso da purificação.

Além disso, a fermentação extrativa demonstrou ser viável para a extração de lipases. Sendo assim, novos estudos envolvendo a otimização das variáveis envolvidas no processo, a possível execução de batelada alimentada, bem como a potencial aplicação de reagentes que não prejudiquem o meio ambiente e reciclagem de fases (ciclo), são pontos fundamentais no êxito da integração de produção e purificação enzimática para redução de gastos do processo, *scale up* e potencial aplicação industrial.

CAPÍTULO 8 - REFERÊNCIAS BIBLIOGRÁFICAS

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CAPÍTULO 9 - APÊNDICE A – Fermentação Extrativa

Após a otimização da extração por sistema bifásico aquoso, o melhor resultado encontrado foi selecionado. Sendo assim, o meio básico de cultura, contendo 0,2% peptona, 0,2% nitrato de sódio, 0,2% (p/p) de ureia, 0,4% (p/p) de extrato de levedura, 2% (p/v) de azeite de oliva, 2% (p/v) tween 80 e 2% (p/v) de glicerol. As fermentações extrativas foram realizadas em frascos de Erlenmeyer de 250 ml com 50 g de meio de fermentação contendo o sistema bifásico aquoso composto por 20% (p/p) de PEG 6000 e 8% (p/p) de sulfato de amônio, onde a massa final foi atingida pela adição de tampão fosfato de sódio. O meio foi esterilizado e o microrganismo foi inoculado. A fermentação ocorreu por 144 horas a 25ºC e sob agitação de 200 rpm. A cada 24 horas, um frasco foi retirado e deixado em descanso para separação de fases durante 30 minutos, onde o volume das fases foi mensurado. Então, uma alíquota de cada fase formada foi retirada para quantificação de proteínas e atividade enzimática no meio.

A atividade específica (A_e) foi calculada pela razão de concentração da atividade lipolítica (C_{lipase}) pela concentração de proteínas da amostra ($C_{proteína}$):

$$A_e(U/mg\ de\ proteína) = \frac{C_{lipase}}{C_{proteina}} \quad [1]$$

Sendo assim, o fator de purificação (FP) foi dados pela razão entra as atividades específicas inicial e final:

$$FP = \frac{A_{e\,inicial}}{A_{e\,final}} \quad [2]$$

O log do coeficiente de partição enzimática (K_e) foi obtido pela razão entre as atividades enzimáticas (U) encontradas nas fases superior (*fs*) e inferior (*fi*).

$$\log K_e = \frac{[atividade\ enzimática]_{fs}}{[atividade\ enzimática]_{fi}} \quad [3]$$

O rendimento da extração da lipase (Y) na fase superior foi calculado de acordo com a seguinte equação (Ooi *et al.*, 2011):

$$Y(\%) = \frac{100}{1+[\,{}^{1}/_{(V_R \times K_e)}]} \quad [4]$$

onde V_R é dado pela razão entre os volumes das fases superior e inferior.

Devido ao fato da fermentação convencional ocorrer a pH 6 e a atividade enzimática ótima ter sido registrada em pH 7,4, ambos foram testados nas fermentações extrativas. Para

ambos ensaios, os melhores resultados de partições enzimáticas (Fig. 1) e fatores de purificação (Tabela 1) foram alcançados no quinto dia de fermentação. As melhores condições para o meio em pH 6 envolveram log de coeficiente de partição enzimática igual a 8,63, rendimento de 82,76% e fator de purificação de 1,29 vezes. Já os ensaios em pH 7,4 apresentaram valores superiores, sugerindo que 5 dias de fermentação promoveram fator de purificação 1,61 vezes, rendimento de 86,8% e log de coeficiente de partição enzimática igual a 9,59. Isso demonstra que, apesar da otimização prévia do meio de cultivo ter utilizado pH 6, o pH ótimo da enzima para a integração de produção e purificação forneceu melhores resultados. A integração dos processos de produção e purificação envolve o uso de uma única operação unitária capaz de substituir duas ou mais operações. Consequentemente, o número total de etapas de recuperação e purificação pode ser diminuído e o rendimento pode aumentar sem comprometer a pureza do produto, uma vez que se essa etapa visa alcançar objetivos de processos semelhantes (Benavides *et al.*, 2011).

Diferentes sistemas aquosos bifásicos já foram testados para integração da produção e purificação de lipases de diferentes microrganismos. Pan *et al.* (2010) estudaram fermentação extrativa para produção de lipase por *Serratia marcescens* ECU1010 utilizando sistema de ponto de nuvem composto por agentes tensoativos não-iônicos com uma proporção de Triton X-114 para Triton X-45 4:1 em solução aquosa, onde o fator de purificação 1,3 vezes da lipase foram alcançados no processo.Ooi *et al.* (2011) utilizaram sistema de duas fases aquosas composto por 9,6% (p/p) de PEG 8000 e 1,0% (p/p) Dextran T500, forneceu as melhores condições para a produção de lipase extrativa de *Burkholderia pseudomalle*, gerando rendimento próximo ao encontrado neste estudo (92,1%).

A queda nos valores registrados no sexto dia de fermentação podem ser explicados pelo fato do aumento da concentração celular, juntamente com o alto peso molecular do PEG, causam elevação na viscosidade da fase superior, limitando a transferência de oxigênio e, assim, retarda o crescimento celular juntamente com a produção da lipase. Sendo assim, é possível observar que a fermentação extrativa para lipase de *M. australis* levou à separação da enzima dos demais componentes do meio, no entanto, há necessidade de otimização das condições tanto de cultivo quanto de composição do sistema bifásico para aprimoramento da técnica a fim de se obter uma melhor extração.

Figura 1: Log de coeficiente de partição enzimática (log Ke) no processo de fermentação ezxtrativa com pH 6 e pH 7,4.

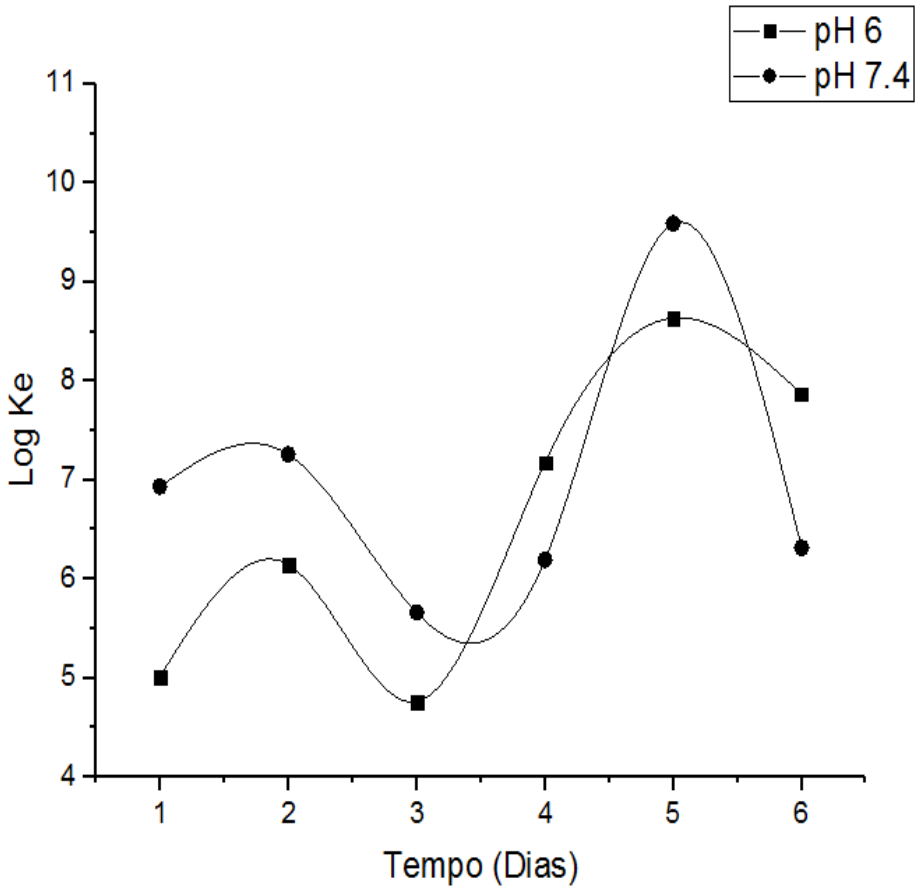

Tabela 1: Parâmetros de extração encontrados durante a fermentação extrativa em pH6 e pH 7,4

Tempo (Dias)	pH 6		pH 7,4	
	Fator de Purificação	Rendimento Enzimático (%)	Fator de Purificação	Rendimento Enzimático (%)
1	0,94	87,53	1,06	81,25
2	1,10	79,35	1,24	87,99
3	0,89	82,64	1,11	77,97
4	1,22	76,13	1,01	91,77
5	1,29	82,76	1,61	86,48
6	0,96	81,38	1,19	77,82

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