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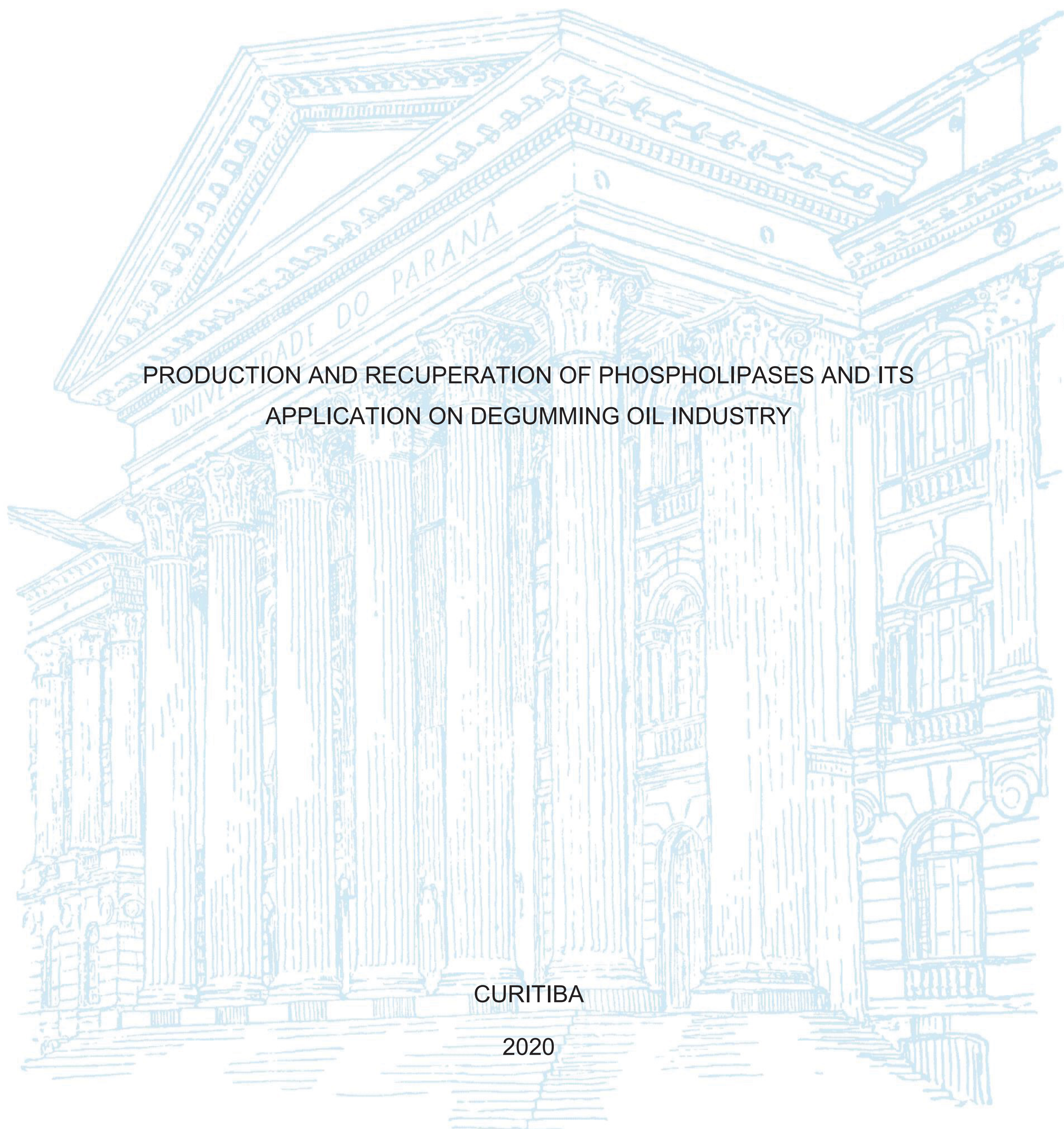

PRODUCTION AND RECUPERATION OF PHOSPHOLIPASES AND ITS APPLICATION ON DEGUMMING OIL INDUSTRY

CURITIBA

2020

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PRODUCTION AND RECUPERATION OF PHOSPHOLIPASES AND ITS APPLICATION ON DEGUMMING OIL INDUSTRY

Dissertação apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do grau de Mestre em Engenharia de Bioprocessos e Biotecnologia.

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CURITIBA

2020

Catalogação na Fonte: Sistema de Bibliotecas, UFPR
Biblioteca de Ciência e Tecnologia

R278p Razera, Giovanni

Production and recuperation of phospholipases and its application on degumming oil industry [recurso eletrônico] /Giovanni Razera. – Curitiba, 2018.

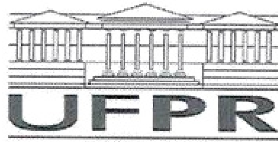
Dissertação - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, 2018.

Orientadora: Luciana Porto de Souza Vandenberghe. Coorientador: Carlos Ricardo Soccol.

1. Biodiesel. 2. Óleos vegetais como combustível. 3. Ácidos graxos. 4. Transesterificação. I. Universidade Federal do Paraná. II. Vandenberghe, Luciana Porto de Souza. III. Soccol, Carlos Ricardo. IV. Título.

CDD: 665.37

Bibliotecária: Vanusa Maciel CRB-9/1928



MINISTÉRIO DA EDUCAÇÃO
SETOR TECNOLOGIA
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **GIOVANNI RAZERA** intitulada: **PRODUCTION AND RECUPERATION OF PHOSPHOLIPASES AND ITS APPLICATION ON DEGUMMING OIL INDUSTRY**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 06 de Abril de 2018.

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RESUMO

A busca por uma sociedade *ecofriendly* torna o biodiesel o combustível do futuro, devido a sua disponibilidade e biodegradabilidade. Óleos de diversas origens são as matérias primas para a produção do biodiesel, especialmente aqueles oriundos de oleaginosas (soja, palma e canola). O óleo contido nestas sementes é composto, majoritariamente, por fosfolipídeos (também conhecidos por gomas). Estes fosfolipídeos podem causar problemas para os motores de combustão devido ao teor de fósforo e reduzem a taxa de produção do biodiesel devido ao sequestro de ácidos graxos. Fosfolipases agem sobre fosfolipídeos, clivando-os. Elas são utilizadas na indústria do refinamento de óleo, para reduzir o teor de fósforo, que oxida o óleo. Na indústria do biodiesel, as fosfolipases são empregadas para liberar mais ácidos graxos, aumento o rendimento da reação de transesterificação. Atualmente, há fosfolipases que são utilizadas no processo de degomagem de óleos. Tais enzimas são produzidas e comercializadas sob altos custos. Logo, o objetivo deste projeto é produzir tais enzimas de forma alternativa, para viabilizar a possibilidade das indústrias se beneficiarem da degomagem enzimática de óleo. A linhagem *Aspergillus brasiliensis* LPB 266 foi selecionada dentre outras 73 linhagens e produziu fosfolipase com atividade de 8200 U/mL após 7 dias de fermentação, atingindo produtividade de 48,81 U/mL.h, em meio de cultivo contendo lecitina de soja como indutor. Micro e ultrafiltração foram utilizadas para a purificação da fosfolipase. A determinação de massa molecular apontou enzima com massa molecular entre 30 e 55 kDa. Boas perspectivas são esperadas para a aplicação e degomagem de óleos.

Palavras-chave: Fosfolipase. Óleo de palma. Degomagem Enzimática.
Aspergillus brasiliensis. Lecitina de Soja.

ABSTRACT

The search for an environment friendly society makes biodiesel a promisor fuel for the future, due to its availability and biodegradability. Edible oils are the feedstock for biodiesel production, obtained from vegetable seeds, such as soybean, rapeseed and palm. The high amount of oil content of the seed is mainly composed by phospholipids (also known as gums). These phospholipids may cause problems to engines due to its content of phosphorus and decrease biodiesel yield due to dragging of fatty acids. Phospholipases act on phospholipid molecules, cleaving them. They are used in the oil refinement that is conducted for phosphorous content reduction, which causes the oxidation of oil. In biodiesel industry, they are employed to release more fatty acids, increasing its yield. To date, there are phospholipases that are widely used in oil degumming process. These enzymes are already commercially produced, but at high costs. The aim of this project is to produce such enzymes in an alternative way, to make feasible the possibility for industries to use enzymatic oil degumming. The strain *Aspergillus brasiliensis* LPB 266 was screened from other 73 strains and produced phospholipase with 8200 U/mL activity in 7 days fermentation, reaching productivity of 48,81 U/mL.h, in a medium based with soybean lecithin (inducer). Microfiltration and ultrafiltration were employed for phospholipase purification and molecular weight determination (between 30-55 kDa). Good perspectives are observed for its application in oil degumming.

Keywords: Phospholipase. Palm oil. Enzymatic Degumming. *Aspergillus brasiliensis*. Soybean Lecithin.

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1 INTRODUCTION

Biodiesel is a very important study field to date. Its characteristics, such as environment friendly, non-toxic, less pollutant than petroleum diesel, smoother combustion than petroleum diesel and biodegradability make it a valuable renewable fuel in a world where petroleum source is limited. Soybean, rapeseed and palm oil come up as the main alternatives to biodiesel production. They show a great content of oil. Palm is the only crop that produces two kinds of oil, one from the palm kernel and other from its mesocarp. Its world production increases each year.

Edible oils, used for the transesterification reaction may present a significant amount of phospholipids, also called gums. Degumming (the removal of gums) process can occur through different methods. The enzymatic degumming is an alternative to acid, caustic soda and wet degumming processes (GIBON; DE GREYT, 2007). The addition of water simply removes away the hydratable phospholipids (dragging neutral oil with them), allowing the non-hydratable phospholipids to remain on oil phase (DAYTON; GALHARDO, 2008). The acid degumming (with the application of citric or phosphoric acid under high mixture yield) converts non-hydratable phospholipids into hydratable ones, easily removed by water washing. It is not completely efficient, because phospholipids remain in the oil phase (CLAUSEN, 2001), besides the danger of keeping a phosphoric acid tank in industry. The caustic refining removes phospholipids, but also the free fatty acids, through the addition of acid and basic solution, which forms sodium soaps (DAYTON; GALHARDO, 2008), non-desirable on biodiesel industry. The main concern of all practices is to reduce phosphorus content, but always taking with them some amount of oil, resulting in a negative economic output. The enzymatic approach comes with a new vision about the waste of oil during biodiesel production. In order to minimize losses, the enzyme attacks phospholipids, releasing fatty acids, which are then converted to biodiesel. For the phosphate groups, they are removed by the aqueous phase in enzyme catalyzed transesterification reaction (CLAUSEN, 2001). In this way, the use of enzymes is doubly important.

Transesterification of free fatty acids (FFA), 10 to 28 carbons backbone coupled to a methyl group at one end, and to a carboxylic group at another, from

edible oils is the most common reaction to produce biodiesel (HAMA; KONDO, 2013). Soybean, rapeseed, sunflower and palm oil are considered edible oils (HAMA; KONDO, 2013). In 2015, the fifteen biggest biodiesel producing countries included United States of America, Brazil, Germany, France, Argentina, Netherlands, Indonesia, Thailand, Malaysia, Belgium, Colombia, Spain, China, Canada and India, which produced, together, 23,1 billion liters of biodiesel (STATISTA, 2016). From 45,9 million tonnes of palm oil produced in 2009-2010 (USDA, 2015), 5% was designated for energy purposes, which are linked with biodiesel production (USDA, 2015).

Palm oil trees are found within tropical areas, Malaysia and Indonesia. Palm trees are also found in South America, in Brazil (KUSUM et al., 2011). Palm oil trees can reach an average oil yield of 3,74 tonnes/hectare/year, extracted from mesocarp and palm kernel (USDA, 2007). Palm oil is mainly composed by palmitic, oleic and linoleic acid, respectively (GEE, 2007).

Edible oils present phospholipids, a glycerol backbone coupled to two fatty acids (first and second position) and one phosphoric group (third position) (LI et al*.,* 2015). There is a broad range of phospholipids, depending on which group (choline, ethanolamine, inositol or others) they are attached to the phosphate group. Phospholipids are classified into two categories: hydratable phospholipids, as phosphatidyl choline (PC), phosphatidylinositol (PI), lysophosphatidylcholine (LPC) and NHP, as phosphatidyl ethanolamine (PE) and phosphatidic acid (PA) (VAN NIEUWEHUYEN, 2008). Phospholipids are also called gums and the process of removing it is called degumming.

Oil degumming is extremely important. In Europe and in the United States of America, there are regulations about phosphorus content in biodiesel (4 and 10 ppm, respectively) (EN 14214, 2003; D6751-12, 2013). Exhausting converter engines may be damaged if high content phospholipids biodiesel is utilized (TYSON et al., 2006). Also, in process with the action of lipases, phospholipids may interfere in the reaction by playing substrate analogue (LAI *et al,* 2005). One possibility for oil degumming is the addition of phosphoric or citric acid (acid degumming), where NHP is converted into hydratable phospholipids. However, this process is not totally efficient (CLAUSEN, 2001). In the caustic oil degumming, soap is formed, dragging phospholipids and FFA. Through

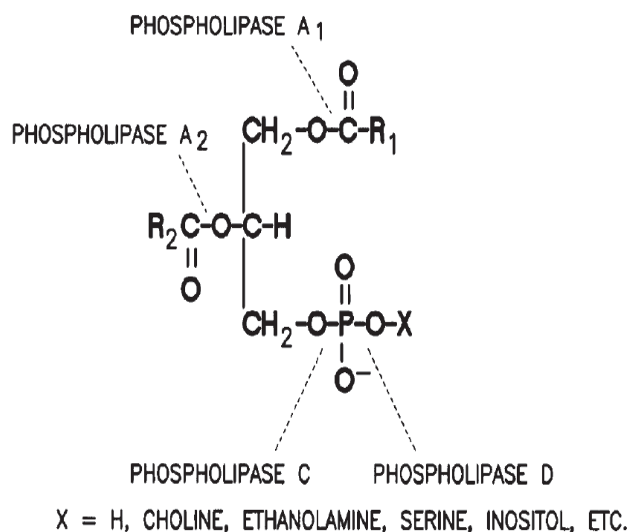
enzymatic oil degumming, the polar group (phosphoric group) is efficiently removed by water and the fatty acid chains remain in the oil, increasing FFA yield (DIJKSTRA, 2010). Degumming and transesterification, during biodiesel production, can occur at the same time, in the same place (LI et al., 2015), which makes the process cheaper and faster.

Enzymes that catalyze phospholipids hydrolysis are called phospholipases. They are classified in phospholipases A_1, A_2, B, C or D, according to its cleavage site on phospholipid molecule. For the biodiesel industry, phospholipases A_1, A_2 and C are the most indicated enzymes for obtaining a higher content of FFA. Phospholipases A_1, according to IUBMB, have an EC number 3.1.1.32 (DE MARIA et al*.,* 2007), releasing fatty acids and lysophospholipids after its action on *sn* 1 position of the phospholipid molecule. There is a triad at the catalytic site, composed by serine, aspartic acid and histidine (COWAN AND NIELSEN, 2009).

Phospholipases A_2, with EC number 3.1.1.4 releases free fatty acid chains lysophospholipids after the hydrolytic catalysis on position *sn* 2 (BURKE; DENNIS, 2009). For a long time, these enzymes were only known as the major part of the venom from snakes. Within this classification, sub-classifications were made to better understand the behavior of phospholipases A_2. They might be grouped as secreted PLA_2 ($sPLA_2$), platelet acting factor (PAF), calcium independent ($iPLA_2$), acetyl hydrolase/oxidized lipid lipoprotein associated ($LpPLA_2$), cytosolic PLA_2 ($cPLA_2$) and lysossomal ($lPLA_2$) (Underwood *et al,* 1998).

Phospholipases C, found in prokaryotes and eukaryotes (KATAN, 1998), whose EC number is 3.1.1.3 (DE MARIA et al*.,* 2007), are part of the phosphoinositide-specific phospholipases C superfamily. Its action on phospholipid molecules results in diacylglycerol and a phosphate ester group release (DAYTON; GALHARDO, 2008). Phospholipases C are effective on PC and PE (CESARINI et al., 2014). FIGURE 1 summarizes the cleavage sites on a phospholipid molecule, of phospholipases A_1, A_2 and C.

FIGURE 1 - PHOSPHOLIPASES'S A_1, A_2 AND C SITE OF ACTION ON A PHOSPHOLIPID MOLECULE



SOURCE: Dayton; Galhardo (2008).

There are commercial phospholipases available for the enzymatic oil degumming, but this practice shows high costs, as enzymes are responsible for up to 90% of total process costs (GHALY et al., 2010). Filamentous fungi from genus *Aspergillus* and *Fusarium* are widely recognized as phospholipase producers (CLAUSEN et al., 2001). In accordance to that, Lima et al. (2000) affirmed that fungi are the most utilized phospholipase producers, especially *Aspergillus, Trichoderma* and *Penicillium*.

In general, synthetic media employed are composed by a carbon source (mainly glucose), a nitrogen source (peptone is widely used) and minor components, as calcium, potassium and magnesium salts. Clausen et al. (2000) and Hasida et al. (2000) considered lecithin as a synonym for phospholipids. A medium containing soy lecithin as phospholipase inducer could present great advantages as it enhances phospholipase production and has lower costs. Lecithin is a phospholipid, composed of phosphoric acid with choline, glycerol or other fatty acids usually glycolipids or triglycerides. A major source of lecithin is soybean oil, which is largely produced. Soybean (*Glycine max*) is one of the world's main agriculture crops with a production in 2015/2016 of 307,2 million

tons. Brazil is the second producer with 100 million tonnes (USDA, 2016). This fact is surely a positive reason for using of soybean lecithin as phospholipase's inducer.

Besides its application in the biodiesel industry, phospholipases are also employed in the bakery industry, for enzymatic treatment of hydrolyzed starch, cheese making, vegetal oil refining, egg yolk and pharmaceutical industry (SHIMIZU et al*.,* 2006; DE MARIA et al*.,* 2007). Its application in different sectors of the industry increases each year, due to biotechnological tools, such as cloning and engineered proteins (BOJSEN et al*.,* 2000).

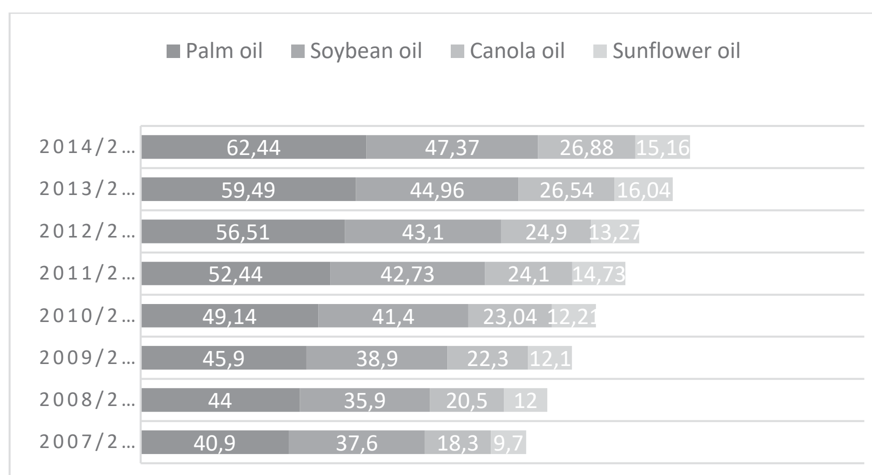
1.1 OIL TRANSESTERIFICATION AND THE QUALITY OF BIODIESEL

1.1.1 BIODIESEL PRODUCTION

Biodiesel is mainly produced through transesterification of edible oils (HAMA; KONDO, 2013). For biodiesel production, the seed is received from the farm. It follows the sterilization (mainly through humid heat). The process of sterilization is also valuable for disrupting seed cell wall, which might be complemented with cellulolytic/hemi-cellulolytic enzymes. This last treatment makes the extraction of oil through pressing easier. The recovered oil is submitted to a transesterification process, turning into biodiesel (HAMM et al., 2013). Oil sources for biodiesel production might come from micro algae (ABBASZAADEH et al., 2012), animal fats (GOG et al., 2012) or edible oils, as soybean, rapeseed, sunflower and palm (HAMA; KONDO, 2012). The annual oil production from different cultivars is presented in FIGURE 2. As it can be seen, the palm oil production showed the biggest increase among all other oils up to date, in production rate, which demonstrates its actual importance.

However, the enzyme's costs correspond up to 90% of the total process (GHALY et al., 2010). Another problem is that the crude oil, obtained either from solvent extraction or compressing, presents high content of impurities such as phospholipids, sterols, tocopherols, trace metals and odorants (NIELSEN AND CHRISTENSEN, 2006), which have to be removed to achieve a higher product yields (CESARINI et al., 2014).

FIGURE 2 - ANNUAL PRODUCTION OF OIL FROM DIFFERENT CULTIVARS IN MILLION TONES FROM 2007 TO 2015



SOURCE: USDA (2015)

1.1.2 OIL TRANSESTERIFICATION AND ITS PROBLEMS

The treatment of gum to recover oil is an old practice (PAULITZ et al., 1986). It is reported that when phosphate esters are hydrated, they bring the phospholipids to the oil-aqueous surface, dragging with them, neutral oil, resulting in the formation of an emulsion (gum), composed by phospholipids, neutral oil and water (HITCHMAN, 2009). The enzymatic oil degumming, which is the hydrolysis of phospholipid molecules increases biodiesel yield (DIJKSTRA, 2010). This process allows the liberation of hydrophobic fatty acid chains to the oil phase and capture of phosphatides in the aqueous phase. In this way, enzymes disrupt these gum structures, releasing free fatty acids and reducing phosphate content. Thus, it is very useful for increasing biodiesel production. Besides, the enzymatic degumming process avoids soap formation and does not have its activity affected by the water content of the oil (DIZGE et al., 2009).

According to Europe and United States legislation for biodiesel, the final phosphorous content must be in the range of 4 and 10 ppm, respectively (EN 14214, 2003; D6751-12, 2013). That is why the degumming process is vital to the biodiesel industry. Degumming becomes, for certain cases, necessary for the transesterification process because phospholipids interfere with the lipase and its substrate contact, reducing or even finishing the reaction (WATANABE et al.,

2002; DU et al., 2004; LAI et al., 2005) due to its structural similarity, acting as a substrate analogue. For biodiesel, if the phosphorous content is high, complex problems at the exhaust converter engines may happen (KALELI, 2005; TYSON et al., 2006).

1.2 PALM OIL AS A POTENTIAL SOURCE FOR BIODIESEL PRODUCTION

1.2.1 THE PALM TREE

The palm oil tree is originated from Africa, more specifically from the region of Guinea, area between Angola and Gambia. It is a perennial tree crop whose seeds have high oil content. Its life cycle is about 25 years, with male and female inflorescence at the axil of leaves (AKPANABIATU et al., 2001). Palm oil trees grow up to 20 meters, in tropical countries, within 10° N and S of the Equator, such as Indonesia, Malaysia (main palm oil producers), Central and South America, South East Asia and East Africa (KUSUM et al., 2011). A palm tree bears 8-12 fruit bunches annually, each weighing 15-25 kg and containing 1000-3000 fruits (MPOC, 2015). The fruit is a drupe (EKPA 1995).

1.2.2 OIL PRODUCTION AND COMPOSITION

The palm oil tree (*Elaeis guineensis*) produces an average of 3,74 tonnes of oil/ha, almost seven times higher than the oil production from rapeseed and almost eleven times higher than the soybean oil production. TABLE 1 summarizes this information.

TABLE 1 – COMPARISON BETWEEN THE FOUR WORLD MAJOR SOURCES OF EDIBLE OIL

OIL CROP	AVERAGE OIL YIELD (TONNES/HECTARE/ YEAR)	TOTAL AREA (MILLION HECTARE)	% AREA
Soybean	0,38	92,63	42,27
Sunflower	0,48	22,95	10,47
Rapeseed	0,67	27,29	12,45
Palm	**3,74**	**9,86**	**4,50**

Source: USDA (2007)

Two oils are obtained from the palm oil fruit: one from the fleshy mesocarp (60% of the fruit) (BOCKISH, 1998) and from palm kernel, usually in a ratio of ten to one. Different oil composition may be observed between palm oil (rich in oleic and palmitic acid) and palm kernel oil (rich in lauric acid) (AKPANABIATU et al., 2001). At room temperature, palm oil is semi-solid (GEE 2007), constituted by the solid part, palm stearin and the liquid part, the olein, which is mostly used for cooking, while stearin is extensively used in food processing. Palm kernel oil is used in the food formulation industry and oleochemical industry. There are differences between these oil fractions composition, resumed in TABLE 2. Considering all fractions of palm oil, palmitic, oleic and linoleic acid are the major fatty acids.

TABLE 2 – COMPOSITION OF FATTY ACIDS IN PALM OIL

Fatty acids	Palm oil %	Palm olein %	Palm stearin %
Lauric (12:0)	0,24	0,27	0,18
Myristic (14:0)	1,11	1,09	1,27
Palmitic (16:0)	44,14	40,93	56,79
Stearic (18:0)	4,44	4,18	4,93
Oleic (18:1)	39,04	41,51	29
Linoleic (18:2)	10,57	11,64	7,23
Linolenic (18:3)	0,37	0,4	0,09
Arachidonic (20:0)	0,38	0,37	0,24

SOURCE: Gee (2007)

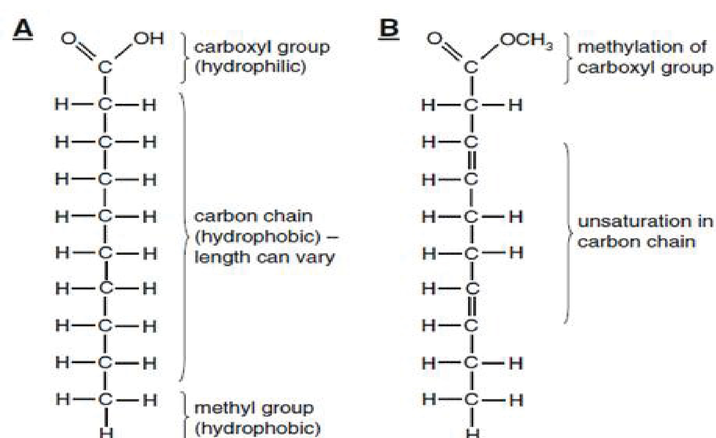
1.3 PHOSPHOLIPASES PRODUCTION AND APPLICATION FOR PALM OIL PHOSPHOLIPIDS HYDROLYSIS

1.3.1 FATTY ACIDS AND PHOSPHOLIPIDS

Free fatty acids (FFAs) are present in various organisms, consisting of carbon atoms (10 to 28) backbone attached to hydrogens (DESBOIS; SMITH, 2010) with a methyl group at one end and a carboxylic group at another. Thus,

free fatty acids are amphipathic molecules, since the carboxylic group is hydrophilic and the carbon backbone is hydrophobic. FFAs with less than eight carbon atoms on its backbone are considered short fatty acids, while above sixteen carbon are considered long fatty acids. The chemical structures of fatty acids are represented in FIGURE 3.

FIGURE 3 - FATTY ACID CHAIN STRUCTURE. (A) SATURATED FREE FATTY ACID, (B) INSATURATED FREE FATTY ACID



SOURCE: Desbois; Smith (2010).

Phospholipids are molecules that are present in all live organisms and they are part of the cellular membrane. They consist of two fatty acid chains (on position 1 and 2) and a phosphoric group (on position 3) coupled to a glycerol backbone (HASIDA et al., 2000; LI et al., 2015).

Phospholipids can be classified into two main categories: hydratable phospholipids and non-hydratable phospholipids (NPL). Phosphatidyl ethanolamine (PE) and phosphatidic acid (PA) are considered as NPL whereas phosphatidyl choline (PC), lysophosphatidylcholine (LPC) and phosphatidylinositol (PI) are part of the hydratable phospholipids group (VAN NIEUWEHUYEN, 2008). Phosphatidic acid has two hydroxyl groups with great affinity for ions, especially divalent ones, as calcium and magnesium. Once they form salts, they become neutral and stable on the oil phase. That is why all the phosphatidic salts are also considered as NPL (NIELSEN, 1960).

The phospholipids A_1 had its activities measured by the hydrolysis of phosphatidylcholine (PC) to lysophosphatidylcholine (LPC) in many organisms (AOKI et al., 2002).

Palm oil (*Elaeis guineensis*) has a high content of linoleic acid and the unsaturated (cis configuration) fatty acids are predominant, which is quite common for plants. The major phospholipids in palm oil are phosphatidylcholine, phosphatidylethanolamine (together, they represent 60% of total phospholipid content), phosphatidylglycerol and phosphatidylinositol (GOH et al., 1982).

1.4 PHOSPHOLIPASES – CATALYSIS AND MECHANISMS

Phospholipases are enzymes whose activity is to cleave phospholipids. Phospholipids vary according to which molecule it is attached to the phosphate group. In this way, if a choline is in the 'X' position (FIGURE 4), it is a phosphatidylcholine. If it is an ethanolamine, it is a phosphatidylethanolamine and so on.

FIGURE 4 - PHOSPHOLIPID MOLECULE AND PHOSPHOLIPASES ACTION

X = choline (phosphatidyl choline or PC)
X = ethanolamine (phosphatidyl ethanolamine, PE)
X = inositol (phosphatidyl inositol or PI)
X = hydrogen (phosphatidic acid or PA)

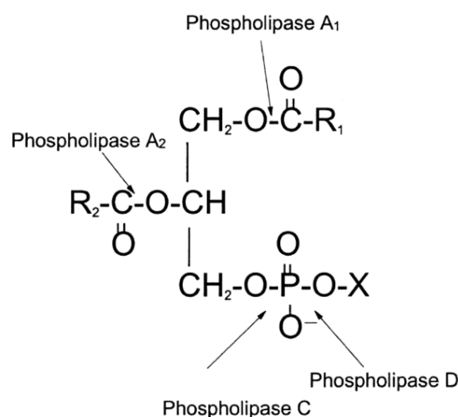
SOURCE: De Maria et al. (2007).

According to the IUBMB (International Union of Biochemistry and Molecular Biology), phospholipases are classified in: phospholipases A_1 (PLA_1) EC (Enzyme Commission) number 3.1.1.32, phospholipases A_2 (PLA_2) EC number 3.1.1.4, phospholipases C (PLC) EC number 3.1.1.3 and phospholipases D (PLD) EC number 3.1.14 (DE MARIA et al., 2007). However, enzymes may have a broad range of substrate specificity, therefore, an enzyme classified as 3.1.1.32 (typical phospholipase A_1) may also have lipase activity, whose EC number is 3.1.1.3 (BORNSCHEUER; KAZLAUSKAS, 2004; KAZLAUSKAS, 2005). This explains, for example, the classification of phospholipases C that are

also classified with the EC number 3.1.4.11, due to its phosphatidylinositol phospholipase action.

The numeric classification refers to where, within the phospholipid molecule, the enzyme acts, position *sn* 1, *sn* 2 or at the phosphate group, shown in FIGURE 5.

FIGURE 5 - PHOSPHOLIPID MOLECULE AND THE ACTION POSITION OF THE DIFFERENT PHOSPHOLIPASES

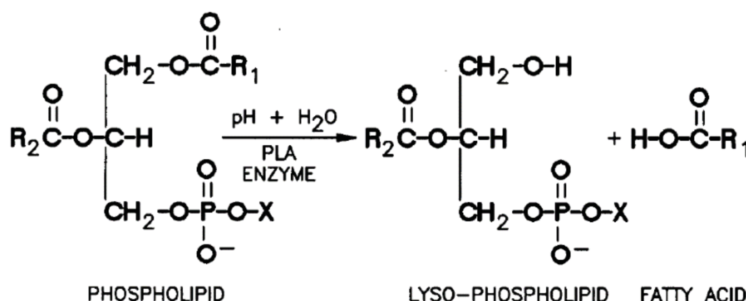


SOURCE: Clausen (2001).

1.4.1 PHOSPHOLISPASES A_1

The catalytic site of PLA_1 is composed by the serine, histidine and aspartic acid triad that cleaves the *sn* 1 position on a phospholipid, releasing lysophospholipid and free fatty acid (CLAUSEN, 2001; COWAN; NIELSEN, 2009) as shown in FIGURE 6.

FIGURE 6 - ACTION MODE OF PLA_1 TOWARDS A PHOSPHOLIPID MOLECULE



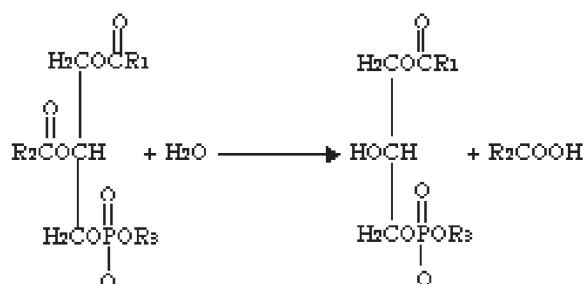
SOURCE: Dayton; Galhardo (2008).

PLA_1 from *Staphylococcus* strains are powerful biotechnology tools, as they show stereoselectivity in organic chemistry reactions (JAEGER et al., 1999). The activity of PLA_1 is increased by the presence of the ion Ca^{2+}, with EDTA acting as an inhibitor (as it chelates ions) (DE MARIA et al., 2007). Although it is such an interesting enzyme, its three-dimension structure is not known. So, models based on the homology with *Bacillus stearothermophilus* lipase were created, sharing the α/β common fold to lipases/esterases (TYNDAL et al., 2002).

1.4.2 PHOSPHOLIPASES A2

A huge progress has been made in the phospholipase A_2 enzymes field in the last 25 years (BURKE; DENNIS, 2009). This enzyme catalyzes the hydrolysis of fatty acid from the position *sn* 2 of phospholipids, releasing free fatty acids and lysophospholipid (AALRUST et al., 1993; SIX; DENNIS, 2000; SCHALOSKE; DENNIS, 2006; MIYAJI et al, 2007; BURKE; DENNIS, 2009). FIGURE 7 shows how phospholipase A_2 cleaves phospholipids.

FIGURE 7 - ACTION MODE OF PLA_2 TOWARDS A PHOSPHOLIPID MOLECULE



Source: Uthe; Magee (1971).

Until the beginning of the 20th century, it was found that phospholipases A_2 (PLA_2s) were the major components of snake venoms. Based on this, a classification system within 15 groups came up (SCHALOSKE, 2006) and numerous subgroups appeared by the year 2000 (SIX; DENNIS, 2000). The 15 groups might be arranged into five main categories: secreted PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), calcium independent ($iPLA_2$), platelet acting factor (PAF)

acetyl hydrolase/oxidized lipid lipoprotein associated ($LpPLA_2$) and lysossomal ($lPLA_2$) (UNDERWOOD et al., 1998).

The $sPLA_2$ was the first type of phospholipase discovered, extracted from snake's venoms, scorpions, synovial fluid, part of pancreatic juice and mammalian tissues (SIX; DENNIS, 2000). It shows a conserved histidine at the catalytic site, it has a low molecular weight (13-15 kDa), large number of disulfide bridges (seven bridges for pancreatic pig phospholipase, snake venom and human synovial fluid, five for bees) (DE MARIA et al., 2007) and requires very low concentrations of the ion Ca^{2+}, for optimum catalytic activity (BURKE; DENNIS, 2009).

The mechanism of hydrolysis involves water activation by the dyad histidine/aspartic acid in a Ca^{2+} dependent manner, so the proton form H_2O molecule is abstracted and latter a nucleophilic attack occurs at the *sn* 2 bond (VERHEIJ et al., 1981; LOMBARDO et al., 1986; YU; DENNIS, 1991). The negative charge of the phosphate oxygen interacts, by coordination, with the calcium ion, orientating properly the lipid substrate for hydrolysis (SCOTT et al., 1990). No specificity is known about fatty acid preference at *sn* 2 position (MURAKAMI; KUDO, 2000; KUDO et al., 1993).

The majority of $sPLA_2$ presents interfacial activation, which means that phospholipases have increased activity towards large lipid aggregates, in solution instead of monomeric forms (PLUCKTHUN, 1985; CARMAN et al., 1995; GEHL et al., 1995). It is more active at the lipid-aqueous interface than in solution (SCOTT et al., 1990). The enzyme does not show any kind of allosteric system that changes its conformation, it is well established by the disulfide bridges, robust, rigidly maintained (ACHARI et al., 1987), as confirmed by a crystal structure analysis. Therefore, the proposal is that the substrate reaches the catalytic site through a hydrophobic channel whose opening is at the interfacial binding surface. It is the aggregation that imposes to phospholipid the conformation required for full susceptibility to PLA_2 action (BARLOW et al., 1988).

The cytosolic PLA_2 ($cPLA_2$) is characterized by a serine and aspartic acid dyad at the catalytic site and Ca^{2+} requirement for active enzyme. The calcium ion does not activate the enzyme itself, but recruits the phospholipase to the membrane of the cell (Ca^{2+} dependent translocation of C2 domain) and

messengers as ceramide 1-phosphate (STAHELIN et al., 2007), biphosphate (SIX; DENNIS, 2003) and phosphatidylinositol (KRAMER, 1989) activate the enzyme using a site directed alteration on its charged residues (SIX; DENNIS, 2009). Its average molar mass is within 60 and 100 kDa (MIYAJI et al., 2007). Besides the calcium ion dependent lipid bonding domain, a core α/β hydrolase region is required for full activity of the enzyme (DESSEN et al., 1999). The enzymatic regulation is also controlled by the phosphorylation on residues 505,515 and 727 (HIRABAYASHI, 2004; BURKE; DENNIS, 2008).

The name PAF acetyl hydrolase/oxidized lipid ($LpPLA_2$) is due to its activity to cleave acetyl group from *sn* 2 position of PAF and another lipoprotein. Although, the action of this enzyme is not restricted to platelet active factor molecule; it can also cleave oxidized chains up to nine carbon atoms (SIX; DENNIS, 2000). Different from other phospholipases A_2, this enzyme can access substrate in aqueous phase (GELB et al., 2000). Another difference from the other groups is the presence of a triad on the active site, composed by serine, histidine and aspartic acid, instead of the regular dyad found on the rest of PLA_2s. $LpPLA_2$ shows also the interfacial activation (MIN et al., 1999).

The lysossomal PLA_2 is the newest type. It conserves the serine, histidine and aspartic acid triad at the catalytic domain and four cysteines for catalytic activity (BURKE; DENNIS, 2009). Another enzyme, the OMPLA (a phospholipase A) is one the few enzymatic components of the outer membrane of the gram-negative bacteria. Initially classified as PLA_1, latter it was stated that has a PLA_2, lyso-PLA_2 and lyso-PLA_1 activity (DEKKER, 2002).

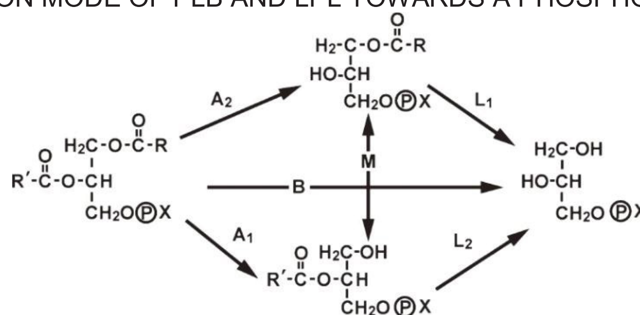
1.4.3 PHOSPHOLIPASES B

These enzymes are responsible hydrolytic cleavage of *sn*1 and *sn*2 acyl-ester bonds within a phospholipid molecule and are found in animal tissues and microorganisms (FUJINO et al., 2006). Even sharing the same activity, their optimum pH, molecular weight, substrate specificity and biological relevance might differ among producer species (FUJINO et al., 2006) Their EC number is 3.1.1.5 (CLAUSEN et al., 2000).

Phospholipases B are also known as lysophospholipases (LOFFLER et al., 1999) although there are controversies about this nomenclature (SAITO, 2014). Lysophospholipid, the substrate for lysophospholipase, is a molecule which was previously deacylated, *sn*1 or *sn*2. Therefore, lysophospholipids are called the enzyme which act on this substrate, while, phospholipases B can act simultaneously on both positions (*sn*1 and *sn*2) (SAITO, 2014). FIGURE 8 shows how phospholipase B and lysophospholipase are supposed to act. According to Methods Enzymol. Vol 197 (1991), phospholipases are divided in 5 sections, PLA_1, PLA_2, LPL, PLC, and PLD. Phospholipases B is included in the section of LPL.

A possible mechanism for the phospholipase B activity may involve an active site and two substrate-binding sites: site I for diacyl phospholipids and Site II for lysophospholipids. The following steps occur sequentially in the hydrolysis of phospholipid: (1) Binding of phospholipid to Site I; (2) Transfer of the acyl group at *sn*-2 to the catalytic site and simultaneous binding of the resultant lysophospholipid to Site II; (3) Transfer of *sn*-2 acyl group to water molecule; (4) Transfer of *sn*-1 acyl group to catalytic site; (5) Transfer of *sn*-1 acyl group to water molecule. Hydrolysis of lysophospholipid would involve direct binding to Site II, followed by steps 3–5 (SAITO, 2014).

FIGURE 8 - ACTION MODE OF PLB AND LPL TOWARDS A PHOSPHOLIPID MOLECULE



SOURCE: Saito (2014).

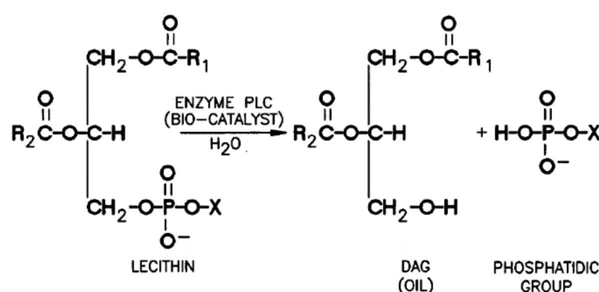
1.4.4 PHOSPHOLIPASES C

The phospholipases C (PLC) belongs to the superfamily called phosphoinositide-specific phospholipases C. They are found in prokaryotes and eukaryotes (KATAN, 1998). Their activity is different depending on the sources.

PLCs from eukaryotes are involved in signal transduction cascades, while from prokaryotes, it works as a virulence factor. Bacterial PLCs have only one domain (30-35 kDa), those from eukaryotes show different domains and have around 80-150 kDa (DE MARIA et al., 2007).

Cofactors as zinc and calcium are needed. The action of these enzymes results in the cleavage of a phospholipid into a diacylglycerol (DAG) and a phosphate ester group (DAYTON; GALHARDO, 2008). The PLCs are selective for phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (HITCHMAN, 2009; DJKISTRA, 2010; CESARINI et al., 2014). Their action does not increase the free fatty acid content, but the DAG (diacylglycerol) content, as shown in FIGURE 9.

FIGURE 9 - ACTION MODE OF PLC TOWARDS A PHOSPHOLIPID MOLECULE.



SOURCE: Dayton; Galhardo (2008).

1.4.5 PHOSPHOLIPASES D

Phospholipases D (PLD) are known for their presence in bacteria, fungi, animals, but they were first found in plants, such as carrots, cabbage and castor beans. Their functions are linked to the metabolism of phospholipids, nucleases, toxins and proteins from virus envelope with no biological function known (LISCOVITCH et al., 2000; MCDERMOTT et al., 2004).

The geometry and charge of the aggregate are important for the hydrolysis with PLDs due to the interfacial activation (ULBRICH-HOFMANN et al., 2005). PLD accepts various phospholipids as substrate (phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, phosphatidylinositol) (PAPPAN; WANG, 1999; WAITE, 1999), but the

unsaturation degree of the fatty acid chain affects its activity (ABOUSALHAM et al., 1997).

1.5 SOURCES FOR PHOSPHOLIPASES PRODUCTION

Generally, phospholipases are produced using artificial medium, containing carbon sources, nitrogen sources and various salts. Lysophospholipases are produced through submerged fermentation (LOFFLER et al., 1999), under aerobic conditions, from 30 to 120 hours, at 28 °C. Submerged fermentation is also employed for bacteria, such as *Bacillus cereus*, at 37 °C, for 28 hours (ZHAN et al., 2013).

1.5.1 PHOSPHOLIPASES A1

PLA_1 from *Staphylococcus*, *Staphylococcus hyicus* show high tolerance degree for lipid compound with variable chain lengths (SIMONS et al., 1996), using it as substrate. These microorganism groups are recognized as phospholipase producers (VAN OORT et al., 1989).

Genes encoding for PLA_1 have been isolated from *Serratia sp*. and expressed in *Escherichia coli* (SONG et al., 1999). Since then, many phospholipases were identified in fungi, such as *Aspergillus niger* (ALBERMAN et al., 2003), *Fusarium sp.* (CLAUSEN, 1998; TSUTSUMI et al., 2002), *Aspergillus oryzae* (SANKYO, 1998; SHIBA 2001) and *Rhizopus oryzae* (AALRUST et al., 1993). PLA_1 have been cloned from non-cultivable organisms through metagenomics (GRAMATIKOVA et al., 2005).

It is known that *Aspergillus oryzae* strain SANK 11870 or the strain available at Institute of Fermentation (IFO) number 30102; *Aspergillus niger*, strain ATCC 9642 or IFO 4407; *Aspergillus usamii mut. shiro usamii*, strain available under Institute of Applied Microbiology (IAM) number 2414 or IFO 6082; *Aspergillus awamori*, IAM 2112 or IFO 4033; *Aspergillus fumigatus*, IAM 2034; *Aspergillus sojae*, IAM 2666; *Aspergillus phoenicis*, IAM 2215 and *Aspergillus wentii*, IAM 2133 are PLA_1 producers (STRINGER et al., 2004). The

authors used the "wheat bran" culture technique (inoculating a solid or liquid grain-based medium with spores of the chosen strain, followed by culturing). The medium can be formed by equal parts of water and grains, which can be rice, wheat, corn, soybeans, sesame seeds, or cottonseed cake. Once the water and the grain have been mixed, the resulting medium is boiled for at a suitable temperature, 120°C for 15 minutes, in order to sterilize it. The medium is then cooled before use. The inoculated medium is suitably cultured at temperatures between 18 and 32°C, preferably from 4 to 8 days.

According to Clausen et al (2001), *Fusarium oxysporum* was cultivated in YPD medium for 4 days at 30°C. However, for *Fusarium culmorum* a different media is necessary, containing peptone, peptidase, meat and yeast extract, dextrose and olive oil.

1.5.2 PHOSPHOLIPASES A2

The truffle *Tuber borchii* secretes PLA_2 (SORGANI et al., 2001). A specie from *Tuber gender*, *Tuber albidum* (CBS 272.72), also produces PLA_2 and can be cultured on X-agar at 20°C, as recommended by the CBS in List of Cultures, 1996 (Stringer et al., 2004).

Fusarium graminearum ATCC 20334 produces a phospholipase that belongs to the PLA_2 (SORGANI et al., 2001). It was cultured in Vogel's minimal medium (DAVIS; DE SERRES, 1970), at 28°C in shaking culture (STRINGER et al., 2004).

1.5.3 PHOSPHOLIPASES B

Phospholipases B (PLB) can be found in *Penicillium notatum* (MASUDA et al., 1991), *Aspergillus niger* (Bulletin G999), *Corticum centrifugum* (UEHARA et al., 1979), *Candida utilis* (FUJINO et al., 2006) and *Schizosaccharomyces pombe* (OISHI et al., 1996).

1.5.4 PHOSPHOLIPASE C

The microorganisms *Acinetobacter calcoaceticus* (LEHMAN, 1971), *Aeromonas liquifaciens, Aeromonas punctata, Aeromonas salmonicida, Pseudomonas caviae, Pseudomonas aeruginosa, Pseudomonas chlororaphis, Pseudomonas fluorescens, Pseudomonas reptilivora, Pseudomonas viscosa, Xanthomonas begoniae, Xanthomonas pbaseoli, Xanthomonas vesicatoria* (ESSELMAN et al., 1961), *Pseudomonas aureofaciens* (LIU, 1961), *Bacillus albolactis, Bacillus prausnitzii* (COLMER, 1948), *Bacillus cereus* (CHU, 1948), *Bacillus anthracis, Bacillus mycoides* (MCGAUGHEY et al., 1948), *Bacillus alvei, Bacillus lentus, Bacillus macerans, Bacillus polymyxa* (KNIGHT et al., 1950), *Clostridium bif ermentans* (HAYWARD, 1943), *Clostridium baemolyticum, Clostridium novyi* (BARD et al., 1948), *Clostridium oedematiens* (PHILIPS et al., 1963), *Clostridium perfringens* (MACFARLANE et al., 1941), *Clostridium sordellii* (MACFARLANE, 1948), *Staphylococcus pyogenes* (MAGNUSSON et al., 1962) are recognized as phospholipase C producers. *Salmonella gallinarum, Salmonella weltevredem* and *Klebsiella pneumoniae* produce phospholipase C (SINGH et al., 1998).

The production sources and enzyme activity conditions of different phospholipases A1, A2 and B are shown in TABLE 3.

TABLE 3 – PHOSPHOLIPASE ACTIVITY FROM DIFFERENT MICROORGANISMS

Microorganism	Substrate	Conditions	Activity	Reference
Thermomyces lanuginosus DSM 4109	Rapeseed oil	50 °C; pH 4,5; 24 hours	10000 LU/g	Holm et al 2011
Aspergillus oryzae(PLA$_1$) SANK 11870	Lecithin (SPL-White)	37 °C, 10 min.	1170 U/g (crude) and 72100 U/g (pure)	Hattori et al., 1993
Aspergillus niger (PLA$_1$) ATCC 9642	Lecithin (SPL-White)	37 °C, 10 min.	119 U/g (crude) and 2100 U/g (pure)	Hattori et al., 1993
Aspergillus niger (PLA$_2$ + PLB)	Soybean oil	40 °C; pH<3; 20 hours	60 LU/g (PLA$_2$) and 1001 LLU/g (PLB)	Loffler et al., 1999

Bacillus cereus (PLA$_1$)	Rapeseed oil	35 °C; pH 7,5; 3 hours	1195,25 U/mg	Zhang et al., 2013

TABLE 3 – PHOSPHOLIPASE ACTIVITY FROM DIFFERENT MICROORGANISMS

Microorganism	Substrate	Conditions	Activity	Reference
Hyphozyma sp. (PLB)	Rapeseed oil	35 - 40 °C; pH 3,5 – 5; 3 hours	1 U/g	Hasida et al., 2000
Fusarium oxysporum (PLA_1)	Rapeseed oil	40-45 °C; pH 5; 6 hours	1000 LU/mg	Clausen et al., 2000
Fusarium culmorum (PLA_1)	Rapeseed oil	40-45 °C; pH 5; 6 hours	200 U/mg	Clausen et al., 2000

SOURCE: The author (2018)

1.6 PHOSPHOLIPASES FOR OIL DEGUMMING FOR BIODIESEL INDUSTRY

Phospholipases have applicability in a broad range of areas, such as pharmaceutical (related to inflammation response and digestion) (SHIMIZU et al., 2006), bread making, cheese making, treatment of starch hydrolyzed, egg yolk industry (emulsifiers) and refining of vegetable oils (degumming) (DE MARIA et al., 2007) The industrial usage of phospholipases has increased due to the ability to clone genes encoding for enzymes and expressing them in microbial hosts (CLAUSEN, 2001) and the protein engineered, which turns regular process into more profit ones (BOJSEN et al., 2000).

In the phospholipase application, NPL on the oil phase are converted to hydratable phospholipids, through the liberation of fatty acid chains to oil phase, releasing the phosphate group, which is extracted on the aqueous phase. The hydratable phospholipids, the ones that easily dissolve in water, have their fatty acid chains also released to the oil phase, remaining just the phosphate group on aqueous phase (CLAUSEN, 2001).

The most common phospholipases used are PLA and PLC, due to their ability to cleave phospholipids in their respective sites of cleavage. Phospholipases D are not used for biodiesel industry because their site of action is the side chain of phosphate group; therefore, the phospholipid molecule

remains almost the same. Regarding to lysophospholipases, they are active on lysophospholipids, which means that it is only active in an environment containing PLA.

For a faster process, degumming and transesterification can occur simultaneously, in the same tank (CESARINI, et al., 2014; LI et al., 2015).

1.7 COMMERCIAL PHOSPHOLIPASES

The commercial enzyme Lecitase Ultra®, by Novozymes, is a hybrid between the homologous genes of lipase production from *Thermomyces lanuginosus* and phospholipase A_1 from *Fusarium oxysporum*. The final compound has the activity of the phospholipase with the stability of the lipase (DE MARIA et al., 2007; DJKISTRA, 2010). Lecitase Novo®, also produced by Novozymes, is based in a phospholipase A_1 from *Fusarium oxysporum*. Both are used in industry for oil degumming.

Tests with the commercial enzyme formulation of Lecitase Ultra®, a phospholipase A1 from a genetic modified *Aspergillus oryzae* were performed (LI et al., 2015). The lipase for transesterification and FAME formation is from a genetic modified *Aspergillus niger*. Providing degumming and transesterification simultaneously, the following data was obtained. With soybean oil, Li et al (2015) tested different phospholipid concentration towards the reduction of phosphorus content and FAME yield. In all cases, phosphorus content was reduced, but the greatest reduction of phosphorus and higher FAME yield achieved was achieved with the less concentrated sample. The lowest phosphorus reduction rate and FAME yield corresponded to the highest phospholipid amount sample. The explanation about the decrease on FAME yield is due to the solubility of alcohol (in this case, methanol) in phospholipids micelles. Once the concentration of phospholipids increased, more methanol was solubilized by its micelles, lowering this way the reaction rate.

The commercial enzyme Rohalase®, produced by Abenzymes, consists of a PLA_2 from *Trichoderma reseei* (FARR; PROCTOR, 2013). The company DSM produces the GumZyme, a PLA_2 and Lyzomax, a lipid acyltransferase (type A_2).

Different commercial enzymes and their application are presented in TABLE 4. All data in which FAME yield was measured were obtained by treating samples simultaneously with lipase for transesterification.

TABLE 4 – COMMERCIAL ENZYME'S APPLICATION ON OIL TO OBTAIN FREE FATTY ACIDS

Enzyme	Substrate	FAME Yield %	Phosphorus content Ppm	Producer	Reference
Purifine[a]	Soybean oil	90,8	12,8 (from 823)	Diversa	CESARINI et al. (2014)
Purifine[a]	Soybean oil	-	70,9 (from 746)	Diversa	DAYTON; GALHARDO (2008)
Lecitase ® Ultra	Soybean oil	-	31,7 (from 746)	Novozymes	DAYTON; GALHARDO (2008)
G-Zyme® [c]	Soybean oil	-	85 (from 190)	Enzyme Biosystems	LOFFLER et al. (1999)
Lecitase ® Ultra	Soybean oil	98,2	8 (from 823)	Novozymes	CESARINI et al. (2014)
Lecitase ® Ultra + Purifine[a]	Soybean oil	-	2,3 (from 547,9)		DAYTON; GALHARDO (2008)
Lecitase ® Ultra + Purifine[a]	Soybean oil	96,6	4,6 (from 823)		CESARINI et al. (2014)
Lecitase ® Ultra + LLPL-2[b]	Soybean oil	97,8	6 (from 823)		CESARINI et al. (2014)
PLB preparation from Corticium species	Soybean oil	-	1 (from 130)	Amano Pharmaceutical Co. Ltd.	AALRUST et al. (1993)
PLC preparation	Soybean oil	-	45 (from 130)	Amano Pharmaceutical Co. Ltd.	AALRUST et al. (1993)
PLD preparation	Soybean oil	-	48 (from 130)	Sigma Chemie Gmbh	AALRUST et al. (1993)

TABLE 4 – COMMERCIAL ENZYME'S APPLICATION ON OIL TO OBTAIN FREE FATTY ACIDS

Enzyme	**Substrate**	**FAME Yield %**	**Phosphorus content ppm**	**Producer**	**Reference**
Lecitase ® Ultra	Soybean oil	92,19	3,29 (from 447,75)	Novozymes	LI et al. (2015)

SOURCE: The author (2018).

[a] Phospholipase C; [b] Lysophospholipase from *A. niger*, produced by Novozymes; [c] Commercial lyso-phospholipase

Data obtained from tests with the analysis of the concomitant use of PLA_1 and PLC are shown in TABLE 5 (DAYTON; GALHARDO, 2008). The enzymes were both commercial forms of PLA_1 and PLC (Lecitase Ultra® and Purifine®, respectively). The substrate was crude soybean oil. The best result was obtained using 45 °C, for 1 hour, with pH 4,3 with the ratio $2PLA_1$:PLC.

TABLE 5 - PLA_1 AND PLC (LECITASE ULTRA® AND PURIFINE®, RESPECTIVELY) ANALYSIS AND RESULTS FOR CRUDE SOYBEAN OIL DEGUMMING

Enzyme	P content before reaction (ppm)	P content after reaction (ppm)	Temperature (°C)	pH	Time - hours
PLA_1	746	31,7	45	4,5	4
PLC	746	70,9	45	4,5	1
PLC + PLA_1	560,1	13,2	45	4,5	1
PLC + PLA_1	560,1	10,5	45	7	4
PLC + PLA_1	560,1	3,2	45	4,5	4
PLC + PLA_1	547,9	2,4	45	4,3	1
PLC + PLA_1	547,9	2,5	45	4,3	4
PLC+ PLA_1	547,9	2,3	55	4,3	1
PLC+ $2PLA_1$	547,9	7	45	4,3	1

SOURCE: Dayton; Galhardo (2008).

1.8 LAB SCALE PRODUCED PHOSPHOLIPASES

Aspergillus niger phospholipases (PLA_2 and PLB) showed high efficiency as biocatalysts in wet degummed of soybean oil, where the phosphorus content was reduced from 190 ppm to 4,2 ppm, in 20 hours of enzyme action (LOFFLER et al., 1999). To check if only citric acid treatment was efficient for disrupting phospholipids micelles of NHP, another experiment was carried out. The addition

of whey protein (non- enzymatic compound) was used to proof the ability of citric acid to decrease phosphorous content on oil phase. The phosphorus content was reduced from 190 ppm to 121 ppm, in 20 hours of enzyme action. Data showed that only citric acid was not an efficient degumming agent.

In another experiment, enzymes from *Aspergillus niger* (ATCC 9642 and IOF 4407) and *Aspergillus oryzae* (SANK 11870 and IFO 30102) were compared it to a commercial PLA_2, on lecithin powder. Authors analyzed that lysophospholipid formation with the commercial enzyme was lower than with the isolated enzyme (HATTORI et al., 1993).

Utilizing *Fusarium oxysporum* DSM 2672 as a phospholipase A_1 producer, on crude rapeseed oil, the results showed that the enzyme is effective for the degumming process at low rates (0,9 mg of phospholipase / kg oil) when compared to other enzyme concentrations (1,6 and 3,2 mg of phospholipase / kg oil), because, for the same period, the phosphorus degumming was similar (CLAUSEN et al., 2000).

Also, phospholipases from *Fusarium culmorum*, with crude rapeseed oil as substrate, were effective as a degumming agent, within 6 hours, reducing phosphorus content from 254 ppm to 7 ppm (CLAUSEN et al., 2000).

Each phospholipase has its best activity parameters, such as temperature and pH. It is known that PLAs acts preferentially on pH 4,5, while PLC shows an optimum pH around 7. PLAs start the denaturation process above 50°C, PLC, above 65°C. Time required for the enzyme action is also different within these two enzymes: 1 hour, generally, for PLC and 4 hours for PLA_1.

According to Holm et al (2011), different enzyme concentrations and reaction times affected degumming and transesterification of rapeseed oil. The longest the time they reacted, the higher was the amount of enzyme, better results were achieved concerning phosphorus content and FAME yield.

Several phospholipases applications are presented produced with different substrates in TABLE 6.

TABLE 6 – APPLICATION OF SEVERAL PHOSPHOLIPASES PRODUCED BY DIFFERENT MICROORGANISM USING DIFFERENT SUBSTRATES

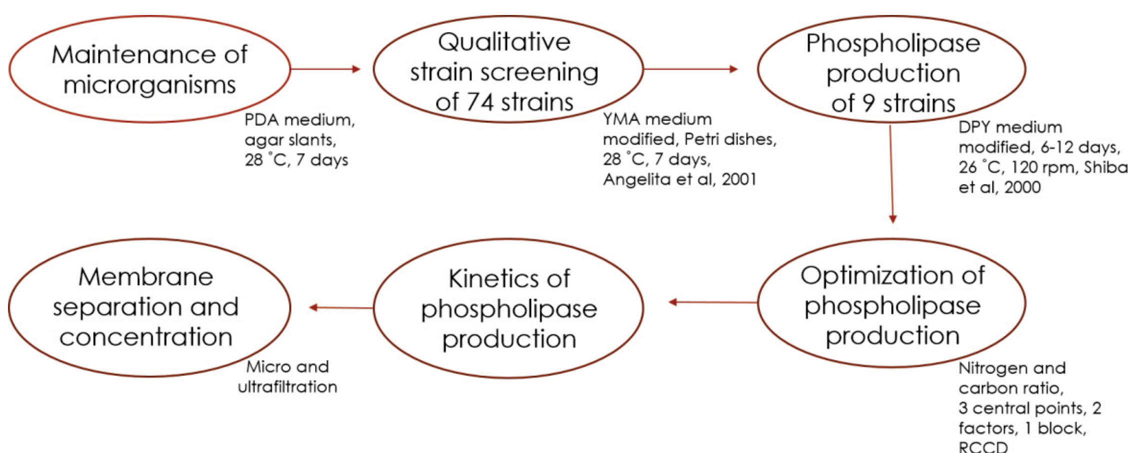
Microorganism	Substrate	Initial P value (ppm)	Final P value (ppm)	FAME Yield %	Time (hours)	Reference
Thermomyces lanuginosus (30ppm)	Rapeseed oil	-	26	94	24	HOLM et al. (2011)
Thermomyces lanuginosus (100 ppm)	Rapeseed oil	-	12	86	24	HOLM et al. (2011)
Corticium sp.	Soybean oil	130	1	-	3	AALRUST et al. (1993)
Hyphozyma sp.	Rapeseed oil	100	1	-	3	HASIDA et al. (2000)
Aspergillus niger	Soybean oil	190	4,2	-	20	LOFFLER et al. (1999)
Fusarium oxysporum	Rapeseed oil	609	10	-	6	CLAUSEN et al. (2002)
Fusarium culmorum	Rapeseed oil	254	7	-	6	CLAUSEN et al. (2002)

SOURCE: The author (2018)

2 MATERIAL AND METHODS

Phospholipase process development passed through the qualitative and quantitative screening of 74 strains of filamentous fungus. The optimization of enzyme production was then carried out including the study of the effect of nitrogen sources and medium conditions. The recovery and separation of the enzyme was performed using microfiltration and ultrafiltration membranes. All steps are presented in FIGURE 10.

FIGURE 10 - PHOSPHOLIPASE PRODUCTION AND RECOVERY STEPS



SOURCE: The author (2018)

2.1 MICROORGANISMS

Seventy-four strains from genus *Aspergillus* sp., *Rhyzopus sp*., *Trichoderma sp*. and *Giberella sp*., from the strain bank of Bioprocess Engineering and Biotechnology Laboratory of UFPR were tested. Strains were cultured on agar slants containing PDA (Potato Dextrose Agar) at 28ºC for 7 days. Strains were periodically renovated.

2.1.1 QUALITATIVE STRAIN SCREENING FOR PHOSPHOLIPASE PRODUCTION

Strains were grown in Petri dishes containing modified YMA medium with the presence of rodamin B, according to method proposed by Hankin and Anagnostakis (1975) and Siloto (2001). Soy lecithin was added as inducer to YMA medium as it is a rich source of phospholipids. It was tested according to IUPAC 2.421 (DIEFFENBACHER et al., 1992). Plates were incubated at 28°C, for 7 days. The halo formed after the production of phospholipase was measured. The ratio between the measure of the colony formation and the decolored halo formed around it was determined. All experiments were made in triplicate.

2.1.2 QUANTITATIVE STRAIN SCREENING FOR PHOSPHOLIPASE PRODUCTION

2.1.2.1 INOCULUM PREPARATION

Nine strains were pre-selected for the fermentative tests. Inoculum of each strain was prepared in Erlenmeyer flasks with 150 mL of PDA medium that were incubated at 28ºC for 7 days. Spores were suspended in 100 mL of a 0,1% Tween 80 solution. The obtained spore suspension was used to inoculate the medium for phospholipase production in a 10% v/v ratio.

2.1.2.2 PHOSPHOLIPASE PRODUCTION IN SUBMERGED FERMENTATION

Fermentation process was conducted in Erlenmeyers flasks, with a DPY medium (2% dextrin, 1% polypeptone, 0,5% yeast extract, 0,5% KH_2PO_4, 0,05% $MgSO_4.7H_2O$), as described by Shiba et al. (2000) and supplemented with 0,005M $CaCl_2$ and 2,5% of soy lecithin. Fermentation process took place in 250 mL Erlenmeyer flasks. Erlenmeyer flasks (250 mL) with 80 mL of DPY medium were inoculated and incubated for 12 days, at 26°C in a rotary shaker at 120 rpm. All experiments were made in triplicate.

2.1.2.3 OPTIMIZATION OF PHOSPHOLIPASE PRODUCTION

2.1.2.3.1 INFLUENCE OF NITROGEN SOURCE ON PHOSPHOLIPASE PRODUCTION

DPY medium, which was added of different nitrogen sources (urea, ammonium sulphate, yeast extract, peptone and lecithin), was tested, keeping the same concentration of nitrogen in the fermentation medium. Fermentation was carried out in 250 mL Erlenmeyer flasks with 80 mL of DPY medium. Flasks were incubated for 7 days, at 26°C in a rotary shaker at 120 rpm. All experiments were made in triplicate. Phospholipase activity was measured as described below.

2.1.2.3.2 INFLUENCE OF CARBON AND NITROGEN RATIO ON PHOSPHOLIPASE PRODUCTION

The influence of the carbon/nitrogen ratio on phospholipase production was studied using a rotational central composite design (RCCD) with the support of the Software Statistica 7 and Response Surface methodology. The experimental design was composed of 2 factors, 13 essays and 3 central points, in 1 block. Different nitrogen/carbon ratio combinations were proposed by the generated matrix (TABLE 7). All experiments were made in triplicate. Phospholipase activity was measured as described below. Data was used to create a mathematic model and a contour graph.

TABLE 7 - CODED AND UNCODED VALUES OF THE STUDIED FACTORS IN RCCD EXPERIMENTAL DESIGN FOR PHOSPHOLIPASE PRODUCTION OPTIMIZATION

Factors	-1,41421	-1	0	1	1,41421
Dextrose (g/L)	5,86	10	20	30	42,43
Ammonium sulphate (g/L)	1,75	3	6	7,5	10,61

SOURCE: The author (2018)

2.1.2.4 RECOVERY AND SEPARATION OF PHOSPHOLIPASE BY MICROFILTRATION AND ULTRAFILTRATION

After submerged fermentation, the medium containing phospholipase was filtrated through microfiltration through a Vivaspin Vacuum Filtration System

PES 0,22µm (KASVI®), with total capacity of 250 mL, connected to a vacuum bomb.

The filtrate from the microfiltration system was ultra-filtrated in a Vivaspin 6 (GE Healthcare®) ultrafiltration system, with molecular weight cutoff between 10 and 100 kDa. 5 ml samples were centrifuged at 1000g for 20 minutes. Different fractions of the retained and permeate were analyzed for phospholipase activity, protein concentration and specific activity determination.

2.2 ANALYTICAL METHODS

2.2.1 DETERMINATION OF PHOSPHOLIPASE ACTIVITY

Phospholipase activity was determined according to Yang *et al* (2006). The amount of enzyme that releases 1 µmol of titrated FFA per minute is considered as one unit of phospholipase (U). An emulsion containing 10% of phospholipid and 4% polyvinyl alcohol solution was emulsified at a volume ratio 1:4 at 20000 rpm for 10 min. Enzymatic reaction occurred with 4 mL emulsion, 5 mL 0,05 M citric acid buffer and 1 mL of enzyme solution that were incubated at 40°C for 15 min. The reaction was interrupted with the addition of 95% ethanol solution (15 mL) after incubation and, the liberated fatty acid was titrated with 0,05 M NaOH. Blanks were measured with heat-inactivates enzyme, in with an enzyme sample was kept at 100 °C for 15 min. Experiments were carried out in triplicate.

2.2.2 DETERMINATION OF PROTEIN CONTENT

Protein concentration was determined by Bradford Method (Bradford, 1976). An amount of 150 µL of microfiltrated extract was added to 150 µL of the Quick Start Bradford 1x Dye Reagent, produced by Bio-Rad Company. Solutions were place on an ELISA essay, with its absorbance measured.

2.2.3 PHOSPHORUS CONTENT ANALYSIS

Phosphorus content analysis was carried with 0,1 g of MgO that was placed in a porcelain dish and heated. A mass of 5 g of soy lecithin was added to the porcelain dish and ignited on an electric furnace until reach white ashes. Phosphorus content of soy lecithin was determined according to IUPAC 2.421 (DIEFFENBACHER et al., 1992). All tests were made in triplicate.

2.2.4 SDS PAGE

The enzymatic extracts ultrafiltrated (from permeated 50 kDa cutoff) was analysed by SDS-PAGE (*sodium dodecyl sulphate polyacrylamide gel electrophoresis*), in gel with 0,1 mm thickness and 15% concentration, according to Laemmli (1970).

3 RESULTS AND DISCUSSION

3.1 QUALITATIVE STRAIN SCREENING FOR PHOSPHOLIPASE PRODUCTION

Seventy-four strains, which were potential phospholipase producers, were tested according the method proposed by Hankin and Anagtomaskis (1975). A soy lecithin-enriched medium (YMA medium) was employed, as it is a rich source of phospholipids. All strains were grown on this medium and the halo formation was measured. From all tested strains, 9 of them were positive for phospholipase production. TABLE 8 summarizes the difference between the distance from the center of the colony to the halo edge and the center of the colony to its edge. All presented data are mean values and essays were performed in triplicate.

Among all tested strains, nine of them were observed as phospholipase producers with halo formation (TABLE 8). *Aspergillus brasiliensis* LPB 266 is a very good phospholipase producer presenting a 16 mm halo formation, followed by *A. flavus* LPB 335 (12 mm), *A. brasiliensis* LPB 355 (11 mm), *A. niger* LPB 3C (10 mm) and *A. oryzae* LPB 532 (10 mm). The nine best strains were selected for the quantitative phospholipase screening in submerged fermentation.

TABLE 8 - MEASUREMENT OF HALO FORMATION ON YMA MEDIUM BY POTENTIAL PHOSPHOSLIPASE PRODUCING STRAINS

Strain	**Difference between halo and colony diameter (mm)**
Aspergillus niger LPB 1278	7 ± 0,4
Aspergillus niger LPB 3C	10 ± 0,54
Aspergillus niger LPB 3	9 ± 0,61
Aspergillus niger LPB 326	7 ± 0,67
Aspergillus flavus LPB 335	12 ± 0,53
Aspergillus flavus LPB 325	9 ± 0,77
Aspergillus brasiliensis LPB 355	11 ± 0,22
Aspergillus brasiliensis LPB 266	16 ± 0,45
Aspergillus oryzae LPB 532	10 ± 0,56

SOURCE: The author (2018)

These results are superior to those found by Silva (2011), who tested 2 strains of *Starmerella sp*.2 that formed a 1,4 mm and 1,3 halo each after incubation at 28˚C for 48 hours. Silva et al (2014) showed that *Trichoderma harzianum* produced, in 7 days and at 27˚C and 38˚C, a halo formation of 0,7 and 1,1 mm, respectively. According to Siloto (2001), the best strain for phospholipase production was observed to be from the genus *Aspergillus,* without halo measurement. Takemori *et al* (2012) also used the observation of halo formation, without measurement, as a high-throughput assay for phospholipase production.

3.2 QUANTITATIVE STRAIN SCREENING FOR PHOSPHOLIPASE PRODUCTION

3.2.1 PHOSPHOLIPASE PRODUCTION IN SUBMERGED FERMENTATION

Nine strains of phospholipase producers, which were screened before, were tested in submerged fermentative essays. The strain *A. brasiliensis* LPB 266 showed the best phospholipase activity reaching a production of 7555,55 U/mL after 12 days with a productivity of 26,23 U/mL.h. Phospholipase production reached 4044,44 U/mL (productivity of 14,04 U/mL.h) for *A. brasiliensis* LPB 355 and 2311,11 U/mL (productivity of 8,02 U/mL.h) for *A. niger* LPB 1278. The lowest phospholipase production was verified for *A. niger* LBP 3C, which reached 888,89 U/mL (productivity of 3,09 U/mL.h).

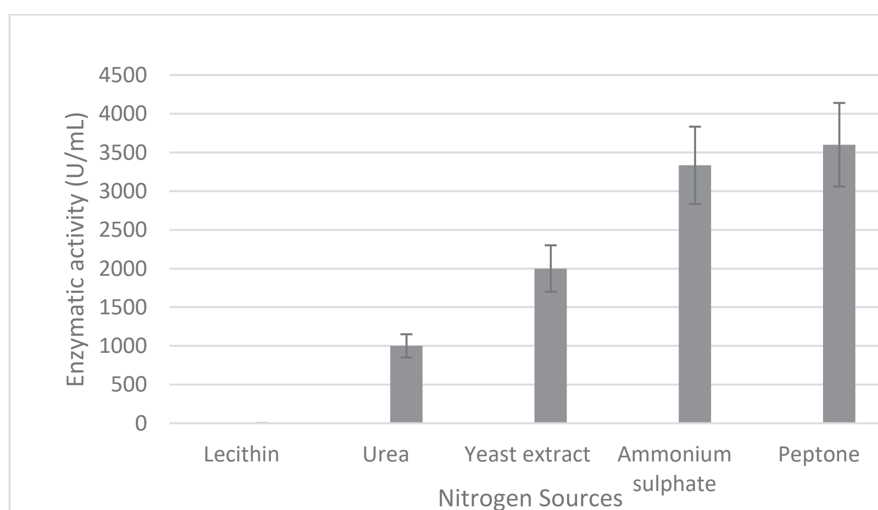
The results obtained in this work are very promising. This fact is probably due to the medium composition based on soybean lecithin and calcium ions that favor the synthesis of the enzyme. There are phospholipases C and phospholipases A_2 which requires calcium ions, to be effective on the cleavage of phospholipid molecules, as described by De Maria et al. (2007), Six and Dennis (2000) and Burke and Dennis (2009). Loffler et al. (1999) utilized soybean fractions in the medium composition due to its inducer role, increasing phospholipase production.

3.2.2 OPTIMIZATION OF PHOSPHOLIPASE PRODUCTION

3.2.2.1 SELECTION OF NITROGEN SOURCE

The influence of nitrogen source on phospholipase production was evaluated by submerged fermentation in 7 days. Urea, ammonium sulphate, yeast extract, peptone and soybean lecithin (without other nitrogen addition) were studied as nitrogen sources (FIGURE 11). The best result was obtained for peptone, which promoted a phospholipase activity of 3600 ± 453,25 U/mL (21,43 U/mL.h). Phospholipase production reached 3333,33 ± 332,59 U/mL (19,84 U/mL.h) with ammonium sulphate, 2000 ± 332,59 U/mL (11,9 U/mL.h) with yeast extract and 1000 ± 435,47 (5,95 U/mL.h) with urea. No significant enzyme production was obtained with lecithin without nitrogen source addition, proving that the fungus needs a more equilibrated C/N ratio for growth and the enzyme secretion.

FIGURE 11 - INFLUENCE OF DIFFERENT NITROGEN SOURCES ON FERMENTATION FOR PHOSPHOLIPASE PRODUCTION



SOURCE: The author (2018)

Hasida et al. (2000) reached a phospholipase activity of 1 U/g supplementing its culture medium with peptone and yeast extract as nitrogen sources, while Clausen et al. (2000) reached 53 U/mL with medium containing yeast extract, trypsin digested casein and meat extract as nitrogen sources.

Takemori et al. (2012) utilized a medium containing only peptone and yeast extract, no carbon source, reaching a phospholipase acivity of 6 U/mL. It shows the importance of nitrogen for biomass formation and, consequently, phospholipase production. However, these are high cost nitrogen sources that have a significant impact on medium production costs of the enzyme.

Considering standard deviation, it was observed that peptone and ammonium sulphate gave similar results on phospholipase production and they were both the best sources of nitrogen. Due to economic reasons, $(NH_4)_2SO_4$ was chosen for further essays.

3.2.2.2 INFLUENCE OF CARBON AND NITROGEN RATIO ON PHOSPHOLISPASE PRODUCTION

After selecting the best nitrogen source for phospholipase production, the carbon and nitrogen ratio was analyzed with the support of a Rotational Central Composite Design (RCCD). The experimental design was composed by 2 factors, 13 essays, with 3 central points in 1 block. The matrix was generated by the program STATISTICA 7 as indicated in TABLE 9. The response surface methodology was employed.

TABLE 9 - INFLUENCE OF CARBON/NITROGEN RATION OF PHOSPHOLIPASE PRODUCTION RCCD (2 FACTORS, 13 ESSAYS, WITH 3 CENTRAL POINTS IN 1 BLOCK)

Essay	Dextrose (g/L)	Ammonium sulphate (g/L)	Phospholipase activity U/mL
1	-1	-1	1444,44 ± 358,47
2	-1	1	3711,11 ± 268,96
3	1	-1	1311,11 ± 452,03
4	1	1	3755,56 ± 198,36
5	-1,41421	0	2866,67 ± 269,88
6	1,41421	0	2911,11 ± 408,21
7	0	-1,41421	0
8	0	1,41421	8200,00 ± 510,29
9	0	0	4555,56 ± 278,32
10	0	0	4644,44 ± 187,53
11	0	0	4822,22± 347,89

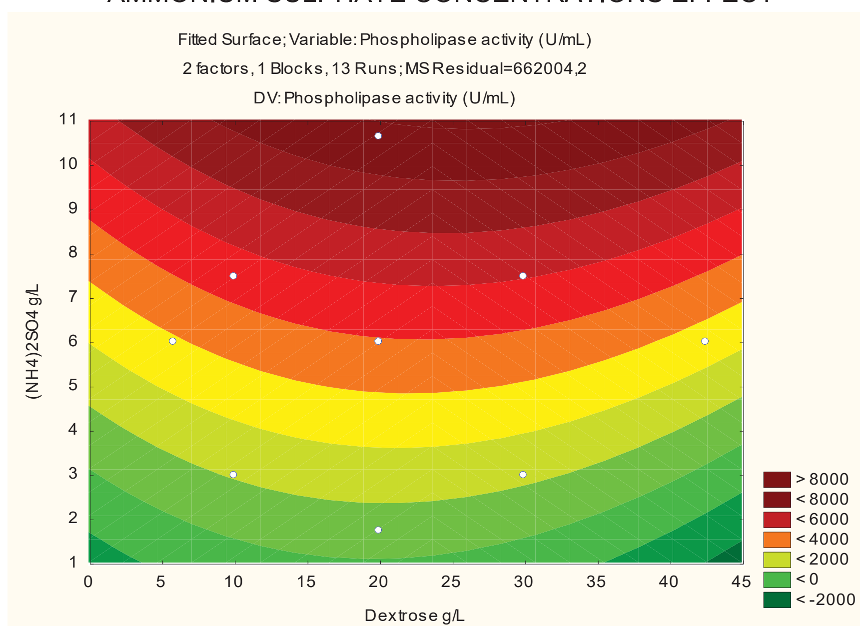
TABLE 9 - INFLUENCE OF CARBON/NITROGEN RATION OF PHOSPHOLIPASE PRODUCTION RCCD (2 FACTORS, 13 ESSAYS, WITH 3 CENTRAL POINTS IN 1 BLOCK)

Essay	Dextrose (g/L)	Ammonium sulphate (g/L)	Phospholipase activity U/mL
12	0	0	4200,00 ± 297,14
13	0	0	4066,67 ± 110,19

SOURCE: The author (2018)

According to TABLE 9, it is possible to notice that phospholipase activity increases with lower C/N ratios in the fermented medium. The best phospholipase production (8200 U/mL ± 510,29) was reached with 20 g/L of dextrose and 10,61 g/L of ammonium sulfate (FIGURE 12). Phospholipase activity between 4822,22 U/mL and 4200 U/mL was obtained with 20 g/L of dextrose and 6 g/L of ammonium sulfate. It is possible to notice that, based on phospholipases activities obtained, 3755,56 U/mL and 3711,11 U/mL (4 and 2 essays, respectively) higher C/N ratios do not favor the enzyme synthesis. It suggests that nitrogen content and more equilibrated C/N ratios significantly affect phospholipase synthesis, but not necessarily the biomass production.

FIGURE 12 - CONTOUR PLOT OF THE RCCD EXPERIMENTAL DESIGN FOR PHOSPHOLIPASE PRODUCTION OPTIMIZATION – STUDY OF DEXTROSE AND AMMONIUM SULPHATE CONCENTRATIONS EFFECT

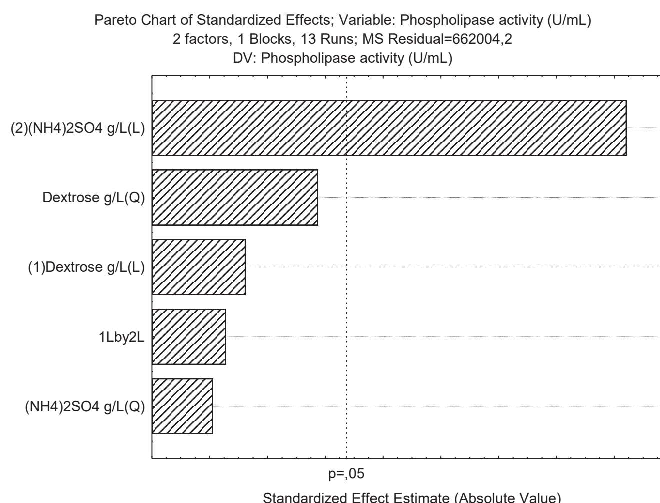


SOURCE: The author (2018).

Clausen et al. (2000), with a medium with C/N ratio approximately of 0,23 presented a phospholipase activity of 53 U/mL, while Zhan et al (2013) reached a phospholipase activity of 1,87 U/mL with a higher carbon/nitrogen ratio (around 2,14). The C/N ratio of Hasida et al. (2000) phospholipase production is the highest one among the other searched on this article (3,55) with the lowest phospholipase activity (1 U/g). Therefore, the relation between nitrogen and carbon, favoring higher nitrogen content is important to increase phospholipase production and its activity.

According to Pareto chart (FIGURE 13), it is possible to verify the strong effect of ammonium sulphate concentration on phospholipase production ($p < 0,05$). Dextrose did not affect the enzyme synthesis significantly neither the interaction between the two factors. Ammonium sulphate was the only factor that affected phospholipase activity.

FIGURE 13 - PARETO'S CHART SHOWING THE EFFECT OF DEXTROSE, AMMONIUM SULPHATE CONCENTRATION AND THEIR INTERACTION ON PHOSPHOLIPASE ACTIVITY



SOURCE: The author (2018).

According to ANOVA (TABLE 10), the R^2 value obtained is 0.903, which means that more 90.3% of the response variable variation is explained by the generated model, with a satisfactory adjustment of the quadratic model with the experimental data.

The coefficient of determination (R^2) measures the proportion of total variability explained by the model. It is suggested that for a good-fitting model R^2 should be close to 1, and at least 0.80 (XIANGLI et al., 2008).

TABLE 10 - ANOVA ANALYSIS OF PHOSPHOLIPASE PRODUCTION IN DIFFERENT CONDITIONS ACCORDING TO RCCD WITH 2 FACTORS AND 13 ESSAYS

ANOVA; R-sqr =,90323; Adj:,83411
Dependent Variable: Phospholipase activity (U/mL)

	SS	df	MS	F	p
(1) Dextrose g/L(L)	255656	1	255656	0,38618	0,554002
Dextrose g/L(Q)	2329363	1	2329363	3,51865	0,102797
(2) $(NH_4)_2SO_4$ g/L(L)	34428527	1	34428527	52,00651	0,000176
$(NH_4)2SO_4$ g/L(Q)	2230	1	2230	0,00337	0,955337
1L by 2L	52826	1	52826	0,07980	0,785743
Error	4634029	7	662004		
Total SS	47887559	12			

SOURCE: The author (2018).

The finding quadratic correlation, which represents phospholipase activity, is given by EQUATION 1.

EQUATION 1 – Mathematical model for phospholipase activity

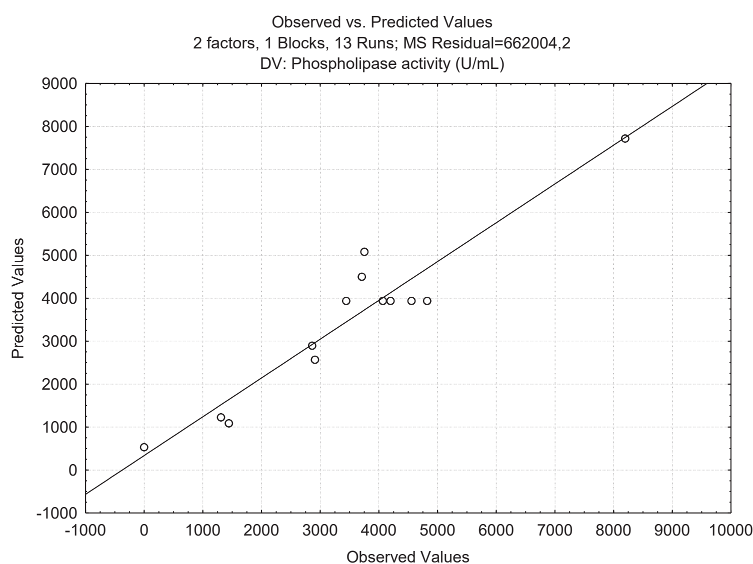
$$\text{Phospholipase activity (U/mL)} = 139{,}47A - 3{,}69A^2 + 687{,}87B + 1{,}91B^2 + 4{,}96AB - 2166{,}98$$

Where: A is the dextrose concentration (g/L) and B is the ammonium sulphate concentration (g/L)

SOURCE: The author (2018).

The predicted values are in reasonable agreement with the experimental data (FIGURE 14). The data points of the response are relatively close to the 45° line, which demonstrates that the experiments come from a normally distributed population. Moreover, the good distribution of data indicates that the choice of the operating parameters and their levels was acceptable.

FIGURE 14 - COMPARISON OF PREDICTED AND ACTUAL VALUES OF PHOSPHOLIPASE ACTIVITY



SOURCE: The author (2018).

The strain *A. brasiliensis* LBP 266 reached a very high productivity of 48,81 U/mL.h. It is superior to the phospholipase productivity obtained by Clausen *et al* (2000), which was 0,44 U/mL.h. Takemori et al. (2012), with a recombinant microorganism, reached 0,29 U/mL.h while Hasida et al. (2000) was only 0,01 U/mL.h. These differences might be attributed to the medium composition and the presence of an effective inducer. According to Loffler et al. (1999), the addition of phospholipids, as soybean lecithin, increases phospholipase production due to its strong inducer role. Several authors have worked in the production of phospholipases by different fungal strains, including recombinant strains, as indicated in TABLE 11. However, the productivity of the phospholipase produced by *Aspergillus brasiliensis* LPB 266 is largely higher.

TABLE 11 - PRODUCTION OF PHOSPHOLIPASE BY DIFFERENT MICROORGANISMS USING DIFFERENT FERMENTED MEDIA

Microorganism	**Productivity**	**Phospholipase activity**	**Reference**
Hyphozyma sp.	0,01 U/g.h	1 U/g	Hasida *et al*, 2000
Asrpegillus oryzea in	0,15 U/mL.h	38,7 U/mL	Shiba *et al*, 2001

Saccaharomyces cerevisea			

TABLE 11 - PRODUCTION OF PHOSPHOLIPASE BY DIFFERENT MICROORGANISMS USING DIFFERENT FERMENTED MEDIA

Microorganism	Productivity	Phospholipase activity	Reference
Aspergillus oryzae (SANK 11870)	0,05 U/mL.h	5,9 U/mL	Hattori *et al*, 1995
Fusarium oxysporum DSM 2672	0,44 U/mL.h	53,0 U/mL	Clausen *et al*, 2000
Recombinant *Kluyveromyces lactis* GG799	0,03 U/mL.h	1.87 U/ml	Zhang *et al*, 2015
Recombinant *Escherichia coli* BL21	0,29 U/mL.h	6 U/mL	Takemori *et al*, 2012
Aspergillus brasiliensis LPB 266	48,81 U/mL.h	8200 ± 510,29 U/ml	This work

SOURCE: The author (2018).

3.2.3 CONCENTRATION AND PURIFICATION OF PHOSPHOLIPASE

Phospholipase enzymatic extract was microfiltrated through a Vacuum Filtration System PES 0,22µm (KASVI®). After microfiltration, samples were purified and concentrated through Vivaspin 6 (GE Healthcare®) ultrafiltration membranes devices from 100 kDa until 30 kDa (TABLE 12).

TABLE 12. PURIFICATION AND CONCENTRATION OF PHOSPHOLIPSE FROM *ASPERGILLUS BRASILIENSIS* 266 THROUGH MICROFILTRATION AND ULTRAFILTRATION SYSTEMS

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Microfiltration 0,22 µm	0,1276	5066,66	39663,90		100,00
Permeated 100 kDa membrane	0,1268	4533,33	35754,66	1,00	99,37

TABLE 12. PURIFICATION AND CONCENTRATION OF PHOSPHOLIPSE FROM *ASPERGILLUS BRASILIENSIS* 266 THROUGH MICROFILTRATION AND ULTRAFILTRATION SYSTEMS

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Permeated 50 kDa membrane	0,1215	5600,00	46075,37	1,28	95,22
Retained 30 kDa membrane	0,0997	8266,66	75363,90	2,10	78,13

SOURCE: The author (2018).

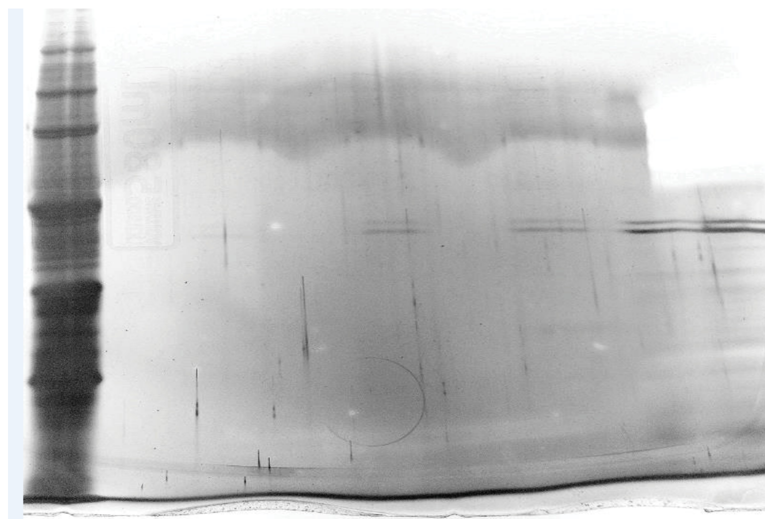
According to data above, it was observed that phospholipase have a molecular weight between 30 and 50 kDa, which was detected by the higher specific phospholipase activity that was retained by the 30 kDa membrane (75363,90 U/mg), followed by the specific phospholipase activity of the 50 kDa permeated (46075,37 U/mg).

UF was performed to concentrate and enrich phospholipase in four steps. As a result of this technique, 2,1-fold purification with 78% yield was observed. A high phospholipase purification fold was obtained from Vivaspin 6 ultrafiltration membranes. These are low volume systems that are employed in preliminary purification essays. Generally, industrial ultrafiltration membranes can treat much higher volumes and are re-used several times. These systems seem to be a very good alternative for the enzyme purification and concentration for further oil degumming process application.

3.2.4 SDS PAGE

Aspergillus brasiliensis LPB 266 phospholipase molecular weight was determined by SDS PAGE. Four different concentrations (125, 250, 500 µg and crude extract) were tested. Each sample was tested in duplicated. The bands shown on the gel (FIGURE 15), in comparison with the markers, indicates that the *Aspergillus brasiliensis* LPB 266 phospholipase has a molecular weight between 30 and 55 kDa.

FIGURE 15 - SDS PAGE ANALYSIS OF PHOSPHOLIPASE MOLECULAR WEIGHT



SOURCE: The author (2018).

A phospholipase with a molecular weight between 35 and 50 kD is close to what is found on literature, especially for phospholipases produced by fungi, as Hattori *et al* (1995) described a 37 kDa phospholipase molecular weight, Shiba *et al* (2014) with a 36 kDa phospholipase and Clausen *et al* (2000) with a 30 kDa enzyme. This result indicated that, it is a phospholipase, but further studies, as an enzymatic identification test should be performed.

The enzyme identification by MALDI-TOF, formulation and accelerated/ normal stability tests will certainly be performed thinking of the application of the enzyme in oil degumming, as response for VALE S.A. industrial demand. Very good perspectives are being viewed for this enzymatic bioproduct.

4 CONCLUSIONS AND PERSPECTIVES

Among all the information, it is known that enzymatic degumming is applicable to biodiesel industry, in order to increase fatty acid yield. Besides that, the enzyme utilized on the process can be produced from a microorganism cultured in laboratory, eliminating the expenses with the commercial enzyme, becoming a more profitable process.

With all information as bases for understanding the enzymatic degumming process, microorganisms, substrates and phosphorous content that were reported by different authors, they may be analyzed and confronted with each other, in order to find the best microorganism and the best substrate/conditions for the reaction.

Based on this work, an efficient phospholipase production process from *Aspergillus brasiliensis* LBP 266 was developed. Optimization essays led to the definition of a medium composition that allowed very high enzyme activity (8200 U/mL) and productivity of 48,81 U/mL.h with soybean lecithin as a low-cost inducer. A quadratic model was defined with R^2 = 0,903, which means that more than 90.3% of the response variable variation is explained by the model. Preliminary purification and concentration studies were carried out by micro and ultrafiltration systems allowing the identification of the molecular weight range of the enzyme between 30 and 50 kDa. 78% of the proteins were recovered with 2,1-fold concentration. Ultrafiltration showed to be a good strategy for enzyme recuperation and purification. Promising perspectives are viewed for this process, which will be continued with the scale-up of the enzyme production and purification. Finally, a stable liquid or powdered enzymatic formulation, after accelerated and normal stability studies, will be defined for its application in oil degumming.

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