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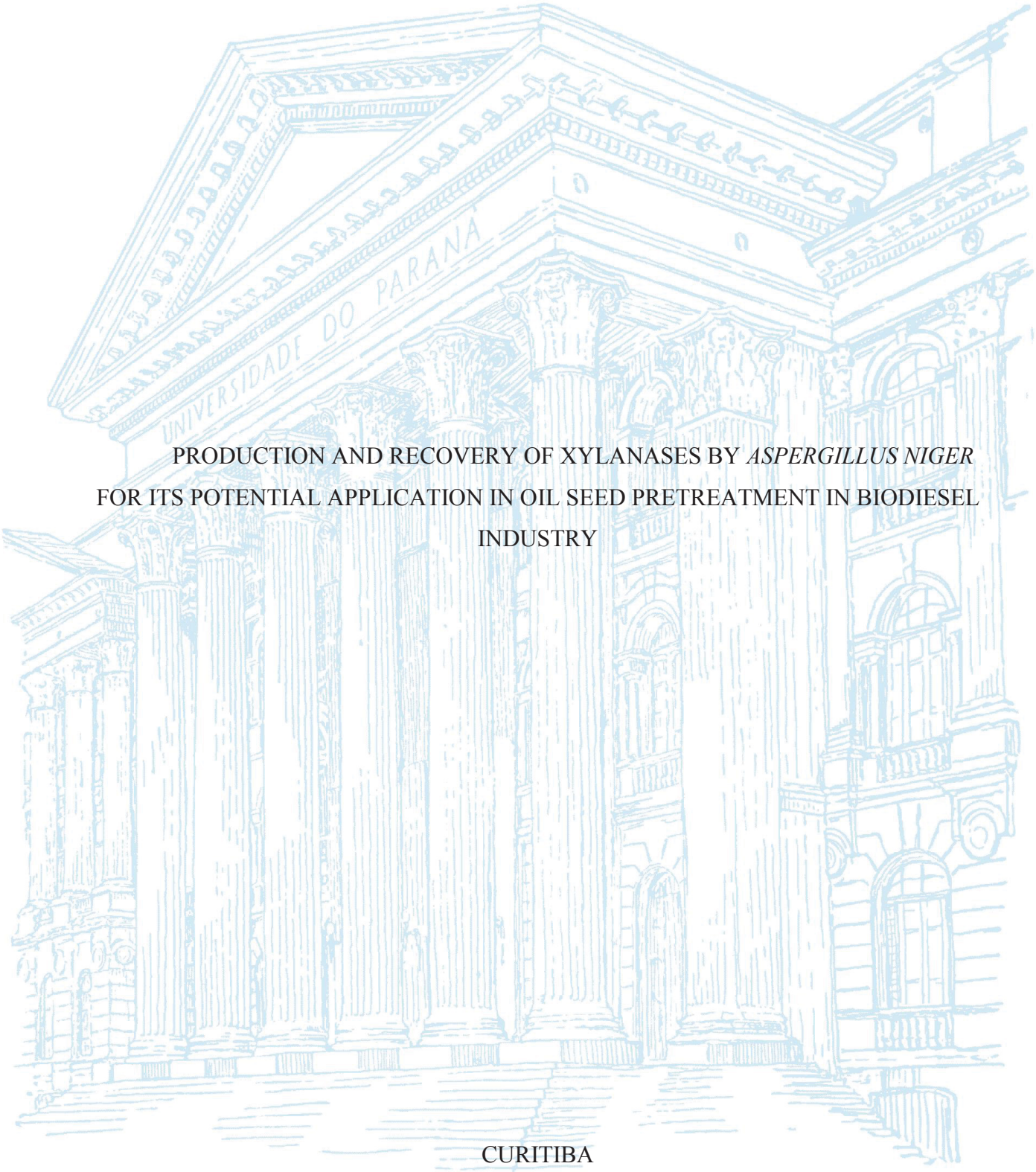
KIM KLEY VALLADARES DIESTRA



PRODUCTION AND RECOVERY OF XYLANASES BY *ASPERGILLUS NIGER* FOR ITS POTENTIAL APPLICATION IN OIL SEED PRETREATMENT IN BIODIESEL INDUSTRY

CURITIBA

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PRODUCTION AND RECOVERY OF XYLANASES BY *ASPERGILLUS NIGER* FOR ITS POTENTIAL APPLICATION IN OIL SEED PRETREATMENT IN BIODIESEL INDUSTRY

Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Engenharia de Bioprocessos e Biotecnologia, no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná.

Orientadora: Profa. Dra. Luciana Vandenberghe
Co-Orientador: Prof. Dr. Carlos Ricardo Soccol

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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 **KIM KLEY VALLADARES DIESTRA** intitulada: **"Production and Recovery of Xylanases by *Aspergillus niger* for its Potential Application in Oil Seed Pretreatment in Biodiesel 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, 09 de Março de 2018.

LUCIANA PORTO DE SOUZA VANDENBERGHE
Presidente da Banca Examinadora (UFPR)

JULIO CESAR DE CARVALHO
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CRISTINE RODRIGUES
Avaliador Externo (UFPR)

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RESUMO

O biodiesel é uma alternativa ao uso de diesel fóssil, não tóxico e menos poluente. A produção de biodiesel ocorre com o uso de óleos extraídos de sementes oleaginosas, como soja, colza e palmeira. A extração de óleos de sementes oleaginosas é uma etapa economicamente importante na produção de biodiesel. A extração aquosa assistida por enzimas (EAAE) tem ganho importância como uma alternativa para extração de óleo, usado na indústria de biodiesel. As enzimas comerciais aplicadas para este fim são xilanases, celulases e outras hemicelulases; as xilanases catalisam a hidrólise das hemiceluloses, o segundo composto mais importante da parede celular das sementes oleaginosas. Por outro lado, o Brasil é um país no qual a indústria agrícola desempenha um papel importante na economia, gerando uma grande quantidade de resíduos com potencial para a produção de hemicelulases. O objetivo deste trabalho foi otimizar a produção de xilanases, utilizando diferentes resíduos agroindustriais brasileiros para futura formulação e aplicação no pré-tratamento de extração de sementes oleaginosas, na indústria do biodiesel. Este trabalho foi dividido em duas partes; o primeiro tem a revisão bibliográfica focada na produção e uso de enzimas hemicelulolíticas como ferramenta potencial no pré-tratamento de oleaginosas, para o aprimoramento da extração de óleo em processos de produção de biodiesel. A segunda contém os ensaios experimentais para otimização da produção e recuperação da xilanase produzidas por Aspergillus niger. De acordo com os resultados, um eficiente processo de produção de xilanase foi desenvolvido por Aspergillus niger LPB BC em SmF usando resíduos agroindustriais (bagaço de cana e farelo de soja) também foram identificados 2 tipos diferentes de xilanases: beta-xilanase de 27 kDa e uma endo-1,4-beta xilanase parcial de 15 kDa. Perspectivas promissoras são vistas para este processo, que será continuado com a análise de escalonamento da produção da enzima e definição de uma mistura enzimática para hidrólise eficiente de sementes oleaginosas.

Palavras chave: Xilanase, bagaço de cana, Aspergillus niger, biodiesel, extração de óleo.

ABSTRACT

Biodiesel is an alternative to the use of diesel fossil, non-toxic and less pollutant. The production of biodiesel occurs with the use of oils, extracted from oleaginous seeds such as soybean, rapeseed and palm fruit. The extraction of oils from oilseeds leads to an important economic step in the production of biodiesel. Enzyme-assisted aqueous extraction (EAAE) is gaining importance as an alternative for extraction of used oil in biodiesel industry. The commercial enzymes applied to this purpose are xylanases, cellulases and other hemicellulases; the xylanases catalyze the hydrolysis of hemicelluloses, the second most important compound of the cell wall of oleaginous seeds. On the other hand, Brazil is a country in which the agricultural industry plays a great role in the economy, generating a large amount of waste that has potential for the production of hemicellulases. This work's objective was the optimization of xylanases production, using different Brazilian agro-industrial waste for future formulation and application in the pretreatment of oil seed extraction, in the biodiesel industry. This work was divided in two parts; the first has the bibliographic review focused on the production and use of hemicellulolytic enzymes as a potential tool in oilseed pretreatment for oil extraction enhancement in biodiesel production processes. The second part content the experiment assays for the production optimization and recovery of xylanase by Aspergillus niger. According to the results, an efficient xylanase production process was developed by Aspergillus niger LPB BC in SmF using agro-industrial waste (sugarcane bagasse and soybean meal) also were identification 2 types different xylanases: beta-xylanase of 27 kDa and partial endo-1,4 - beta xylanase of 15 kDa. Promising perspectives are viewed for this process, which will be continued with the analysis of scale-up of the enzyme production and define an enzymatic blend for efficient enzymatic hydrolysis of oleaginous seed.

Keywords: Xylanase, sugarcane bagasse, Aspergillus niger, biodiesel, oilseed extraction.

SUMÁRIO

1. INTRODUÇÃO

Nas últimas décadas o consumo de petróleo aumentou, devido ao crescimento da população mundial e consequente industrialização, o que resultou no esgotamento das reservas de combustíveis fósseis e no aumento do preço do petróleo, Bhuiya et al. (2016). O biodiesel, que é uma mistura de ácidos graxos alquílicos ésteres, é um combustível à base de óleo que tem sido sugerido como um possível substituto para o diesel de petróleo, especialmente no transporte. O biodiesel tem propriedades semelhantes às do diesel convencional, Taher; Al-Zuhair (2017), reduz as emissões de gases de efeito estufa, Atadashi et al. (2012) é um combustível renovável, não tóxico e biodegradável, Divya; Tyagi (2006).

A maioria do biodiesel comercial é produzida a partir de óleos vegetais refinados, como óleo de soja nos Estados Unidos e no Brasil, óleo de semente de colza e girassol na Europa, óleo de palma no sudeste da Ásia e óleo de coco nas Filipinas, Yustianingsih et al. (2009). A extração de óleo das sementes ou frutos é uma das etapas mais determinantes durante a produção de biodiesel, o que afeta os custos do processo final, a produção e pode gerar problemas ambientais.

A extração com solvente orgânico é o processo mais convencional e eficiente usado para extrair óleo das sementes. No entanto, compreende o uso de solventes inflamáveis, como o hexano, que precisa de algumas atenções para evitar sua volatilização, altos custos de operação e influência indesejável na qualidade dos alimentos, Zhang et al. (2012). Um processo alternativo para a extração de óleo de sementes ou frutos é o uso de enzimas como celuloses, hemiceluloses e proteases, para facilitar a extração de óleo de muitas sementes e frutos que fja foi realizado a escala laboratorial e numa escala, Domínguez et al. (1994) e Rosenthal et al. (1996).

A extração aquosa assistida por enzimas (EAAE) vem ganhando importância como alternativa para a extração simultânea de óleo e proteína. Acredita-se que seja ambientalmente amigável, seguro e barato, Latif; Anwar (2009). Este método é o mais utilizado e difundido para a extração de óleos com o uso de enzimas. A fase aquosa permite a solubilidade e mobilização das enzimas para degradar as paredes celulares das sementes e frutos oleaginosos. Também permite o controle de diferentes parâmetros como concentração enzimática, manter o pH e temperatura desejada dentro do sistema.

As hemicelulases é um grupo diversificado de enzimas que hidrolisam hemiceluloses, um dos grupos mais abundantes de polissacarídeos na natureza, Robl et al. (2013) e Shallom; Shoham (2003). As hemicelulases consistem em um grupo de enzimas capazes de aumentar o

rendimento de produção de açúcares redutores durante a hidrólise enzimática de substratos lignocelulósicos. As xilanases catalisam a hidrólise de hemiceluloses em conjunto com outras enzimas acessórias como α-L-arabinofuronosidase, esterases hemicelulolíticas, β-mananases, α-glucoronidases, β-xilosidases e pectinases. Numerosos microorganismos são capazes de produzir hemiceluloses por diferentes técnicas de fermentação, como fermentação em estado sólido (SSF) ou fermentação submersa (SmF). Embora os fungos sejam naturalmente os principais produtores de hemiceluloses, essas enzimas são necessárias para a degradação do material orgânico e, portanto, reduzem os resíduos de lignocelulose para os açúcares a serem utilizados como nutrientes em seu crescimento e proliferação.

Por outro lado, o Brasil é um país no qual a indústria agrícola desempenha um papel importante na economia, sendo o maior produtor de cana-de-açúcar com uma produção anual de 739.300 TMT, Maciel et al. (2008) e Sheth (2017). O país é o segundo maior produtor de soja com uma produção anual de 86,8 MMT, James Karuga (2017) e um dos produtores de óleo de palma em crescimento com uma produção anual de 410 TMT, GPOP (2017). Essas indústrias geram uma grande quantidade de resíduos que causam poluição no meio ambiente, sendo os principais resíduos gerados: bagaço de cana na produção de açúcar, farelo de soja e cachos vazios de óleo de palma (OPEFB) na extração de óleo de soja e dendê, respectivamente. Estes resíduos agroindustriais têm potencial para produção de hemicelulases, Alberton et al. (2009) e Goelzer (2015 e Maciel et al. (2008) por seu alto teor de proteína e lignocelulose, reduzindo assim seu impacto negativo sobre o meio ambiente e o custo de produção da enzima.

Por essa razão, o objetivo principal deste trabalho foi a otimização da produção de xilanases utilizando diferentes subprodutos obtidos de processos agroindustriais brasileiros, para futura formulação enzimática e aplicação no pré-tratamento de extração de sementes oleaginosas. indústria de biodiesel.

2. OBJETIVOS

2.1 Objetivo geral

O estudo principal deste trabalho foi a otimização de produção de xilanases utilizando diferentes resíduos produzidos pela indústria agrícola do Brasil (bagaço de cana e o farelo de soja), para uma futura aplicação no pré-tratamento, assistido por enzimas, da extração de óleo de sementes oleaginosas para a indústria de biodiesel.

2.2 Objetivos Específicos

a) Triagem qualitativa e quantitativa de fungos produtores de xilanase.
b) Otimização da produção de xilanase pela cepa selecionada em fermentação submersa utilizando resíduos agroindustriais.
c) Estudo cinético da produção de xilanase em frascos Erlenmeyer e reator de tanque agitado (STR).
d) Recuperação e purificação parcial de xilanase por membranas de microfiltração e ultrafiltração.
e) Identificação molecular da xilanase.
f) Caracterização de xilanases e estudos preliminares de estabilidade enzimática.

3. ARTIGO 1

Enzymatic Preparations: Production and Potential Application for Oil Seed Pretreatment in Biodiesel Industry: A review

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Abstract

Biodiesel is an alternative to the use of fossil diesel, non-toxic and less pollutant. The production of biodiesel occurs with the use of oils, extracted from oleaginous seeds such as soybean, rapeseed and palm fruit. For this reason, the extraction of oils from oilseeds leads to an important economic step in the production of biodiesel. Different technologies to improve oil extraction have been developed and applied, but one of the most used and developed is the enzyme-assisted aqueous extraction (EAAE). EAAE is an environmentally friendly technology that takes advantage of the degradation efficiency of the enzymes, in this case hemicelluloses preparations, which specifically degrade different structures that are present in vegetable cell walls to improve oil extraction from oilseeds. The enzymes used in this process are produced commercially, but with high costs. The use of agro-industrial residues or the improvement of the enzymes producing strains could be an interesting solution for viable enzymatic preparations' production for responding to the demand of the biodiesel industry.

Keywords: Hemicellulases preparations, Biodiesel, Oilseed, Enzymatic oil extraction

Introduction

Petroleum consumption has increased over the last few decades due world's population growth and consequent industrialization, which has resulted in depletion of fossil fuel reserves and increasing petroleum price Bhuiya et al. (2016). Energy consumption is inevitable for human existence. There are various reasons for the search of an alternative fuel that is technically feasible, environmentally acceptable, economically competitive, and readily available. The first foremost reason is the increasing demand for fossil fuels in all sectors of human life, such as transportation, power generation, industrial processes, and residential consumption Kafuku; Mbarawa (2010). Fortunately, concerns about the environment are growing and the scientific community is being challenged to improve existing alternatives to petroleum-derived fuels and to create renewable fuels, which will likely be an important product in bio-based economies Bergmann et al. (2013). In the 1930s and 1940s vegetable oils were already used as diesel fuels from time to time, but only in emergency situations. Recently, due of the increases in crude oil prices, limited resources of fossil oil and environmental concerns, there has been a renewed focus on vegetable oils and animal fats for biodiesel production Ma; Hanna (1999).

Biodiesel is a mono alkyl ester of fatty acids. Oil sources for biodiesel production might come from micro algae Abbaszaadeh et al. (2012), Bergmann et al. (2013), animal fats Gog et al. (2012) or edibles oils, as soybean, rapeseed, sunflower an oil palm Chattopadhyay et al. (2011 e Hama; Kondo (2013 e Öner; Altun (2009), but it is mainly produced through transesterification of edible oils Hama; Kondo (2013). It is biodegradable and nontoxic, has low emission profiles and so is environmentally beneficial Balat (2006). The degree in which this substitution will depend on how economically competitive biodiesel is, which could happen with the use renewable and abundant feedstock as oil sources, the largest component of biodiesel's price Bergmann et al. (2013). For biodiesel production from oil seed, seeds are received from the farm and pass through sterilization. This process is also important 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. However, the use of enzymes may cause serious impact on biodiesel final costs. New alternatives for enzymatic preparations production could favor its application in seed pretreatment and reduce the biofuel's price.

The production of biodiesel output rose fractionally, from 22.4 billion liters (5.9 billion gallons) in 2011 to 22.5 billion liters (5.94 billion gallons) in 2012. Biodiesel now accounts for more than 20 percent of global biofuel production. Oleaginous seeds and fruits are still the main

source of oil for biodiesel production Ron Kotrba (2014), among these seeds the most important are soybean, oil palm, rapeseed, and sunflower. Different studies are carried out to improve the extraction of oils from these seeds. Among these studies Enzyme-assisted aqueous extraction (EAAE) proves to be one of the best options, having very good extraction results De Moura et al. (2008 e Passos et al. (2009) and being environmentally friendly Latif; Anwar (2009). This enzyme extraction technology is based on the pretreatment of cellulose, hemicellulose and protein structure of the cell walls and organelles that contain the lipids or oils, which are employed in biodiesel production.

This review is focused on the production and use of hemicellulolytic enzymes as a potential tool in oilseed pretreatment for oil extraction enhancement in biodiesel production processes, as a response for the biodiesel industry demand.

Biodiesel

Biodiesel reduces carcinogenic compound emissions by approximately 85% compared with diesel fuel and is essentially free of sulfur, metals, and polycyclic aromatic hydrocarbons Atadashi et al. (2012). The world production of biodiesel is increasing; the prospects of production for the year 2024 are 39 billion liters in the world, generating a greater development in the technologies that entails all the production of bioethanol and a greater investment in the research. This increase also influence the majority of policies of each country, increases the obligatory of the use of biodiesel and higher investments on production and research about the subject OECD/FAO (2015).

Renewable energy sources are vitally important for Brazil's energy matrix. Sugarcane production for energy purposes (i.e., bioethanol) has a long-standing tradition in Brazilian agro culture. However, Brazil is currently not only a large producer of soybeans and other oilseeds and fruits, but also an important producer and consumer of biodiesel Bergmann et al. (2013). Moreover, the demand for biodiesel is increasing due to governmental policies and alternatives to soybean such as palm fruit, castor beans and other, which are candidate feedstocks for biodiesel production, are also appearing Saturnino et al. (2005).

In 2010, Brazil became the second largest producer of biodiesel with a production of 2.4 million m^3, behind Germany the largest world biodiesel producer with a production of 2.8 million m^3 Ron Kotrba (2014). This was as a result of strong investments made in biodiesel research and production due to a governmental program Bergmann et al. (2013). In 2011, despite Brazil's increase in biodiesel production to 2.6 million m^3 ANP (2014), it was surpassed by the United States, which produced 3.7 million and Argentina, which produced 2.7 million

of m^3 of biodiesel. Germany maintained the production of 2.8 million m^3 Ron Kotrba (2014). In 2016, total Brazilian biodiesel production is projected at 3.87 billion liters, down three percent compared to the revised estimate for 2015 (4.01 billion liters). The projections take into account the current recession of the Brazilian economy and the consequent decrease of diesel consumption, according to updated information released by the Petroleum Agency USDA (2016). Brazil's energy policy obliges a fuel mix of 7% biodiesel and seeks to increase the percentage of biodiesel by 1% per year to reach a blend of 10% biodiesel in the fuel for the year 2019. The final idea is to reach a final concentration of 20% of biodiesel blend. This will depend on the advance in the manufacture of cars and the resistance of their engines for this level of mixing, which certainly would imply in a significant increase of biodiesel production Umbrabio (2016).

Oilseed as biodiesel oil sources

Most commercial biodiesels are produced from refined vegetable oils, such as soybean oil in USA and Brazil, rapeseed and sunflower seed oils in Europe, palm oil in Southeast Asia, and coconut oil in the Philippines Yustianingsih et al. (2009). In order to reduce biodiesel's cost, a number of efforts have been made. The use of low cost feedstocks, simplified processes, and utilization of by-products from biodiesel production are expected to reduce production costs.

Considerable research has been done on vegetable oils as sources for biodiesel production, which include palm oil, soybean oil, sunflower oil, coconut oil, rapeseed oil and castor oil (Table 1). Animal fats, although frequently mentioned, have not been studied to the same extent as vegetable oils. Some methods applicable to vegetable oils are not applicable to animal fats due to some natural properties differences. Oils from algae, bacteria and fungi also have been investigated Shay (1993).

Vegetable oils are produced on an industrial scale using processes that depend on both the physical and the chemical characteristics of the feedstock. All seeds contain fat, but few of them contain fat in quantities large enough to make its extraction convenient Santori et al. (2012). Soybean oil dominates the world oilseed production while rapeseed is second position (**Table 1**). The oil content in soybean and rapeseed is 21% and 35%, respectively. Despite the lesser availability, palm oil is an interesting source for biodiesel production due to its lower price and relatively high oil content (40%). This oil also gives highest oil yield per area per year as compared to other oils Issariyakul; Dalai (2014).

Table 1: World oilseed production, average oil price and oil content of various oilseeds, adapted from European Biofuels (2016 e Issariyakul; Dalai (2014)

Oil crops	Oil content (%)	Oilseed Production (million metric tons)	Average oilseed price (U.S.D/metric tons)	Average oil price (U.S.D /metric tons)	Production (tons/yr)	Yield (kg/ha/yr)
Rapeseed	35	46.72	375	82	70,954,407	600-1000
Soybean	15-22	235.77	254	684	308,436,057	300-450
Sunflower	44-51	30.15	n/a	n/a	867,659	280-700
Palm	40	10.27	n/a	655	52,821,076	2500-4000
Rice bran	10-25	1.4	n/a	500	n/a	n/a
Castor bean	39.6–59.5	2	463	987	n/a	1.07

U.S.D: United States Dollar

Soybean oil

Today soybean is the world's largest oilseed in terms of total production and international trades with a production of 312 million metric tons. It is widely cultivated in a number of countries, with the major producers being the USA (34%), Brazil (31%), Argentina (18%) and China (4%) USDA (2015). The oil content in soybean seed ranges from 15% to 22% depending on environmental conditions during seeds maturity and is composed of five principal fatty acids: palmitic acid (13%), stearic acid (4%), oleic acid (18%), linoleic acid (55%) and linolenic acid (10%) (Kinney & Clemente, 2005) (Titipong Issariyakul, 2014). Soybeans are the world's largest oilseed crop, with a production of about 56% of the world's total oilseeds European Biofuels (2016). Brazil, soybean is responsible for over 80% of all biodiesel produced (Moreira, Piovesan, Gonçalves, & Alves, 2013).

Rapeseed oil

Due to its ability to tolerate low temperature, *Brassica* crops were among the few vegetable oil sources that could be cultivated in cold climate regions. These seeds have oil content over 40% in which the dominant fatty acids include oleic acid, linoleic acid, and erucic acid (Titipong Issariyakul, 2014). The oil content of the rapeseed at maturity is known to vary among cultivars, and plant breeders have expended a great effort to increase the oil content in the mature seeds of modern oilseed. In 2011, the European biodiesel represented 43% of the world production and the rapeseed oil was 68% the totality oil production in the European Union EUBIA (2014).

Palm oil

The oil palm, *Elaeis guineensis*, is proven to be the species that produces the largest quantity of oil per hectare planted, averages of 3,700 kg are suggested for Brazil in about 86 thousand hectares of plantation Ministerio do meio Ambiente (2006). The most productive regions are located in Southeast Asia especially Malaysia and Indonesia, which together account for around 80%. Palm is the most efficient oil-producing plant per area per year Issariyakul; Dalai (2014). In face of the edaphoclimatic requirements, it presents limitations for its dissemination in the Brazilian territory prevailing restricted production areas in the states of Pará and Bahia Saturnino et al. (2005), in the North and Northeast of Brazil, respectively. There are generally two types of oil derived from palm including palm oil from mesocarp and palm kernel oil from kernel inside the seed. Palm oil is more saturated than soybean oil and rapeseed oil as its major fatty acids include palmitic, stearic, oleic, and linoleic. Palm kernel oil is more saturated than palm oil as it mainly contains lauric, myristic, andoleic Issariyakul; Dalai (2014). The greatest difficulty of this species is related to the impossibility of storage as much as palm fruits must be processed in 48 hours. For this reason, the company Agropalma, in the State of Pará, Brazil, implemented a production unit of biodiesel to process its acidic residue obtained from the processing of this fruit, the installed capacity is 15 million liters per year. Brazil has an oil palm production of 98.91 million metric tons in 2015 USDA (2015).

Other seed oil

Sunflower with high oil content is one of the most prominent oilseed crops for biodiesel production. The annual sunflower seed production is estimated at around 25 million tons versus the global sunflower oil output estimated at 10 million tons produced from over 80% of the global sunflower seed production. The average oil content of the seed is 40–50% Marvey (2008). Sunflower oil is composed of linoleic, oleic, and linolenic acids, and their presence affects the oxidative oil stability. Sunflower oil has approximately 70% linoleic acid and is highly susceptible to lipid oxidation GBOGOURI et al. (2013 e Saydut et al. (2010). In 2011, the area used for sunflower cultivation was 66,400 ha with a total production of 83,100 tons. The central region of Brazil is the largest producer generating 64,000 tons in 2010/2011; most of the production is destined for bird food. A reduced number of companies produce biodiesel from sunflower seed oil Bergmann et al. (2013).

Rice bran contains 10-25% of lipids being thus the main source of rice oil Hata et al. (2008 e Zullaikah et al. (2005). The majority of oil components are triacylglycerides with palmitic, oleic, and linoleic as major fatty acids. The rice bran oil production is non-edible.

Therefore, rice bran is a potential low-cost feedstock and it would be an excellent candidate for biofuels production through biorefining Issariyakul; Dalai (2014).

The castor bean has approximately 39.6–59.5% of oil, but the yield is only 470 of oil/ ha Saturnino et al. (2005). In 2011, Brazil had 128,200 ha of castor bean planted area and a production of 24,800 t of seeds Bergmann et al. (2013). The company Petrobras Biofuels opened a pilot plant for producing biodiesel from castor bean oil in the city of Candeias in the state of Bahia, Northeast region of Brazil promoting the cultivation of this seed.

Oilseed and its composition

Plant cell walls are amazingly complex amalgams of carbohydrates, proteins, lignin, water, and incrusting substances such as cutin, suberin, and certain inorganic compounds that vary among plant species, cell types, and even neighboring cells Showalter (1993). To extract the lipid reserves stored in cells, it is necessary to be able to cross several barriers: first the extra cellular walls (or secondary cell walls), then the cell wall, which is the most important barrier. It is composed mostly of cellulose, hemicellulose and pectin (**Fig 1**), and, finally, oleosomes. Each cell wall has its own constituents, sometimes organized in a complex structure, and synthesized and maybe degraded in a natural manner by specific enzymes Ricochon; Muniglia (2010).

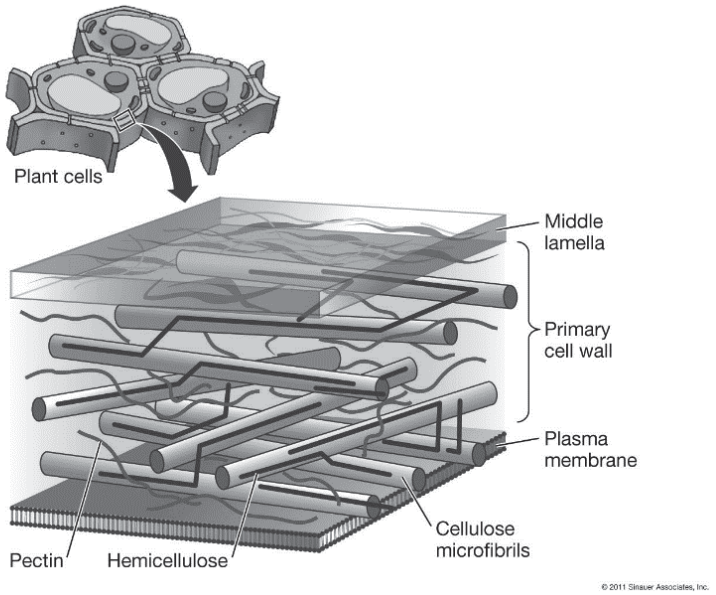

Fig 1: Composition of the cell wall Sinauer Associetes (2011)

Cellulose is a linear chain of several hundred to over nine thousand of β- (1 → 4) -linked D-glucose units. In the cell wall, the molecules of cellulose are assembled in parallel rows: microfibrils with a diameter from 5 to 12 nm. They are constituted from 36 to 1,200 molecules of cellulose held together by hydrogen bonds between the hydroxyl groups of the nearby glucose residues.

Hemicelluloses are linear or branched out polysaccharides bound to the celluloses microfibrils by hydrogen bonds or connected to the lignin by covalent bonds, so they form a complex and solid structure around plant cells. This complex polymer requires a set of enzymes able to work together. Oligosaccharides forming hemicelluloses are various and, consequently, many are the possible connections. Because enzymes are mostly specific for an unique type of bond, the use of a mixture of enzymes with different activities is required for the complete degradation of hemicelluloses Ricochon; Muniglia (2010).

Pectins are a linear chain of β-(1→4)-linked D-galacturonic acid. Lateral chains of neutral sugar can be attached to the rhamnose. The neutral sugars are mainly D-galactose, L-arabinose and D-xylose; the types and the proportions of neutral sugars vary with the origin of pectin. The ratio of esterified to non-esterified galacturonic acid determines pectins' are classification as with more or less half of galacturonic acid esterified.

Oil extraction for biodiesel production

Biodiesel process involves different steps: the oil seed/fruit pretreatment, oil extraction and oil transesterification for biodiesel production itself (**Fig 2**). First seeds are stored under certain conditions to avoid some problems such as mold formation, lipolytic enzyme activation, and others. Seeds' water content is maintained at 5–7% wt by means of drying in hot-air furnaces Santori et al. (2012). The sterilization and threshing of the crop or seed is the next step, which eliminates some impurities and empty brunches, leaving only the seeds suitable for the extraction process (Hamm, Hamilton, & Calliauw, 2013).

Oil extraction from the seeds/fruits is one of the most determinant steps during biodiesel production, which will affect final process' costs, production yields and generate or not environmental problems. Some methods of oil extraction from seeds/fruits are:

i. The mechanical extraction by pressure, consists on the application of a pressure of around 30-40 bar and temperatures close to 95°C, or also the use of pressures around 400 bar in combination with temperatures up to 120-155°C Amalia Kartika et al. (2006 e Willems et al. (2008). The press type depends on the raw material. Seeds with high oil content are usually fully or partially treated by mechanical means.

ii. The chemical extraction with the use of solvents is carried out when the oil within the cells is difficult to extract, demanding a very slow diffusion process. The diffusion process is also influenced by the type of the used solvent Harrington; D´Arcy-Evans (1985). The most common solvent that is employed in this process is n-hexane Nash; Frankel (1986). The mechanical method produces better-quality oil while the chemical one ensures greater yields. In practice, the two systems are often combined.

iii. The new method used in the oil extraction from seed oil is the use of different enzyme such as hemicelluloses, celluloses or proteases. This method allows the degradation of cell wall and facilitates the recovery and extraction of oils. The rupture of the cell walls of plants releases the organelles or oily bodies. An alternative approach to favor the oil release from the cell is the partial hydrolysis of the cell walls by means of appropriate enzymes. The choice of enzymes depends on seed's composition itself.

The oil recovery is one of the final process steps, which passes through transesterification. Vegetable oils for biodiesel production must be suitably pretreated (degumming with phospholipases) before entering the transesterification process whenever feedstock quality and refinement cannot otherwise be adequately guaranteed Santori et al. (2012). The transesterification involves different solvents such as ethanol or methanol.

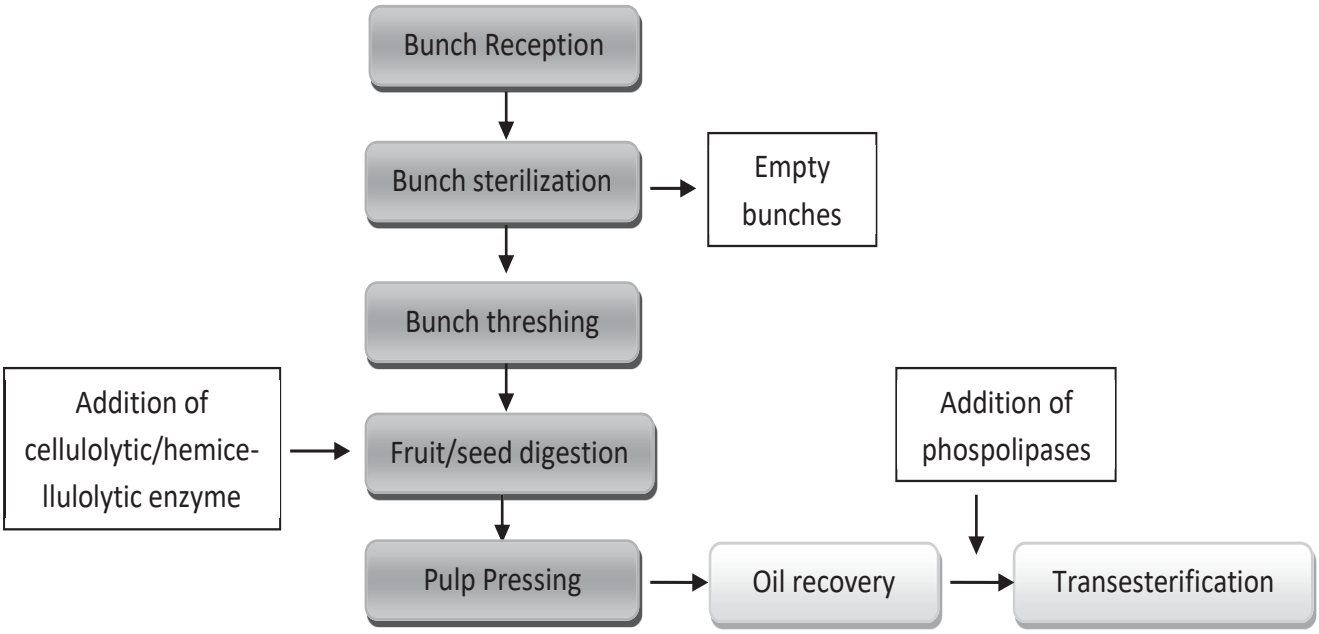

Fig 2. Steps of biodiesel process production: from seed/fruit pretreatment to oil recover for oil transesterification. Source: (Hamm et al. 2013)

Organic solvent extraction

Organic solvent extraction is the conventional and efficient process used to extract the oil from seeds. However, it comprises the use of flammable solvents, such as hexane, that needs some attentions and leads to its volatilization, high running costs, and undesirable influence on the quality of the food Zhang et al. (2012). The use of hexane for extracting oil has recently become the focus of concerns with respect to its safety and environmental effects; these concerns have increased with the presence of hexane in the group of hazardous air pollutants Rosenthal et al. (1996).

Enzymatic oil extraction

An alternative process for oil extraction from seeds/fruits is the use of enzymes such as celluloses, hemicelluloses and proteases, to ease extract oil from many seeds/fruits that has been attempted in the laboratory and/or at pilot industrial scale level Domínguez et al. (1994 e Rosenthal et al. (1996). The enzymatic pretreatment of seeds/fruits, prior to oil extraction, helps in degrading cell wall of oilseeds or the proteins components, which form the cell and lipid body membrane, thus facilitating oil release from the seeds.

The process usually consists of an aqueous (enzymatic) extraction of the oil from seeds/fruits, followed by separation through centrifugation of the aqueous and solid phases. The enzymatic process mainly hydrolyzes the structural polysaccharides, which results in substantial energy savings and higher yields. The enzymatic technology has been applied in industrial scale in order to enhance the yield of extra virgin olive oil Mariano et al. (2009). Protein may be recovered in the aqueous or solid phase, depending on the selected conditions Zhang et al. (2007). Despite these advantages, this method presents some problems, such as the formation of an emulsion that necessitates a mechanical de-emulsification operation in the downstream process Rosenthal et al. (1996).

In the last decades, different researchers have worked on the use of enzymes in the pretreatment of oleaginous grains. The different proposed methods promote optimized oil extraction yields and high quality of the cake obtained. This interest was born due to the worldwide growth of oil production, for edible, pharmaceutical, biofuel and other uses. Enzymatic oil extraction is then becoming an important alternative to hexane oil extraction. With this process it is possible to eliminate not only the use of hexane but also some refining steps can also be omitted. The challenge of producing high quality oil can be met through the innovative enzymatic extraction process Latif; Anwar (2009). The aqueous enzymatic extraction method, which is thought to be environmentally friendly, cheap and safe Latif;

Anwar (2009 e Rui et al. (2009). Depending on the nature of the oleaginous fruits or grains, the type of treatment varies with the composition and characteristics of cell walls. The application of enzymatic treatments then requires an effective strategy for each case, thus being the variables defined according to each type of process Domínguez et al. (1993). As another advantage of the enzymatic pretreatment of oleaginous fruits/seeds is the transformation of complex molecules of lipoproteins and lipopolysaccharides (not extracted with the oil) in more simple ones, providing more oil during the extraction process Smith et al. (1993).

Different researchers have been worked on aqueous enzyme-assisted oil extraction from different seeds such as Kalahari melon with 71.55% oil recovery Nyam et al. (2009), sunflower with 39.7% oil recovery Latif; Anwar (2009 e Sineiro et al. (1998), peanut with 91.98% oil recovery Jiang et al. (2010), *jatropha curcas* with 74% oil recovery Shah et al. (2005 e Winkler et al. (1997), grape seed with an increment of 163% oil recovery comparing to the conventional extraction (Passos et al.2009), evening primrose improved 12% of the yield oil recovery Collao et al. (2007), white pitaya with 7.78% wt/wt oil recovery Rui et al. (2009), rapeseed with 76% oil recovery Zhang et al. (2007), soybean with 96% oil recovery De Moura et al. (2008 e Santos; Ferrari (2005) and canola with 26% oil recovery Latif et al. (2008).

The in which oil extraction is based on the insolubility of oil in water rather than on the dissolution of oil, is one alternative. AEP offers several advantages over conventional solvent extraction as less capital investment, inherently safe operation, and simultaneous production of edible oil and protein rich fractions with less protein damage De Moura et al. (2008). Nevertheless, low oil recovery is one of the major challenges for this process, which may be overcome by using selected enzymes. Enzyme-assisted aqueous extraction (EAAE) is gaining importance as an alternative for simultaneous extraction of oil and protein. It is thought to be environmentally-friendly, safe and cheap Latif; Anwar (2009). Different studies have been carried out with EAAE by different researchers (**Table 2**). This method is the most used and diffused for the extraction of oils with the use of enzymes. The aqueous phase allows the solubility and mobilization of the enzymes to the degradation of plant cell walls in oleaginous fruits and oil seeds. It also allows the control of different parameters such as enzymatic concentration, pH and temperature maintenance within the system. In the last years, new models are being evaluated as the use of sonication and the use of microwaves to improve the recovery of oil after the action of enzymes Rui et al. (2009 e Shah et al. (2005).

The main factors that affect the hydrolytic process are particle size, moisture, hydrolysis time and the enzyme/ substrate mass ratio Mariano et al. (2009). Incubation time was the most significant reaction factor on oil yield of melon seed Liu et al. (2014 e Nyam et al. (2009), the

enzyme concentration, liquid/solid ratio, time were the most significant factor on oil extraction of bayberry kernel Zhang et al. (2012). Together with the incubation temperature, all these factors are determinant to obtain better results in the final yield of oil extraction from oleaginous fruits or seeds.

Enzymes used in the oil extraction from seed or fruit

Some enzyme preparations, having cellulases, hemicellulases, pectinases and proteases activities, are quite effective in different seed/fruits oil extraction processes Latif; Anwar (2009). To extract the lipid reserves stored in cells, it is necessary to be able to cross several barriers: first the extra cellular walls (or secondary cell walls), then the cell wall, and finally oleosomes. Each cell wall has its own constituents, sometimes organized in a complex structure, and synthesized and degraded in a natural manner by specific enzymes Ricochon; Muniglia (2010). In this way, the use of different enzymes is necessary in the extraction of oils from oily fruits or seeds. These enzymes must degrade each cell wall constituent to release the lipids captured therein, cellulases, xylanases, mannanases, pectinases and other enzymes are used for this purpose in order to degrade and break down the walls, membranes and organelles that encapsulate lipids.

The complete degradation of cellulose requires three types of enzymes: two cellulases (EC 3.2.1.4 and EC 3.2.1.91) and a β-glucosidase (EC 3.2.1.21). The first cellulase, called 1,4-β-cellobiosidase, is able to hydrolyse the intermolecular β- (1 → 4) -glucosidic bonds. The second cellulase can hydrolyse the cellulose from the extremities of glucosidic chains. They consequently form either glucose or cellobiose. Finally, β-glucosidases can hydrolyze the cellobiose molecules Ricochon; Muniglia (2010).

The hemicellulases are a diverse group of enzymes that hydrolyze hemicelluloses, one of the most abundant groups of polysaccharide in nature Robl et al. (2013 e Shallom; Shoham (2003). These enzymes have many biotechnological applications and their structure/function relationships are a subject of intense research. Hemicellulases, such as pectinases and xylanases, degrade hemicelluloses and lignin residues and stimulate cellulose hydrolysis by removal of the non-cellulosic polysaccharides that coat the cellulose fibers. The hemicellulases consists of a group of enzymes capable of increasing the yield of reducing sugars during enzymatic hydrolysis of lignocellulosic substrates. The definition of the accessory enzymes has evolved over time Robl et al. (2013). Xylanases catalyze the hydrolysis of hemicelluloses together with other accessory enzymes are currently considered to be α-L-arabinofuronosidase, hemicellulolytic esterases, β-mannanases, α-glucoronidases, β-xylosidases and pectinases.

Several studies have shown that cellulase enzymes supplementation can improve the enzymatic hydrolysis of lignocellulosic biomass, in terms of speed and hydrolysis yield Gusakov et al. (2007).

During the past year, new high-resolution structures of catalytic and non- catalytic domains of hemicellulases have been elucidated, and, together with biochemical studies, they reveal the principles of catalysis and specificity for these enzymes Shallom; Shoham (2003). Xylanases (EC 3.2.1.8) hydrolyse β-1,4 linked chains of xyloses, producing small xylooligomers. Lateral chains of glucuronic acids or of arabinoses fixed to this xylosidic skeleton can hide the action of these enzymes Shallom; Shoham (2003).

β-Mannanases (EC 3.2.1.78) hydrolyse hemicelluloses composed of mannoses and liberate β-1,4-manno-oligomers, which can be then hydrolysed in mannose by β-mannosidase (EC 3.2.1.25) Shallom; Shoham (2003).

Pectin ester hydrolases are also known as pectin methyl esterases. These enzymes catalyse the de-esterification of methoxyl groups forming pectic acids. These enzymes act in a preferential way on methyl esters of galacturonate units which are near the same non-esterified units. These actions on pectin chains prepare the substrate to supplementary enzymes.

Proteases correspond to any enzyme, which conducts proteolysis, that is to say, any enzyme, which begins protein catabolism by hydrolysis of the peptide bonds linking amino acids together in the polypeptide chain. In theory, using proteases could be interesting for extracting oil. It would allow the proteins of cell wall structures to break, as well as oleosins, which stabilize oleosomes. Moreover, their use in an aqueous extraction process only promotes appearance and stabilization of the emulsions which they try to reduce.

Commercial enzymes are commonly used for oil extraction processes with EAAE methodology. In synthesis, these commercial preparations contain the above mentioned enzymes, such as cellulases, xylanases, mannanases, pectinases and in many cases proteases. Concentrations, dilutions and mixtures of these formulations have been studied for each type of oleoginous fruit or seed (**Table 2**).

Table 2: Different enzymes used in the oil extraction treatment from oilseed or fruit oil with the percent of oil recover.

Seed or fruit oil	Enzyme	Treatment	% Oil extracted	Time of incubation	T° of incubation	Reference
Soybean	Protex 6L	EAAE	96	1 h	50 °C	De Moura et al. (2008)
	Protex 7L	EAAE	93	15 min	50 °C	
	Alcalase 2.4L	EAAE	44.6	4 h	55 °C	
Sunflower	Protex 7L	EAAE	28.3 ± 0.4	2 h	45 °C	Latif; Anwar (2009)
	Kemzyme	EAAE	32.2 ± 0.3	2 h	45 °C	
	Alcalase 2.4L	EAAE	26.6 ± 0.3	2 h	45 °C	
	Viscozyme L	EAAE	39.7 ± 0.4	2 h	45 °C	
	Natuzyme	EAAE	35.5 ± 0.6	2 h	45 °C	
Pequi fruit	Pectinase and CMCase	EAAE	20[a]	1.5 h	45 °C	Mariano et al. (2009)
Peanut	Alcalase 2.4L	EAAE	79.32	5 h	60 °C	Jiang et al. (2010)
	Nutrase	EAAE	60.08	5 h	55 °C	
	Protamex	EAAE	48.89	5 h	40 °C	
	As1398	EAAE	66.36	5 h	45 °C	
	Protozyme	EAAE	55.02	18 h	40 °C	
Canola	Natuzyme	EAAE	22.7 ± 0.5	2 h	45 °C	Latif et al. (2008)
	Multifect CX 13L	EAAE	26.0 ± 0.4	2 h	45 °C	
	Protex 7L	EAAE	23.4 ± 0.5	2 h	45 °C	
	Multifect Pectinase FE	EAAE	22.2 ± 0.4	2 h	45 °C	
Sesame	Protex 7L	EAAE	18.7 ± 0.3	2 h	45 °C	Latif; Anwar (2011)
	Kemzyme	EAAE	16.5 ± 0.8	2 h	45 °C	
	Alcalase 2.4L	EAAE	24.8 ± 0.4	2 h	45 °C	
	Viscozyme L	EAAE	21.4 ± 1.2	2 h	45 °C	
	Natuzyme	EAAE	16.8 ± 0.3	2 h	45 °C	
Kalahari Melon Seed	Neutrase 0.8L	EAAE	68.58 ± 3.39	31 h	58 °C	Nyam et al. (2009)
	Flavourzyme 1000L	EAAE	71.55 ± 1.28	36 h	50 °C	
Grape seed	Cocktail (cellulose, protease, xylanase and pectinase	EAAE	17.5[b]	2 h	40 °C	Passos et al. (2009)
White Pitaya	Cellulase, pectinase amylase and protease	MAEE	7.78 ± 0.21(wt/wt) %	12 h and 15 min with microwave	50 °C	Rui et al. (2009)
***Jatropha curcas* L.**	Alkaline protease	AEOE	74	6 h and 5 min ultrasonication	50 °C	Shah et al. (2005)
	Pectinex Ultra SP-L, Promozyme, Cellulase and Protizyme	EAAE	49	18 h	50 °C	
	Protease Alcalase	EAAE	85.6	2 h	50 °C	Winkler et al. (1997)
Rapeseed	Alcalase 2.4L	EAAE	73-76	2.5-3 h	48 °C	Zhang et al. (2007)
Bayberry	Cellulase and protease	EAAE	31.15	4 h	51.6 °C	Zhang et al. (2012)
Palm Fruit	Tannase, cellulase and pectinase	EAAE	90-93	0.5 h	50 °C	Teixeira et al. (2013)
Mustard	Protex 6L	EAAE	91	n/a	n/a	Tabtabaei; Diosady (2013)

[a]Calculate in improving in the extraction productivity of oil. [b]Increment reached 163% respect to the conventional extraction.
EAAE (Enzyme-assisted aqueous extraction), MAEE (microwave and aqueous enzymatic extraction), AEOE (Aqueous enzymatic oil extraction and sonification)

Production of hemicellulolytic enzymes applied to oil extraction

In nature, lignocellulosic materials are degraded by a consortium of microorganisms (fungus, bacteria, and other) that synthesize many hydrolytic enzymes able to degrade these substrates. Several microorganisms are able of produce hemicelluloses by different fermentation techniques such as solid-state fermentation (SSF) or submerged fermentation (SmF). Some examples of these enzymes and their processes are presented in **Table 3**.

Generally, a single hemicellulase gene encodes multiple hemicellulases, and hemicellulase multiplicity may arise from posttranslational modifications, such as differential glycosylation, proteolysis or both. Therefore, each enzyme produced by different microorganisms under different culture conditions is going to be unique as well as its biochemical and catalytic properties. However, crude multi-enzyme blends (enzyme preparations), obtained from a single fungus strain, are not ideal for biotechnological applications. This is because individual enzyme activities are not expressed at sufficient levels, or the enzyme complexes are not well balanced in terms of the individual enzymes (Gusakov et al. 2007).

As the volumes of enzymes involved in the application are rather large, the cost of enzymes becomes a critical factor in the adaptation of this technology on a commercial scale. Generally, downstream processing has high impact on enzyme or enzymatic preparation costs. The pretreatment of oilseeds to facilitate oil recovery, apparently, does not demand high purity levels of enzyme preparations. Therefore, it should be possible to develop simple and efficient strategies for enzyme production, recovery and purification, which are efficient, economical and suitable for such applications Kalia et al. (2001).

Table 3 Hemicellulase production by different microorganisms, substrate/support and fermentation techniques

Enzyme	Microorganism	Carbon Substrate	Fermentation	Activity	Reference
Xylanase	*Streptomyces viridosporus* T7A	Sugarcane bagasse, Napier grass, soybean meal	SmF / SSF	24.31 U ml^{-1} / 390.4 U g^{-1}	Alberton et al. (2009)
	Aspergillus niger LPB 326	Sugarcane bagasse and soybean meal	SSF	3099 U g^{-1}	Maciel et al. (2008)
	Colletotrichum graminicola	Wheat bran	SSF	378.1 U g^{-1}	Zimbardi et al. (2013)
	Bacillus licheniformis P11(C)	Xylan / Wheat bran	SmF	30 U ml^{-1} / 28 U ml^{-1}	Bajaj; Manhas (2012)
	Trichoderma reesei SAF3	Wheat brn	SSF	290.7 U g^{-1}	Kar et al. (2013)
	Aspergillus niger	*Jatropha curcas* seed-cake	SSF	6087 U g^{-1}	Ncube et al. (2012)
	Bacillus halodurans	Wheat bran	SSF	1685 U g^{-1}	Gupta et al. (2015)
	Aspergillus niger	Brewer's spent grain	SmF	5.49 U ml^{-1}	Izidoro; Knob (2014)
	Penicillium sp.	Coffee cherry husk	SSF	9475 U g^{-1}	Murthy; Naidu (2012)
	Kluyveromyces lactis	Galactose	SmF	273 IU ml^{-1}	Thomas et al. (2014)
	Penicillium glabrum	Brewer's spent grain	SmF	48.54 U ml^{-1}	Knob et al. (2013)
	Aspergillus niger	Rice husk	SmF / SSF	6500/ 5200 U g^{-1}	Membrillo Venegas et al. (2013)
Mananase	*Pichia pastoris*	Corn Steep Liquor Powder Dextrose	SmF	513 U ml^{-1}	Zheng et al. (2012)
	Aspergillus niger	Rise husk	SSF	119.91 U g^{-1}	Ibrahim et al. (2012)
	Penicillium oxalicum	Robusta coffee residues	SmF	53.77 U ml^{-1}	Chantorn et al. (2009)
	Bacillus halodurans	Locust bean gum	SmF	67.8 U ml^{-1}	Vijayalaxmi et al. (2013)
	Pichia pastoris	BMGY medium	SmF	50030 U ml^{-1}	Katrolia et al. (2012)
	Cellulosimicrobium sp.	Locust bean gum	SmF	7109 U mg^{-1} [a]	Kim et al. (2011)
Pectinase	*Aspergillus niger* DMF 27	deseeded sunflower head+ glucose	SmF	18.9 U ml^{-1} endo-pectinase 30.3 U ml^{-1} exo-pectinase	Patil; Dayanand (2006)
	Aspergillus niger DMF 45	deseeded sunflower head + sucrose	SSF	19.8 U g^{-1} endo-pectinase 45.9 U g^{-1} exo-pectinase	
	Aspergillus spp.	Wheat bran and orange peel	SSF	32.9 U g^{-1} endo-pectinase 33.4 U g^{-1} exo-pectinase	Heerd et al. (2012)
	Aspergillus terreus NCFT 4269.10	Banana peels	SmF	1000 U ml^{-1}	Sethi et al. (2016)
	Penicillium pinophlilum	Orange bagasse and molokhia stalk	SSF	3269.6 U g^{-1}	Ahmed; Mostafa (2013)

Conclusions

The use of enzymes has great potential to increase the productivity, efficiency and quality of oil extraction for biodiesel production. Although basic information on most enzymatic processes is available, it is generally restricted to laboratory-scale studies and because of the high costs of enzymes it is often not feasible. That is is why the use of agribusinesses together with the application of enzymatic extracts that do not have high purity are a possible outlet in the reduction of costs of production and application of enzymes or enzyme preparations. In addition to that, the processes catalyzed by enzymes need to be explored and exploited more thorough the light of the benefits that can derive from their use. Systematic investigation of process engineering and economic evaluation of these processes seems necessary before venturing into enlargement studies.

4. ARTIGO 2

Production and recovery of xylanases by *Aspergillus niger* for its potential application in oil seed pretreatment in biodiesel industry

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Abstract: Enzyme-assisted aqueous extraction (EAAE) is gaining importance as an alternative for extraction of oil used in biodiesel industry. One of the commercial enzymes applied to this purpose are xylanases, cellulose and other enzyme in its composition. Xylanases catalyze the hydrolysis of hemicelluloses, the second most important compound of the cell wall of oleaginous seeds; this is the reason because xylanases are very important in the extraction of oils for EAAE. The use of low cost raw material for enzyme production in an environmental and economic solution. Xylanase production by submerged fermentation with solids in suspension was investigated. The strain *A. niger* LPB BC was screened for its high capacity of xylanases production. Sugarcane bagasse and Soybean meal were employed for enzyme production in submerged fermentation reaching initially 28 U ml^{-1}. Xylanase production media was optimized with two-step optimization with a Plackett-Burman and RCCD surface response model with five factors: substrate concentration, inoculum rate, medium pH, concentration of K_2HPO_4 and $CuSO_4*5H_2O$. A fermentation kinetic was conducted with optimal data in both Erlenmeyer flask and stirred tan reactor (STR): 2.69 % w/v substrate concentration, $4.33*10^6$ spores ml^{-1}inoculum rate, 5.96 medium pH, 3.186 g l^{-1} and 0.327 g l^{-1} of K_2HPO_4 and $CuSO_4*5H_2O$ concentration respectively; where xylanase activity reached 52 U ml^{-1}after four days with a productivity of 13.22 U ml^{-1} day^{-1}. Xylanase production process was efficient with 1.93-fold argumentation of enzymatic activity after optimization. The enzymatic extract, with not only xylanase, but also cellulase activities, has great perspective to be employed for oilseed pre-treatment for oil extraction in biodiesel industry.

Keywords: Xylanase, sugarcane bagasse, *Aspergillus niger*, submerged fermentation, oilseed extraction.

Introduction

Petroleum consumption has increased over the last few decades due world's population growth and consequent industrialization, which has resulted in depletion of fossil fuel reserves and increasing petroleum price Bhuiya et al. (2016). The first foremost reason is the increasing demand for fossil fuels in all sectors of human life, such as transportation, power generation, industrial processes, and residential consumption Kafuku; Mbarawa (2010). Fortunately, concerns about the environment are growing and the scientific community is being challenged to improve the existing alternatives to petroleum-derived fuels and to create renewable fuels, which will likely be an important product in bio-based economies Bergmann et al. (2013). Biodiesel, which is a mixture of fatty acids alkyl esters, is an oil-based fuel that has been suggested as a possible replacement to petroleum diesel, especially in transportation. Biodiesel has similar properties to those of conventional diesel Taher; Al-Zuhair (2017), reduces emissions of greenhouse gases, carcinogenic compound emissions by approximately 85% compared with diesel fuel, is free of sulfur, metals, and polycyclic aromatic hydrocarbons Atadashi et al. (2012) renewable, non-toxic and biodegradable fuel Divya; Tyagi (2006),. Other benefits of biodiesel include improved lubricity, which enhances the performance and life of the engine Demirbas (2007). For all of the above features, biodiesel has received increasing attention on industrial scales.

Most commercial biodiesels are produced from refined vegetable oils, such as soybean oil in the United States and Brazil, rapeseed and sunflower seed oils in Europe, palm oil in Southeast Asia, and coconut oil in the Philippines Yustianingsih et al. (2009). Vegetable oils are produced in industrial scale using processes that depend on both the physical and chemical characteristics of the feedstock. All seeds contain fat, but few of them contain fat in quantities large enough to make its extraction convenient Santori et al. (2012). Soybean oil dominates the world oilseed production while rapeseed is in second position; soybean and rapeseed have 21% and 35 % of oil content respectively. Despite the lesser availability, palm oil is an interesting source for biodiesel production due to its lower price and relatively high oil content (40%). This oil also gives highest oil yield per area per year as compared to other oils Issariyakul; Dalai (2014).

Oil extraction from the seeds/fruits is one of the most determinant steps during biodiesel production, which affects final process' costs, production yields and generate or not environmental problems. To extract the lipid reserves stored in plant cells, it is necessary to be able to cross several barriers: first the extra cellular walls (or secondary cell walls), then the cell wall, which is the most important barrier, which is mainly composed of cellulose, hemicellulose

and pectin, and, finally, oleosomes. Each cell wall has its own constituents, sometimes organized in a complex structure, and synthesized and, maybe, degraded by specific enzymes Ricochon; Muniglia (2010).

Organic solvent extraction is the conventional and efficient process used to extract the oil from seeds. However, it comprises the use of flammable solvents, such as hexane, that needs some attentions and leads to its volatilization, high running costs, and undesirable influence on the quality of the food Zhang et al. (2012). The use of hexane for extracting oil has recently become the focus of concerns with respect to its safety and environmental effects; these concerns have increased with the presence of hexane in the group of hazardous air pollutants Rosenthal et al. (1996).

An alternative process for oil extraction from seeds/fruits is the use of enzymes such as celluloses, hemicelluloses and proteases, to facilitate extract oil from many seeds/fruits that has been attempted in the laboratory and/or at pilot industrial scale level Domínguez et al. (1994 e Rosenthal et al. (1996). The enzymatic pretreatment of seeds/fruits, prior to oil extraction, helps in degrading cell wall of oilseeds or the proteins components, which form the cell and lipid body membrane, thus facilitating oil release from the seeds. The enzymatic process mainly hydrolyzes the structural polysaccharides, which results in substantial energy savings and higher yields. The enzymatic technology has been applied in industrial scale in order to enhance the yield of extra virgin olive oil Mariano et al. (2009). Protein may be recovered in the aqueous or solid phase, depending on the selected conditions Zhang et al. (2007). Despite these advantages, this method presents some problems, such as the formation of an emulsion that necessitates a mechanical de-emulsification operation in the downstream process Rosenthal et al. (1996).

The aqueous extraction process (AEP), in which oil extraction is based on the insolubility of oil in water rather than on the dissolution of oil, is one alternative. AEP offers several advantages over conventional solvent extraction as less capital investment, inherently safe operation, and simultaneous production of edible oil and protein rich fractions with less protein damage De Moura et al. (2008). Nevertheless, low oil recovery is one of the major challenges for this process, which may be overcome by the use of selected enzymes. Enzyme-assisted aqueous extraction (EAAE) is gaining importance as an alternative for simultaneous extraction of oil and protein. It is thought to be environmentally-friendly, safe and cheap Latif; Anwar (2009). This method is the most used and diffused for the extraction of oils with the use of enzymes. The aqueous phase allows the solubility and mobilization of the enzymes to the degradation of plant cell walls in oleaginous fruits and oil seeds. It also allows the control of

different parameters such as enzymatic concentration, pH and temperature maintenance within the system. In the last years, new models are being evaluated as the use of sonication and the use of microwaves to improve the recovery of oil after the action of enzymes Rui et al. (2009 e Shah et al. (2005).

These enzymes must degrade each cell wall constituent to release the lipids captured therein, cellulases, xylanases, mannanases, pectinases and other enzymes are used for this purpose in order to degrade and break down the walls, membranes and organelles that encapsulate lipids. Hemicellulases is a diverse group of enzymes that hydrolyze hemicelluloses, one of the most abundant groups of polysaccharides in nature Robl et al. (2013 e Shallom; Shoham (2003). These enzymes have many biotechnological applications and their structure/function relationships are a subject of intense research. Hemicellulases, such as pectinases and xylanases, degrade hemicelluloses and lignin residues and stimulate cellulose hydrolysis by removal of the non-cellulosic polysaccharides that coat the cellulose fibers. Several studies have shown that cellulases supplementation can improve the enzymatic hydrolysis of lignocellulosic biomass, in terms of speed and hydrolysis yield Gusakov et al. (2007). The hemicellulases consists of a group of enzymes capable of increasing the yield of reducing sugars during enzymatic hydrolysis of lignocellulosic substrates. The definition of the accessory enzymes has evolved over time Robl et al. (2013). Xylanases catalyze the hydrolysis of hemicelluloses together with other accessory enzymes, which are currently considered such as α-L-arabinofuronosidase, hemicellulolytic esterases, β-mannanases, α-glucoronidases, β-xylosidases and pectinases.

During the past year, new high-resolution structures of catalytic and non- catalytic domains of hemicellulases have been elucidated, and, together with biochemical studies, they reveal the principles of catalysis and specificity for these enzymes Shallom; Shoham (2003). Xylanases (EC 3.2.1.8) hydrolyse β-1,4 linked chains of xyloses, producing small xylooligomers. Lateral chains of glucuronic acids or of arabinoses fixed to this xylosidic skeleton can hide the action of these enzymes Shallom; Shoham (2003).

Several microorganisms are able of produce hemicelluloses by different fermentation techniques such as solid-state fermentation (SSF) or submerged fermentation (SmF). Although fungi are naturally the major producers of hemicelluloses because these enzymes are necessary for the degradation of organic material and thus reduce lignocellulose residues to sugars to be used as nutrients in their growth and proliferation.

Brazil is a country in which the agricultural industry plays a great role in the economy, being the largest producer of sugarcane with an annual production of 739,300 TMT Maciel et

al. (2008 e Sheth (2017). The country is the second largest producer of soybeans with an annual production of 86.8 MMT James Karuga (2017) and one of the growing oil palm producers with an annual production of 410 TMT GPOP (2017). These industries generate a high amount of waste causing a pollution in the environment, being the main wastes generated: sugarcane bagasse in the production of sugar, soybean meal and oil palm empty frit bunches (OPEFB) in the oil extraction from soybean an oil palm respectively. They agro-industrial wastes have potential for hemicellulases production Alberton et al. (2009 e Goelzer (2015 e Maciel et al. (2008) for its high protein and lignocellulose content thus reducing its negative impact on the environment and the cost of enzyme's production.

Materials and methods

Xylanases production process development was first carried out through the qualitative and quantitative screening of 13 strains of filamentous fungus. The next step was the application of different agro-industrial sub-product (sugarcane bagasse and soybean meal) as substrate for enzyme production and the optimization of process´s condition with the study of the effect of carbon and, nitrogen sources and medium composition. Finally, the recovery and purification of xylanase was performed using microfiltration and ultrafiltration membranes. All steps are presented in Fig 1.

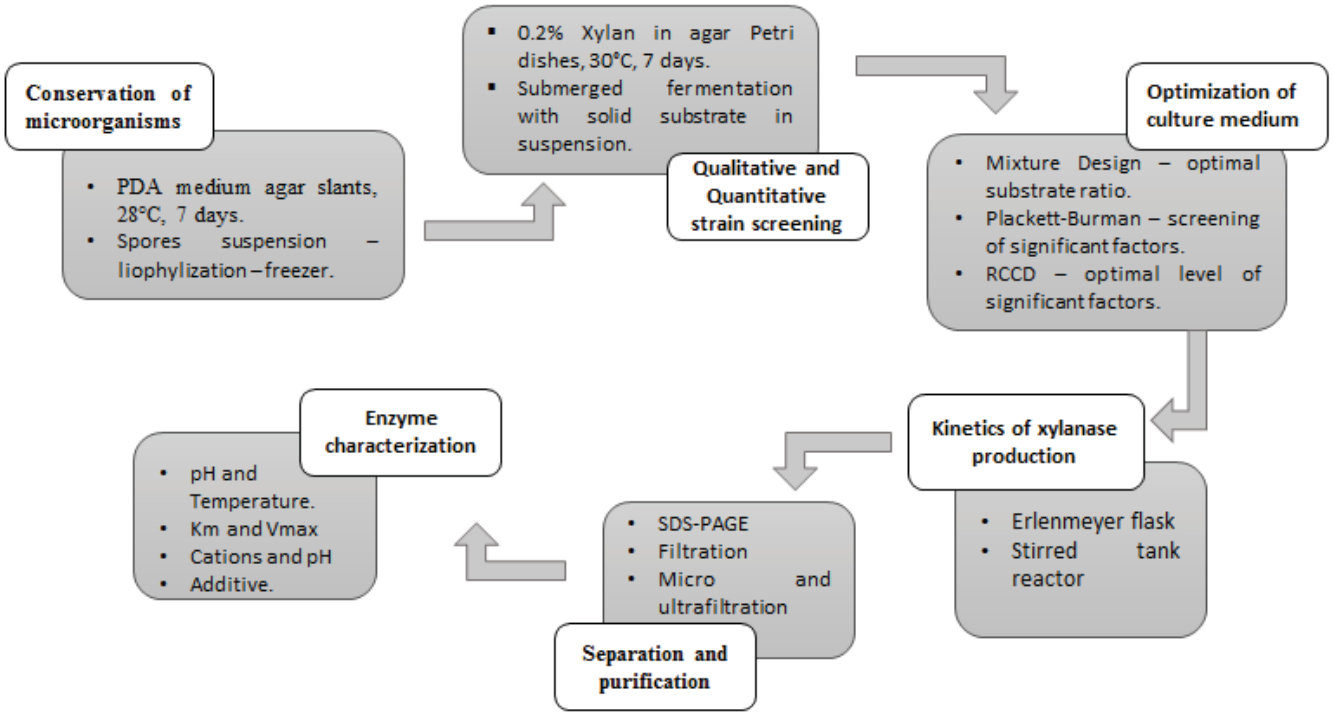

Fig 1: Step of xylanases production and recovery process development

Microorganisms

Thirteen strains from the genus *Aspergillus sp.* and *Rhyzopus sp.* from the strain bank of the Bioprocess Engineering and Biotechnology Laboratory of UFPR were tested. Strains were cultured on agar slants containing PDA (Potato Dextrose Agar) at 30ºC during 7 *days*. Strains were periodically renovated.

Qualitative strain screening for xylanase production

The qualitative test of xylanase production using the thirteen strain was carried out using the xylan-agar plate screening method Whitaker et al. (2003). Mediuem with 0,2% of Beechwood xylan (Megazyme, Denmark) as the sole carbon source was prepared and distributed in Petri dishes. Each strain was seeded in the center of the plates, which were incubated at 30°C, for 7 days. Xylanase production naturally degraded the xylan of the medium forming the halo was measured. The ratio between the halo measurement of the colony formation and the discolored halo formed around it was determined. All experiments were made in triplicate.

Quantitative strain screening for xylanase production

Nine strains pre-selected in the qualitative screening and two more strain (*A. oryzae* NRRL 1808 and *R. oryzae* LPB 28627) were employed in fermentation tests. Inoculum of each strain was prepared in Erlenmeyer flasks with 30 ml of PDA medium that were incubated at 30ºC during 7 days. Spores were suspended in 30 ml of a 0,1% Tween 80 solution. The obtained spore suspension was used to inoculate the medium for xylanase production.

Xylanase production was performed by mixing in an Erlenmeyer flask (250 ml), 5 g of substrate (65% of sugarcane bagasse and 35% of soybean meal), and mineral salt solution. The mineral salt solution was composed by (g l^{-1}): K_2HPO_4, 1.5; $MgSO_4*7H_2O$, 0.3; $CuSO_4*5H_2O$, $CaCl_2*2H_2O$, 0.005; $NaNO_3$, 3; $FeSO_4*7H_2O$, 0.009; $CoSO_4*7H_2O$, 0.02 $ZnSO_4*7H_2O$, 0.002; and $MnSO_4*H_2O$, 0.012 (Maciel et al. 2009; Goelzer 2015). The medium was autoclaved (15 min at 121°C) and then inoculated with a spore inoculum rate of 10^6 spores ml^{-1}. After medium homogenization, the flasks were incubated at 120 rpm and 30°C. The time of fermentation of all experiment was fixed in four days according to literature. All experiments were made in duplicate.

Optimization of substrate mixture ratio for xylanase production

Two strain that were previously chosen, were tested for xylanase production by submerged fermentation using three alternative hemicellulosic substrates (soybean meal, sugarcane bagasse and oil palm empty frit bunches) with 5% w/v at different ratio mixture of substrate. Submerged fermentation was carried out as it was descried before. The influence of different substrate ratio on xylanase production was evaluated using a Mixture Design. The Software Statistica 8 was employed. The experimental design was composed of 3 factors, 10 essays, in 1 block. Different soybean meal/sugarcane bagasse/palm residues ratio combinations were proposed by the generated matrix (Table 1). All experiments were carried out in duplicate. Xylanase activity was determined as described below. Data was used to create a mathematical model and a contour graph.

***Table 1**: Mixture design for substrate ratio optimization xylanase production*

Factors / Essays	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Sugarcane bagasse	1.00	000	000	0.33	0.33	000	0.67	0.67	000	0.33
Empty fruit Bunches	000	1.00	000	0.67	000	0.33	0.33	000	0.67	0.33
Soybean meal	000	000	1.00	000	0.67	0.67	000	0.33	0.33	0.33

Screening of significant fermentation parameters on xylanase production

Plackett-Burman (PB) designs whit 7 factors and 8 runs were used for the screening of significant factors on xylanase production. The studied factors were grouped in two factorial design. PB1 salts and nitrogen source (sodium nitrate, urea, ammonium sulfate, potassium hydrogen phosphate, magnesium sulfate, copper sulfate, and trace element) (Table 2) all experiment in duplicate. PB2 sugars source and physicochemical parameters (glucose, saccharose, inoculum ratio, medium pH, sugarcane bagasse and soybean meal particle size) (Table 3). All experiment were carried out with *A. niger* LPB BC. Xylanase activity was measured as described below. Data was used to create a mathematic model and Pareto chart graph.

***Table 2:** Composition of nutrient solution for xylanse production in submerged fermentation*

Factors (g l^{-1})	**Level**	
	0	**1**
Sodium nitrate	0	3
Urea	0	1.5
Ammonium Sulfate	0	1.5
Potassium hydrogen phosphate	0	2
Magnesium Sulfate	0	0.3
Copper Sulfate	0	0.4
Trace element*	0	1X

*Trace element (g l^{-1}): $FeSO_4*7H_2O$, 0.009; $CoSO_4*7H_2O$, 0.02 $ZnSO_4*7H_2O$, 0.002; and $MnSO_4*H_2O$, 0.012

Table 3: *Sugars source and physicochemical parameters*

Factors	Level	
	0	**1**
Substrate concentration (% w/v)	2.5	5
Glucose (g l^{-1})	0	10
Saccharose (g l^{-1})	0	5
Inoculum rate (spores ml^{-1})	10^6	10^7
Medium pH	5	6.5
Sugarcane Bagasse particle size (mm)	0.35-0.85	0.85-1.18
Soybean meal particle size (mm)	0.17-0.35	0.35-0.85

Influence of level significant factors on xylanase production

The influence of five factors on xylanase production was studied. A rotational central composite design (RCCD) was used with the support of the Software Design expert 10 and Response Surface Methodology. The experimental design was composed of 5 factors, 54 essays and 4 central points per block, in 3 blocks. Different factors combinations were proposed by the generated matrix (Table 4).

Table 4: *Coded and uncoded values of the studied factors in RCCD experimental design for xylanase production optimization*

Factors	Level				
	-2	**1**	**0**	**1**	**2**
Substrate concentration (% w/v)	1.5	2	2.5	3	3.5
Inoculum rate (spores*10^6 ml^{-1})	1	2.5	5	7.5	10
Medium pH	5	5.5	6	6.5	7
Potassium hydrogen phosphate (g l^{-1})	2	2.5	3	3.5	4
Copper Sulfate (g l^{-1})	0.25	0.3	0.35	0.4	0.45

Kinetics study of xylanase production in Erlenmeyer flask and stirred tank reactor

The kinetics study of xylanase production was performed in Erlenmeyer flasks (250 ml) and stirred tank reactor (STR), under optimal condition. The medium was autoclaved (15 min at 121°C). After medium homogenization, flasks were incubated at 120 rpm at 30°C for 144 hours. Samples were withdrawn each 12 to 24 hours. All experiment were made in triplicate. Xylanase and CMCase activities, reducing sugars concentration and pH were determined.

A 10.5 L-BioFlo 110 fermenter (New Brunswick, Edison, NJ, USA), was employed for xylanase production with a volume of 5 L using previously optimized conditions. Temperature, agitation and the aeration rate were maintained at 30°C, 200 rpm and 1.0 v/v min, respectively, during fermentation. Samples were withdrawn each 24 hours. Silicone oil (Dow Chemical Inc., USA) was added to control foaming if necessary.

SDS-PAGE gel and Zymogram:

The enzymatic extract filtrate was microfiltrated and ultrafiltrated in a Vivaflow® 200 system (Sartorius Company) using membranes with molecular weight cutoff of 100 kDa (separation and permeate recovery) and 5 kDa (retentate concentration). Concentrated samples were mixed with loading buffer, denaturant and not denaturant Schägger (2006), and subjected to electrophoresis Laemmli (1970) at 30 V for approximately 1 h. Then, 10 % (w/v) SDS-PAGE gels were submitted to 80 mA for, approximately, 2 h using a running buffer containing glycine 192 mM, 0.1% (w/v) SDS and Trizma Base 25 mM. Samples with denaturant buffer were boiled for 3 min prior to electrophoresis. After SDS-PAGE gel was fixed, stained and destained Schägger (2006).

Similarly, two sets of samples (boiled and not boiled) were prepared for zymogram analysis that were prepared with 10% (w/v) SDS-PAGE gel containing 0.5 % (w/v) of Beechwood xylan (Megazyme, Denmark), whichwas washed thrice with distilled water and, then, incubated at 50˚C, with a 0.5% xylan solution in citrate phosphate buffer pH 5.8 (0.05 M) for 1.5 hours. After it was stained in 1% (w/v) Congo red for 1.5 h and distained with 1 M NaCl.

Enzyme identification by MALDI-TOF/TOF

Protein bands obtained on SDS-PAGE were digested with trypsin (Promega, USA) Shevchenko et al. (1996). After incubation at 37øC for 18 hours, aliquots of the hydrolyzed sample were mixed in HCCA "α-cyano-4-hydroxycinnamic acid" matrix solution and pipetted into the MALDI plates where they remained until complete drying at room temperature. The mass spectrum (MS) was obtained by means of a MALDI-TOF / TOF Autoflex II spectrophotometer (Bruker Daltonics, Germany) operating in a reflective mode and detection of positive ions with acceleration voltage of 20 kV. Some signals present in the MS mass spectrum were subjected to positive reflective LIFT MS / MS mode, fragmentation was induced by LID laser induced dissociation. The peaks were converted into lists of monoisotopic masses with the aid of the computational software FlexAnalysis 3.0 (Bruker Daltonics).

Protein identification was performed using the National Sequence Center for Biotechnology Information (NCBI) and the Mascot program. The tolerated parental ions mass was 200 ppm, with the search criteria defined as: parental error (200 ppm), trypsin digestion, 1 cleavage site lost, methionine oxidation selected as the modifying variable, and fragmentation errors of 0.5 Da.

Recovery and separation of xylanase by microfiltration and ultrafiltration

After submerged fermentation, crude enzymatic extract was filtrated through Whatman filter paper n°1, connected to a vacuum bomb. The enzymatic extract filtrate was then microfiltrated and ultrafiltrated in a Vivaflow® 200 (Sartorius Company) system and Vivaspin® 6 (GE Healthcare) ultrafiltration system, using different membrane cutoffs (between 10, 30, 50 and 100 kDa). Different fractions of the retentate and permeate were analyzed for xylanase activity, protein concentration and specific activity determination.

Xylanase characterization and definition of enzymatic parameters

The influence of pH and temperature interaction on enzyme activity was studied using a rotational central composite design (RCCD) with the support of the Design expert 10 and Response Surface methodology. The experimental design was composed of 2 factors, 20 essays and 3 central points, in 1 block. Different factors combinations were proposed by the generated matrix (Table 5). Xylanase activity was measured as described below. With the optimal parameters the enzymatic kinetic, with different substrate concentrations of xylan, were calculating Km and Vmax parameters with the support of the GraphPad Prism 6.

Table 5: *Coded and uncoded values of the studied factors in RCCD experimental design for xylanase activity optimization*

Factors	Level				
	-2	**1**	**0**	**1**	**2**
Buffer Citrate-Phosphate pH	5	5.5	6	6.5	7
Temperature °C	40	45	50	55	60

Effect of metal ions and pH on partially purified xylanase activity

Effect of metal ions on xylanase activity was evaluated using solutions prepared with Co^{+2}, Mn^{+2}, Cu^{+2}, Fe^{+3}, Zn^{+2}, Hg^{+2}, Mg^{+2}, Al^{+3}, Li^{+}, Ba^{+2}, Na^{+}, NH_4^{+}, Ca^{+2} and K^{+} at the concentration of 10 mM. Inhibition/activation degree of xylanase activity was expressed as residual activity as percentage of that was compared to control sample (100% activity) incubated without any additive. Effect of pH from 3 to 7.5 was also evaluated with different buffer citrate-phosphate concentrations.

Stabilizing effect of different additives on partially purified xylanase activity

Six groups of additives: salts, sugars, polyols, acids, amino acids and polyethylene glycol were evaluated on enzyme stability. 5 ml of partially purified (micro and ultrafiltrated) enzymatic extract was individually added of different additives, in amber flasks with 10 ml of capacity. Flasks were incubated 50° C for 3 days (activity accelerated conditions). Enzyme

activity was then measured for the 0 day (initial) and after 3 days (final). Essays were carried out in duplicate.

Determination of xylanase and cellulose activities

Xylanase and cellulase activity was assayed using 1% Beechwood xylan (Megazyme, Denmark) and 2% Carbometilcellulose (Sigma, U.S.A.) respectively, in 0.05 M citrate-phosphate buffer (pH 5.3) (Bailey et al. 1992). The release of reducing sugars was determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). A unit of enzymatic activity (U) was defined as the amount of enzyme producing 1 μmol of xylose per minute. Enzyme was expressed as units per ml of enzymatic extract (U ml^{-1}). Enzymatic blanks were measured for each essay. Experiments were carried out in triplicate.

High performance liquid chromatography (HPLC)

The concentration of reducing sugars (glucose and fructose), organic acids (citric, succinic, lactic, acetic and propionic acids) and ethanol was determined in intervals of 24h. Samples were centrifuged at 6000 g for 15 min and filtered through 0.22 μm pore size filter (Millipore Corp., Billerica, MA, USA). The filtered samples were injected into HPLC system equipped with an Aminex HPX 87 H column (300 by 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector (HPG1362A; Hewlett-Packard Company, São Paulo, Brazil). The column was eluated with a mobile phase containing 5 mM H2SO4 at 60 ºC and a flow rate of 0.6 ml min^{-1}

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 Protein dye Bradford reagent, produced by Sigma-Aldrich Company. Solutions were place on an ELISA essay, with its absorbance measured.

Determination of metal ions composition

For metal content determination, an ICP-OES (Varian, Model ES 720, Palo Alto, CA) was used simultaneously with axial arrangement and solid-state detector. The torch was aligned horizontally and vertically with a Mn^{+2} standard solution concentration of 5.0 mg l^{-1}. The optical system of the ICP OES was calibrated with multi-element stock solution of scanned patterns. Spectral lines were selected considering the absence of interferences and appropriate sensitivity

for determining elements in high and low concentrations. The operation conditions were as follows: power of 1.10 kW, plasma gas flow of 1.5 l min^{-1}, auxiliary gas flow of 1.5 l min^{-1}, nebulizer pressure of 180 kPa, triplicate time read of 3 s, stabilization time of 15 s, sample delay of 30 s, pump speed of 15 rpm and sample washing time of 3 s.

Results and Discussion

Qualitative strain screening for xylanase production

Eleven strains which, were tested Whitaker et al. (2003) for xylanase production. All strains were grown on plates with xylan (0.2% w/v) as unique carbon source and the halo formation was measured Table 6 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. From all tested strains (Table 6), 9 of them were positive for xylanase production (difference between halo and colony > 5 mm). *Aspergillus niger* LPB 599 and BC are probably very good xylanase producers presenting a 5 mm halo formation, followed by *A. niger* LPB 1960 and 334 (5.83 mm). The strains *A. niger* NRRL 599 and LPB BC showed great potential for xylanase production, as it was pointed out by Maciel (2006).

The observation of the diameter of the halo formed by xylanase producing strains is very useful to select those with high levels of polysaccharide degradation activity. This method allows a rapid screening of strains with productive potential of xylanases Meddeb-Mouelhi et al. (2014 e Ten et al. (2004).

***Table 6**: Measurement of halo formation on plates of xylan 0.2% w/v by xylanase producing strains*

Strain	Difference between halo and colony diameter (mm)
***Aspergillus niger* LPB BC**	7.33 ± 0,58
***Aspergillus niger* LPB 28**	1.17 ± 0,76
***Aspergillus niger* LPB 3**	0.83 ± 0,29
***Aspergillus niger* NRRL 3536**	5.67 ± 0,58
***Aspergillus niger* NRRL 326**	6 ± 1
***Aspergillus niger* NRRL 599**	7.67 ± 0,58
***Aspergillus niger* NRRL 2270**	5.33 ± 0.58
***Aspergillus niger* NRRL 334**	5.83 ± 0,29
***Aspergillus niger* NRRL 335**	5.5 ± 0,5
***Aspergillus niger* NRRL 328**	7.17 ± 1,04
***Aspergillus niger* NRRL 1960**	5.83 ± 0,29

Quantitative strain screening for xylanase production

Xylanase production of nine strain was tested in submerged fermentation using alternative substrate according to different reported: sugarcane bagasse Alberton et al. (2009 e Di Marco et al. (2017) and soybean meal Goelzer (2015 e Maciel et al. (2008) were employed as carbon source and inducers of xylanases production with addition of other nutritive components. Two others strains were added (*A. oryzae* NRRL 1808 and *R. oryzae* LPB 28627) to the group. The strain *A. niger* NRRL 599 showed the best xylanase activity reaching a production of 37.09 U ml^{-1}, followed by *A. niger* LPB BC (27 U ml^{-1}) and *A. niger* NRRL 328 (24.12 U ml^{-1}) with also high production. The lowest xylanase production was verified for *A. oryzae* NRRL 1808, which reached 3.74 U ml^{-1} and *R. oryzae* LPB 28627 with 3.61 U ml^{-1} (Fig 2).

The results are in agreement with the data obtained in the qualitative selection, which showed that the strains LPB BC and NRRL 599 have a high xylanases' production profile. This fact is probably due to the medium composition based on sugarcane bagasse and soybean meal that favor the growing of fungi, synthesis and induction of the enzyme. Different microorganisms such as bacteria and fungi are xylanase producers Beg et al. (2001). Fungi are naturally the main producers of hemicellulases as in this case of xylanases. One of the most evaluated and reported genera is the genus *Aspergillus sp.* Nair; Shashidhar (2008 e Polizeli et al. (2005), which shows high productivities. Within the genus *Aspergillus* strains of *A. niger* are widely used in the production of this type of enzymes, due to their high productivities and also because they are considered GRAS. Those characteristics are surely favorable for future enzyme's application Schuster et al. (2002).

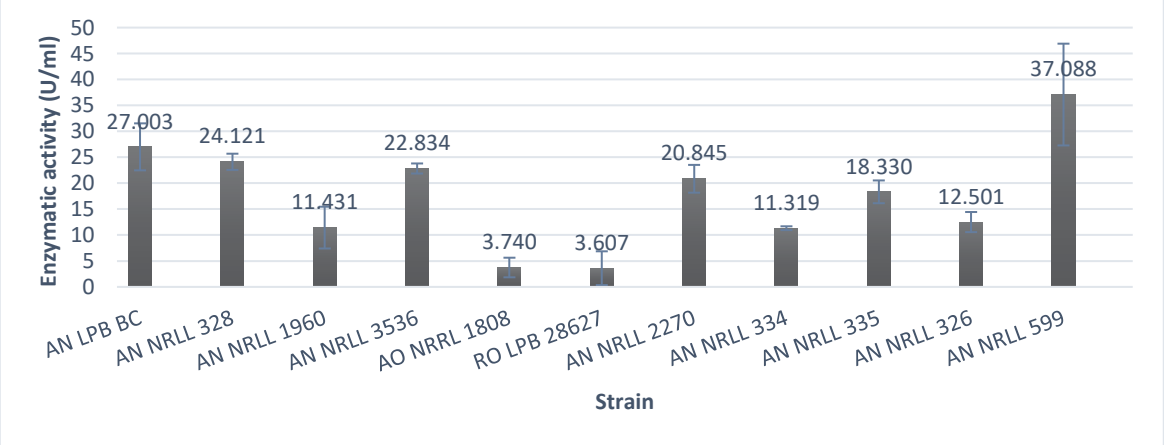

***Fig 2**: Xylanase production in submerged fermentation by different strain.*

Optimization of substrate mixture and their ratio for xylanase production submerged fermentation

A. niger NRRL 599 and BC, which were selected for their high xylanase productivities, were used in the study of substrate ratio definition. Three hemicellulosic substrates were tested with the support of Mixture design. The two strains showed a considerable difference in enzymatic production behavior (Table 7). *A. niger* NRRL 599 was very unstable in terms of xylanase production with high activity variation. In this way *A. niger* LPB BC was chosen for future studies and analyzed from this step.

According to the results (Table 7), it is possible to notice that xylanase activity increases with the increase of sugarcane bagasse and soybean meal ratio in the fermented medium. The best xylanase production by *A. niger* LPB BC (31.141 ± 8.89 U ml^{-1}) was reached with 66.67% of sugarcane bagasse and 33.33% of soybean meal. This result is in accordance with different works about xylanases production using sugarcane bagasse, 24.31 U ml^{-1}Alberton et al. (2009), 8.4 U ml^{-1} Bocchini et al. (2005) and 8.99 U ml^{-1} Di Marco et al. (2017).

***Table 7**: Enzymatic activity for AN LPB BC and AN NRRL 599 strain in different mixture substrate*

Run	Substrate (% w/v)			Xylanase Enzyme Activity (U ml^{-1})	
	Sugarcane Bagasse	Oil Palm empty fruit bunches	Soybean Meal	AN LPB BC	AN NRRL 599
1	5.00	0.00	0.00	0.840 ± 0.002	0.176 ± 0.16
2	0.00	5.00	0.00	0.260 ± 0.002	0.239 ± 0.007
3	0.00	0.00	5.00	6.759 ± 2.48	0.859 ± 0.07
4	1.67	3.33	0.00	0.308 ± 0.03	0.350 ± 0.08
5	1.67	0.00	3.33	2.230 ± 0.03	14.875 ± 3.96
6	0.00	1.67	3.33	1.879 ± 1.02	3.733 ± 0.61
7	3.33	1.67	0.00	0.139 ± 0.18	1.300 ± 1.58
8	3.33	0.00	1.67	31.141 ± 8.89	5.974 ± 0.21*
9	0.00	3.33	1.67	8.711 ± 1.03	10.893 ± 0.67
10	1.67	1.67	1.67	6.486 ± 0.16	26.253 ± 12.17

According to the results showed in Figure 3, the model that best fits our experimental design is the cubic, with a R^2 value obtained is 0.9505, which means that more than 95.05% of the response variable is explained by the generated model, with a satisfactory adjustment 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).

Equation 1 gives the finding cubic correlation, which represents xylanase activity whit the influence of different substrate ratio. The estimate of the highest production of xylanases according of model was 29.54 U ml^{-1} with a ratio of 73.77% of sugarcane bagasse, 26.23% of Soybean meal and 0% of Oil Palm empty brunch. The experimental activity of xylanases obtained was 28.89 U ml^{-1} with the optimal factors, this represent a 97.83% of predict.

Xylanase activity (U ml^{-1}) = 0.84003224726815*A+.25952999463736*B+6.759214*C-
1.4673889315398*A*B+57.987334693647*A*C+8.036870637066*B*C
+240.60775462533*A*B*C-2.4510539340168*A*B*(A-B)
+208.46886019365*A*C*(A-C) +60.739603887066*B*C*(B-C)
(**Equation 1)**

Where: A is Sugarcane Bagasse, B is Oil Palm Empty Bunches and C Soybean Meal

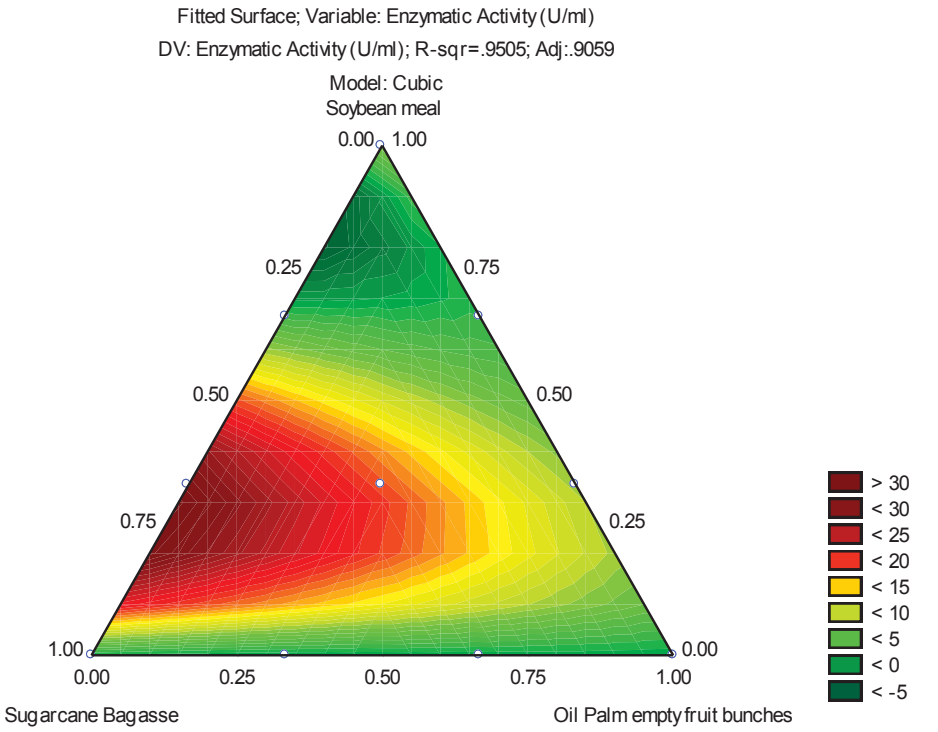

***Fig 3**: Contour plot of the Mixture experimental design for xylanase production optimization – Study of agro industrial waste substrate ratio.*

From the results obtained, the production of xylanases may be induced by the high presence of hemicellulose in the sugarcane bagasse, 27-32% Esa (2014). Then it could be considered that the high protein content 44-50% Heuzé et al. (2017) present in the soybean meal is probably simulating the growth and development of the fungus. In the absence of a simple and digestible carbon source, the polysaccharides present in the sugarcane bagasse, such as cellulose and hemicellulose, will induce the production of enzymes that reduce these polysaccharides in simple sugars to be consumed by the fungus.

On the other hand, the use of Oil palm empty fruit brunches does not have a significant influence in xylanases production. This can be due to the constitution of this type of waste that has a low percentage of hemicellulose 19% and on the contrary has a high amount of cellulose 30.5% Coral Medina et al. (2015) and lignin that make difficult the degradation of hemicelluloses.

Influence of nitrogen and carbon sources and different salt on xylanase production

In the optimization of culture media, one of the most important factors is the evaluation of the different variables that may influence production. One of the main methods for the evaluation of these variables is the use of Plackett-Burman designs as a first step of optimization. Some factors are already known to influence the production of xylanase. The most studied variables are sodium nitrate, potassium hydrogen phosphate, magnesium sulfate, iron sulfate, waste substrate concentration, pH of the basal medium Ghanem et al. (2000), cobalt sulfate, copper sulfate, inoculum size Park et al. (2002), glucose and other sugars Pérez-Rodríguez et al. (2014). To avoid overlapping effects on some variables that may affect the production of xylanases, these factors were separated into two groups: the study of the influence of carbon source with salts and the study of the influence of nitrogen sources and physical chemical factors (pH, particle size and inoculum ratio) on xylanase production

According to the screening of significant factors of the first group using PB it was possible to identify the most significant for xylanases production. In Pareto's chart (Fig 4A) it is possible to verify the strong effect of potassium hydrogen phosphate and copper sulfate presence on xylanase production ($p< 0,05$). Salts' composition of the medium acts as important cells' osmosis controllers. Several salts or micronutrients are redox-active and can act sometimes as active enzyme cofactors Hänsch; Mendel (2009). In the case of xylanases, different authors found that cooper sulfate and potassium hydrogen phosphate affect enzyme production positively Ghanem et al. (2000 e Maciel (2006 e Park et al. (2002). On the other hand, other salts as magnesium sulfate have positive effect Maciel et al. (2008) or not Pérez-Rodríguez et al. (2014) on xylanase production. According to results, magnesium sulfate is not significant, which is probably due to the micronutrients' composition of sugarcane bagasse and soybean meal. Micronutrient analysis was carried out for the used substrates, sugarcane bagasse and soybean meal, showing its profile (Table 8). It was possible to observe a high content of magnesium, iron, calcium, aluminum and sodium and other micronutrients, which could explain why the medium does not need to be supplemented with these components.

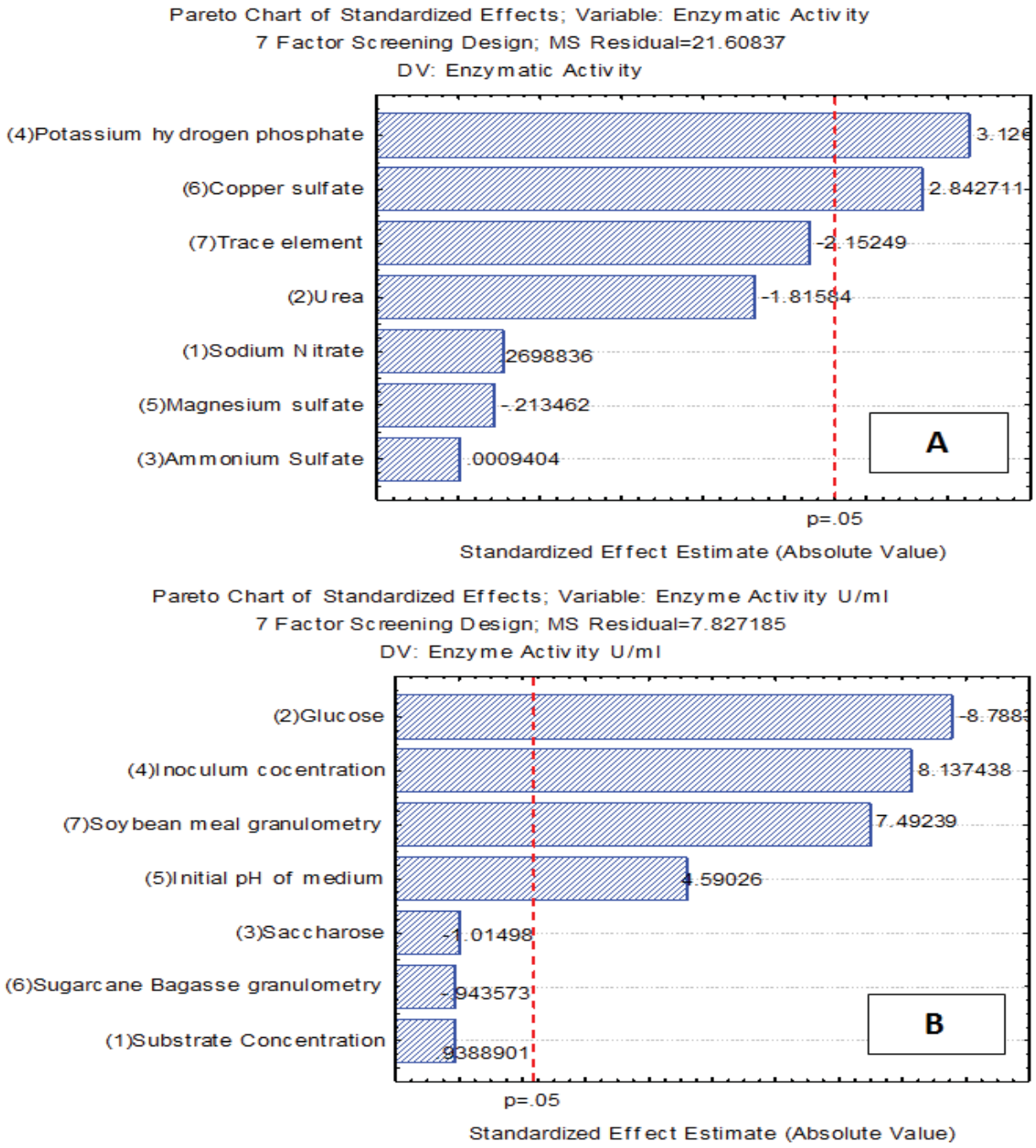

Fig 4: *Pareto's chart showing the effect of: Nitrogen source and salts (A). Carbon source and physicochemical factor (B).*

The use of soybean meal in submerged fermentation also would explain the no significance of the tested nitrogen sources (urea, sodium nitrate and ammonium sulfate) because it has high protein content. The natural protein composition of the medium certainly helps fungal growth. In addition, some amino acids of its structure could help in the synthesis of new enzymes.

The study of the influence of carbon sources and physico-chemical factors showed that the inoculum rate, soybean meal particle size and medium pH (Fig 4B) have positive influence on xylanase production, were; conversely the presence of glucose decrease the enzymatic synthesis ($p< 0.05$). The same can be observed in ANOVA analysis.

The inoculum rate is one of the main factors that influence xylanase production because it plays an important role in fermentation processes. The excessive increase of inoculum rate is sometimes negative since overcrowding of spores can inhibit germination and development of microorganisms Ghanem et al. (2000).

Finally, the negative (not significant) influence of glucose and saccharose medium supplementation on xylanases production, can be explained by the fact that these sugars promote sugar growth and metabolism deviation for another biomolecules production. The hemicellulose, of both substrates, induces the enzymatic synthesis adequately.

***Table 8**: Metal composition of waste substrate*

Metals (mg kg^{-1})	Substrate	
	Sugarcane Bagasse	Soybean meal
Al	519.11 ± 79.82	554.505 ± 57.03
Ba	2.305 ± 0.77	2.915 ± 0.21
Ba	ND	ND
Cd	ND	ND
Ca	747.03 ± 50.3	2720.79 ± 41.24
Co	ND	ND
Cu	5.435 ± 0.26	17.38 ± 0.68
Fe	978.06 ± 93.95	182.58 ± 25.96
P	213.01 ± 7.39	10242.45 ± 76.16
Li	32.075 ± 1.48	25.3 ± 5.01
Mg	427.39 ± 10.88	3216.93 ± 51.04
Mn	ND	ND
Mo	ND	4.08 ± 0.23
Ni	ND	ND
K	3763.855 ± 49.22	76225.15 ± 12.09
Se	ND	ND
Na	717.535 ± 42.62	873.9 ± 12.28
V	2.28 ± 0.31	ND
Zn	16.195 ± 0.06	75.605 ± 5.013

**ND: not dectected*

According to the results, it was observed that the most influential factors in xylanase's production are: Inoculum ratio, soybean meal size particle, medium pH and concentration of copper sulfate and potassium hydrogen phosphate. All these factors, except for soybean meal size particle, will be evaluated in RCCD to find the optimal levels.

Influence of significant-factors level on xylanase production

Statistically planned experiments reduce its number by developing a specific design, which also minimizes the error in determining the values for significant parameters Rao et al. (2008). Four of the five significant factors that had positive influence on xylanase production were tested in a RCCD. Even if the substrate concentration did not show a clear influence on xylanase production (Fig. 4B), this factor was included in this step of optimization.

Xylanase activity was markedly different ranging from 0.642 to 55.283 U ml^{-1}. According to this analysis, a quadratic model (R-square = 0.85, p-value < 0.05 and F-value = 9.37) could be well adjusted to explain the variation of xylanase activity as a function of factors. Significant factors and their interactions were identified and considered for selecting the best fits, in this case A (Substrate concentration), C (Initial pH), E (Copper Sulfate), AC, AE, BC, A2, C2 are significant model terms (Table 9).

***Table 9**: ANOVA for Response Surface Quadratic model DCCR of Xylanase production*

Source	Sum of Squares	df	Mean Square	F-value	p-value
Block	643.93	2	321.97		
Model	10181.46	20	509.07	9.37	< 0.0001
A-Substrate Concentration	*2989*	*1*	*2989*	*55.04*	*< 0.0001*
B-Inoculum rate	*30.85*	*1*	*30.85*	*0.57*	*0.4567*
C-Medium pH	*724.42*	*1*	*724.42*	*13.34*	*0.0009*
D-Potassium Hydrogen Phosphate	*175.22*	*1*	*175.22*	*3.23*	*0.0822*
E-Copper Sulfate	*425.75*	*1*	*425.75*	*7.84*	*0.0087*
AB	*1.45*	*1*	*1.45*	*0.027*	*0.8713*
AC	*294.13*	*1*	*294.13*	*5.42*	*0.0267*
AD	*34.62*	*1*	*34.62*	*0.64*	*0.4307*
AE	*1160.34*	*1*	*1160.34*	*21.37*	*< 0.0001*
BC	*252.46*	*1*	*252.46*	*4.65*	*0.0389*
BD	*58.74*	*1*	*58.74*	*1.08*	*0.3064*
BE	*63.37*	*1*	*63.37*	*1.17*	*0.2883*
CD	*1.63*	*1*	*1.63*	*0.03*	*0.8634*
CE	*135.82*	*1*	*135.82*	*2.5*	*0.1239*
DE	*7.11*	*1*	*7.11*	*0.13*	*0.72*
A^2	*1110.33*	*1*	*1110.33*	*20.45*	*< 0.0001*
B^2	*54.89*	*1*	*54.89*	*1.01*	*0.3225*
C^2	*2202.26*	*1*	*2202.26*	*40.56*	*< 0.0001*
D^2	*43.86*	*1*	*43.86*	*0.81*	*0.3757*
E^2	*1.04*	*1*	*1.04*	*0.019*	*0.8906*
Residual	1683.37	31	54.3		
Lack of Fit	*1485.79*	*22*	*67.54*	*3.08*	*0.0425*
Pure Error	*197.58*	*9*	*21.95*		
Cor Total	12508.76	53			

From these observations, the equation for xylanase activity is given from the predicted equation 2. A three-dimensional diagram response surface was plotted with the interactions of the most significant studied variables (Table 9): substrate concentration (Figure 5A); copper sulfate concentration (Figure 5B); and initial pH (Figure 5C).

Xylanase activity ($U.mL^{-1}$) = -1151.27052 - 11.045*A + 8.736*B + 388.588*C + 37.866*D -123.165*E - 0.170*A*B + 12.127*A*C - 4.160*A*D + 240.867*A*E - 2.247*B*C + 1.084*B*D + 11.258*B*E + 0.904*C*D - 82.409*C*E - 18.851*D*E - 23.079*A^2 - 0.238*B^2 - 32.504*C^2 - 4.587*D^2 - 70.765*E^2 **(Equation 2)**

Where: A is Substrate concentration, B is Inoculum rate, C is Initial pH of Medium, D is Potassium Hydrogen Phosphate and E is Copper Sulfate

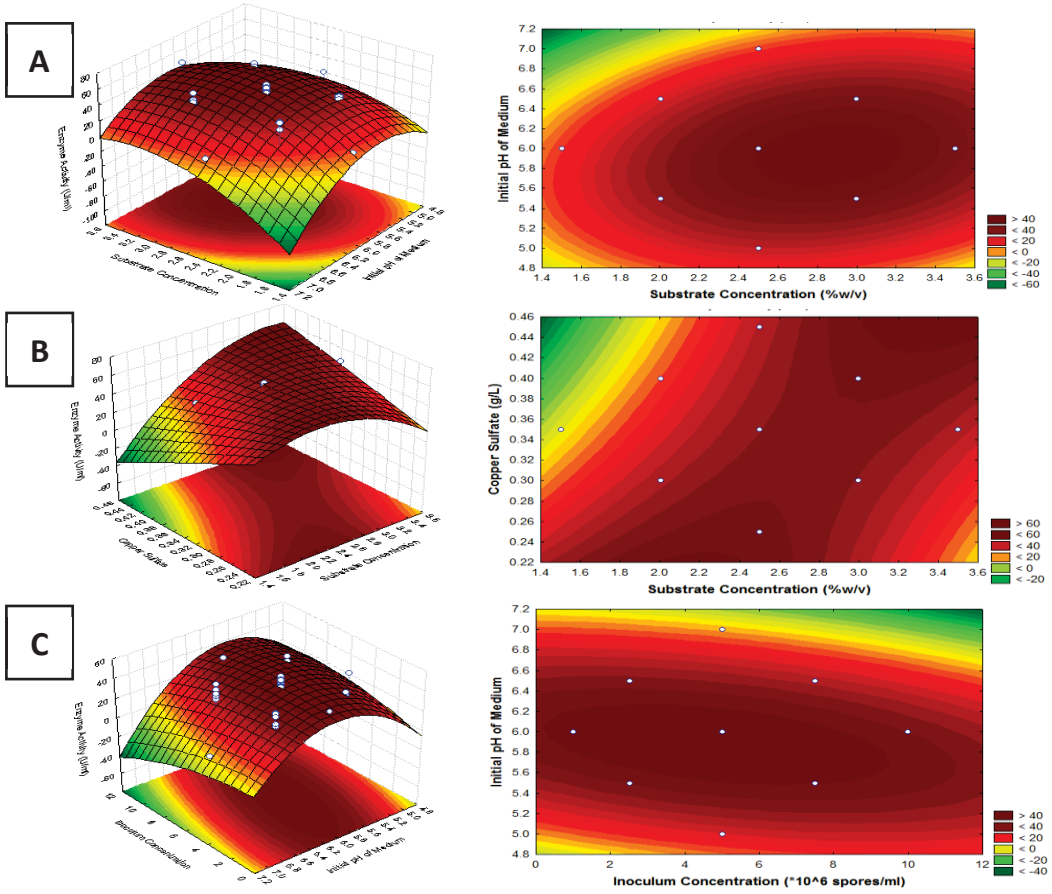

Fig 5: *Three-dimensional response surface plot of the RCCD experiment and contour plot of the calculated response for the studied factors and their influence on xylanase production.*

The optimum levels for each studied variable was: 2.69% w/v substrate concentration, inoculum rate of 4.33*10^6 spores ml^{-1}, 5.96 of Initial pH, 3.186 g l^{-1} of potassium hydrogen phosphate and 0.327 g l^{-1} of copper sulfate. The highest xylanase activity (51.48 U ml^{-1}) was reached on the 4th day of cultivation. The maximum value of enzyme activity 52.87 U ml^{-1} (1965.43 U g^{-1} of substrate). Validated up to 102.7% confidence.

Kinetics of xylanase production in Erlenmeyer flasks and stirred tank reactor (STR)

A kinetics study of xylanase production was followed, first in Erlenmeyer flasks, for the validation of optimized process conditions. The time course profile of xylanase activity is shown in Fig. 6. The strong correlation between experimental and statistical results confirms the validity of the response model and the existence of an optimal point. The highest xylanase activity of 52.87 U ml^{-1} (13.22 U ml^{-1} day^{-1} of productivity) was obtained after 4 days of fermentation. During the cultivation, there was a decrease of sugar concentration that could indicate that sugars were assimilated by the microorganism. Other enzymes, such as cellulases (CMCases) were produced (5.92 U ml^{-1} on 3th day) almost concomitantly, which is explained by the cellulosic composition of sugarcane bagasse Duenas et al. (1995). Pectinases and mannanases production was not significant. The presence of mainly two enzymes, xylanases and cellulases, will lead to the formulation of an enzyme complex with different potential application perspectives, such as oil seed pre-treatment for further oil extraction, our focus and animal feed.

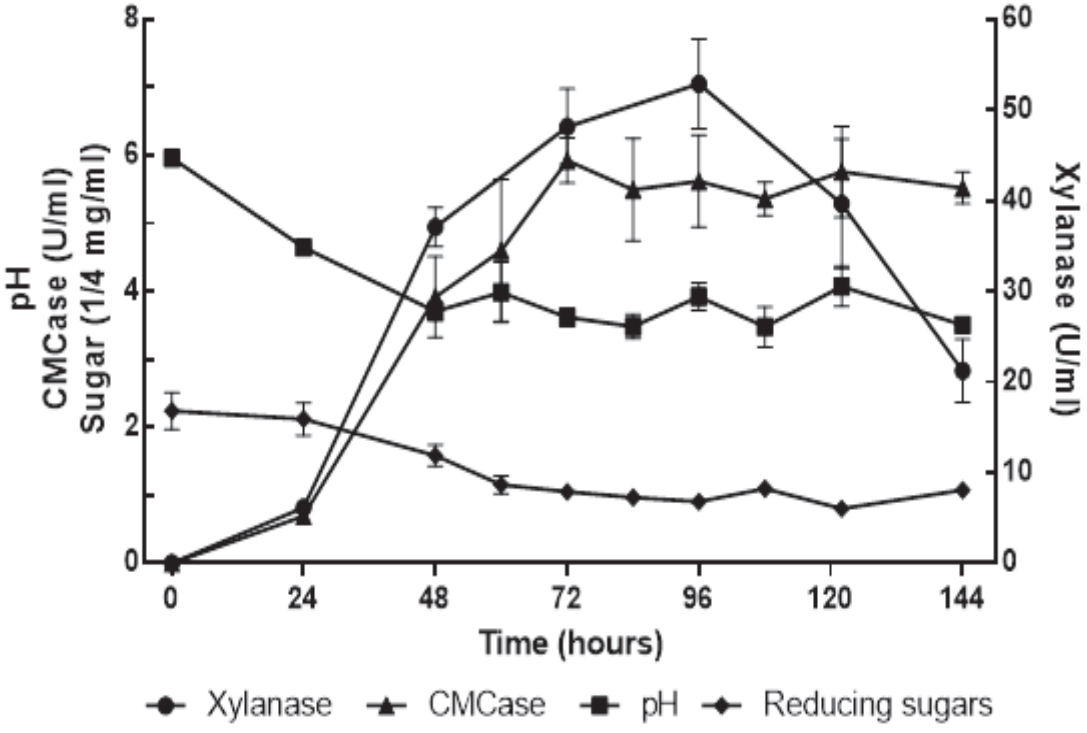

Fig 6: *Time course profile of xylanase production by Aspergillus niger LPB BC with the optimal conditions.*

The highest enzyme production occurs when sugar concentration reached very low levels. HPLC analysis (data not shown) indicated the presence of glucose and fructose this could be the result of the action of the enzymes hemicellulose and cellulose, thus releasing that are consumed by the fungi. Citric acid and other organic acids were also detected. This is normal since these metabolites are excreted by the fungus *A. niger* due to its metabolism. Niu et al. (2016). Concerning the pH, it can be seen that it decreases due to the production of organic acids during fermentation.

Table 10: *Production of Xylanase by different microorganisms using different fermented media*

Microorganism	Substrate	Fermentation	Productivity	Xylanase Activity	Reference
Coprinellus disseminatus MLK-07	Wheat bran	SmF	3.42 U $ml^{-1}day^{-1}$	30.32 U ml^{-1}	Lal et al. (2015)
Aspergillus fumigatus SD5A	Corncob	SmF	7.22 U $ml^{-1}day^{-1}$	50.55 U ml^{-1}	Elegbede; Lateef (2017)
Penicillium echinulatum S1M29	Wheat bran and soybean meal	SmF	6.1 U $ml^{-1}day^{-1}$	30.5 U ml^{-1}	Dos Reis et al. (2015)
Stenocarpella maydis	Xylan	SmF	3 U $ml^{-1}day^{-1}$	18.02 U ml^{-1}	Hernández-Domínguez et al. (2014)
Paenibacillus sp. AR247	Sugarcane bagasse	SmF	4.49 U $ml^{-1}day^{-1}$	8.99 U ml^{-1}	Di Marco et al. (2017)
Tuber maculatum	Xylan	SmF	2.19 U $ml^{-1}day^{-1}$	13.15 U ml^{-1}	Bedade et al. (2017)
A.niger SBS57	Xylan	SmF	107.57 U $g^{-1}day^{-1}$	753 U g^{-1}	Nair; Shashidhar (2008)
Aspergillus fumigatus SBS58			86.86 U $g^{-1}day^{-1}$	608 U g^{-1}	
Aspergillus sydowii SBS45			77.57 U $g^{-1}day^{-1}$	543 U g^{-1}	
Penicillium citrinum SBS26			105.57 U $g^{-1}day^{-1}$	739 U g^{-1}	
A. Niger LPB BC	Sugarcane bagasse and Soybean meal	SmF	13.22 U $ml^{-1}day^{-1}$ 491 U $g^{-1}day^{-1}$	52.87 U ml^{-1} 1965.4 U g^{-1}	This Work

The strain *A. niger* LBP BC reached a very high productivity of 13.22 U $ml^{-1}day^{-1}$ (491 U $g^{-1}day^{-1}$). It is superior to the mean observed in Table 10 compared con different authors. These differences might be attributed to the medium composition and the presence of an effective inducer. Several authors have worked in the production of xylanases by different fungal strains and bacteria, including recombinant strains, as indicated in Table 10. However, the productivity of the xylanase produced by *Aspergillus niger* LPB BC largely is higher.

Optimized conditions of xylanase production were then used in a 5 liter-STR to evaluate the effect of a different aeration system. Erlenmeyer flasks present aeration by diffusion whereas STR system has forced aeration, which can significantly affect growth, mycelia formation and, consequently, xylanase production. In fact, the highest xylanase activity (26.25 U ml^{-1}) was obtained after 3 days of fermentation. During submerged fermentation, there was a decrease on xylanase production and pH (Fig. 7)

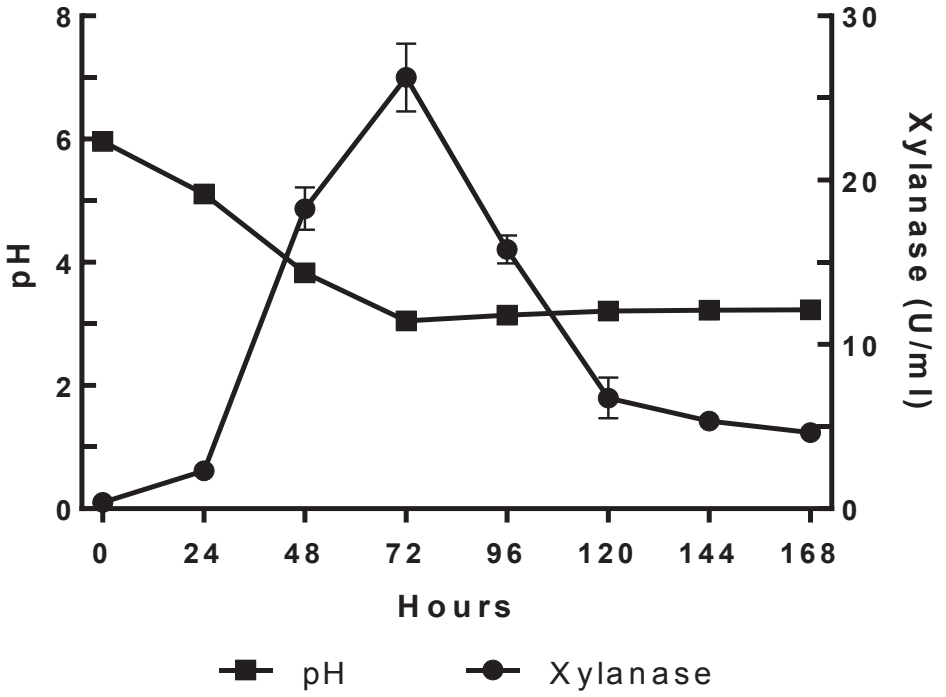

Fig 7: *Time course profile of xylanase production by Aspergillus niger LPB BC in STR*

A clear decrease is observed in xylanase production in STR. This may be due to the difference in mass transfer and shearing promoted by the bioreactor impellers on fungal mycelia. *A. niger* is a filamentous fungus that can be affected by forced agitation and aeration. In this way, with the change on mycelia formation behavior, the fungal metabolism was changed. It was possible to observe the decrease of pH and the presence citric acid in high quantities and other organic acids (data not shown) that causes the acidification of the medium during the late growth phases of fungi in batch culture. pH is a very important factor that influences the expression of extracellular enzymes Niu et al. (2016 e Peñalva et al. (2002). In this way, future assays will be performed to optimize STR xylanase production for further process scale-up.

SDS-PAGE gel and Zymogram:

SDS-PAGE was performed to determine xylanase's molecular weight. Fig 8 shows the protein profile of the purified and concentrated enzymatic extract between 100 kDa and 5 kDa. The induction of xylanase production generates a unique profile, as it can be seen in different bands indicating the presence of more than one protein.

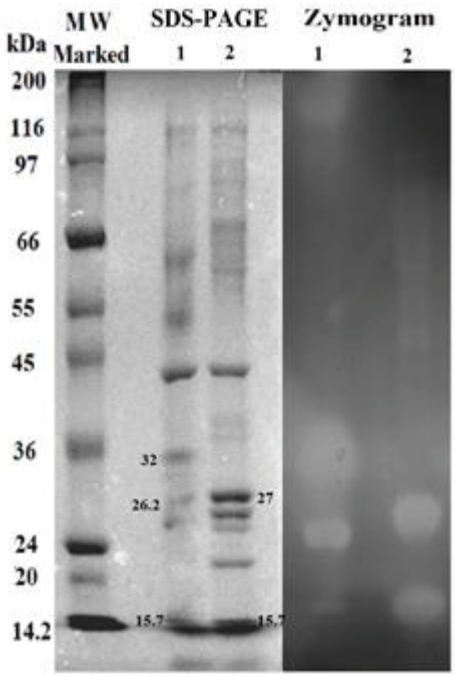

Fig 8: *SDS-PAGE and zimogram. Lane 1 MW marked, samples not denatured (1) and, samples with denatured buffer and heat treatment (2).*

The secretome of *A. niger* is strongly bound to the substrate and components of the culture medium. From the bands observations it can be deduced that there are enzymes, which participate in the degradation of hemicelluloses and celluloses from sugarcane bagasse Borin et al. (2015). proteases due to the high content of proteins of soybean meal, and other proteins that are involved in the metabolism of *A. niger*.

Comparing the enzymatic sample treated with non-denatured and denatured buffer loading, it is possible to observate a clear difference in the bands' pattern. Denaturant buffer and heat treatments may cause denaturation of proteins and generating, from quaternary structures (polymers of proteins), simple monomers, which allows a better migration and distribution into the gel Schägger (2006). This fact can be seen in Figure 8 where the band pattern is situated below 45 kDa with better resolution.

Xylanase activity was examined using a zymogram staining technique in polyacrylamide gel; a clear zone on the agar gel indicated the presence of xylanase (Fig.8 zymogram lane 1 and 2). Zymogram analysis revealed at least two xylanases in the enzyme extract both in denatured and non-denatured treatment. The possible molecular mass of xylanases in non-denatured treatment are 32 kDa, 26.2 kDa and 15.7 kDa, and in denatured and heat treatment are 27 kDa and 15.7 kDa.

It is possible that the 26.2 and 27 kDa bands present in both treatments are the same. On the other hand, a greater intensity of the 15.7 kDa band is observed after the denaturant and heat treatment; this may be due to the fact that the band is a protein monomer and, when the enzymatic extract is denatured, the 32 kDa band (possible xylanase dimer) is separated into monomers of approximately 15.7 kDa. Xylanases may are also allozymes, products of different alleles of the same gene Wong et al. (1988) or even isoenzymes with different genes, but with the same enzymatic action Loera Corral (2002). Multiple xylanases may be artifacts arising from the degeneration of microbial culture filtrates. Contrarily, each of the multiple xylanases may be a distinct gene product produced by a microorganism to enhance its utilization of xylan Wong et al. (1988). This would explain the absence of 32 kDa band and greater intensity of 15.7 kDa band in the sample with denaturation and heat treatment.

As initially indicated, xylanases are a very diverse family with different molecular weights. There are reports of xylanases with 123 kDa (trimers of 41 kDa) in *Pseudomonas sp*.Sharma PK; Chand D (2012), 22.5 kDa in *A. niveus* Sudan; Bajaj (2007), 32 kDa in *A. versicolor* Carmona et al. (2005) and 22 kDa *R. oryzae* Bakir et al. (2001).

The MALDI-TOF/TOF result indicate that 26.2 and 27 kDa bands have an indented or high homology with beta-xylanase produce by *A. niger*, 15 kDa band have a high homology with partial endo-1,4- beta xylanase; for other hand the 32 kDa bands have a low homology with glycoside hydrolase family 62, enzyme that specifically cleave either α-1,2 or α-1,3-L-arabinofuranose side chains appended to the backbone of xylan. Finally, the high-resolution band close to 45 kDa is a protease of the aspartyl protease family.

Recovery and separation of xylanases by microfiltration and ultrafiltration

The enzymatic extract filtrate was microiltrated and ultrafiltrated in a Vivaflow® 200 (Sartorius Company) system and in a Vivaspin 6 (GE Healthcare®) ultrafiltration system, with molecular weight cutoff between 10, 30, 50 and 100 kDa. Different fractions of the retentate

and permeate were analyzed for xylanase activity, protein concentration and specific activity (Table 11 and Table 12)

Table 11: *Purification and concentration of xylanase for ultrafiltration membranes by Vivflow 200 system.*

Purification Steps		Xylanase Activity ($U.mL^{-1}$)	Protein ($mg.mL^{-1}$)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity ($U.mg^{-1}$)	Purification fold	Yield (%)
Microfiltration Permeate		27.044	0.082	1500	40566.4	123.016	329.766	1	100.00
50 kDa	Retentate	36.644	0.208	300	10993.0	62.466	175.986	5.0	27.099
	Permeate	19.543	0.046	1200	23451.9	54.824	427.765	1.3	57.811
30 kDa	Retentate	38.296	0.179	250	9573.97	44.695	214.205	6.0	23.601
	Permeate	19.727	0.037	950	18740.5	35.316	530.649	1.6	46.197
10 kDa	Retentate	18.206	0.037	150	2730.84	5.598	487.867	10.0	6.732
	Permeate	16.055	0.035	800	12843.9	28.378	452.603	1.9	31.662

Table 12: *Purification and concentration of xylanase for ultrafiltration membranes by Vivaspin 6*

Purification Steps		Xylanases Activity	Total Activity (U)	Total Protein (mg)	Specific Activity ($U.mg^{-1}$)	Purification fold	Yield (%)
Microfiltration	Permeate	28.58	142.9	0.376	380.154	1	100.00
100 kDa	Permeate	37.68	169.6	0.242	701.079	1.84	118.65
50 kDa	Permeate	33.07	148.8	0.243	613.226	1.61	104.12
30 kDa	Retentate	19.07	85.8	0.246	348.783	0.92	60.05
	Permeate	22.06	99.3	0.140	**707.455**	1.86	69.45
10 kDa	Retentate	13.99	63.0	0.088	**716.530**	1.88	44.06
	Permeate	12.00	54.0	0.078	688.030	1.81	37.79

According to data above, enzymatic activity is observed in all fractions, that could be explained because the molecular mass of this enzyme complex varies from 7-90 kDa Maciel et al. (2009 e Uday et al. (2016). However, the highest enzymatic activity is found in the retentate of 50 kDa membrane (36.64 U ml^{-1}) and 30 kDa retentate (38.29 U ml^{-1}) and a high specific activity in permeate of 30 kDa (530.6 U mg^{-1}) in Vivaflow 200 system. On the other hand, the specific activities in 30 kDa permeate (707.5 U mg^{-1}) and in retentate of 10 kDa (716.5 U mg^{-1}) could be explained by the high concentration of xylanases in this fraction in Vivaspin 6 system.

According to the previous SDS-PAGE analysis, xylanases' molecular weigh are probably between 10 kDa and 35 kDa. That would be supported by the high specific activity found between 30 kDa and 10 kDa in the Vivaspin 6 system and the high xylanase activity in retentate of 30 kDa and the high specific activity under 30 kDa in Vivaflow 200 system.

However, the high concentration in the retentate of 50 kDa membrane in Vivaflow 200 is explained. For this reason, samples of 50 kDa retentate and under 100 kDa was tested in SDS-PAGE at a concentration of 10% (Fig. 9) showed that that the profile of permeate from 100 kDa in Vivaspin 6 and permeate and retentate from 100 kDa in Vivaflow 200 are the same. According to the protein molecular weight, which is marked in the profile of 100 kDa retentate in Vivaflow 200, show some protein bands under 100 kDa that, in theory, should not happen. Maybe that is because ultrafiltration membrane's fouling. The secretome of *A. niger,* which was previously described, is very diverse and may produce different proteins and enzymes such as cellulases, proteases, peptidases and other hemicellulases Gouka et al. (1997 e Oda et al. (2006). These proteins could correspond to those bands that were shown by the SDS-PAGE gel and are the resultant of ultrafiltration membrane fouling. Therefore, small proteins that should normally pass through the pores of the membranes are trapped in the retentate fraction. Possibly, for this reason, the fraction that was obtained from the 50 kDa have a high xylanase activity concentration.

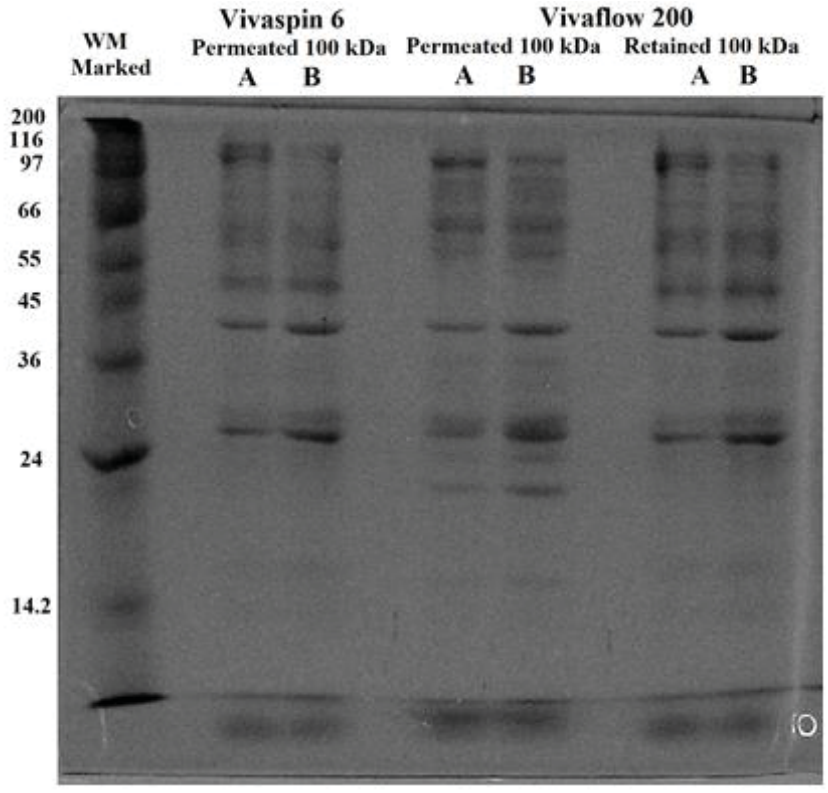

Fig 9: *SDS-PAGE 10%, samples not denatured (A) and samples denatured (B)*

Xylanase characterization and definition of enzymatic parameters

The most influential factors in the activity of an enzyme are the pH and the temperature. These factors were evaluated with One-factor-at-a-time (OFAT) optimization; the first variable evaluated was the pH with a fixed temperature (50°C). The best xylanase activity was found with pH 6 (Figure 10A). After that, the influence of temperature was evaluated at pH 6 constant. It can be seen that maximal xylanase activity occurs between 50 and 55 °C (Figure 10B).

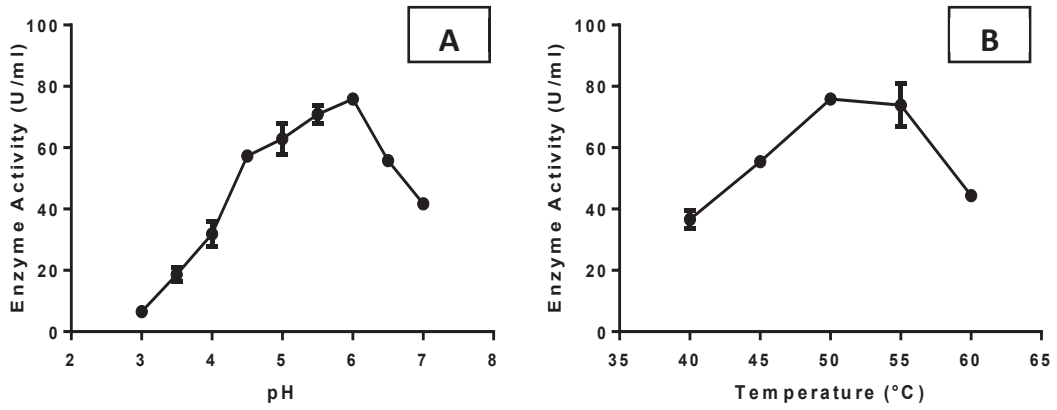

Fig 10: *Xylanase activity of A. niger LPB BC (A) influence of pH and (B) Effect of reaction temperature*

In the second optimization, a RCCD model was defined with 50°C and pH 6 as central points, The Model F-value of 26.21 and R-squared of 0.9035. Equation 3 gives the finding quadratic correlation, which represents xylanase activity. The estimate of the highest production of xylanases according to the model was 72.47 U ml^{-1} with 50.69°C and 5.81 of pH (Figure 11). The experimental activity of xylanases obtained was 72.89 U ml^{-1} with the optimal factors, this represent a 100.58% of prediction.

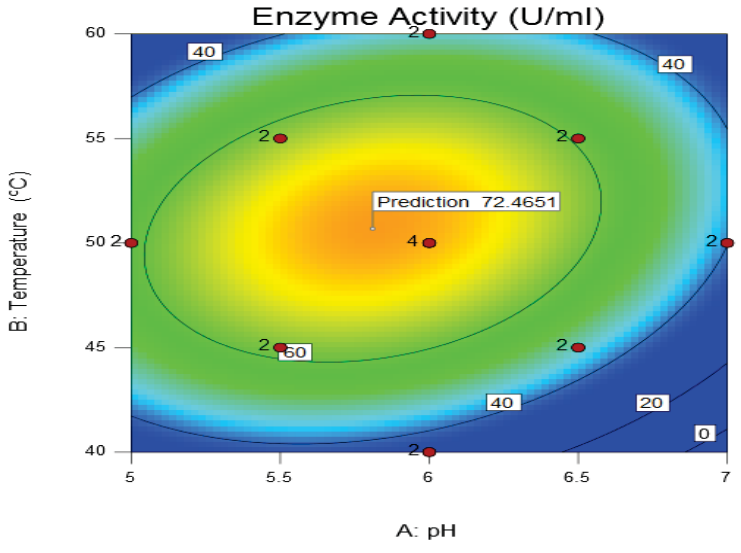

Fig 11: *Contour plot of the calculated response surface of Xylanase activity*

Xylanase activity (U ml^{-1}) = -1180.76649+202.99336*A+26.17806*B+1.05260*A*B-22.05889*A^2-0.31855*B^2 **(Equation 3)**

Where: A is pH and B temperature.

Generally acidic xylanases have optimum temperature range from 50 to 60°C and most fungal xylanases have optimum pH for xylan hydrolysis in the range of 4.5 to 6.0 Maciel (2006). The enzyme has optimal activity at 50.69°C and pH of 5.81(Fig. 11). This temperature was approximately comparable to the xylanase formed by *Aspergillus fischeri* Senthilkumar et al. (2004), *Aspergillus terreus* Ghanem et al. (2000), *Aspergillus niger* Levasseur et al. (2005).

Kinetic of partially purified extract of Xylanase

The effect of substrate (xylan of Beechwood) concentration on the activity was examined using different concentrations. The profile of kinetic of xylanase is shown in Fig. 12. The analysis of different parameter of xylanases kinetic was performed using GraphPad Prism 6 with Michaellis-Menten model, giving a Km of 8.344 (mg xylan)/ml and a Vmax of 129.8 U ml^{-1}, indicating high affinity and high catalytic power of the enzyme for the substrate. The Km values of xylanases were shown to range between 0.15 and 49.5 mg.mL-1 Beg et al. (2001).

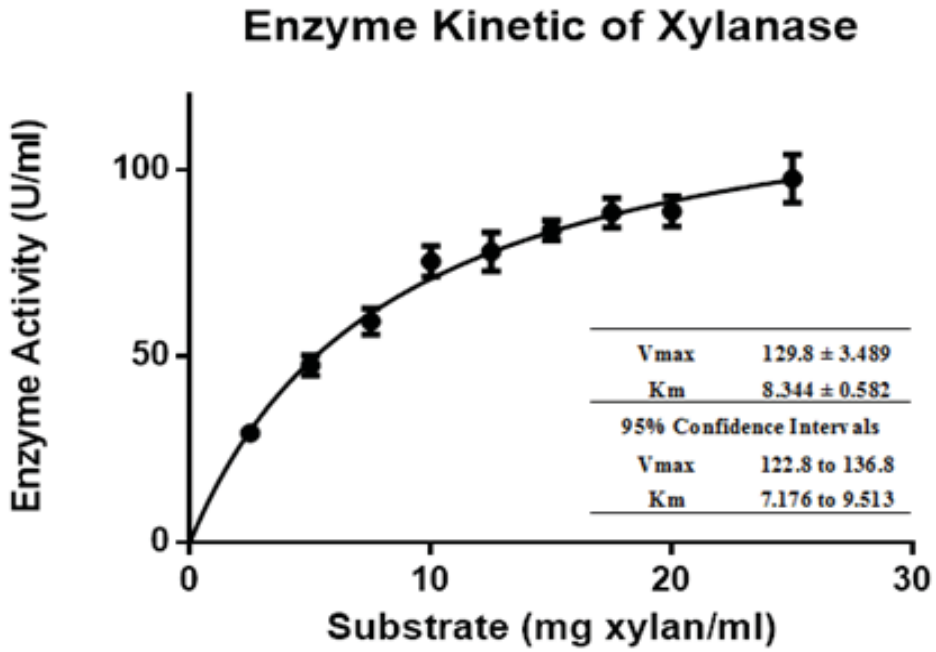

Fig 12: *The profile of kinetic of xylanase*

The K_m reported in this study is higher than that reported for *A. niger* BRFM281 (7.1 mg ml^{-1}) Levasseur et al. (2005) and that of *A. fischeriwas* Fxn1 0.53 mg ml^{-1} Senthilkumar et

al. (2004) but lower than *A. niger* FCUP1 (10.41 mg ml^{-1}) Benedetti et al. (2013). These differences could be due to a certain extent, which is attributed to different xylanases, xylan, pH or temperatures used in the xylanase assay and to the methods used in determination of sugar concentration.

Effect of metal ions and pH on partially purified xylanase activity

Previously mentioned references have revealed that xylanases activity depend on their producers and metal ions. The influence of certain inhibitors and activators on xylanase activity was studied. The xylanase activity was stimulated (Fig. 13) by Co^{+2}, Mn^{+2} and Cu^{+2} up to 182.37%, 188.91% and 181.14%, respectively, and was inhibited by Fe^{+3}, Zn^{+2}, Hg^{+2} and Mg^{+2} up to 86% 88%, 87% and 82.7%, respectively. Enzyme activity was not significantly affected by Al^{+3}, Li^{+}, Ba^{+2}, Na^{+}, NH_4^{+}, Ca^{+2} and K^{+}. However, the results reported in this work differ from other reports. As an example, Mg^{+2} active and Cu^{+2}, Li^{+} and Hg^{+2} inactived xylanase activity from *Cladosporium oxysporum* Guan et al. (2016), Co^{+2} and Mn^{+2} activated while Hg^{+2} inactivated xylanase activity from *Thermomyces lanuginosus* Knob et al. (2013)Cesar; Mrša (1996), Cu^{+2}activated and, Fe^{+3}, Mn^{+2} and Hg^{+2} inactivated xylanase activity from *A. ficuum* Lu et al. (2008).

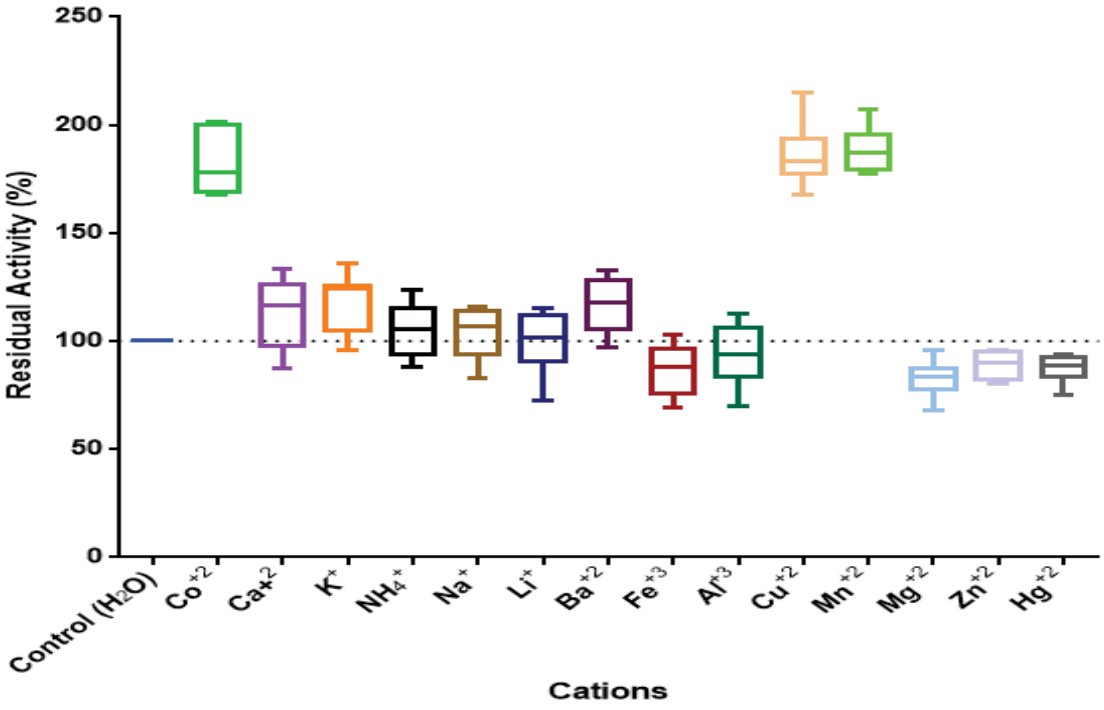

Fig 13: *Residual activity of Xylanase with different cations*

Catalytic pH markedly affects enzymes activity by dissociating their bind with the substrate, affecting catalysis and, even, destroying their molecular structures. In Fig. 14, is possible to observe that xylanase activity from A. niger LPB BC is favored near neutral condition (5.8-7 pH) and pH 6 stimulates a greater residual activity. On the other hand, pH 3 also leads to high residual activity that could be due to the possibility of existing two or three different xylanases in the same enzymatic extract produced by A. niger LPB BC. That is why the enzyme reacts differently with a large range of pHs. In this way, the pH profiles showed that xylanases produced by A. niger LPB BC can favorably act under different pHs ranging from 3.0 to 7.5.

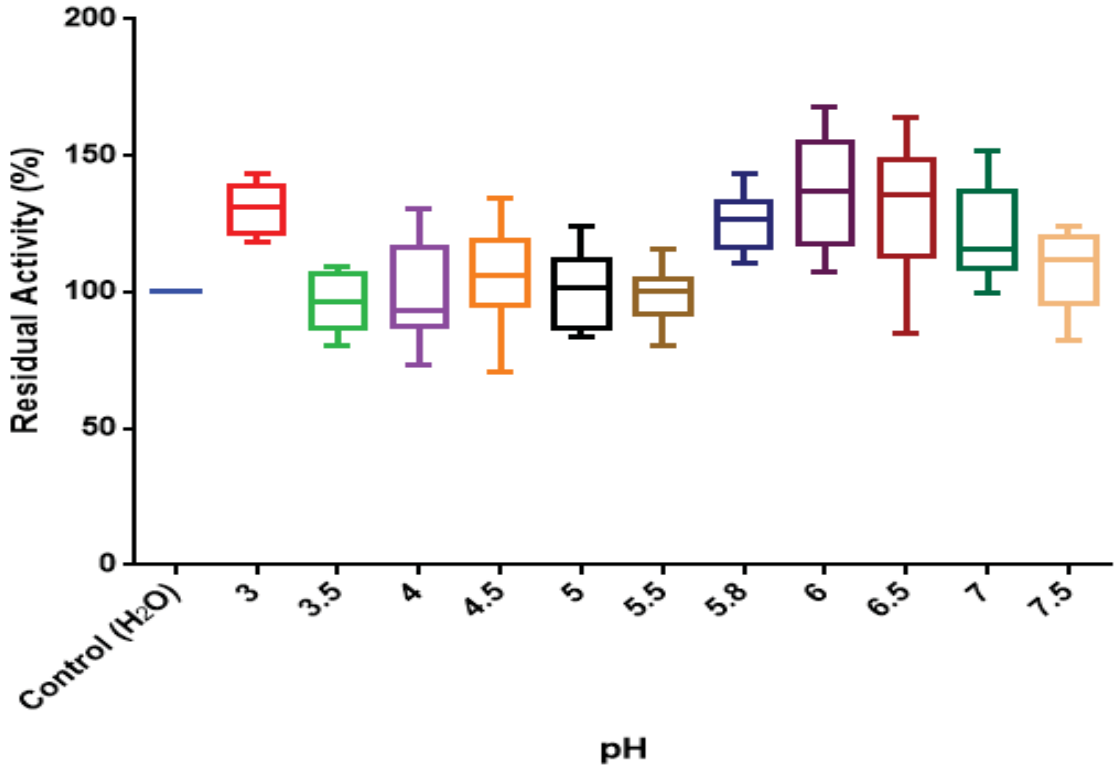

Fig 14: *Residual activity of Xylanase with different pH*

Stabilizing effect of different additives on partially purified xylanase activity

The addition of stabilizing agents is the simplest and cheapest method to achieve enhanced activity stabilization of enzymes through time. Various types of additives have been reported in the literature such as polyols, sugars, metals, surfactants, and salts (Table 13). New additives are screened every day, which reflect the effectiveness of this method. However, some of these additives may interfere with the final use of the enzyme due to its incompatibility with reaction system, for example, mainly in the pharmaceutical sector Nirmal; Laxman (2014).

***Table 13**: Stabilizing effect of different additives on partially purified xylanase activity.*

Additives		Concentration % (w/v)	Residual Xylanase activity (%)
Control	Enzyme extract	-	7.244 ± 1.7
Salts	Sodium chloride	1	4.250 ± 0.4
	Sodium citrate	1	22.283 ± 0.3
	Potassium sorbate	0.15	48.122 ± 4.1
	Sodium benzoate	0.25	66.988 ± 3.3
Sugars	Glucose	5	36.751 ±17.6
	Sucrose	5	**99.252 ± 1.1**
	Raffinose	5	65.074 ± 1.6
	Dextrose	5	74.268 ± 31.7
Polyols	Glycerol	20*	73.514 ± 13.8
	Mannitol	5	12.284 ±2.0
	Sorbitol	20	79.475 ± 13.0
	Xylitol	20	**100.91 ± 1.3**
Acids	Benzoic acid	0.1	8.191 ± 1.2
	Citric acid	1	16.504 ± 1.2
	Ascorbic acid	0.1	17.318 ± 0.8
Amino acids	Methionine	1	9.820 ± 3.1
	EDTA	0.5	3.705 ± 1.1
Polyethylene glycol	PEG 1000	2.5	7.716 ± 0.2
	PEG 8000	2.5	17.078 ± 3.8

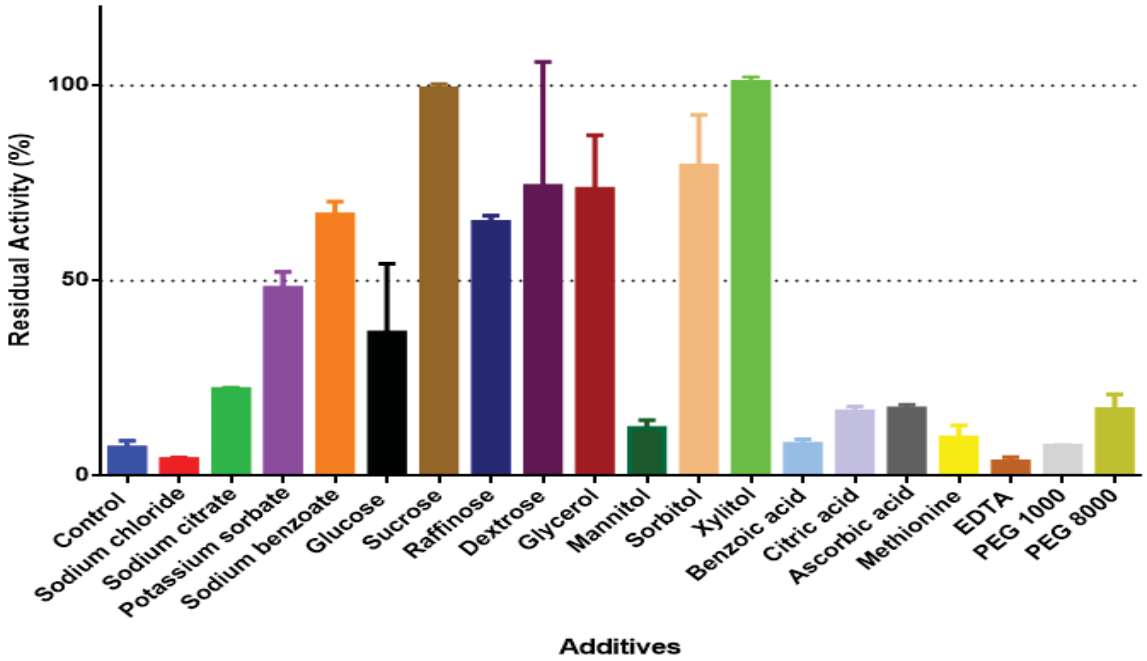

Fig 15: Stabilizing effect of different additives on partially purified xylanase activity

According to the results obtained in this work, (Fig. 15) xylitol and sucrose are the best additives for better stabilization of the produced xylanase, keeping residual activity at 100 % under accelerated conditions. This is an excellent result. Contrarely, the best additive from the salts' group was sodium benzoate that keeped only 66.99% of residual xylanase activity. These results are similar to those reported by Goelzer 2015. It is essential to point out that each enzyme reacts differently with the employed stabilization additives, that is why they must be tested preliminarly to a final enzymatic formulation definition.

Conclusion

An efficient xylanase production process from *Aspergillus niger* LPB BC was developed in submerged fermentation with solids in suspension, which is the innovative characteristic of this process. Optimization essays led to the definition of a medium composition that allowed very high enzyme activity (52.87 U ml^{-1}) and productivity of 13.22 U ml^{-1} day^{-1}. The use of sugarcane bagasse and soybean meal is an efficient alternative to reduce the costs of xylanase production by *Aspergillus niger* LPB BC, since these materials are often available in tropical countries like Brazil, as an inexpensive source of components that propitiate the fungi growth and the enzyme production. SDS-PAGE and zimogram allowed to determine the molecular mass of different xylanases produced by the same strain. Ions such as Co^{+2}, Mn^{+2}, Cu^{+2} generate an increase in xylanolytic activity and a high resistance to different pH is observed. For other hand additives such as xylitol, sucrose and sodium benzoate are possible conservatives and stabilizers of xynalolytic activity.

Promising perspectives are viewed for this process, which will be continued with the analysis of scale-up of the enzyme production, Identify the type of xylanase produced by maldi tof tof, purification and identification of other concomitant produced enzymes to define an enzymatic blend for efficient enzymatic hydrolysis of oleaginous seed. Finally, a stable liquid or powdered enzymatic formulation will be defined for its application.

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5. CONSIDERAÇÕES FINAIS

A otimização de produção de xilnases por *A. niger* BC, utilizando resíduos industrias como o bagaço de cana e o farelo de soja, deram ótimos resultados, mas é necessário também saber que essa produção vai depender muito das características dos resíduos, e a produção não sempre vai ser mantida devido a isso.

As xilanases son um grupo muito diverso é por isso que dentro de um mesmo caldo fermentativo podemos encontrar mais de um tipo de enzima, o gênero *Aspergillus* é um dos maiores produtores de esta enzima devido a sua adaptavilidade a se desenvolver em ambiente ricos em resíduos vegetais, estas enzimas vao a agir e degradar o xylan em diferentes partes do polissacarídeo, tanto na cadeia principal como nas cadeias laterais.

6. RECOMENDAÇÕES PARA TRABALHOS FUTUROS

Para trabalhos futuros e recomendavel terminar a fase de preparo enzimático e finalmente obter uma formulação final, por outro lado o estudo de esta enzima para fututras aplicações e caracterização de enzimas produzidas dentro do caldo fermentativo.

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