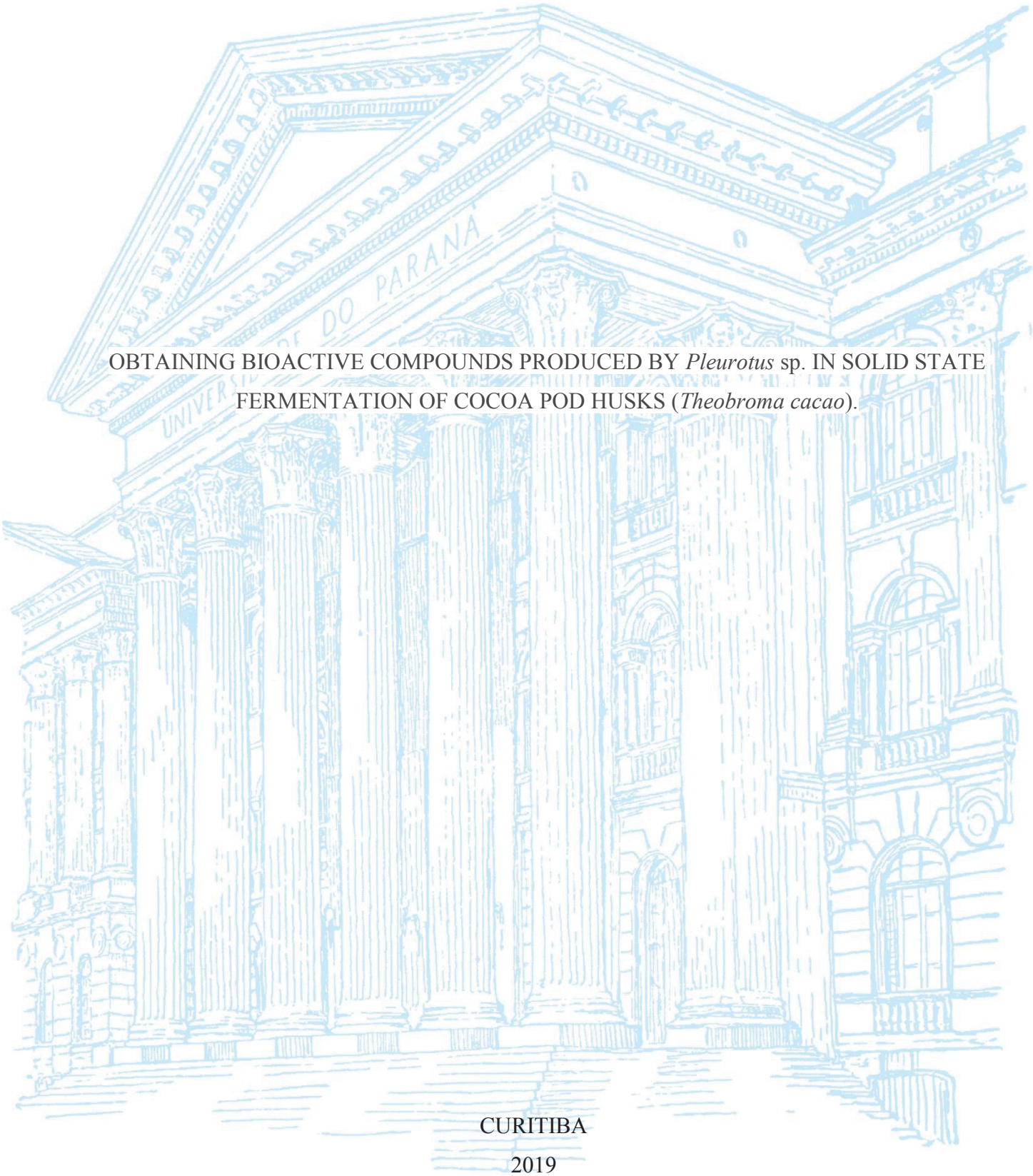
UNIVERSIDADE FEDERAL DO PARANÁ

LUIS FERNANDO PÉREZ FALCÓN



OBTAINING BIOACTIVE COMPOUNDS PRODUCED BY *Pleurotus* sp. IN SOLID STATE FERMENTATION OF COCOA POD HUSKS (*Theobroma cacao*).

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OBTAINING BIOACTIVE COMPOUNDS PRODUCED BY *Pleurotus* sp. IN SOLID STATE FERMENTATION OF COCOA POD HUSKS (*Theobroma cacao*).

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

Orientadora: Prof. Dra. Adriane Bianchi Pedroni Medeiros

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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 tese de Doutorado de **LUIS FERNANDO PEREZ FALCON** intitulada: **"Obtaining bioactive compounds produced by Pleurotus sp. in solid state fermentation of cocoa pod husks (Theobroma cacao)"**, 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 doutor 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, 15 de Fevereiro de 2019.

ADRIANE BIANCHI PEDRONI MEDEIROS
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DEDICATORY

A mi hija María Fernanda,
quien es la razón de mi
vida.

En memoria de mis queridos padres Irma y Luis, quienes siempre permanecerán conmigo en mi corazón y en mi mente.

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RESUMO

A casca de cacau (*Theobroma cacao* L.) é o principal subproduto da indústria de chocolate, correspondendo a 80% do fruto do cacau em massa. A geração de toneladas de resíduos pela agroindústria do chocolate é um problema não somente ambiental, mas também econômico, já que esses possuem um valor agregado não explorado. Considerando sua composição e valor nutricional, a casca de cacau possui potencial para ser utilizada na indústria alimentícia, farmacêutica, cosmética e biotecnológica. Neste trabalho a casca de cacau foi utilizada como substrato para o cultivo de *Pleurotus* spp. A casca de cacau proveniente da Bahia e do Pará foi caracterizada físico-quimicamente, determinando-se sua atividade antioxidante, teor total de polifenóis, pectina, açúcares e ácidos orgânicos. Nas fermentações em estado sólido (FES) foram testadas 10 cepas de *Pleurotus* spp. provenientes de distintas instituições e países. A FES foi realizada com a casca do Pará durante 30 dias, a 28 ± 2 °C. Foi avaliado o efeito da degradação da casca, a atividade das enzimas pectinase e lacase produzidas durante o cultivo de *Pleurotus* spp. e a produção de metabólitos secundários. Foi também determinado o teor total de polifenóis, atividade antioxidante e açúcares redutores. A cepa que apresentou melhor desempenho foi a PL22E (*Pleurotus* sp.). Esta cepa foi submetida à FES e fermentação semi-sólida. Os extratos obtidos da casca fermentada foram testados em relação a sua atividade antifúngica com 3 espécies de *Aspergillus (A. westerdijkiae, A. carbonarius e A. niger*) e também antividade antagonistica contra *Moniliophthora perniciosa.* Foi observado que *Pleurotus* spp. possui potencial para ser utilizada no controle biológico na agricultura.

Palavras-chave: *Pleurotus* sp.. Casca de cacau. Fermentação em estado solido. antioxidantes. *Moniliophthora perniciosa.*

ABSTRACT

The cocoa husk (*Theobroma cacao* L.) is the main by-product of the chocolate industry, accounting for 80% of the cocoa fruit. The generation of tons of waste by the chocolate industry is not only an environmental problem, but also economic, since it has an unexploited added value. Considering its composition and nutritional value, the cocoa husk has the potential to be used in the food, pharmaceutical, cosmetic and biotechnological industries. In this work the cocoa husk was used as substrate for the cultivation of *Pleurotus* sp. The cocoa husks from Bahia and Pará were characterized physicochemically, determining its antioxidant activity, total content of polyphenols, pectin, sugars and organic acids. In the solid-state fermentations (SSF) 10 strains of *Pleurotus* sp. from different institutions and countries were tested. SSF was performed with the Pará cocoa pod husk until 30 days, at 28 ± 2 °C. The degradation effect on the husks, the activity of the pectinase and laccase enzymes produced by *Pleurotus* sp. and the production of secondary metabolites were evaluated. It was also determined the total content of polyphenols, antioxidant activity and reducing sugars. The strain that showed the best performance was PL22E (*Pleurotus* sp.). This strain was submitted to SSF and semi solid-state fermentation. The extracts obtained from the fermented cocoa pod husk were tested for their antifungal activity with 3 species of *Aspergillus* (*A. westerdijkiae*, *A. carbonarius* and *A. niger*) and also antagonistic activity against *Moniliophthora perniciosa*. It was observed that *Pleurotus* sp. has potential to be used in biological control in agriculture.

Keywords: *Pleurotus* sp.. Cocoa pod husk. Solid-state fermentation. Antioxidants. *Moniliophthora perniciosa.*

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SUMMARY

1. INTRODUCTION

At the global level there is concern about the care and conservation of the environment, and its naturals resources. Finding solutions to deal with the problems generated by the industrial sectors such as agro-industry is an important issue. This sector generates thousands of tons of waste that are used as fertilizer for agriculture.

The agro-industry is one of the main sectors that stimulates the economy of the countries, and is characterized by agricultural land, livestock productivity and forestry (FOUNTOULAKIS *et al*., 2008); it also generates large amounts of agro-industrial waste, with significant negative impacts on the environment, such as generation of water pollution, odors, among others; is necessary a proper management to avoid environmental problems (PELLERA; GIDARAKOS, 2017).

The agro-industrial wastes are organic and inorganic components, which can be used to obtain bioactive compounds or biofuels, thus improving the added value. They can be used approach it as a usable waste for the production of new functional products that contain fiber, antioxidants or other important components to maintain good health, thus reducing pollution, and improving the return of companies and of the country. Agro-industrial sectors such as the processing of fruits, chocolate, wine, coffee, among others are rich in organic compounds that through biological, chemical or physical processes, can provide bioactive compounds of nutraceutical importance.

Another important agroindustrial sector is the chocolate industry. Its main residue are cocoa pod husks. For the production of each ton of dry cocoa beans, 10 tons of pod husks are generated; the discarding of which constitutes a serious problem for the industries (KALVATCHEV *et al*., 1998). Thus, only 10 to 20% of cocoa fruits mass is exploited; the remaining fraction is rejected (BARAZARTE *et al*., 2008). Cocoa pod husks are rich in fibers, minerals, contain antioxidants such as polyphenols and other bioactive compounds (BONVEHÍ; JORDÀ, 1998).

The cocoa industry in Brazil had produced 238141 tons/year of cocoa beans, in average (FAOSTAT, 2018), from 2007 to 2016. Approximately 80% of the fruit is constituted by cocoa pod husks, used mainly as soil fertilizer for agriculture, representing a waste of a valuable source of nutrients (BONVEHÍ; JORDÀB, 1998; VRIESMANN *et al*., 2011) that could be used for obtaining bioactive compounds, food, drugs and cosmetics industries, with the development of healthy products, with high quality and competitive prices.

The genus *Pleurotus* constitutes a group of mushrooms with high nutritional value, great nutraceutical potential, and recognized for important applications in the biotechnological, health, environmental, agricultural, pharmacological, cosmetic and other important sectors of the industry (COHEN *et al*., 2002; GUILLAMÓN *et al*., 2010; KHAN; MOUSUMI, 2012).

The aim of this study was to obtain bioactive compounds with properties such as antioxidant and antifugic, from the use of the cocoa pod husk through the processes of fermentation in solid state by different species of the fungus *Pleurotus*.

The research developed is framed within the Biocau Project (Researchers from Brazilian universities in proposing solutions to the problematic of the cocoa production chain), whose main objective is to evaluate the potentialities of the use of cocoa pod husk as a source of raw material for the production of bioactive compounds and biomolecules of high added value.

2. OBJECTIVES

2.1. GENERAL OBJECTIVE

To identify and assess bioactive compounds of cocoa pod husks fermented by *Pleurotus* sp.

2.2. SPECIFIC OBJECTIVES

- To characterize the cocoa pod husk (macronutrients, micronutrients, polyphenols, antioxidant capacity, etc.) from the North (Pará) and northeast (Bahia) of Brazil.
- To determine the pectin content in the cocoa husk and evaluate the pectinase activity of *Pleurotus* strains.
- To determine the lignin content in the cocoa pod husk and evaluate its degradation by *Pleurotus*, and also evaluate the laccase activity.
- To determine the antioxidant activity and phenolic compounds in cocoa husk and *Pleurotus* fermented husk extract.
- To perform semi-solid culture with *Pleurotus* and test the raw extract for antioxidant and antifungal activity.

3. BIBLIOGRAPHIC REVIEW

3.1. COCOA

The cocoa trees scientific name is *Theobroma cacao* L., which is a tropical plant, native to Central and South America, that belongs to the family Malvaceae (LEÓN, 2000). The cocoa is grown mainly in Mexico, Central America, Ecuador, Brazil, Ghana, Ivory Coast, Nigeria, Ceylon and Java (OETTERER *et al*., 2006).

The fruit of the cocoa is the most appreciated raw material, which is obtained pulp, butter, liquor, nuts and cocoa powder; It is used mainly in the production of chocolate (ENRIQUEZ; PAREDES, 1989).

FIGURE 1 - COCOA FRUIT VARIETIES.

Criollo cocoa

Forastero cocoa

Trinitarian cocoa

Source: https://www.barry-callebaut.com/ (2018).

There are mainly three varieties of cocoa: criollo (cocoa smooth taste and better quality), forastero (occurs in greater amounts than the other types of cocoa) and trinitarian (is a hybrid of criollo and forastero), (CENTRO DE COMERCIO INTERNACIONAL, 2001). In Table 1, it is presented the characteristics of the different cocoa varieties.

TABLE 1 - CHARACTERISTICS OF THE DIFFERENT COCOA VARIETIES

CHARACTERISTICS	CRIOLLO	FORASTERO	TRINITARIO
Pod husk:			
Texture	Soft, crinkly	Hard, Smooth	Mostly hard
Colour	Red	Green	Variable
Beans:			
Average no, per Pod	20 to 30	30 or more	30 or more
Colour of Cotyledons	White, ivory or very pale	Pale to deep Purple	Variable, White beans rarely
Agronomic:	Purple		
Tree vigour	Low	Vigorous	Intermediate
Pest and disease Susceptibility	Susceptible	Moderate	Intermediate
Quality:			
Fermentation need	Maximum 1 to 3 days	Normally 5 Days	4 to 5 days
Flavour	Weak choclolate; mild and nutty	Good; Chocolate	Good; chocolate; full cocoa
Fat contente	Low	High	Medium
Bean Size (g/100beans)	85	94	91

Source: Afoakwa (2014).

According to Hebbar *et al*. (2017), there are 22 known species of the genus *Theobroma*, *Theobroma cacao*, is the only species that is widely cultivated outside its natural range, which is produced with the chocolate and its derivatives. According to Figueira *et al*. (2002) and Hebbar *et al*. (2011) (cited by AFOAKWA, 2014), by its morphological characteristics, these species are subdivided into 6 sub-sections:

- Andropetalum (*T. mammosum*)
- Glossopetalum (*T. angustifolium, T. canumanense, T. chocoense, T. cirmolinae T. gradiflorum, T. hylaeum, T. nemorate, T. obovatum, T. simiarum, T. sinuosum, T. stipulatum, T. subincanum*)
- Oreanthes (*T. bernoullis, T. glaucum, T. speciosum, T. sylvestre, T. velutinum*)
- Rhytidocarpus (*T. bicolor*)
- Telmatocarpus (*T. gileri, T. microcarpum*)
- Theobroma (*T. cacao*).

3.1.1. Production of cocoa

There is important world production of cocoa bean, which represent 20% of the total weight of the fruit and the rest is the waste (80% of the weight of the cocoa). The production of the cocoa bean in thousands of tons from the year 2010 to 2015, is presented in the Table 2:

TABLE 2 - PRODUCTION OF COCOA BEANS (THOUSAND TONS).

	2010/2011	2011/2012	2012/2013	2013/2014	2014/2015
AFRICA	**3224**	**2929**	**2836**	**3199**	**3073**
Cameroon	229	207	225	211	232
Cote d`Ivoire	1511	1486	1449	1746	1796
Ghana	1025	879	835	897	740
Nigeria	240	245	238	248	195
Others	220	113	89	97	109
AMERICA	**516**	**655**	**622**	**727**	**763**
Brazil	200	220	185	228	230
Ecuador	161	198	192	234	250
Others	201	237	246	265	283
ASIA & OCEANIA	**526**	**511**	**485**	**447**	**400**
Indonesia	440	440	410	375	325
Papua New Guinea	48	39	41	36	36
Others	39	32	34	36	39
WORLD TOTAL	**4266**	**4095**	**3943**	**4373**	**4236**

Source: ICCO (2012/2013, 2013/2014, 2014/2015 and 2015/2016).

The main cocoa producer continent is Africa that corresponds to almost 3/4 of the world production. Cote d'Ivoire is the most representative. In America the main producer of cocoa is Brazil followed by Ecuador. In Asia and Oceania, the largest producer is Indonesia.

Brazil is the fifth major producer of cocoa in the world, with Bahia and Pará being the main producing states (MERCADO DO CACAU, 2018). In Table 3, cocoa production is shown in terms of beans weight, cultivation area and average yield for hectare.

TABLE 3 - PRODUCTION, GROWING AREA AND AVERAGE YIELD OF THE COCOA BEAN AND COCOA POD HUSK (CPH) FROM BRAZIL (2008 TO 2017).

Characteristics	Year									
	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Production (ton)	202030	218487	235389	248524	253211	256186	273793	278299	213843	235809
Waste of CPH (ton)	808120	873948	941556	994096	1012844	1024744	1095172	1113196	855372	943236
Area (ha)	641337	635975	660711	680484	684333	689276	704122	702841	720053	590813
Average yield (hg/ha)	3150	3435	3563	3652	3700	3717	3888	3960	2970	3991

Source: Modified of FAOSTAT (2018).

From the whole cocoa residues weight, 80% corresponds to fruit residues and the remaining 20% come from the processing of beans. From Table 3, it is possible to infer that more than 800 thousand tons of cocoa pod husks were produced during 2008; and there was a 135 thousand tons increase in this generation up to 2017. This important volume of residues could be commercially used by the food, drugs and cosmetics industries (MAKRIS *et al*., 2007; BALASUNDRAM; SUNDRAM; SAMMAN, 2006).

3.1.2. Cocoa processing

Cocoa is the fundamental raw material for the production of chocolate and other derivatives such as butter and cocoa powder.

The agroindustrial production of cocoa can be divided into 4 stages: cultivation, harvest, post-harvest and transformation.

a) The stage of cultivation

This stage is related to the sowing of cocoa, including biotechnological processes such as intervarietal hybridizations through the technique of grafting, in vitro culture and others. Seed selection, establishment of nurseries, selection and preparation of soil, proper application of pruning, weed removal and disease prevention. All these constitute the important processes of this stage, which influences the yield of the plant and the quality of its fruits (AFOAKWA, 2014; PÉREZ, 2015).

b) The harvest stage

At this stage, the ripe fruits of the cocoa tree (Figure 2) are harvested. Expertise is required in order not to damage the tree or the fruits. It is essential to do it applying the right technique and at the right time, so as to maintain the quality of the grains for the required processes (AFOAKWA, 2014).

FIGURE 2 - COCOA TREE.

Source: Medeiros (2018).

c) The post-harvest stage

This is the stage where the almonds are extracted from the cocoa cob, then separated and taken to a fermentation process. This process can be carried out in sacks or in wooden or plastic crates; this stage is important because the chemical reactions generated by the precursors of aroma and flavor that give the sensory characteristics are produced. After fermentation, which usually takes place for a period of 3 to 8 days, depending on the variety of cocoa bean, proceeds to drying (LIENDO, 2015).

In Figure 3, selected and clean cocoa fruits are presented, followed by the extraction of almonds (Figure 4).

FIGURE 3 - COCOA FRUITS.

Source: Medeiros (2017)

FIGURE 4 - EXTRACTION OF ALMONDS.

Source: Medeiros (2017).

The drying of the cocoa beans can take place in drying zones (Figure 5), in artificial chambers, in sliding carts or in wooden tendals, the purpose of which is to reduce the water content, for proper conservation for processing or export purposes (LIENDO, 2006).

FIGURE 5 - OUTDOOR DRYING OF COCOA.

Source: Medeiros (2018).

d) Processing stage

This stage requires greater technological resources, including the processes of cleaning, roasting, husking, crushing, grinding and pressing (PÉREZ, 2015).

Toasting is the thermal process carried out in a toaster in order to develop the characteristics of aromas and flavor preformed during fermentation; husking is used to remove the husk that covers the grain, since this part is neither edible nor desirable in subsequent processes. The crushing of the husked grain produces what is known as nib, fragments of

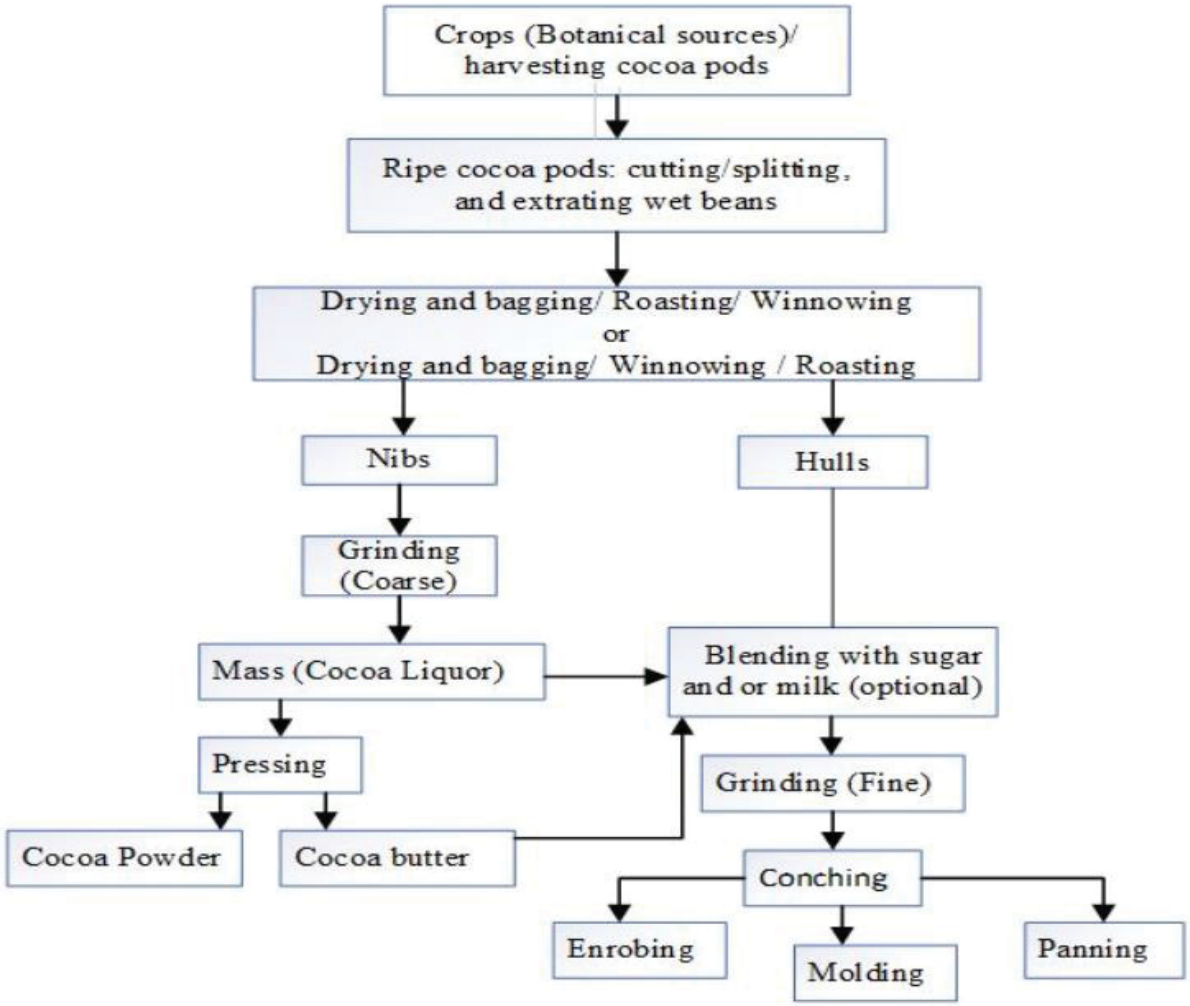
roasted cocoa beans that facilitate the subsequent milling process (AFOAKWA, 2014; PÉREZ, 2015).

The milling process forms a paste, known as cocoa liquor, which contains all the edible components of the grain and is the basis for obtaining derivatives, known as fatty solids (butter) and non-fat solids (cocoa cake, cocoa powder), obtained through a separation process with large pneumatic presses. Finally, the cocoa powder, with which the chocolate drinks are prepared, is produced through the fine grinding of the cocoa cake (AFOAKWA, 2014; PÉREZ, 2015).

Cocoa powder, butter and liquor are used in a wide variety of processes in the food, pharmaceutical and cosmetics industries (PÉREZ, 2015; LIENDO, 2006).

In the Figure 6, the flowchart of cocoa processing is presented until the chocolate and its derivatives are obtained.

FIGURE 6 - FLOWCHART OF COCOA PROCESSING FOR CHOCOLATE PRODUCTION.



Source: Pérez (2015).

Each stage in the processing of cocoa and the manufacture of chocolate is essential to obtain a high-quality product. As well as the methods used in each step in the process, as well as the ingredients used, have a direct effect on the characteristics and quality of the final product, which is why it must be strictly controlled (PÉREZ, 2015).

3.1.3. Agro-industrial wastes: Cocoa pod husk

It is defined as agro-industry to productive and economic activities of a particular biological product, which goes along the production chain from the primary activities of production (agricultural, livestock or forestry), for the activities of transformation and to the marketing of the product (SAVAL, 2012).

The agro-industry generates in its productive activity thousands of tons of waste (Table 4), which pollute the environment; many of the industries don't know what to do with their waste; which in most cases is used as a fertilizer in the agricultural lands, missing out on a great opportunity to give a higher added value to produce new products that benefit society, reduce pollution and give greater profitability to the employer.

TABLE 4 - WASTE GENERATED FROM COCOA POD HUSK (CPH) FROM THE PRODUCTION OF COCOA BEANS.

CPH	***ANNUAL PRODUCTION* (thousand tons)**				
	2010/2011	**2011/2012**	**2012/2013**	**2013/2014**	**2014/2015**
WORLD TOTAL	17064	16380	15772	17492	16944

Source: Modified of ICCO (2012/2013, 2013/2014, 2014/2015 and 2015/2016).

Table 4, shows the cocoa pod husk (CPH) residues generated in the world production of cocoa beans.

In the Table 5, shows the chemical composition of the cocoa pod husk (CPH) is show:

TABLE 5 - CHEMICAL COMPOSITION OF CPH (g/kg dry matter).

Component (g/kg D.M.)	Donkoh et al. (1991)	Okal et al. (1984)	Bonvehí and Jordà (1998)	Vriesmann et al. (2012)
Dry matter	946			
Crude protein	76.6	74		
Ether extract	43.7	82		
Crude fibre	325			
Total lipids			24.76	
Ash	101	100	107	
Acid detergent fibre	414	564		
Neutral detergent fibre	522	644		
Hemicellulose	108	80		
Starch			12.060	
Pectin				101
Metabolizable energy (MJ/kg D.M.)	4.72			
Calcium	8.12	8.8	3.52	
Phosphorus	4.36	4.8	7.36	
Magnesium	6.89	7.1	9.93	
Iron	0.33	0.4	6.04	
Potassium	71.8	73.6	17.40	
Manganese	0.15	0.1		
Zinc	0.1	0.1	0.53	
Copper	5	4.2	0.46	
Chromium			0.09	
Sodium	0.17	0.2	9.64	
Vit. B1			0.002	
Vit. B2			0.002	
Vit. B6		tr.		
Vit. D		tr.		
Theobromine			0.011	
Caffeine			0.001	
Polyphenols			7.364	
Tannins			1.814	
Non-Tannins			5.443	
Gentisic acid			5.870	
Caffeic acid			1.494	
Phytic acid			10.459	

Dry matter (D. M.), Traces (tr.)
Source: Okal *et al*. (1984); Donkoh *et al*. (1991); Bonvehí and Jordà (1998); Vriesmann et al. (2012).

The majority of agroindustrial residues is rich in cellulose and hemicellulose and has around 75-80% of these compounds. They can be degraded by biological, physical and/or chemical processes to sugars and its be subsequently converted into various

products (MUÑOZ-MUÑOZ *et al*., 2014). Some agro-industrial wastes are presented in Table 6:

TABLE 6 - MAIN COMPONENTS OF SOME AGRO-INDUSTRIAL RESIDUES (LIGNOCELLULOSIC WASTES).

Agro-industrial Residues	Cellulose (wt%)	Hemicellulose (wt%)	Lignin (wt%)
Barley hull	34.0	36.0	19.0
Barley Straw	33.8	21.9	13.8
Cocoa Pod Husk	35.0	11.0	14.6
Corn cobs	33.7	31.9	6.1
Corn stalks	35.0	16.8	7.0
Cotton	90.0	10.0	0.0
Cotton stalk	31.0	11.0	30.0
Coffe Pulp	35.0	45.0	17.0
Jute fibers	49.0	20.0	24.0
Oat Straw	58.5	14.4	21.5
Oilseed rape	27.0	21.0	14.0
Nutshells	28.0	25.0	35.0
Rice Straw	39.4	27.1	17.5
Rye Straw	36.2	19.0	9.9
Sorghum Straw	32.0	27.0	18.0
Soya stalks	37.6	30.5	19.0
Sugarcane bagasse	40.0	27.0	10.0
Sunflower stalks	42.1	29.7	13.4
Winter rye	30.0	24.0	16.0
Wheat bran	12.0	37.0	10.0
Wheat Straw	32.9	24.0	8.9

Source: Modified from Nigam *et al*. (2009), Mussato *et al*. (2012), Mansur *et al.* (2014) and Gopinath *et al.* (2016).

The residues of cocoa can be used in accordance to their nature, for various products: activated carbon, biogas, cocoa gum, compost, dietary fiber, essential oils, methyxanthine, particle boards, pectic enzyme, polyphenols, theobromine (Table 7).

TABLE 7 - THE BY-PRODUCTS THAT MAY BE PRODUCED FROM COCOA WASTE.

By-products of cocoa waste	Source
Activated carbon	Pod husk
Biogas	Pod husk
Cocoa gum	Pod husk, leaves, and chupons
Compost	Pod husks
Dietary fiber	Shells
Essential oils	Leaves, chupons, flowers and pod husk
Methylxanthine	Shells, leaves, beans and chupons
Particle boards	Shells
Pectic enzyme	Pod husk
Polyphenols	Shells, leaves, pod and branches
Theobromine	Shells and beans

Source: Oddoye *et al*. (2013).

In the Biocau Project (Integrated Network of Graduate Programs in Biotechnology for Development Scientific, Technological, Innovation and Human Resource Training in the Production Chain of Cacau; team formed by UFPR, UFPA and UEFS), several studies have been carried out to obtain organic acids (citric acid, cinnamic acid, ellagic acid, propionic acid), polysaccharides with industrial value (pectin), enzymes (lacase, pectinase), antioxidants (polyphenols) among others, obtained from the cocoa pod husk.

3.2. GENERALITIES OF THE FUNGI

The fungi are eukaryotic organisms, adapt to any medium or surface of the nature, reproduce by spores that are spread by wind and water (INSTITUTO NACIONAL DE LA BIODIVERSIDAD, 2017). The fungi are heterotrophs, possess a thick wall called chitin that gives them strength and stiffness; their bodies are made up of hyphae, the branching and cross-linking of them are known as mycelium (COMISIÓN NACIONAL PARA EL CONOCIMIENTO Y USO DE LA BIODIVERSIDAD, 2017).

There are more than 150000 different species of fungi in the earth, only 10% of these species have been studied to date (SHARMA *et al*., 2013).

According to Stamets (1993), the fungi can be classified into three groups saprophytes, parasites and mycorrhizae. They are responsible for decomposition, stabilization and metabolism of cellulose, hemicelluloses, lignin and other organic compounds (FLOUDAS *et al*., 2013 and WIN *et al.*, 2013; as cited by WRIGHT *et al*., 2016).

Ruggiero et al. (2015), classifies the fungi in five divisions:

- **Ascomycetes** (division: Ascomycota): Develop asci with ascospores.
- **Basidiomycetes** (division: Basidiomycota): Develop basidiocarps called mushrooms that produce basidia with basidiospores.
- **Glomeromycetes** (division: Glomeromycota): The mycorrhiza symbionts of plants with glomerosporas multinucleated cells.
- **Zigomycetes** (division: Zygomycota): The molds that form zigospores.
- **Quitridiomycetes** (division: Chytridiomycota *sensu lato*): The microscopic fungi with zoospores and gametes uniflagellates.

The following Table 8, general characteristics that distinguish some fungi are shown (DEACON, 2006):

TABLE 8 - GENERAL CHARACTERISTICS OF SOME OF THE FUNGI.

Character	**Fungi**
Growth habit	Hyphal tip growth or budding yeast
Nutrition	Heterotrophic, absorb soluble nutrients
Cell Wall	Typically contains chitin
Nuclei	Usually haploid; nuclear membrane persists during division
Histones	Histones 2B
Microtubules	Sensitive to benximidazoles and griseofulvin
Lysine synthesis	Synthesized by AAA pathway
Golgi cisternae	Unstacked, tubular
Mitochondria	Plate or diks-like cisternae
Translocated carbohydrates	Polyols (mannitol, arabitol, etc.), trehalose
Sorage compounds	Glycogen, lipids, trehalose
Mitochondrial codon usage	UGA codes for tryptophan
Membrane sterols	Ergosterol

AAA, alpha-amino adipic acid pathway.

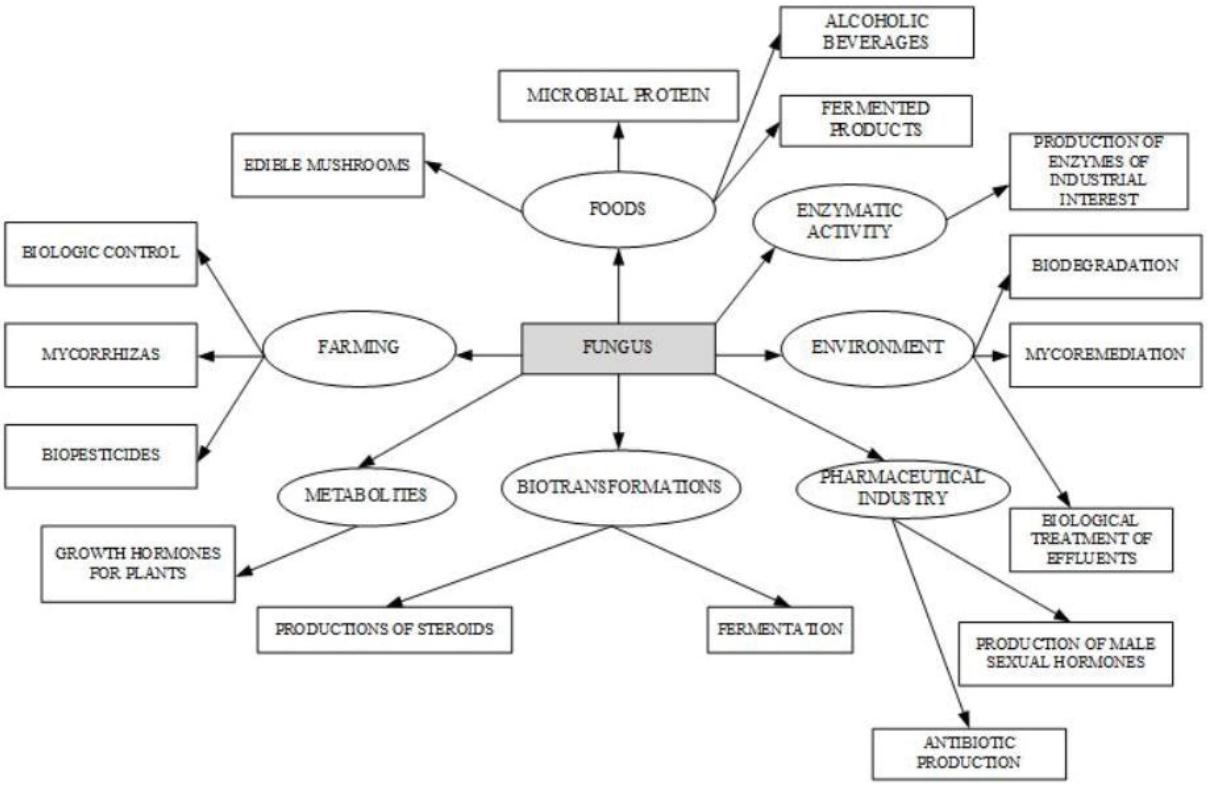
Source: Modified of Deacon (2006).

3.3. FUNGI AND THEIR POTENTIAL APPLICATIONS

Fungi are responsible for the production of different organic acids (citric acid, succinic acid, propionic acid, etc.), drugs (antibiotics), enzymes (xylanases, pectinases, celluloses, laccases and amylases), products for biological control in the agriculture, ethanol, fermented beverages among others of industrial importance (ESPOSITO; ACEVEDO, 2010).

The potential uses of fungi in different sectors of industry are shown in Figure 7.

FIGURE 7 - POTENTIAL USE OF FUNGI.



Source: Esposito and Azevedo (2010).

Fungi are used to produce food (bread, mycoproteins), fermented beverages (wine, beer), recombinant proteins, vitamins and antibiotics (KAVANAGH, 2005); as presented in the Table 9:

TABLE 9 - INDUSTRIAL APPLICATIONS OF FUNGI.

Product	**Example**
Biomass	Production of baker's yeast
	Brewer's yeast tablets
	Single-cell protein (mycoprotein)
Cell components	Proteins (native or recombinant)
Products	Antibiotics (penicillin)
	Vitamins (B12)
Catabolite products	Ethanol
Bioconversion	Breakdown of range of carbohydrates

Source: Kavanagh (2005).

To achieve better productivity of products obtained by fermentation with mushrooms, it will depend on the fermentation system and nature of the fungus (filamentous or yeast) used, as well as the scale of production to obtain the required product (KAVANAGH, 2005); as presented in the following Table 10.

TABLE 10 - FUNGAL FERMENTATION SYSTEMS AND PRODUCTS.

Fermentation type	**Example**	**Product**
(1) Solid	Cultivation of *Agaricus bisporus*	Mushrooms
(2) Batch	Brewing, wine making	Beer, wine
(3) Fed batch	Cultivation of *Saccharomyces cerevisiae*	Baker's yeast
(4) Cell recycle batch	Wine making (Italy)	Summer wines
(5) Continuous	Cultivation of *Penicillium graminerium*	Mycoprotein

Source: Kavanagh (2005).

The production of edible fungi is of great economic and social significance, gaining great importance at the global level by the biotechnological processes developed for food of good nutraceutical quality and with medicinal properties (antibiotic, anti-cancer, anti-diabetic that reduce cardiovascular disease, cholesterol, hypertension), (CHANG; MILES, 2004).

The ligninolytic enzymes of white-rot fungi have very important applications such as the bio-whitening of pulps, dyeing of textiles, bioremediation of contaminated industrial waste, biotransformation of pharmaceutical products and other intermediate products, improvement of food properties, cosmetics, medicine, among others (VILLALBA *et al*., 2010).

3.3.1. The genus *Pleurotus*

The genus *Pleurotus* belongs to the class of basidiomycete, classified taxonomically as follows (RODRÍGUEZ, 1996):

Kingdom: Fungi
Group: Eumycota
Class: Basidiomycetes
Subclass: Holobasidiomicetidae
Order: Agaricales
Family: Trichoiomataceae
Genus: *Pleurotus*

According to Guzman (2000), the species of the genus *Pleurotus* are characterized by presenting fan-shaped or funnel-shaped basidiomes. Their caps or hats can have a great variety of colors, from white, whitish, cream, yellowish, pale brown to dark, grayish brown, bluish gray, pink, or orange pink, sometimes with viscous surface. Some species have veils, then leave remains on the edge of the crown and occasionally form an annular zone in the stipe. The "flesh" is spongy, white in colour to whitish, with a pleasant smell of fungus or flour. Its sheets are adhered to the foot, white, whitish, pink to orange pink. The stipe or foot is absent or may present lateral, short or long (SALMONES; MATA, 2017).

As for their microscopic characteristics, they present cylindrical to subcylindrical spores, occasionally ellipsoidal, thin-walled, smooth and hyaline (SALMONES; MATA, 2017). The hifal system can be monomithic or dimithic and the hymenophore and cap weft is irregular. It is considered a ligninolytic genus, since it grows on wood, although it has occasionally been found parasitizing some cacti and agave trees (GUZMÁN, 2000).

The fungus of the genus *Pleurotus* mushroom is more popular in China and the second most sold in the world market (BELLETINI *et al*., 2016), it is known as "White rot fungus" (TSUJIYAMA; UENO, 2013) because it produces white mycelium on lignocellulosic substrates (SAVOIE *et al*., 2007), by having lignocellulosic enzymes that degrade these substrates.

According to Cohen et al. (2002), the genus *Pleurotus* constitutes a group of edible ligninolitic fungi with medicinal and nutraceutical properties, with important biotechnological and environmental applications. *Pleurotous* with its fruiting body and mycelium has therapeutic

effects to fight the cancer, high cholesterol, high blood pressure, and diabetes in humans (WASSER *et al*, 2002).

Chang, and Buswell (1996), consider that the *Ganoderma, Lentinula* and *Pleurotus* are the triad of the nutraceutical properties, pharmacological and functional food.

The production of basidiomycetes fungi and their respective enzymes, it is affected by factors such as the composition of the medium, pH, temperature, aeration speed, etc. (VELIOGLU; UREK, 2015; COGORNI *et al*., 2014; AHMED *et al*., 2013).

In the following Table 11 the range of different parameters (particle, pH, substrate, temperature, relative moisture and light) for a better crop and productivity for certain species of *Pleurotus* is shown.

TABLE 11 - PARAMETERS OF GROWTH OF SOME SPECIES OF *Pleurotus.*

Species	Particle (mm)	Ph	Substrate moisture (%)	Temperature (°C)	Relative moisture (%)	Light (Lux)
P. ostreatus	0.92 - 50	5.5	60 – 80	7 – 30	31 - 100	15 - 2000
***P.* spp. ***	50 - 60	6.5 - 7	50 – 75	18 – 30	60 - 97	200 - 640
P. eryngii				25		80 - 100
P. sajur – caju	47.6 - 100	5.5 - 7	70 – 80	20 – 25	85	15 - 600
P. sapidus						15 - 135
P. cornucopiae	50 - 100	6.5 - 7	70 – 72	20 – 25	85	

Source: Modified from Pineda-Insuasti *et al*. (2014),
*Bellettini *et al*. (2016).

3.3.1.1. *Pleurotus* sp.

Pleurotus sp. (Figure 8) is one of the most studied basidiomycetes of white-rot fungus, for its important ligninolytic properties, because it produces cleavages of cellulose, hemicellulose and lignin in wood (MACHADO *et al*., 2016; BELLETTINI *et al.*, 2016).

The species of *Pleurotus* are saprophytes that use grass, wood, agro-industrial waste, etc. as substrates to obtain sources of carbon, nitrogen, vitamins and minerals for their development and growth of the fungus (BELLETTINI *et al*., 2016).

Pleurotus produce extracellular enzymes to decompose lignocellulosic substrates. Cellulases, xylanases and ligninasesare used in the production of beer, bread, starch, in the production of leather and in the textile industry. In addition, they are used in the discoloration of industrial synthetic dyes (SUWANNAWONG *et al*., 2010; WANG et al., 2012; LIM et al., 2013).

FIGURE 8 - *Pleurotus* sp.

Source: https://www.forestryimages.org/browse/detail.cfm?imgnum=1406224 (2018).

3.4. BIOACTIVE COMPOUNDS FROM MUSHROOMS

Bioactive compounds are substances that have beneficial effects or may be harmful to human health, and are produced by plants or microorganisms such as fungi, yeasts and bacteria (DÍAZ-GODÍNEZ, 2015).

The latest researches on mushrooms have highlighted their nutraceutical, antioxidant, antimicrobial, anti-inflammatory, anti-cancer, anti-diabetic, anti-cardiovascular disease, properties (SHARMA *et al*., 2013). Below is presented the biological activity and the bioactive components of *Pleurotus* (Table 12).

TABLE 12 - BIOLOGICAL ACTIVITY AND/OR *Pleurotus* BIOACTIVE COMPOUNDS.

Fungal strain	**Active principle/ constituents/extracts**	**Activity Reported**
Pleurotus pulmonaris	Methanolic extract	Antioxidant, antitumor, and anti-inflammatory
Pleurotus tuberregium	Crude methanolic and water extracts	Antioxidant
Pleurotus ostreatus	Water and 30% ethanolic extract	Antioxidant, Hypocholesterolemic
Pleurotus eryngii	Ethanolic extracts	Antiallergic
Pleurotus cornucopiae	Dietary fiber	Hypocholesterolemic
Pleurotus ostreatus, P. eryngii, P. tuberregium,	β-glucans	Prebiotics

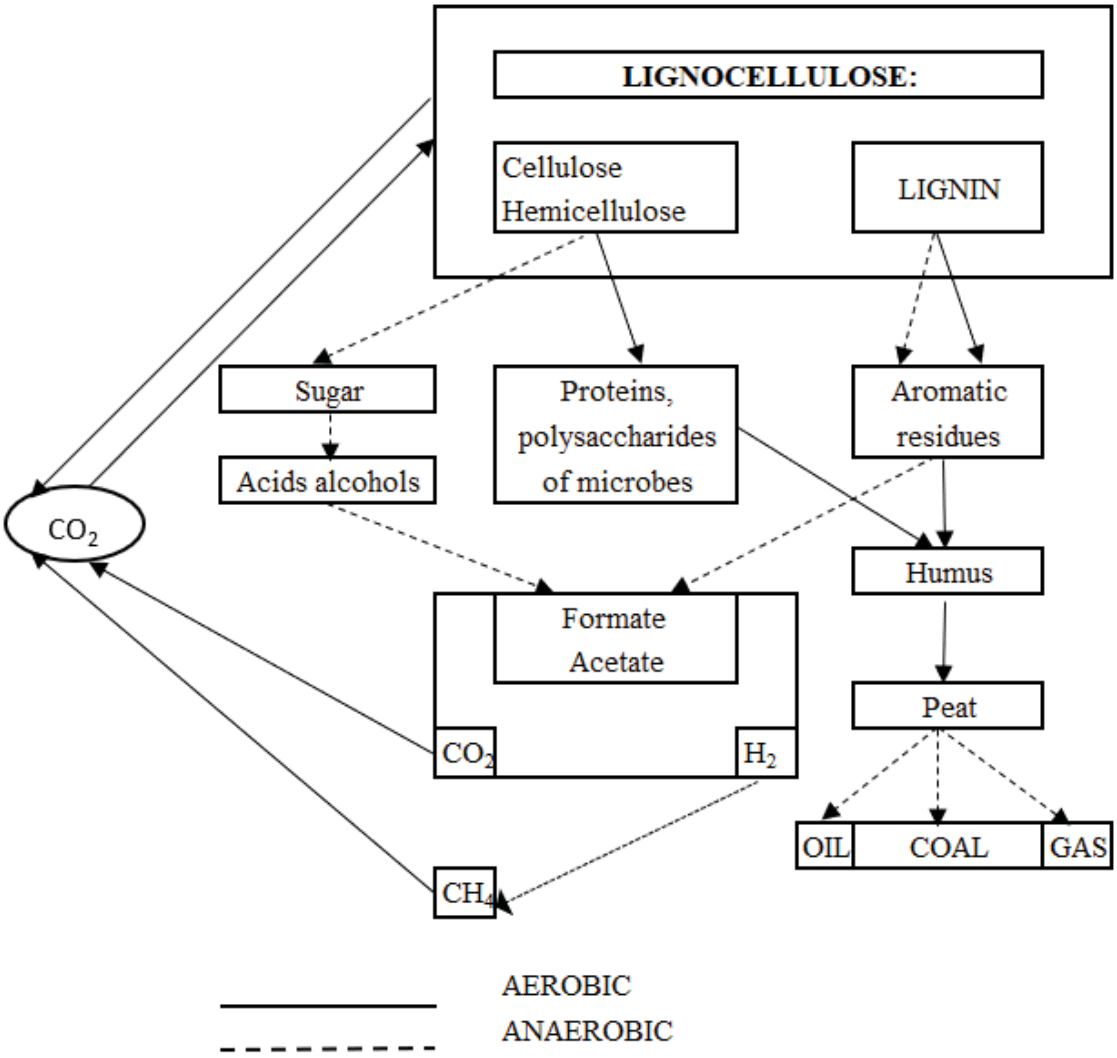
Source: Díaz-Godínez (2015).

As can be seen in Table 12, the *Pleurotus* fungi have compounds or produce biologically active metabolites that benefit the health of the people, that can be consumed as food or be used for therapeutic purposes (DÍAZ-GODÍNEZ, 2015).

3.5. BIODEGRADATION OF LIGNOCELLULOSIC MATERIALS BY FUNGI

The lignocellulosic components are produced by photosynthesis in plants, they make up around 50% of their biomass (SÁNCHEZ, 2009), and their degradation is important for the global carbon cycle to take place (Figure 9) (TUOMELA et al., 2000); this lignocellulosic material is mainly made up of cellulose, hemicellulose, lignin, salts, and minerals (FERRAZ, 2010).

FIGURE 9 - GLOBAL CARBON CYCLE.



Source: Tuomela *et al.* (2000).

The Basidiomycota class are the main fungi that degrade lignocellulosic materials, act in white decomposition (they decompose the components of wood) and in brown/cast brown decomposition (they degrade mainly polysaccharides) (FERRAZ, 2010; SÁNCHEZ, 2009).

The action of the white decomposition fungi on the lignocellulosic materials results in a whitened appearance and break down in the sense of the vegetable fibers. In the case of brown decomposition by fungi, the lignocellulosic material is brown in color and breaks easily into cubes, in a transverse direction to that of plant fibers (KIRK; CULLEN, 1998; RODRÍGUEZ *et al.,* 1997).

The biodegradation of lignocellulosic material occurs in extracellular form, by the action of enzymes and extracellular compounds of low molar mass; for this the lignocellulosic components must be degraded in simple forms to be transported by the cell wall and the intracellular metabolism of the fungi involved (FERRAZ, 2010).

3.5.1. Cellulose biodegradation

Cellulose is the main component of the cell wall and is the most abundant constituent in lignocellulosic materials, giving structure and support to plants (AGBOR *et al*., 2011; FERRAZ, 2010).

Cellulose is a linear homopolymer of D-glucose subunits joined by glycosidic bonds β-1,4 that form cellobiose molecules (PÉREZ *et al*., 2002). Cellulose chains (20-300) are joined together to form microfibers, which join together to form cellulose fibres (AGBOR *et al*., 2011).

The biodegradation of cellulose is due to a group of enzymes such as endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and 1,4-β-glicosidase (EC 3.2.1.21). Endoglycanase enzymes act by releasing smaller units that serve as substrate for exo-glucanases (they hydrolyze fragments of lower molecular mass); and β-glycosidase hydrolyze cellobiose to glucose (SÁNCHEZ, 2009; FERRAZ, 2010).

3.5.2. Hemicellulose biodegradation

Hemicellulose is the second most abundant component of lignocellulosic biomass, it is a linear, branched heteropolymer, with a lower molecular weight than cellulose and is composed of five different sugars (-D-xylose and L-arabinose as pentoses, and D-mannose, D-glucose and D-galactose as hexoses), (MICHELIN *et al*, 2014; AGBOR *et al*., 2011).

Hemicellulose are biodegraded in their monomeric sugars and acetic acid, being xylan the main carbohydrate in hemicellulose, which needs a variety of hydrolytic enzymes to degrade it, for its great heterogeneity in comparison to cellulose (SANCHEZ, 2009).

3.5.3. Lignin biodegradation

Lignin is a structural polymer in the supporting tissues of vascular plants, composed of units of phenylpropane forming an amorphous three-dimensional macromolecule, which confers rigidity to plants and binds plant cells together (resistance to impact, flexure, and compression) (CRAWFORD; CRAWFORD, 1976; FERRAZ, 2010). Lignin also provides rigidity and protection against microbial attack and oxidative stress, and is the third most abundant polymer in nature (AGBOR *et al*., 2011).

The different enzymes involved and reactions produced during lignin degradation are summarized as follows (GUTIÉRREZ; MARTÍNEZ, 1996):

a) There is the formation of an aromatic cationic radical, which later evolves in different ways. The reactions included in the scheme are as follows:

b) The oxidation of non-phenolic units by lignin peroxidase (LiP) is produced,

c) Oxidation of phenol units by MnP/Mn^{2+} or lacasse also occurs,

d) Ether breakage occurs in C_4, giving a phenoxyl radical,

e) The aromatic ring is opened,

f) C_α-C_β breakage and formation of a carbonyl group occurs

g) Demethoxylation/demethylation occurs,

h) Radical repolymerization occurs,

i) Radical reduction is given by enzymes such as quinone-reductase (QR) and cellobiose dehydrogenase (CDH),

j) Quinone/hydroquinone formation occurs from the phenoxyl radical,

k) Quinona-hydroquinone redox cycling (reduction by QR and spontaneous oxidation) is produced,

l) There is a reduction in aromatic aldehydes,

ll) Oxidation of aromatic alcohols by aryl alcohol oxidase (AAO) and formation of H_2O_2,

m) Radicals were generated from O_2,

n) Non-enzymatic attack of lignin by radicals (e.g. hydroxyl).

The enzymes of basidiomycetes (white - rot) such as laccases, phenoloxidases were attributed an important role in the biodegradation of lignin (ligninolitic fungi). These enzymes can degrade non-phenolic lignin in the presence of certain substrates, which act as intermediates or cooxidants, resulting in a C_{α}-C_{β} break similar to that produced by lignin peroxidase (GUTIÉRREZ; MARTÍNEZ, 1996).

There are other species of fungi that are important lignin degraders that produce a subgroup of enzymes called laccases, cuproproteins that do not require peroxide to act (FERRAZ, 2010). According to Kirk and Cullen (1998), the ligninolytic complex is classified according to its oxidation potential: Lignin peroxidase>Manganese peroxidase>Laccase.

3.5.4. Laccase

Laccases (p-diphenol oxygen oxidoreductase, EC 1.10.3.2) are oxidative enzymes of industrial importance, present in higher plants and fungi (Ascomycetes, Deuteromycetes, and Basidiomycetes); Laccases belong to group the blue multicopper oxidases (MCO) (PISCITELLI *et al.,* 2014).

Laccases in higher plants have the function of tissue healing, in addition to the biosynthesis and degradation of lignin; and in fungi it plays a role in physiological vegetative and reproductive processes, as well as in the breakdown of lignin (GOMES *et al*., 2014).

Laccases from fungi are monomeric glycoproteins with an acidic isoelectric point around pH 4 and between 60-70 kDa of molecular mass (GIARDINA; FARACO, 2010).

The relevant biotechnological applications of lacase are due to the capacity of its methylation and demethylation reactions, polymerization and depolymerization with other compounds in the different reactions it acts on (SOLANO *et al*., 2001; VILLALBA *et al*., 2010). In this way, laccase becomes an enzyme of interest in the different sectors of industry.

There are several applications of laccase in different areas of industrial interest, many of which have been patented, as shown in the following Table 13.

TABLE 13 - PATENTS ON TECHNOLOGICAL LACCASE APPLICATIONS.

Nº Patent	Title
CA2103260A1	A process for the delignification of material containing lignocellulose, bleaching and treatment of white water by means of laccases with extended efficiency
WO9745549A1	DNA sequences coding for laccases, and their applicationsin the field of plant lignin content control.
WO9915137A1	Enzymatic foam compositions for dyeing keratinous fibres
US5948121A	Laccases with improved dyeing properties
WO9923887A1	Antimicrobial activity of laccases
WO0015899A1	Methods for deinking and decolorizing printed paper
WO0002464A1	Use of a phenol oxidising enzyme in the treatment of tobacco
US6030933A	Detergent compositions comprising immobilized enzymes
US2001047852A1	Methods for deinking and decolorizing printed paper
PT102779A	Textile materials pretreatment-based whiteness enhancement consists of oxidative bleaching with peroxide after treatment with laccases
US2003205247A1	Use of solutions containing enzymes for cleaning fermentation for storage tanks
WO2004029193A1	Fermentation methods and compositions
US2006054290A1	Novel catalytic activities of oxidoreductasesfor oxidation and or bleaching
WO2006034811A2	Use of laccases as reporter genes
WO2007035481A1	Treatment of wood chips using enzymes
US2007261806A1	Treatment of pulp stocks using oxidative enzymes to reduce pitch deposition
US2008070284A1	Oxidative, reductive, hydrolytic and other enzymatic systems for oxidizing, reducing, coating, coupling or cross-linking natural and artificial fiber materials, plastic materials or other natural or artificial monomer to polymer materials
US6893470B1	Keratinous fibre oxidation dyeing composition containing a laccase and dyeing method using same
US2010018658A1	Laccases for Bio-Bleaching

Source: Modified from Villalba *et al.* (2010).

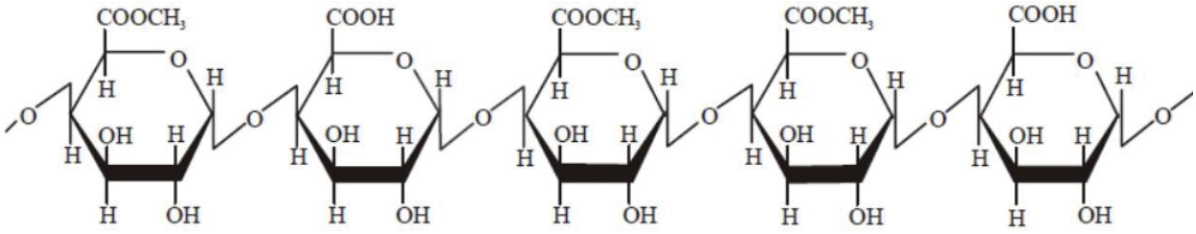
In the Table 14, the different technological applications of lacase are shown, as part of a fermentation mixture, the treatment of wood or pulp, as a gene reporter, for the use of oxidation and bleaching processes, among other uses (VILLALBA *et al.,* 2010).

3.6. PECTIN AND PECTINASE

3.6.1. Pectin

The pectins are carbohydrates belonging to the group of acid heteropolysaccharides, which have a main chain composed of D-galacturonic acid residues linked by ligaments of type α-1; and which, depending on their nature and method of extraction, these have ramifications of residues of rhamnose, arabinosis, galactose and xylose (FERREIRA FILHO, 2014).

FIGURE 10 - PECTIN A POLYMER OF A-GALACTURONIC ACID WITH A VARIABLE NUMBER OF METHYL ESTER GROUPS.



Source: http://std.com.pk/fulltext/?doi=std.2015.9.15 (2018).

Pectin is found in the cell walls of many vegetables, plays an important role during storage, ripening and processing of food; is extracted from vegetable sources (citrus peel, quince, apple, pear, peach, legumes among others) (SCHOLS; COENEN; VORAGEN, 2009); pectin is used by the food industry for having thickening, stabilizing and gelling properties (LAURENT; BOULENGUER, 2003).

Pectin presents potential functional properties which have a positive effect on human health, this is due to its chemical structure that confers the attributed functionality (YAMADA; KIYOHARA; MATSUMOTO, 2003; SCHOLS; COENEN; VORAGEN, 2009).

The American Chemical Society classified the pectic substances into 4 main groups, as presented below (ALKORTA *et al*., 1998; JAYANI *et al*. 2005):

- Protopectin is the water-insoluble pectic substance present in intact tissue. Protopectin by hydrolysis produces pectin or pectic acids.
- Pectic acid is the soluble polymer of galacturonans containing an insignificant amount of methoxyl groups. The normal or acidic salts of pectic acid are called pectates.
- Pectinic acids is the polygalacturonial chain containing> 0 and <75% of methyl galacturonate units. The normal or acidic salts of pectinic acid are known as pectinates.
- Pectin (Polymethyl galacturonate) is the polymer in which at least 75% of the carboxylic groups of the galacturonate units are esterified with methanol. It confers rigidity to the cell wall when it is bound to cellulose.

3.6.2. Pectinase

The pectinases are enzymes that act basically on the substrate pectin, playing an important role in the process of development of the plant (growth, maturation of the fruit and in the modification of its biomass) (VAN BUREN, 1991; FERREIRA FILHO, 2014). The pectinases

are produced by some plants (*Carica papaya, Lycopersicum esculentum, Prunus malus, Vitis vinífera*, etc.) and microorganisms (*Trichoderma, Bacillus, Fusarium, Clostridium, Penicillium, Pseudomonas, Aspergillus, Erwinia*, etc.), (JAYANI *et al.*, 2005; FERREIRA FILHO, 2014).

These pectic enzymes are classified according to the action they perform (hydrolysis or trans-elimination), or preference for the substrate (pectin, pectic acid, or Oligo-D-galacturonate), as well as acting in synergy with other enzymes (cellulases, hemicelluloses and ligninases) for the degradation of the cell wall of the plant (FERREIRA FILHO, 2014).

The pectinolytic enzymes can be divided into three groups, as detailed below (JAYANI *et al*., 2005):

- Protopectinases: degrade the insoluble protopectin and give rise to highly polymerized soluble pectin.
- Esterases: catalyze the de-esterification of pectin by the elimination of methoxy esters. Depolymerases: catalyze the hydrolytic cleavage of α- (1-4) -glucoside bonds in the D-galacturonic acid of pectic substances.

3.7. ANTAGONISM AND ANTIFUNGAL ACTIVITY

3.7.1. Antifungal activity

There are fungi that produce mycotoxins that are toxic chemical compounds, which are present in nature, as well as during the pre and post harvest of vegetables, cereals, grains, nuts, fruits and other foods for human and animal consumption (CENTENO; CARRERA, 2013). Under suitable conditions of mainly humidity and temperature, fungi produce toxic secondary metabolites that contaminate food; therefore, their ingestion, inhalation or cutaneous absorption may cause disease or death of animals and humans (FAO, 2004).

Numerous researches are being carried out to find natural biological substances that have the capacity to prevent the growth of some fungi or even to provoke their death to face this type of diseases (GARCÍA-HERNÁNDEZ *et al*., 2016), these substances are called antifungal or antimicrobial; for that, are evaluated the biological principle of extracts, oils of aromatic and medicinal plants (HERNÁNDEZ- DÍAZ; RODRÍGUEZ- JORGE, 2001).

Phenolic compounds found in plant tissues can inhibit fungal development and the production of mycotoxins (antifungal effect), they are located especially in external tissues and have a protective function of their structure (ALBAYRAK *et al*., 2010).

3.7.2. Microbial antagonism

The microbial antagonism is given by the inhibition, deterioration or death of some species of microorganisms by the action of another; or a relation between two populations in which one of them causes deleterious or negative effects to the other (ANDREWS *et al.*, 1983; ATLAS; BARTHA, 2002).

There are pathogenic microorganisms in crop areas that damage or spoil crops, causing enormous damage to farmers by the loss of their products; thus, researchers have developed biological products to deal with pathogenic microorganisms that cause damage to agriculture, taking advantage of the antagonistic relationships between microbial populations to control plant pathogens due to the antagonistic effect (PÉREZ-Y-TERRÓN *et al*., 2014).

3.8. SOLID-STATE FERMENTATION

The definition of solid-state fermentation is a microbiological bioprocess in substrates that do not contain free water, and that have the property of absorbing or containing water-soluble nutrients or not (PANDEY *et al*., 2000; VINIEGRA-GONZALES, 1997).

The types of fermentation to be used in the industry of the different bioactive compounds are the Solid-State Fermentation (SSF) and Liquid Fermentation or Submerged (SmF) (CHEN; XU, 2004). The comparison is shown in the following Table 14:

TABLE 14 - COMPARISON OF SOLID-STATE FERMENTATION (SSF) WITH SUBMERGED FERMENTATION (SMF).

There is no free water, and the water content of substrate is low	Water is the main component of the culture
Microorganisms absorb nutrients from the wet solid substrate; a nutrient concentration gradient exists	Microorganisms absorb nutrients from the liquid culture; there is no nutrient concentration Gradient
The culture systems consist of three phases (gas, liquid, solid), and gas is the continuous phase	The culture system mainly consists of liquid; the the liquid is the continuous phase
Inoculation size is large, more than 10%	Inoculation size is small, less than 10%
The required oxygen is from the gas phase; low energy consumption	The required oxygen is from dissolved oxygen; there is a larger amount of dissolved oxygen
Microorganisms adsorb on or penetrate into the solid substrate	The microorganisms uniformly distribute in the culture system
At the end of fermentation, the medium is a wet state substrate, and the concentrations of products are high	At the end of the fermentation, the medium is liquid, and the concentrations of products are Low
High production rate and high product yield	Low production rate and low product yield
Mixing is difficult, and the growth of microorganisms are restricted by nutrient diffusion	Mixing is easy, and the growth of microorganisms is not restricted by nutrient diffusion
Removal of metabolic heat is difficult	Temperature control is easy
Heterogeneity	Homogeneity
The fermentation process is hard to detect and control online	The fermentation process can be detected and controled online
Extraction process in simpe and controllable; little waste organic wáter	Extraction process is usually complex; there is large amount of waste organic water
Low water activity	High water activity
Simple fermentation container	Sealed fermentation container
Natural enrichment or artificial breeding strains	Pure strains
Energy consumption and equipment investment are high	Energy consumption and equipment investment are low
Low raw material cost	High raw material cost

Source: Chen and Xu (2004).

The SSF is being used more frequently for its operability in the process and for the generation of bioactive products within their main advantages is increasing oxygenation in the process, higher productivity and lower costs; being their major disadvantages the problema of major impurities in the product and the greater difficulty of control of parameters than in SmF (CHEN; XU, 2004).

In the following Table 15, examples of processes to obtain a given product, using fungi and yeasts, in a solid-state fermentation are shown.

TABLE 15 - EXAMPLES OF USE OF THE PROCESSES OF FERMENTATION IN SOLID STATE.

Process	Product	Micro-organisms
Fermented	Cheeses	*Penicillium*
foods:	Cocoa	*Acetobacter*
	Pozol	*Lactobacillus* spp
	Koji	*Aspergillus oryzae*
Production	Amylases	*Aspergillus*
of enzymes:	Proteases	*Aspergillus*
	Cellulases	*Trichoderma*
	Pectinases	*Aspergillus*
Secondary	Aromas	*Penicillum, Ceratocystis*
metabolites:	Penicillin	*Penicillum*
	Tetracycline	*Streptomyces*
	Aflatoxins	*Aspergillus flavus*
	Alkaloids	*Claviceps*
Organic	Citric acid	*Aspergillus niger*
acids:	Gallic acid	*Aspergillus niger*
	Gibberellic Acid	*Giberella ffujikuroi*
	Lactic acid	*Rhizopus*
Alcohol:	Ethanol	*Saccharomyces*
Production	Ethanol	*Schwanniomyces*
Production	Inoculum	*Penicillium*
of spores:	Biological control	*Trichoderma*
Composting:	Compost	*Mixed microflora*
Silage:	Silage	*Lactobacillus*
Higher fungi:	Pleurotus	*Pleurotus spp*
Biological	Sewage wáter	*Mixed microflora*
Filters:		
Utilization of	Food for livestock	*Mixed microflora*
by-products:		

Source: Modified from Roussos and Perraud-Gaime (1996).

As can be seen in Table 15, various kinds of organic products can be obtained from agro-industrial wastes fermented by fungi.

4. MATERIALS AND METHODS

4.1. MATERIALS

The Forastero variety of cocoa (*Theobroma cacao* L.) was used, origeneted from the states of Bahia and Pará, (north of Brazil). The whole fruit was previously washed, cut and the almonds of cocoa from the husk were separeted, the pod husk was cut into pieces and then placed on trays to be dried in the oven (Orion-Fanem, SP-Brazil) at 60 °C for 24 hours. Then, grinding was conducted in knife mill (Marconi Model MA 580/E, SP-Brazil) and the granulometry of the grinding was analyzed (0.044 to 2 mm of opening diameter), being used for the present investigation the particle size between 0.83 and 2 mm of diameter of the cocoa pod husk (CPH).

4.2. MICROORGANISMS

The microorganisms used were fungi of the genus *Pleurotus* from different species, which belong to different national and international institutions, as shown in this Table 16:

TABLE 16 - SPECIES OF *Pleurotus* USED

CODE OF THE STRAIN	*Pleurotus* SPECIES	LOCAL SOURCE
PL 9	*Pleurotus s*p.	IBSP
PL 20	*Pleurotus sajor-caju*	IBSP
PL 22	*Pleurotus sajor-caju*	IBSP
PL 22E	*Pleurotus sp. (P. ostreatus)*	EMBRAPA
PL 23	*Pleurotus sp.*	IBSP
PL24	*Pleurotus sajor-caju*	IBSP
PL 31	*Pleurotus ostreatus*	INRA/ORSTOM
PL 50	*Pleurotus sp.*	MÉXICO
PL 3501	*Pleurotus ostreatus*	INRA
PL 3824	*Pleurotus ostreatus*	INRA

IBSP: Instituto Botánico de Sao Paulo, Brazil
INRA: Institute National de Recherches Agronomiques, Jouyen-Fosas, France
ORSTOM: Centro de Bordeau, do Centro de Orstom de Montepellier-France
EMBRAPA: Empresa Brasileira de Pesquisa Agropecuaria, Brazil
Source: the author (2018).

4.3. PREPARATION OF THE INOCULUM AND FERMENTATION

The pure strains of *Pleurotus* were inoculated in Petri dishes with Potato Dextrose Agar (PDA) previously prepared (4.2 g of PDA in 100 mL of distilled H_2O, sterilized for 15 minutes at 121°C and then cooled), this was incubated for 7 days at 30°C. Then, with a sterile test tube of 1 cm in diameter a hole was drilled through the surface of PDA with mycelium of *Pleurotus* forming small circles, 3 circles of PDA were introduced in the solution of malt and yeast extract (2 g of malt extract and 2 g of yeast extract in 60 mL of distilled H_2O, sterilized for 15 minutes at 121°C and then cooled sterilized for 15 minutes at 121°C and then cooled), and incubated for 7 days at 30°C and 120 rpm in a shaker.

4.4. FERMENTATION OF COCOA POD HUSK (CPH)

In the development of the thesis, solid state fermentation and semi solid-state fermentation were carried out.

4.4.1. Solid - State Fermentation of CPH

For the Solid State Fermentation of the cocoa pod husk, in a Erlenmeyer of 250 mL, 5g of CPH (0.83 mm diameter) and 5 g of CPH (2 mm diameter) with adequate moisture (according to its absorption capacity) were added, are sterilized for 15 minutes at 121°C and then cooled; Then, the corresponding inoculum was added for each 0.5 g CPH/1mL of *Pleurotus* pellet solution and incubeted at 30°C for 30 days.

This fermentation process was carried out to analyze the degradation of the cocoa pod husk by several *Pleurotus* species. Regarding the enzymatic activity (lacase and pectinase), the fermentation time for *Pleurotus* species (first selection) was 20 days and then for PL22E strain (second selection) was 8 days, in triplicate.

4.4.2. Semi Solid-State Fermentation of CPH

For the Semi Solid State Fermentation of the CPH, a 1 litre solution of deionised water was prepared in an Erlenmeyer with 0.03g of Potassium Phosphate Monobasic Anhydrous (KH_2PO_4) and 0.03 g Magnesium Sulfate Heptahydrated ($MgSO_4.7H_2O$), and 100 ml of this solution was added to erlenmeyers with 5 g CPH and sterilized at 121 C for 15 minutes, then cooled. Then, the corresponding inoculum was added for each 0.5 CPH/1mL of *Pleurotus* pellet solution and taken to the shaker at 30°C and 120 rpm for 8 days, in triplicate.

4.5. THE OBTAINING OF EXTRACTS

4.5.1. Extract from the cocoa pod husk in ethanol

One g of CPH (moisture of 4,8 to 10%) was introduced in 10 mL of absolute ethanol in a dark bottle, was placed in an orbital Shaker (Tecnal, SP-Brasil) at 75 rpm and 25°C for 24 hours, in triplicate. The macerated was leaked in filter paper and was sstored in bottles with identification in the refrigerator for later analysis.

4.5.2. Extract from the cocoa pod husk and its fermented products by LPG

This Liquefied Petroleum Gas (LPG) extractor is a portable equipment. It has certain advantages with respect to the large extraction volume, the cost of the equipment, the easy handling, to the obtaining of some active biocompounds of interest and quality; also having disadvantages as the capacity of extraction, not all the biocompounds of interest are extracted due to their apolarity, but highlighting cost/benefit can be of great utility for the future investigations.

The portable extractor developed by Oliveira (2000) was used. I used 5 g of CPH (moisture of 4,8 to 10%) which was introduced in the portable extractor, it was assembled with its components used in the equpment and as a solvent was used liquefied petroleum gas (LPG) that contains 25% propane and 75 ± 5% of iso-butane n-butane, the pressure exerted on the LPG portable extractor was estimated at 544.93 ± 43.4 kPa (CUNICO *et al*., 2003; BIER *et al*. 2016).

At each cycle of removal 15 g of LPG were used (Volcano Isqueiros Ltda., São Paulo-Brasil) and transferred to the portable extractor, remaining with the sample for 20 minutes at 35°C in the oven (Orion-Fanem, São Paulo-Brasil). The number of cycles of removal by sample it depended on the efficiency determined (BIER *et al*., 2016); for the case of experimentation, was 7 cycles of extractions per sample, which represents 100%. The extract was collected in a vial of 10 mL with pressure plug and scourge (heavy with dry before), dislodge the gas in the bottle (the liquefied gas) and weighed again to know the difference between the weight of bio-compounds extracted and was subsequently performed the respective dilution with absolute ethanol, kept in the refrigerator for later analysis (all trials were made in triplicate).

Then, the analysis of the total polyphenols content between the extraction cycles was carried out together with the statistical analysis, also the accumulated percentage of the extraction cycles was counted. Therefore, to determine the number of cycles required for an optimal extraction yield, the highest cumulative percentage of the extraction cycle and the yield

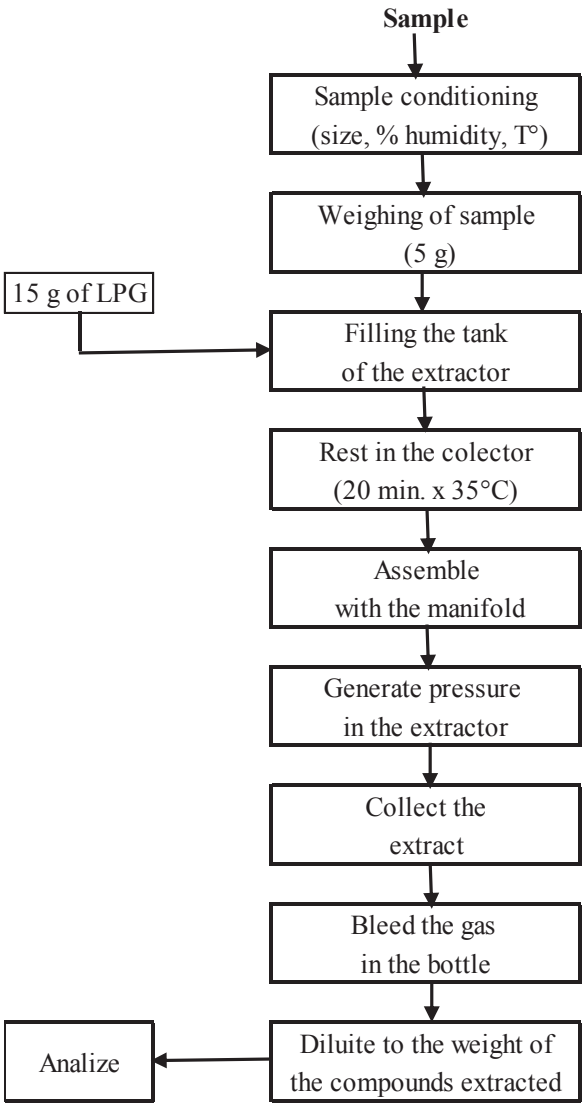
of the total polyphenol content were taken into account, which will then be used to obtain extracts of CPH and its fermented products with different *Pleurotus* strains, (Figure 11, Portable Extractor with LPG).

FIGURE 11 - PORTABLE EXTRACTOR WITH LPG

Source: the author (2018).

In the Figure 12, the process of the removal of the bio-compounds of CPH, using the portable extractor with LPG, is shown.

FIGURE 12 - FLOW DIAGRAM OF THE PROCESS OF EXTRACTION WITH THE PORTABLE EXTRACTOR.



Source: the author (2018).

4.6. COMPOSITIONAL ANALYSIS

a) The moisture content was determined by drying 1 g of samples (cocoa pod husk and its fermented products) is an oven at 80°C for 12 hours, by differences in mass was calculated the percentage of moisture, this was done in triplicate.

b) Absorption capacity, it is the amount of water that can absorb 10 grams of cocoa pod husk (CPH), for this was added in a test tube 10 grams of dried CPH and added 100 mL of water, was left to stand 24 hours. The solution is then filtered and the water absorbed by the sample is calculated per volume.

c)For the determination of the total ashes of the CPH, the crucibles (clean and dry) were weighed, 2.5 g of sample were then added to the crucible, and introduced to the muffle (Q31M, Diadema, Brazil) at 550ºC for the time necessary to get some ashes white or slightly gray, homogeneous. It was cooled in a desiccator and weighed in triplicate, and the calculation was performed to find the % ash.

d)The pH of the cocoa pod husk and its fermented products was determined by using the potentiometer (Ms Tecnopon, São Paulo-Brazil). A gram of sample was weighed and was added ten milliliters of water deionized in the Becker, stirred for 10 minutes and proceeded to measure the pH (INSTITUTO ADOLFO LUTZ, 2008).

e) The content of total reducing sugars of the cocoa pod husk and its fermented products was determined by the method of dinitrosalicylic acid (DNS) of Miller (1959).

The standard curve was prepared, from the concentrations of glucose 0.01, 0.03, 0.06, 0.09 and 0.13 g/L until completing 9 mL with distilled water, then was added 1 mL of reagent DNS mixed and boiled in a water bath for 5 minutes, cooled and the absorbance was measured in the spectrophotometer (S22PC Visible, China) at 540 nm in triplicate. For the analysis of the sample 1 mL was used and 8 mL of distilled water were added, then 1 mL of reagent DNS was added, mixed and boiled in a water bath for 5 minutes cooled and then read the absorbance at 540 nm.

f) The total sugars of the cocoa pod husk and its fermented products were quantified using the method of the anthrone (DREYWOOD, 1946), used to determine the hexoses, aldopentoses, uronic acids whether they are free or being part of the polysaccharides. The carbohydrates in the presence of sulfuric acid and of the reagent antrhone, become dehydrated to form furfur aldehyde producing green reaction products. The protocol of Southgate (1991) was followed that has been modified for the determination of total sugars.

The standard curve was prepared, from the concentrations of glucose 0, 100, 200, 300, 400, 500 and 600 µg/mL up to complete 200 µL with distilled water, then 1800 µL of the reagent anthrone were added, mixed and boiled in a water bath for 3 minutes, cooled and the absorbance was measured in the spectrophotometer (S22PC Visible, Ningbo - China) at 620 nm in triplicate. To 200 µL sample, 1800 µL of the reagent anthrone was added, then homogenized the mixture and boiled in a water bath (100 ° C) for 3 minutes and measured the absorbance at 620 nm in triplicate.

g) For analysis of sugars alcohols and acids from CPH and fermented products by HPLC, first we obtained the extract of sugars, soluble CPH and fermented products (INSTITUTO ADOLFO LUTZ, 2008), one gram of sample was placed in 50 ml of distilled water in a becker and was heated for 10 minutes at 100°C, then cooled and completed with distilled up to 100 mL, then filtered to be used for HPLC. Alcohols and acids were obtained by the maceration method and by the portable extractor, referred to previously.

The extract of CPH and fermented products were microfiltered with 0.22 µm hydrophilic membrane (PVDF) and placed in vials of 1.5 mL to be analyzed by HPLC.

The HPLC system Agilent 1260 Infinity Quaternary LC (Santa Clara, United States), it is coupled with Diode Array Detector Dad, which consists in the mobile phase of H_2SO_4 0.005 M and the fixed column Hiplex H (300 x 7.7 mm), the flow in the column was 0.6 mL/minutes.

The column was maintained at 60°C and equilibrated by pumping the mobile phase through the column for at least 30 minutes, the sample injection was 10 µL.

h) ***The proteins*** of the cocoa pod husk and its fermented products were quantified using the methodology of Bradford, which has been amended for the determination of soluble proteins (BRADFORD, 1976). The method is based on the union of the proteins with the dye, Comassie Blue G-250. The proteins bind to the blue form to form a protein-dye complex with an extinction coefficient greater than the free dye, and can be measured at 595 nm.

The calibration curve of bovine albumin was prepared in a range from 0, 100, 200, 300, 400, 500 and 600 µg/mL, distilled water was added until the final volume in each tube was 200 µL; then add1800 µl of Bradford reagent buffer was added, mixed and homogenized the solution in each tube, and absorbance was measured at 595 nm in triplicate in the spectrophotometer (S22PC Visible, Ningbo - China). For the samples was used of the cocoa pod husk and its fermented products, 200 µL of the solution were mixed with 1800 µL of Bradford reagent buffer, then homogenized the mixture and measured the absorbance at 595 nm, in triplicate.

i) ***The total lipids*** were determined by the method of Bligh and Dyer (1959). 0.5 g of dry sample (CPH) were weighed and 3.2 ml of 4M HCL was added in Falcon tube, homogenized and placed in the oven at 70 °C for one hour. Then, 8 mL of absolute methanol and 4 mL of chloroform were added, and tirred in the Shaker Orbital (Tecnal, SP-Brazil) at 25 °C, 100 rpm for one hour. After, 3ml of sodium sulfate were added to 1.5% (w / v), forming 3 phases, the lower phase is chloroform with oil, the next fraction is biomass, in the upper phase are the polar molecules such as methanol and water. Then, centrifuged (Fanem Mod 206 BL, SP - Brazil) at 2000 rpm

(448 G) for 5 minutes and with a syringe the lower phase where the chloroform is found was extracted with the oil and placed in penicillin bottles (previously dried and weighed), and they were placed in an oven at 40 ° C for 24 hours to evaporate the chloroform, leaving only the lipids, which were weighed to determine their percentage.

j) ***Determination of fatty acids of the CPH of Pará and Bahía by GC-MS***. One mg of lipid fraction of the sample obtained in the extraction of total lipids (according to Bligh and Dyer, 1959) was placed in the vial with 1mg of heptane; then it was eluted with helium as the carrier gas in the VF-5MS chromatography column at 0.8 mL min^{-1} and at 2 mm Torr pressure. The injected volume was 2 μL and the ratio was 1:50. The temperatures of the injector, the collector, the transfer line and the ion source were set at 315, 40, 280 and 300 °C, respectively. The column thermostat was adjusted to the following temperature program:

For an isothermal process at 100 ° C for one minute, 10 °C/min was heated to 200 °C; Isothermal at 200 °C for 2 minutes, heating at 3.5 °C min^{-1} up to 260 °C until reaching a total operating time at 37 °C. The mass spectrum was analyzed every 0.5s in the mass range of 32 to 380 m/z. The FAME components were identified by comparing retention times with analytical standards or by interpreting their mass spectra, whose similarity index was investigated using the NIST spectrum (National Institute of Standards and Technology).

4.7. DETERMINATION OF METAL CONTENT BY OPTICAL EMISSION SPECTROMETRY IN INDUCTIVELY COUPLED PLASMA-ICP-OES

4.7.1. Cleaning of glassworks and preparation of reference solutions

All glassworks used in the preparation of the solutions and the erlenmeyer flask used in the digestion were previously decontaminated in a bath with a solution of acid 10% HNO_3 (m/v), at least 24 and then washed with deionized water before use. The glass used in the preparation of the solutions and the Erlenmeyer flask used in the digestion were previously decontaminated in a bathroom with a solution of acid 10% HNO_3 (m/v), at least 24 and then washed with deionized water before use.

For the preparation of the reference solutions, deionized water was used with the resistivity of 18.2 MΩ cm^{-1}, of a system of purification of water from a Milli-Q, (Millipore, Bedford, MA, USA), coupled to a glass of water distiller model Fisatom, Brazil (534).

4.7.2. Removal of minerals with HNO_3/H_2O_2

The mass of samples of 1 g of material was transferred to a 250 mL volumetric flask, followed by an addition of 5.0 mL of concentrated nitric acid, analytical grade and 4.00 mL of hydrogen peroxide (30%). After starting the boiling the system was left under heating for 40 min. The flask was then removed from the heater, the contents allowed to cool to room temperature, and filtered on filter paper, the volume was made up to 100 mL in a pre-calibrated volumetric flask. The same procedure was carried out for all samples. The three samples of the control (white) were prepared in the same way.

4.7.3 The quantification of the contents of micro and macronutrients by optical emission spectrometry in inductively coupled plasma (ICP-OES)

For the determination of the content of the minerals, we used an ICP-OES, model 720 simultaneously under axial and solid-state detector. The torch is aligned horizontally and vertically with a standard solution of Mn concentration of 5.0 mg L^{-1}. The optical system of the ICP OES was calibrated with multielementar stock solution of patterns tracked. The spectral lines were selected considering the absence of spectral interference and adequate sensitivity for the determination of elements in low and high concentrations. The analysis was carried out in the facilities and equipment belonging to the Laboratory for Analysis of Automotive Fuels - LACAUT / UFPR. The experimental conditions used in the experiments and the spectral lines are presented in Table 17.

TABLE 17 - OPERATIONAL CONDITIONS OF ICP-OES AND RESPECTIVE SPECTRAL AIR LINES

Parameters	**Conventional values**
Power	1.10 Kw
Plasma Gas Flow	15.0 $L.min^{-1}$
Auxiliary Gas Flow	1.50 $L.min^{-1}$
Nebulizer Pressure	180 kPa
Replication Read Time (integration)	3 s
Stabilization Time	15 s
Sample Delay	30 s
Pump Speed	15 rpm
Sample Wash Time	20 s
Number of Replicas	3
Selected Spectral Lines	Nm
	Al (396.152) B (249.772) Ba (455.403) Ca (396.847) Cd (226.502) Co (230.786) Cr (267.716) Cu (327.395) Fe (238.204) K (766.491) Li (670.783) Mg (280.270) Mn (257.610) Mo (202.032) Na (589.592) Ni (230.299) P (213.616) Se (196.026) V (292.401) Zn (213.857)

Source: Modified of Oliveira (2018).

4.8. ANTIOXIDANT ACTIVITY WITH DPPH

The antioxidant activity was determined with 2,2-Dipheniyl-1-picrylhydrazyl (DPPH) of the cocoa pod husk and its fermented products. The effect of antioxidant was based on the transfer of electrons by the action of an antioxidant or radical. The DPPH has rich purple color and is reduced forming diphenyl-picril-hydrazine of yellow color, having a bleaching by the decrease of the absorbance by the reaction (BRAND-WILLIAMS *et al*. 1995).

DPPH reagent, 0.004 g was dissolved in 100mL of absolute ethanol in a volumetric ball protected from light, mixed and poured into a dark amber bottle.

The preparation of the calibration curve, from a solution of ascorbic acid 1mg/L, was made with dilutions in concentrations of 50, 30, 15, 10, 8, 4, 2 and 1 μg/mL with absolute ethanol, with 3 repetitions. Was added 2 ml of the solution of DPPH to 1 mL of each solution of ascorbic acid with ethanol prepared earlier, which was left to stand in a dark environment for 30 minutes and the absorbance was read on the spectrophotometer (S22PC Visible, China) at 517 nm. (MENSOR *et al*., 2001). The percentage of antioxidant activity (%A.A.) is found with the formula presented below, and the concentrations of ascorbic acid with %A. A. were plotted in a graph.

$$\%A.A. = 100 - \frac{(Abssample - Absblank)x100}{Abscontrol\_}$$

Abssample: 1 mL sample plus 2 mL of DPPH, Absblank: 3 mL of absolute ethanol and Abscontrol (-): one mL of absolute ethanol plus 2 mL of DPPH. For the sample was used 1000 μL of the sample solution (diluted in absolute ethanol) and was then added 2 mL of reagent DPPH (with little light), then homogenized the mixture and after 30 minutes was measured the absorbance at 517 nm, in triplicate. The result obtained was inserted in the equation of the line of the standard curve pattern to determine concentrations that were multiplied by their corresponding dilutions; the concentration is expressed in antioxidant capacity equivalent to the Vitamin C (VCEAC), which is a concept of antioxidant capacity based on the natural antioxidant (vitamin C), expressed in mg of ascorbic acid/100 g of sample (KIM *et al*., 2002); it is also the percentage of antioxidant activity of each sample with the formula mentioned above.

4.9. THE TOTAL POLYPHENOLS CONTENT

The total polyphenols content of the cocoa pod husk and its fermented products was determined by the Folin Cioculteu reagent by the methodology of Singleton and Rossi (1965), which has been modified; the total phenolic compounds react with the Folin Cioculteu reagent resulting in color susceptible to be determined with the spectrophotometer. The preparation of the calibration curve was prepared with gallic acid dissolved in absolute ethanol at concentrations of 1, 5, 12.5, 25, 50, 100 and 150 mg/L; then, added 0.5 mL of Folin Cioculteu with the concentrations of gallic acid in triplicate, after 5 minutes add 2.5 mL of Folin and at 60 minutes 2 mL of Na_2CO_3 were added in low light environment and absorbance was read on the spectrophotometer (SP-2000 Visible, Ningbo 315040 - China) to 760 nm. For samples 500 µL of diluted extract was added 2.5 ml of solution Folin Cioculteu (10% v/v), after 5 minutes 2 mL Na_2CO_3 (7.5% w/v) were added and was stored in dark environment for 60 minutes. Then the readings were prepared at 760 nm in triplicate, the result was expressed in mg of gallic acid equivalent / 100 g.

4.10. METHODS TO DETERMINE ANTIOXIDANTS, PHENOLIC COMPOUNDS AND ALKALOIDS BY HPLC

4.10.1. Methods to determine antioxidants and alkaloids

The HPLC system Agilent Technologies 1200 Series Systems (United States) was used, it is coupled with Diode Array Detector Dad; the column used was the Zorhax Eclipse XDB-C18 (4.6 x 150 mm, 5 µm), the flow in the column was 0.7 mL/minutes. The mobile phase consists in acetic acid 2.5% (v/v) and pure methanol according to the following Table 18:

TABLE 18 - MOBILE PHASE MIXING SYSTEM IN HPLC FOR DETERMINE ANTIOXIDANTS AND ALKALOIDS.

Time (minutes)	Acetic acid (Concentration, % v/v)	Methanol
0	95	5
18	95	5
25	75	25
27	5	95
32	95	5

Source: the author (2018).

The column was maintained at ambient temperature and equilibrated by pumping the mobile phase through the column for at least 36 min, the sample injection volume was 10 µL. The absorbance of the injected patterns was measured at the wavelength according to the following: Quercitin (370 nm), Galic acid (275 nm), Rutin (275 nm), Trans-Cinnamic acid (275 nm), p-Coumaric acid (311 nm), Daizein (260 nm), Genistein (260 nm), Genistein (325), Theobromine (275 nm) y Theophylline (275 nm).

The extracts were microfiltered with 0.22 µm hydrophilic membrane (PVDF) and placed in vials to be analyzed by HPLC.

4.10.2. Methods to determine antioxidants and phenolic compounds by HPLC

The HPLC system Agilent Technologies 1260 Infinity (United States) was used, it is coupled with Diode Array Detector Dad; the column used was the Zorhax Eclipse XDB-C18 (4.6 x 150 mm, 5 µm), the flow in the column was 1 mL/minute.

The extracts were microfiltered with 0.22 µm hydrophilic membrane (PVDF) and placed in vials to be analyzed by HPLC.

4.10.2.1. Methods to determine antioxidants

The mobile phase consists in acetic acid 1% (v/v) and pure methanol according to the following Table 19:

TABLE 19 - MOBILE PHASE MIXING SYSTEM IN HPLC FOR DETERMINE ANTIOXIDANTS.

Time (minutes)	Acetic acid (Concentration, % v/v)	Methanol
0	90	10
10	80	20
20	70	30
30	65	35
35	50	50
50	40	60
60	20	80
80	0	100

Source: the author (2018).

The column was kept at 30 °C and balanced by pumping the mobile phase through the column for at least 80 minutes, the sample injection was 10 µL. The absorbance of the injected patterns was measured at the wavelength 280 nm.

4.10.2.2. Methods to determine phenolics compounds

The mobile phase consists in the use of acetic acid 2.5% (v/v) and pure acetonitrile according to the following Table 20:

TABLE 20 - MOBILE PHASE MIXING SYSTEM IN HPLC FOR DETERMINE PHENOLICS COMPOUNDS.

Time (minutes)	Acetic acid (Concentration, % v/v)	Acetonitrile
0	95	5
18	95	5
25	75	25
27	5	95
32	95	5

Source: the author (2018).

The column was maintained at 30 °C and equilibrated by pumping the mobile phase through the column for at least 32 min, the sample injection was 10 µL. The absorbance of the injected patterns was measured at the wavelength 280 nm.

4.11. MORPHOLOGY BY SCANNING ELECTRONIC MICROSCOPY (SEM)

The morphology of cocoa pod husk (Bahía and Pará) and fermented products, 1 g of samples (with PL9, PL20, PL22, PL22E, PL23, PL24, PL31, PL50, PL3502 and PL3824) was dried in an oven at 80°C for 12 hours, was examined by scanning electronic microscopy (SEM) Teskan mod Vega 3LMO (Oxford Instruments), operating at 15 keV. Before examination, the samples were sputter-coated with a thin layer of gold in a vacuum chamber. 1 g of samples (cocoa pod husk and its fermented products) was dried in an oven at 80°C for 12 hours

4.12. FUNCTIONAL GROUPS BY FOURIER-TRANSFORM INFRARED SPECTROSCOPY (FTIR)

The samples of Pará cocoa pod husk and fermented products (with different species of *Pleurotus*) were characterized by FTIR spectroscopy in a VERTEX 70 equipment (Bruker), with the accessory DRIFTS (diffuse reflectance) with 64 scans, 4 cm^{-1} resolution, without the elimination of atmospheric compensation. The samples were previously dried at temperature 100°C for 24 hours, then were ground and mixed to homogeneity in spectroscopic KBr and placed in DRIFTS accessories for the acquisition of the spectra.

4.13. DEGREE OF CRISTALINITY BY X RAY DIFFRACTION (XRD)

Wide-angle X-ray scattering patterns of the samples Pará cocoa pod husk and fermented products (with different species of *Pleurotus*), were obtained in the reflection mode with a SHIMADZU XRD 700 MAXIMA diffractometer and Ni-filtered copper radiation (CuKα, λ = 1.5418 Å). The samples were scanned in the 2° range of 10–40 °, and the generator was operated at 40 kV and 20 mA.

The Cristallinity Index (CrI) was determined by means of the relationship given by equation (TORRES JARAMILLO *et al*., 2017):

$$Cristallinity\ Index = \frac{(Ik - Ia)x100}{Ik}$$

Where:

Ik: Peak intensity at maximum of 2θ between 22º and 23º for cellulose type I and between 20-22º for cellulose type II.
Ia: Peak intensity at minimum 2θ between 15º to 17º for cellulose type I between 12 and13º for cellulose type II.

Consideration was given to the peaks characteristic maximums of cellulose using the diffraction intensities of the structure (flat 2θ = 22.7º) and of the amorphous fraction (plan 2θ = 17,0º) (BORYSIAK; DOCZEKALSKA, 2005; TORRES JARAMILLO *et al*., 2017).

4.14. EXTRACTION OF PECTIN

The Pará CPH was hydrolyzed in an aqueous solution of citric acid (pH = 2.9), in a ratio of 1 g of sample/25 mL of solution, the mixture was heated in a water bath at 97°C for 30 minutes; then, it was centrifuged at 4000 rpm (1792 G) for 30 minutes, filtered with synthetic fabric (TNT) and the supernatant was mixed with absolute ethanol in a ratio of 2 mL of supernatant/1 mL of ethanol. It was refrigerated at 4 °C for 16 hours, the precipitate was filtered and washed 3 times with absolute ethanol to remove impurities and then taken to the oven under vacuum at 60 °C for 24 hours (VRIESMANN *et al.,* 2012; LUO *et al*., 2018).

The yield of pectin was determined using the following formula (COLODEL *et al*., 2018; LUO *et al*., 2018):

$$Yield\ (\%) = \frac{\text{Mass of dried pectin (g)}}{\text{Mass of dried CPH used for extraction (g)}} x100$$

4.15. DETERMINATION OF LIGNIN (ACID-INSOLUBLE LIGNIN METHOD)

In a 50 mL beaker, 1 g of dry sample that is free of extractables was weighed (Sluiter et al., 2008). Then 15 mL of H_2SO_4 (72%, 13°C) was added and shaken vigorously for 1 minute. Then, it was left to rest for 2 h, with frequent agitation at ambient temperature. The resulting mixture was transferred to a 1 L Erlenmeyer flask, and the acid concentration was diluted to 3% (adding 560 mL of distilled water). The flask was boiled for 4 h with a condenser of Reflux. After the indicated time, the mixture in the Erlenmeyer was cooled until the insoluble material was decanted. It was then vacuum filtered using filter paper No. 1, which was previously dried at 100° C. The precipitate was washed with 500 ml of hot water until it was free of acid. The filter paper and its contents were dried in a stove for 4 h at 100 ° C. Next, the filter paper was removed from the oven and allowed to cool in a desiccator. After reaching a constant weight, the filter paper was weighed and the lignin content determined (ASTM D1106-96, 2013; TORRES *et al*., 2015), according to equation:

$$Lignin\ (\%) = \frac{(\mathrm{A} - \mathrm{B})\mathrm{x}100}{\mathrm{C}}$$

A: Filter paper with the weight of the dried residue on the stove (g)

B: Weight of the filter paper dried in the stove (g)

C: Sample weight free of dried extracts on the stove (g)

4.16. ENZYMATIC ACTIVITY

4.16.1. Laccase Enzyme Activity

The Solid-State Fermentated CPH with the *Pleurotus* fungus was previously mixed and 1g (wet basis) was extracted which was placed in a conical tube and completed with the phosphate buffer ($NaH_2PO_4.H_20$, 0.2 M, pH 6.9) in the ratio of 1:10 (w/w), (KARP *et al*., 2015; IANDOLO *et al*., 2011). It was then homogenized in the vortex for one minute and taken to the centrifuge at 4000 rpm (1792 G) for 1 hour. Test tubes were prepared with 100 µL of 2 mM ABTS (2,2'-azino-bis-3 ethylbenzthiazoline-6-sulphonic acid, in solution with 20 mM sodium citrate, pH= 3) with 800 µL of sodium buffer citrate (20 mM, pH 3) and 100 µL of the sample supernatant (GIL *et al*., 2012; KARP *et al.,* 2015).

The activity of the lacase enzyme was measured at 420 nm for one minute (Ɛ=36000 $M^{-1}cm^{-1}$) as the absorbance of oxidized ABTS in the spectrophotometer. A unit of enzymatic activity of Lacasse (U), is defined as the amount of enzyme required to oxidize 1 μmol of ABTS in one minute (CHAN CUPUL *et al.,* 2016) and was expressed in (U/g).

4.16.2. Pectinase enzyme Activity (Polygalacturonase)

The Solid-State Fermentated CPH with *Pleurotus* was previously homogenized and 2g were extracted which were placed in a conical tube and completed with 12 mL of the buffer citrate (0.1 M, pH 4.1), (ROSSI, 2011); then it was homogenized in vortex for one minute and taken to the centrifuge at 4000 rpm (1792 G) for 15 minutes. Then 0.9 mL of pectin solution (0.5% solution with 0.1 M citrate buffer and pH=3) were placed in the test tubes and 0.1 mL of enzyme extract was added; the solutions were incubated for 15 minutes at 50°C, then 1 mL of DNS was added and boiled in the water bath for 5 minutes; then, 5 ml of distilled water were cooled and added to each test tube with the sample and measured in the spectrophotometer at 540 nm (HANDA *et al.,* 2016).

The enzymatic activity of polygalacturonase is measured by the release of reducing sugars by the DNS method. Galacturonic acid is obtained by degradation of pectin. A unit of polygaracturonase is defined as the amount of enzyme released by one mole of D-galacturonic acid (ROSSI, 2011; HANDA *et al*., 2016).

4.17. SELECTION

During the experimental investigation, two selections were made of the strains of *Pleurotus* to ferment the cocoa pod husk of Pará, according to the following:

• Selection 1: The first selection was made from the ten initial strains, in order to work with the strains that presented the best results with the fermented product; the results were considered according to the following criteria in the analyzes: remaining reducing sugar, the degradation of antioxidant activity (VCEAC), the degradation of total polyphenols and morphological growth, compared with the cocoa pod husk of Pará.

• Selection 2: With the strains of the first selection, the second selection was made, according to the best results obtained from the following analyzes: reducing sugars, total polyphenols, enzymatic laccase activities and pectinase; the best strain was selected that was later used for the application.

4.18. ANTIFUNGAL ACTIVITY

4.18.1. Spore suspensions

1.63 g of Sabouraud dextrose agar were weighd and placed in 125 mL Erlenmeyer flasks (3 were used), then 25 mL of deionized water was added and autoclaved (125 °C for 15 minutes). It was cooled and then 0.5 mL of spore suspension (106 spores/mL) of *Aspergillus niger*, *Aspergillus carbonarius* and *Aspergillus westerdijkiae* were inoculated into each Erlenmeyer, which were taken to the stove for incubation at 28± 2°C for 5 days. Then, 30 mL of saline solution was added to each Erlenmeyer with a drop of tween 20 with a magnetic bar, previously autoclaved, and magnetic agitation was performed until the spores were removed. The suspension of spores was placed in a sterile conical, previously identified, and stored at 4 °C for analysis (CILERDZIC *et al*., 2015; CENTENO; CARRERA, 2005).

4.18.2. Extraction and Antifungal activity (Disk difusión method)

The antifungal extracts were obtained from the fermentation in solid state (SSF) and the fermentation in semi solid state (SSSF), after 0, 4 and 8, days of fermentation, in the following way:

- Extract of SSF, 1 g (wet base) of CPH fermented with PL22E was weighed and placed in a test tube (21.5 mm in diameter) and 10 ml of absolute ethanol were added, then the mixture was maintained in shaker at 25 °C at 75 rpm for 24 hours. It was then filtered with Whatman N° 1 paper and stored in a dark vial.

- Extract of SSSF, 10 mL were extracted from the semi-solid fermented CPH, filtered with Whatman N° 1 paper and stored in a dark vial.

On sterile petri dishes (90 mm diameter), 100 µL of the spore suspension was added and spread uniformly with a Digrlaski handle on the surface of the plate, then the warm PDA medium (no more than 40 °C) was added and allowed to cool. Then, the 6 mm diameter Wathman filter paper discs N°1 were impregnated with 10 µL of the extract and placed in the middle of the Petri dishes with PDA inoculated with the spore suspension, in triplicates (DE SOUZA *et al*., 2005; CENTENO; CARRERA, 2005). A control treatment was performed with plates containing only the suspension of spores without the extract. The plates were incubated for ten days at 28 ± 2°C. Inhibition halos were measured using a graduated ruler and expressed

in millimeters (CENTENO; CARRERA, 2005; RASOOLI *et al.*, 2008), the mycelial inhibition (%) was calculated using the following equation (modified of RASOOLI *et al.,* 2008):

$$Inhibition\ (\%) = \frac{T}{C} x100$$

Where:

T: is the colony diameter of mycelium from a Control petri plate (mm), control with absolute ethanol o control with SSSF medium

C: is the colony diameter of mycelium from a Test petri plate (mm)

4.19. ANTAGONISTIC ACTIVITY

Two fungi, *Pleurotus* sp. and *Moniliophthora perniciosa* were used for the antagonistic activity, this procedure was followed:

a) Preparation of PDA-CPH agar

First, the 2.5% (w/v) cocoa pod husk solution was prepared with deionized water, boiled for 10 minutes, then cooled and then filtered with TNT fabric, obtaining the CPH solution.

In a second step, 4 g of Potatos Dextrose Agar (PDA) were placed in an Erlenmeyer with 100 ml of CPH solution, homogenized in a microwave oven and taken to the autoclave to sterilize at 121 °C for 15 minutes.

b) Preparation of Petri control plate inocula

On sterilized Petri dishes (90 mm), a layer of PDA-CPH agar was placed to sufficiently cover the entire surface of the plate, and then left to solidify; a 1 cm circle with *Pleurotus sp* mycelium was placed in the center of the petri dish, then taken to the stove at 28 ± 2 °C for 8 days. With *Moniliophthora perniciosa* the same process was performed; the difference was that it was incubated for 18 days.

c) Preparation of inocula of *Pleurotus spp*. and *Moniliophthora perniciosa* in the same Petri dish with PDA-CPH agar

The preparation of inocula of the fungi is similar to the previous point b), it differs that in each opposite end of the Petri Dish with PDA-CPH agar, 2 circles with mycelium of *Pleurotus spp* and *Moniliophthora perniciosa* were placed, and then was incubated in the stove at 28 ± 2 °C. Here there are two situations, first the confrontation of *Pleurotus* spp. and *Moniliophthora pernicious* to determine the common zone (measured with a

ruler) and the advance of a fungus on the other fungus, for this case, the incubation time was 22 days. For the situation of the confrontation between fungi, when they begin to produce metabolites for protection and defense, their fermentation time was 31 days.

In the second part of the experimentation, the study was carried out using the CPH and the inoculums of the fungi, to carry out the SSF.

- **Preparation of SSF control**

  2.5 g (0.83mm particle size) and 2.5 g (2 mm particle size) of CPH were weighed and placed in an Erlenmeyer (250 mL capacity); then moisture conditioning (according to the absorption capacity) was performed, the Erlenmeyer was autoclaved and sterilized at 121 °C for 15 minutes. The Erlenmeyer was left to cool with the sample and two circles (1 cm in diameter) with mycelium of *Pleurotus* spp. were introduced the flasks were incubated at 28 ± 2 °C for 18 days for fermentation; similarly, it was done for *Moniliophthora perniciosa*.

- **SSF for the confrontation of *Pleurotus sp.* and *Moniliophthora perniciosa***

  The preparation for the confrontation of both fungi is similar to the previous step of SSF Control, only it differs that the 2 circles (mycelium of each fungus) were placed on the surface of the same CPH and incubated at to 28 ± 2 °C for 18 days.

- **Fermentation in liquid medium by fungi**

  In Erlenmeyers flasks (125 mL capacity), 2 g of malt extract and 2 g of yeast extract were placed with 60 mL of deionized water; then autoclaved at 121 °C for 15 minutes, allowed to cool and 3 circles (1 cm in diameter) were introduced with mycelium of *Pleurotus* sp. The flasks were incubated in the shaker at 28 °C for 120 rpm for 22 days. The same procedure was performed for *Moniliophthora perniciosa*.

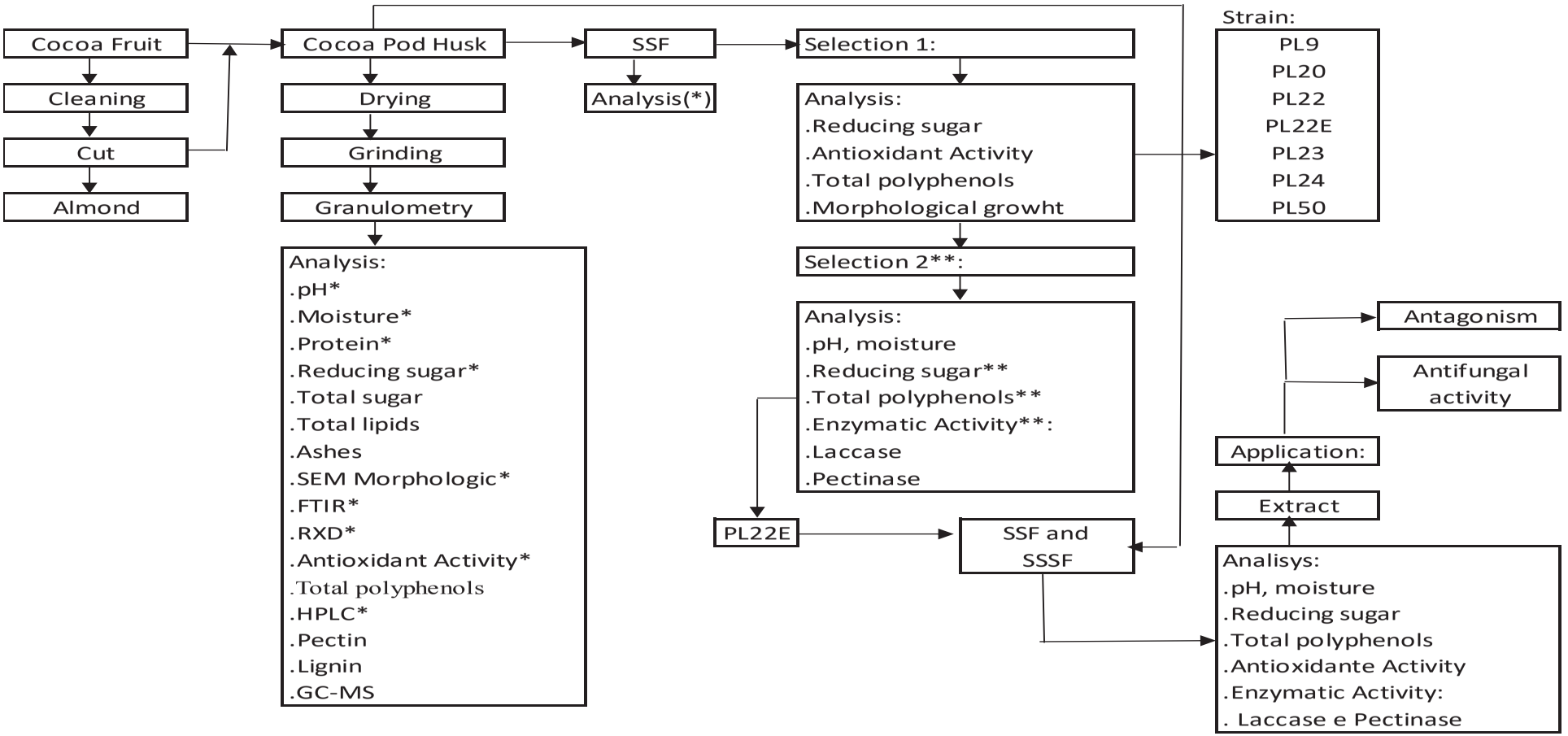
4.20. THE STATISTICAL ANALYSIS OF THE DATA

The data are expressed as mean ± standard deviation of repetitions of the treatments that were made for the analysis. The mean comparisons between the groups were assessed using analysis of variance (ANOVA, $p \leq 0.05$) and for the multiple comparisons between treatments the Tukey test was used with a confidence level of 95%. The data were analyzed with the software Statistica 8 (Stat Soft, Inc., Oklahoma State, USA).

4.21. METHODOLOGY

The experimental research was followed as detailed in the Figure 13:

FIGURE 13 - EXPERIMENTAL METHODOLOGY.



(*) Analysis: pH, Moisture, protein, reducing sugar, total lipids, ashes, SEM morphologic, FTIR, DRX, Antioxidant activity, total polyphenols, HPLC, pectin, Lignin, GC-MS.

(**) Analysis: Reducing sugar, total polyphenols, antioxidant activity, enzymatic (lacasse, pectinase).

SEM morphologic (Morphology by scanning electronic microscopy), FTIR (Fourier-Transform Infrared spectroscopy), (XRD) Degree of cristalinity by x ray diffraction , HPLC (high performance liquid chromatography), GS - MS (Gas chromatography–mass spectrometry), Solid State Fermentation (SSF), Semi Solid State Fermentation (SSSF).

Source: the author (2018).

5. RESULTS AND DISCUSSION

5.1. CHARACTERIZATION OF THE COCOA POD HUSK FROM BAHIA AND PARÁ

The cocoa pod husk from Bahia and Pará were characterized and compared (macronutrients, micronutrients, polyphenols, antioxidant capacity, etc.); The chemical composition of the cocoa pod husk is variable and depends on several factors such as: the geographical region of origin, genetic characteristics, the soil and climate where they develop, their maturity, conditions of growth and harvest, and subsequent storage (ORACZ *et al*., 2015), that influence the composition of the CPH.

5.1.1. Compositional analysis

The factors that can vary the composition of the CPH: variety of the type of cocoa, nutrition of soil, climate and geographical factors and the post-harvest, among others; mainly. In Table 21, the results of the compositional analisys of CPH from Bahia and Pará are shown.

TABLE 21- COMPOSITIONAL ANALYSIS OF COCOA POD HUSK BAHIA AND PARÁ.

Property	***Bahía CPH***	***Pará CPH***
% Moisture	$4.802^{a} \pm 0.154$	$9.89^{b} \pm 0.849$
Ph	$5.468^{a} \pm 0.120$	$5.97^{a} \pm 0.545$
Reducing sugars (g/100 g dry matter)	$13.25^{a} \pm 0.969$	$12.0^{a} \pm 0.933$
Total sugars (g/100 g dry matter)	$33.05^{a} \pm 0.081$	$30.82^{b} \pm 0.195$
Glucose (g/100 g dry matter)	$0.820^{a} \pm 0.095$	$0.702^{a} \pm 0.507$
Fructose (g/100 g dry matter)	$1.460^{a} \pm 0.608$	$0.631^{b} \pm 0.033$
Pectin (g/100 g dry matter)	$2.165^{a} \pm 0.243$	$5{,}828^{b} \pm 0.182$
Lignin (g/100 g dry matter)	$11.869^{a} \pm 0.455$	$11.412^{a} \pm 0.669$
Protein (g/100 g dry matter)	$6.072^{a} \pm 0.540$	$5.15^{b} \pm 0.193$
Total lipid (g/100 g dry matter)	$2.826^{a} \pm 0.092$	$2.485^{a} \pm 0.361$
Ashes (g/100 g dry matter)	$8.215^{a} \pm 0.372$	$8.961^{b} \pm 0.545$

CPH: Cocoa pod husk.
Values showing the same letter, in a same line do not present significant differences ($p>0.05$) and otherwise, if there is a significant difference ($p<0.05$); for 95% of the confidence interval.
Source: the author (2018).

The moisture present in the CPH from Bahía is statistically lower than the humidity of Pará, having significant difference ($p < 0.05$), remain within the humidity range of 3.6 to 7.8 g/100 g for the sample of Bahía (BONVEHÍ; JORDÀB, 1998), being near the moisture of the sample of Pará with 8.5 g/100 g reported by Vriesmann *et al.* (2011); the moisture content depends on the time of harvest, the climate and the conditions of storage (DAUD *et al*., 2013).

The pH was measured for CPH from Bahía and Pará, there was not significant difference ($p > 0.05$) between the two types of CPH.

The values of reducing sugars for CPH of Bahia and Pará were near to 10.4 ± 0.7 g/ 100 g (VRIESMANN *et al*. 2011), there were no statistically significant differences ($p > 0.05$), which indicates that there is presence of monosaccharides or reducing sugars (presenting group aldehyde or ketone) that can be used for biotechnological processes (GUSAKOV *et al*., 2011). Also, the values of total sugars of CPH of Bahia and Pará were near to 32.3 ± 1.8 g/ 100 g reported in the scientific literature (VRIESMANN *et al*., 2011), and the results are within the range of 50 to 400 μg/mL of the method for total soluble sugars (LEYVA *et al*., 2008); there was significant difference ($p < 0.05$) between the two types of CPH.

The sugars glucose and fructose were obtained in CPH of Bahia and Pará, there was not significant difference ($p > 0.05$) in the glucose values between Bahia and Pará, being the values less than 1.11 ± 0.15 g/100 g reported (BONVEHÍ; JORDÀB, 1998); with respect to fructose there was no significant difference ($p < 0.05$) for Bahia and Pará, the vaues obtained were higher than 0.56 ± 0.21 g/100 g (BONVEHÍ; JORDÀB, 1998).

Regarding the pectin obtained, there were significant differences ($p<0.05$) in the pectin extraction yield from Bahia and Pará, Vriesmann (2012) presents a pectin yield range of 3.7 to 10.6 g/100 g of CPH, the pectin extracted from Pará is within this range.

As for the lignin obtained from the CPH of Bahia and Pará, there were no significant differences ($p > 0.05$) between them, and the results obtained are less than the reported values of 14.6 g/ 100 g (SOBAMIWA; LONGE, 1994; MANSUR *et al.,* 2014).

The protein value obtained from the CPH of Bahía was greater than that obtained for the variety from Pará, and there was significant difference ($p< 0.05$) between them. Both results are within the range of 5.7 to 9.7% of crude protein (BLAKEMORE *et al*., 1966), for other researchers the values obtained were less than 8.6 g/100 g (VRIESMANN *et al.,* 2011) and 76.6 g/kg of crude protein (DONKOH et al., 1991), dry sample.

There was no significant difference ($p>0.05$) between the total lipids of CPH from Bahia and Pará, the values obtained are similar to 2.32 ± 0.27 g/100g (BONVEHÍ; JORDÀB, 1998) and are higher than 1.5 ± 0.13 g/100 g (VRIESMANN *et al*., 2011).

Regarding the total ashes of CPH for the state of Bahia and the State of Pará, there was significant difference ($p<0.05$) between them, the values for both cases are within the range of 8.8 to 10.2% (Blakemore et al., 1966); also, the values obtained are greater than 6.7 g/100 g

(VRIESMANN *et al*., 2011) and below 10 g/100 g (BONVEHÍ; JORDÀB, 1998; DONKOH *et al*., 1991).

In the Table 22, it reported the results of the minerals of the cocoa pod husk from Bahia and Pará.

TABLE 22 - THE MINERALS OF COCOA POD HUSK BAHIA AND PARÁ.

Minerals	Bahía CPH mg/kg	Pará CPH mg/kg
Al	42.27 ± 3.08	90.53 ± 37.73
Ba	64.76 ± 1.01	22.45 ± 0.04
B	<0.10	<0.10
Cd	<0.10	<0.10
Ca	2915.68 ± 42.08	3787.16 ± 31.40
Co	<0.10	<0.10
Cu	10.09 ± 0.13	12.26 ± 1.19
Fe	20.70 ± 4.21	47.62 ± 18.01
P	2113.68 ± 18.63	1354.42 ± 22.66
Li	32.49 ± 6.49	34.94 ± 2.43
Mg	3154.43 ± 9.65	2753.72 ± 20.90
Mn	<0.10	<0.10
Mo	<0.10	<0.10
Ni	<0.10	<0.10
K	96351.85 ± 64.56	76423.8 ± 8.77
Na	1146.54 ± 3,3	271 ± 27.76
V	<0.10	<0.10
Zn	56.39 ± 0.01	86.88 ± 0.18

Source: the author (2018).

According to the Table 22, the values obtained for minerals of CPH Bahía and Pará are lower than those reported in the Table 5, except in K and Na whose results are greater. CPH provides an important quantity and quality of minerals to be used in biotechnological processes. The existing differences between the minerals obtained from CPH Bahia and Pará are due to the variety of cocoa, the origin of the fruit, the nutrients in the soil where it has been sown, the climatic conditions, the sowing and harvesting technique, the post-harvest of the fruit, how it arrived in Curitiba as a whole fruit or as a cocoa pod husk (ORACZ *et al*., 2015). These conditions influence the results obtained in the composition of the CPH of Bahia and Para.

5.1.2. Results of determination of organic compounds in cph of bahía and pará by gas chromatography–mass spectrometry (gs-ms)

The organic compounds (fatty acids) obtained from Bahia CPH by GC-MS are presented in Table 23.

TABLE 23 - ORGANIC COMPOUNDS (FATTY ACIDS) OF THE BAHÍA CPH BY GC-MS.

Rt.	%AREA	Name	Characteristics
4.65	37.81	Dimethyl dl-malate	Intermediate cycle product of Piruvato, significantly restores or GSIS
7.97	11.25	Tetradecane	n-tetradecane, main component of petroleum
9.39	11.92	Phenol, 2,4-bis(1,1-dimethylethyl)-	Antibacterial, anti-fungal, anticancer and antioxidant properties
11.5	11.51	5,5-Diethylheptadecane	Does not report pharmacological activity
15.1	15.25	Me. C16:0	Palmitic acid, 16: 0 (hexadecanoic acid), principal fatty acid diet
31.8	12.26	dl-Isopulegol	l-isopulegol, an important component for l-menthol

T.r.: Retention time.
Source: the autor (2018).

Table 23, shows that 6 organic compounds were obtained from the Bahía CPH by means of GC-MS. Through the retention time (R.T., minutes) and the area (%) corresponding to each of them, the type of compounds is determined, standing out mainly three compounds by their bioactive qualities such as phenol, 2,4-bis (1,1-dimethylethyl) that have some antibacterial, antifungal, anticancer and antioxidant properties (PADMAVATHI *et al*., 2014; ROMERO-CORREA *et al.*, 2014; DEHPOUR *et al*., 2012 SATHUVAN *et al.,* 2012; RANGEL-SANCHEZ *et al*., 2013); Dimethyl dl-malate is an intermediate cycle product of Pyruvate, significantly restores Glucose-Stimulated Insulin Secretion (GSIS) in type 2 diabetes (BOUCHER *et al.*, 2004; LU *et al*., 2002; RONNEBAUM *et al*., 2008); Palmitic acid, the main fatty acid in people's diets (MICHA; MOZAFFARIAN, 2010; BENOIT *et al*., 2009); between these three correspond to 64.98% of the total fatty acids.

In Table 24, the organic compounds (fatty acids) obtained in Pará CPH by GC-MS, through the retention time (R.T., minutes) and the area (%) corresponding to each of them, are shown.

TABLE 24 - ORGANIC COMPOUNDS (FATTY ACIDS) OF THE PARÁ CPH BY GC-MS.

R.T.	%AREA	Name	Characteristics
4.598	23.01	Dimethyl dl-malate	Intermediate cycle product of Piruvato, significantly restores or GSIS
6.233	1.29	(-)-Carvone	Monoterpene, used in the pharmaceutical, food and cosmetics industry
9.011	2.78	Eicosane	Acyclic alkane, source of carbon and energy
9.358	4.7	Phenol, 2,4-bis(1,1-dimethylethyl)-	Antibacterial, anti-fungal, anticancer and antioxidant properties
10.375	1.95	Hexadecane <n->	Helps measure the cetane index for better diesel combustion
15.079	9.36	Me. C16:0	Palmitic acid, 16: 0 (hexadecanoic acid), principal fatty acid diet
16.348	1.01	Et. C16:0	Methyl palmitate, saturated fatty acid, coronary risk factor, diabetes, cerebrovascular
18.368	3.49	Me. C18:2n6	Linoleic acid, essential polyunsaturated fatty acid (omega 6)
18.491	4.14	Me. C18:1n9	Oleic acid, monounsaturated fatty acid (omega 9)
19.018	1.74	Me. C18:0	Stearic acid, To make candles, soaps and cosmetics
19.861	1	Ethyl Oleate	Flavoring agents, lubricants and derivatives
33.113	6.43	13-Docosenamide, (Z)-	Amides of fatty acids belong to the family of cerebral lipids.

R.T.: Retention time.
Source: the autor (2018).

In Table 24, the main 12 fat components of Pará CPH are shown, this corresponds to 60.9%; through the retention time (R.T., minutes) and the area (%) corresponding to each of them, the type of compounds is determined, standing out mainly by their bioactive qualities Dimethyl dl-malate is an intermediate cycle product of Piruvato, significantly restores Glucose-Stimulated Insulin Secretion (GSIS) in type 2 diabetes (BOUCHER *et al*., 2004; LU *et al*., 2002; RONNEBAUM *et al.,*2008); (-)-Carvone is a monoterpene used in the pharmaceutical, food and cosmetics industry (DE SOUSA *et al*., 2007; RENTZSCH *et al*., 2011; BICAS *et al*., 2011; CONDE *et al.;* 2011); Eicosane is an Acyclic alkane, source of carbon and energy (RADWAN *et al*., 1995; LINTAS *et al*., 1979); Phenol, 2,4-bis(1,1-dimethylethyl)-, has some antibacterial, anti-fungal, anticancer and antioxidant properties (PADMAVATHI *et al*., 2014; ROMERO-CORREA *et al*., 2014; RANGEL-SANCHEZ et al., 2013); Hexadecane <n-> helps measure the cetane index for better diesel combustion (FOURNET *et al.,* 2001; OUTCALT *et al.,* 2010); Palmitic acid (Me C16:0, hexadecanoic acid) is a principal fatty acid diet (MICHA; MOZAFFARIAN, 2010; BENOIT *et al*., 2009); methyl palmitate (Et. C16:0, saturated fatty acid) implies coronary risk factor, diabetes, cerebrovascular (MICHA; MOZAFFARIAN, 2010); Linoleic acid (Me. C18:2n6), is an essential polyunsaturated fatty acid (omega 6) (HIBBELN, 2006; DOLECEK, 1992); Oleic acid (Me. C18:1n9, monounsaturated fatty acid), is an omega 9 fatty acid (LANDS, 2005); Stearic acid (Me. C18:0), is used for making candles,

soaps and cosmetics (BATISTUZZO, 2006); Ethyl Oleate is used in flavoring agents, lubricants and derivatives (LI *et al*., 2015; MARTINEZ-RUIZ *et al*., 2018; SALUM *et al*., 2008); 13-Docosenamide, (Z)-, amides of fatty acids belong to the family of cerebral lipids (CRAVATT *et al*., 1995).

5.1.3. Morphology of the CPH of Bahia and Pará

The study of morphology helps to understand the distribution and the layout of the fibers in the agricultural waste (DAUD *et al.,* 2013). In the Figures 14, 15, 16 and 17 shows the morphological structure of the CPH observed in an electron microscope to 2000X is shown; as it can be observed in the photography CPH of Bahía fibers are of greater diameter than the fibers of the CPH of Pará, and in both cases that the cell wall has not undergone any change by a chemical hydrolysis or enzymatic reaction, only the effect of the milling process, and granulometry.

FIGURE 14 - BAHIA CPH TO 2000X BY ELECTRONIC MICROSCOPIC SEM.

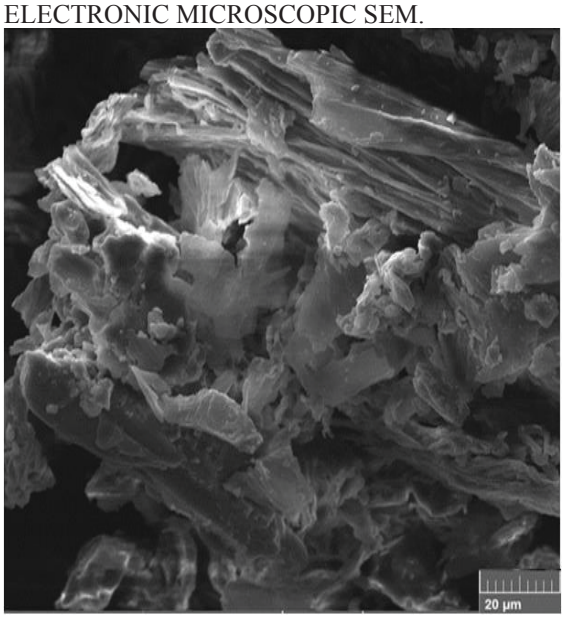
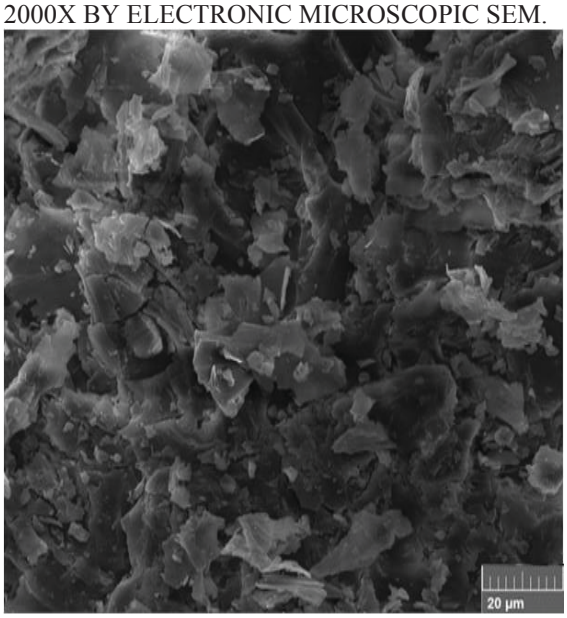


FIGURE 15 - PARÁ CACAO POD HUSK TO 2000X BY ELECTRONIC MICROSCOPIC SEM.



Source: the author (2018).

In the Figure 14, at a scale of measurement of 20 µm, it can be displayed that the particle of CPH of Bahia is long and compact, and protects the content lignocellulosic fibers; a rough and thick layer is produced by the fibers for protective purposes (DAUD *et al*., 2013). In contrast to the Figure 15, at the same scale of measurement, it is noted that the particles of the CPH of Pará are smaller and less compact, shorter fibers (EL-SHEKEIL *et al.,* 2014).

FIGURE 16 - BAHIA CPH, AFTER OF THE EXTRACTION WITH LPG, TO 2000X BY ELECTRONIC MICROSCOPIC SEM.

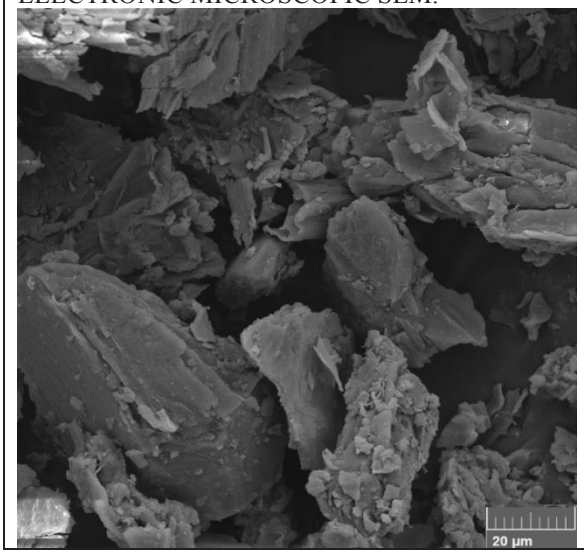
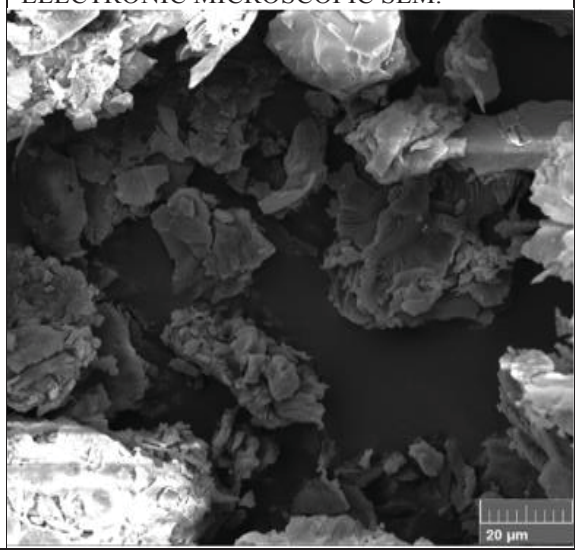


FIGURE 17 - PARÁ CPH, AFTER OF THE EXTRACTION WITH LPG, TO 2000X BY ELECTRONIC MICROSCOPIC SEM.



Source: the author (2018).

In Figure 16, the CPH of Bahia after extraction with LPG, is shown the vegetal tissue is less elongated and more compact after the removal; is lost elongation of the vegetal tissue and contract in the chemical extraction (CHUN *et al*., 2013). In Figure 17, the CPH of Pará after LPG extraction shows shorter and more compact tissue than the CPH before removing compounds.

5.1.4. Number of cycles in the process of extraction with the extractor of LPG for total polyphenols

The number of cycles of removal taking as standard of comparison the total content of polyphenols, which used the portable extractor with LPG and the CPH of Bahía and Pará was determined. In Table 25, the number of extractions and its cumulative percentage (relative) also compares the results obtained in the extraction for CPH of Bahía with CPH of Pará (in wet basis).

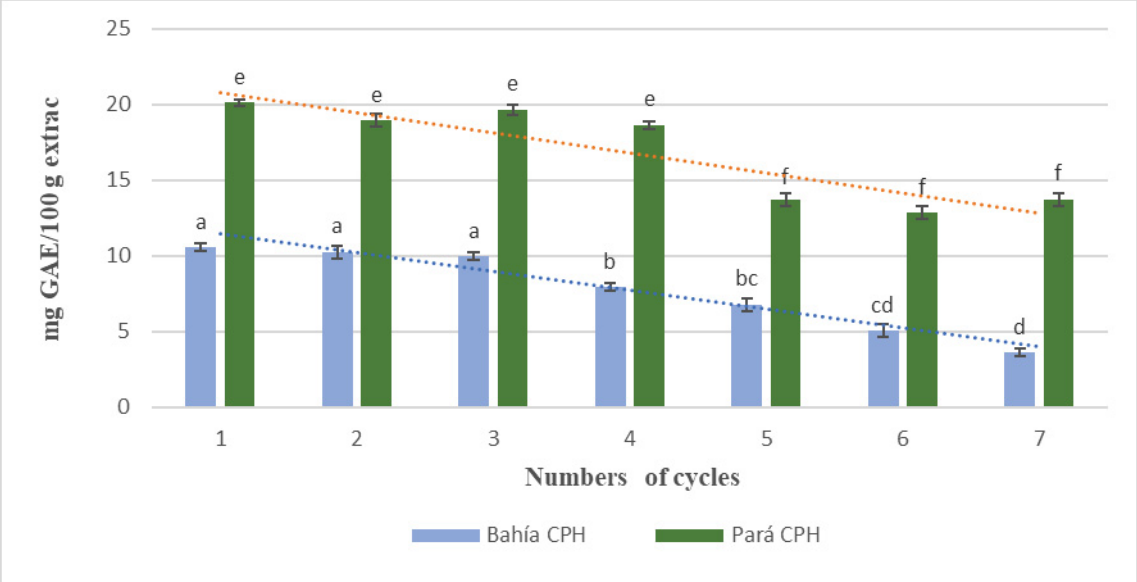
TABLE 25 - COMPARATIVE EFFICIENCY OF LPG EXTRACTION FOR COCOA POD HUSK OF BAHIA AND PARÁ FOR TOTAL POLYPHENOLS CONTENTS.

	Bahía cocoa pod husk		Pará cocoa pod husk	
Number of Extractions N°	Total polyphenols Contents mg GAE/100g	The cumulative percentage of Extraction (%)	Total polyphenols Contents mg GAE/100g	The cumulative percentage of Extraction (%)
1	$10.551^{a} \pm 0.502$	19.486	$20.116^{e} \pm 0.502$	17.081
2	$10.261^{a} \pm 0.870$	38.437	$18.087^{e} \pm 0.870$	33.177
3	$9.971^{a} \pm 0.502$	56.852	$19.681^{e} \pm 0.670$	49.889
4	$7.942^{b} \pm 0.502$	71.520	$18.667^{e} \pm 0.552$	65.740
5	$6.783^{bc} \pm 0.870$	84.047	$13.739^{f} \pm 0.870$	77.406
6	$5.043^{cd} \pm 0.870$	93.362	$12.870^{f} \pm 0.870$	88.334
7	$3.594^{d} \pm 0.502$	100.000	$13.739^{f} \pm 0.870$	100.000

GAE: Gallic Acid Equivalent, % cumulative percentage of extaction (relative).
Values showing the same letter, in a same column, do not present significant differences ($p>0.05$) and other otherwise, if there is a significant difference ($p<0.05$); for 95% of the confidence interval.
Source: the author (2018).

Statistical test (ANOVA) was carried out for CPH of Bahía and Pará, in both cases there was a significant difference between treatments, according to the Tukey test to 95% level range of confidence; with these results, the first 4 extractions were chosen to represent more than 65 percent of the cumulative percentage of the extraction ($p>0.05$), being the most noticeable trend of decline for the removal of total polyphenols from cycle 5, as can be seen in Figure 18.

FIGURE 18 - EFFICIENCY OF LPG EXTRACTION FOR COCOA POD HUSK OF BAHIA AND PARÁ BY TOTAL POLYPHENOLS CONTENTS.



Source: the author (2018).

As can be seen in Table 25 and Figure 18, the CPH of Pará is more efficient in the extraction with respect to the CPH of Bahia with almost double the total polyphenols extracted. As it can be observed, for the extraction of compounds from Pará CPH there were no significant differences in the 4 cycles of extractions and the cumulative percentage of extraction was more than 65%, from there on the values decreased. In addition, in each cycle of extraction in average a time of 40 minutes is used, that also affects productivity. For the extraction of Bahia CPH, in the first three cycles there are no significant differences, and its cumulative percentage in the third cycle was 56.85%, being its efficiency low, so by increasing one more cycle, the value increased to 71.52%. So, for both samples, four cycles were used.

For historical background, the best cocoa in Brazil comes from the state of Pará, for the factors mentioned above that favor the development and quality of the cocoa fruit, as well as the treatment exposed to the pod husk, the conditions of storage, washing, handling, drying temperature, the type of stove used (normal or vacuum) to grinding, granulometry, method of extraction of total polyphenols, among others, which can also influence the results of total polyphenols.

5.1.5. Comparison of ethanol with the LPG for the extraction of total polyphenols and their antioxidant capacity

Comparisons were made between the traditional method of maceration with absolute ethanol (>99% purity) with respect to the use of extrator with LPG, the results of the extraccion of the total content of polyphenols are shown in Table 26 (in wet basis).

TABLE 26 - COMPARATIVE TABLE OF THE TOTAL POLYPHENOLS CONTENTS OF THE CPH OF BAHIA AND PARÁ BY DIFFERENT EXTRACTION METHODS EXPRESSED IN mg GAE/100 g EXTRACT.

Property	**Bahía CPH**		**Pará CPH**	
	LPG extraction	**Ethanol extraction**	**LPG extraction**	**Ethanol extraction**
Total polyphenols Contents mg GAE/100g	$9.681^{a} \pm 1.183$	$8.744^{a} \pm 0.837$	$19.667^{b} \pm 0.663$	$47.874^{c} \pm 0.837$

GAE: Gallic Acid Equivalent
Values showing the same letter, in a same line, do not present significant differences ($p>0.05$) and otherwise, if there is a significant difference ($p<0.05$); for 95% of the confidence interval.
Source: the author (2018).

There was no significant difference ($p>0.05$) in the extraction with LPG and ethanol of total polyphenols for the CPH of Bahia; there was a significant difference ($p<0.05$) between the two extractions for the measurement of polyphenols of Pará CPH, being better the extraction

with ethanol. The values are below (BONVEHÍ; JORDÀB, 1998) reported in the literature 0.68 ± 0.31 GAE g/100 g. The number of cycles of extraction with LPG was of 4, the difference of total content of polyphenols with respect to CPH of Pará obtained in both methods maybe due to the solvent that is submitted to the contact with CPH, In the methods of maceration the solvent is absolute ethanol (polar), while the other method uses LPG (apolar), each one of these methods will extract compounds related to the polarity of the solvent. The polarity of the solvent and the solubilization properties of different solutes are important factors to be considered, it must be in a homogeneous phase, so that both come into contact and the extraction of compounds is more efficient (BORGES *et al*., 2015).

Table 27, shows the comparison between these 2 methods with respect to the antioxidant activity and its concentration expressed in Vitamin C Equivalent Antioxidant Capacity (VCEAC), (in wet basis).

TABLE 27 - COMPARISON OF THE % ANTIOXIDANT ACTIVITY AND VCEAC OF THE POD HUSK OF BAHIA AND PARÁ BY DIFFERENT EXTRACTION METHODS.

Property	Bahía CPH		Pará CPH	
	LPG extraction	Ethanol extraction	LPG extraction	Ethanol extraction
% Antioxidant Activity	$84.137^{a} \pm 1.183$	$56.46^{b} \pm 0.837$	$91.995^{c} \pm 0.522$	$81.63^{d} \pm 0.142$
VCEAC mg AAE/100 g Ext	$1497.517^{a} \pm 0.778$	$1018.275^{b} \pm 2.873$	$1633.550^{c} \pm 1.453$	$1454.094^{d} \pm 2.450$

VCEAC: Vitamin C Equivalent Antioxidant Capacity
AAE: Ascorbic Acid Equivalent
Values showing the same letter, in same line, do not present significant differences ($p>0,05$) and other otherwise, if there is a significant difference ($p<0.05$); for 95% of the confidence interval.
Source: the author (2018).

The values of antioxidant activity (relative to ascorbic acid) are greater in the extraction with LPG, having a significant difference ($p< 0.05$); in contrast, the values of VCEAC are greater in the extraction by LPG that is more concentrated than the extracted by ethanol (there is a significant difference between the two methods ($p<0.05$), taking best representative values than indicated for some fruits (KUSKOSKI *et al.*, 2005); to be obtained better results the CPH of Bahía that the of Pará. The antioxidant activity of the polyphenols of the CPH is determined by its chemical structure and depends on the experimental conditions (substrate, temperature, light, pressure of oxygen and metals); so, there are variability in the effectiveness as antioxidants between groups of phenolic compounds (GARCÍA, 2006; KIM *et al*., 2004).

5.2. COMPOSITIONAL ANALYSIS OF FERMENTED CPH AND SELECTION OF *Pleurotus* STRAINS

It is present a compositional analysis of the fermented products of Pará cocoa pod husk with *Pleurotus* strains.

5.2.1. Compositional analysis of fermented CPH with *Pleurotus* strains

It was carried out the compositional analysis of Solid State Fermentatio of the cocoa pod husk with the different *Pleurotus* strains, after of the 30 days of fermentation.

In Table 28, these analyzes were used to select the first strains that fermented to the cocoa pod husk of Pará, shows the different results of % moisture, pH, reducing sugar, protein, VCEAC and the total polyphenols contents in the fermentation of cocoa pod husk with different strains of *Pleurotus* (in dry matter).

TABLE 28 - SUMMARY OF THE MOISTURE, PH, ANTIOXIDANT CAPACITY (VCEAC) AND TOTAL POLYPHENOLS IN FERMENTED COCOA POD HUSK.

Fermented of CPH and Pleurotus strains	Reducing sugar (g RS/100g*)	Protein (g of protein/100g*)		Antioxidant activity VCEAC (mg AAE/100 g*)		Total polyphenols content (mg GAE/100 g*)	
	Water	LPG	Ethanol	LPG	Ethanol	LPG	Ethanol
Pará (CPH)	$8.71^{f} \pm 0.27$	$5.82^{f} \pm 0.13$	$4.98^{f} \pm 0.03$	$1786.44^{f} \pm 0.43$	$1584.80^{f} \pm 0.42$	$362.08^{cd} \pm 1.23$	$426.5^{g} \pm 0.90$
PL9	$0.84^{ab} \pm 0.24$	$19.00^{d} \pm 0.43$	$18.7^{c} \pm 0.60$	$4617.40^{b} \pm 3.02$	$4463.60^{c} \pm 2.61$	$407.34^{d} \pm 3.29$	$40.63^{ab} \pm 2.18$
PL20	$1.00^{ab} \pm 0.25$	$24.79^{ae} \pm 0.37$	$23.31^{d} \pm 0.42$	$8839.29^{e} \pm 4.75$	$5685.71^{de} \pm 3.17$	$487.88^{a} \pm 4.67$	$38.05^{abd} \pm 2.38$
PL22	$1.08^{abc} \pm 0.20$	$15.37^{b} \pm 0.27$	$14.22^{a} \pm 0.57$	$5500.80^{d} \pm 3.51$	$3560.39^{a} \pm 2.06$	$194.71^{b} \pm 3.38$	$47.39^{ac} \pm 1.41$
PL22E	$2.04^{de} \pm 0.41$	$17.35^{c} \pm 0.24$	$16.84^{b} \pm 0.15$	$6205.48^{a} \pm 0.94$	$4002.34^{b} \pm 0.65$	$233.94^{b} \pm 2.83$	$162.54^{f} \pm 1.80$
PL23	$0.75^{a} \pm 0.11$	$14.54^{b} \pm 0.25$	$14.43^{a} \pm 0.26$	$5187.33^{cd} \pm 2.38$	$3360.42^{a} \pm 1.57$	$356.06^{c} \pm 2.52$	$65.57^{ce} \pm 1.68$
PL24	$1.40^{bc} \pm 0.13$	$22.50^{e} \pm 1.02$	$23.24^{d} \pm 1.06$	$7497.48^{g} \pm 7.66$	$5587.40^{d} \pm 5.68$	$497.00^{a} \pm 5.26$	$78.69^{e} \pm 4.37$
PL31	$2.62^{e} \pm 0.27$	$19.66^{d} \pm 0.61$	$19.05^{c} \pm 0.65$	$4835.74^{bc} \pm 2.78$	$4495.87^{c} \pm 2.41$	$508.33^{a} \pm 3.48$	$15.84^{d} \pm 1.04$
PL50	$1.13^{abc} \pm 0.20$	$25.24^{a} \pm 0.64$	$26.15^{e} \pm 1.08$	$9255.96^{e} \pm 7.97$	$5945.54^{e} \pm 5.33$	$950.05^{f} \pm 7.61$	$44.56^{abc} \pm 4.07$
PL3501	$1.61^{cd} \pm 0.24$	$17.22^{c} \pm 0.67$	$16.97^{b} \pm 0.28$	$6276.11^{a} \pm 2.09$	$3948.72^{b} \pm 1.29$	$1238.97^{g} \pm 5.64$	$53.55^{ac} \pm 1.78$
PL3824	$0.81^{a} \pm 0.19$	$24.00^{ae} \pm 1.27$	$26.84^{e} \pm 0.97$	$6472.55^{a} \pm 5.42$	$6345.25^{g} \pm 4.62$	$752.98^{e} \pm 5.56$	$22.34^{bd} \pm 1.32$

* Extract, CPH: Cocoa pod husk, RS: Reducing sugar, VCEAC: Vitamin C equivalent antioxidant capacity, AAE: Ascorbic acid equivalent, GAE: Gallic acid equivalent. Values showing the same letter, in a same column, do not present significant differences ($p>0,05$) other otherwise, if there is a significant difference ($p<0,05$); for 95% of the confidence interval.

Source: the autor (2018).

The results of the reducing sugars in the fermented products were very low than the values obtained in the coca pod husk, which indicates that not only the fungi have used the sugars for its maintenance, also that the *Pleurotus* have produced other metabolites. The values obtained from the extract with water were between 0.75 and 2.62 mg of reducing sugar / 100 g of dry matter of *Pleurotus* species analyzed. There is no significant difference ($p> 0.05$) between the

reduction sugar remnants of the PL9, PL20, PL22, PL23, PL50 and PL3824 samples; in the other samples the statistical difference is present.

The values obtained from fermentation in soluble protein are higher than the value obtained in the cocoa pod husk of Pará, and similar between the values of extracts extracted with LPG and absolute ethanol, but higher than the values reported in some *Pleurotus* fungi from 16.30 to 16.75 mg / g of dry matter of agro-industrial residues (SÁNCHEZ, 2010). There is not significant difference ($p>0.05$) of soluble protein from the fermented samples extracted with LPG for PL9 and PL31, as also for PL22 and PL23, so too for PL22E and PL3501, as well as for PL20 and PL3824; in the other samples if there are statistically differences between them. There is not statistically difference ($p>0.05$) in soluble protein between the fermented samples extracted with absolute ethanol for PL9 and PL31, as also for PL20 and PL24, so too for PL22E and PL3501, as well as for PL50 and PL3824; in the other samples if there are significant differences ($p<0.05$).

The VCEAC values of the antioxidant capacity of the extract of the fermented products extracted with LPG and absolute ethanol, these were higher than those obtained by cocoa pod husk; due to the degrading effect that suffers the lignocellulosic material in the fermentation process by *Pleurotus* that affects the phenolic components (MINTESNOT *et al*., 2014). There is a statistically difference ($p<0.05$) in the degradation of antioxidant activity among the fermented samples extracted with LPG, with the exception of PL20 and PL50, as also for PL3501 and PL3824. There is not significant difference ($p>0.05$) in the loss of antioxidant activity between the fermented samples extracted with absolute ethanol of PL22E and PL3501, also with PL22 and PL23, and finally with PL9 and PL31; in the other cases there is a significant difference ($p<0.05$) between the samples.

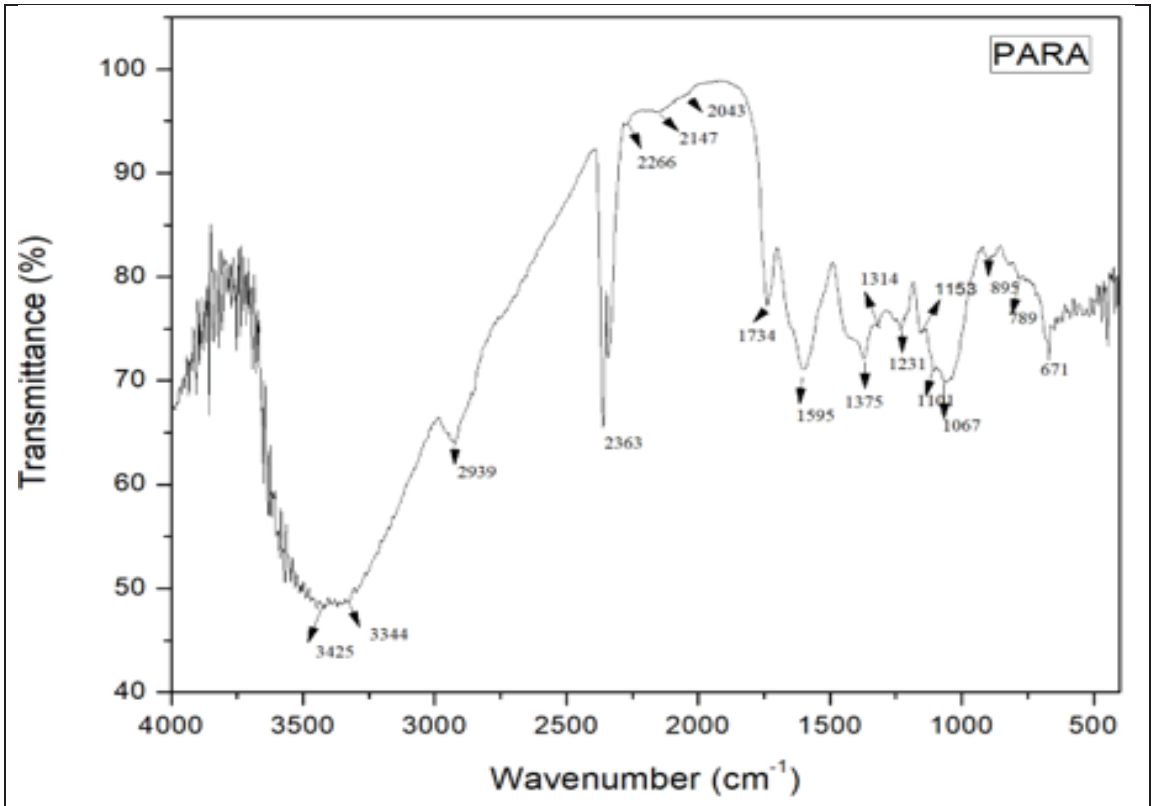
The values of the total polyphenol content of the extracts extracted from the LPG are superior to the result of the extract with absolute ethanol, and these extracts were superior to the total polyphenol content of the cocoa pod husk, except in PL22, PL22E and PL23; in addition, these values are lower than 10.24 to 36 mg of GAE / g of extract of some species of Pleurotus analyzed (BABU *et al*., 2012; YANG *et al*., 2002). There is a significant difference ($p<0.05$) in the degradation of total polyphenols in the fermented ones extracted with LPG, except for those fermented with PL22 and PL22E, as well as those fermented with PL20, PL24 and PL31. There are not significant differences ($p>0.05$) between the fermented samples extracted with absolute ethanol of PL22 and PL3501; in other cases, if there is a significant difference ($p<0.05$). This degradation of polyphenols is due to the presence of ligninolytic

enzymes present in the metabolism of *Pleurotus* that degrade lignocellulosic substrates, so that the lacase enzyme degrades the lignin substrate and generates aromatic compounds. The white rot fungi produce lacase enzyme or ligninolytic peroxidases (LiP and MnP) that oxidize the lignin, generating aromatic radicals (SANCHEZ, 2009).

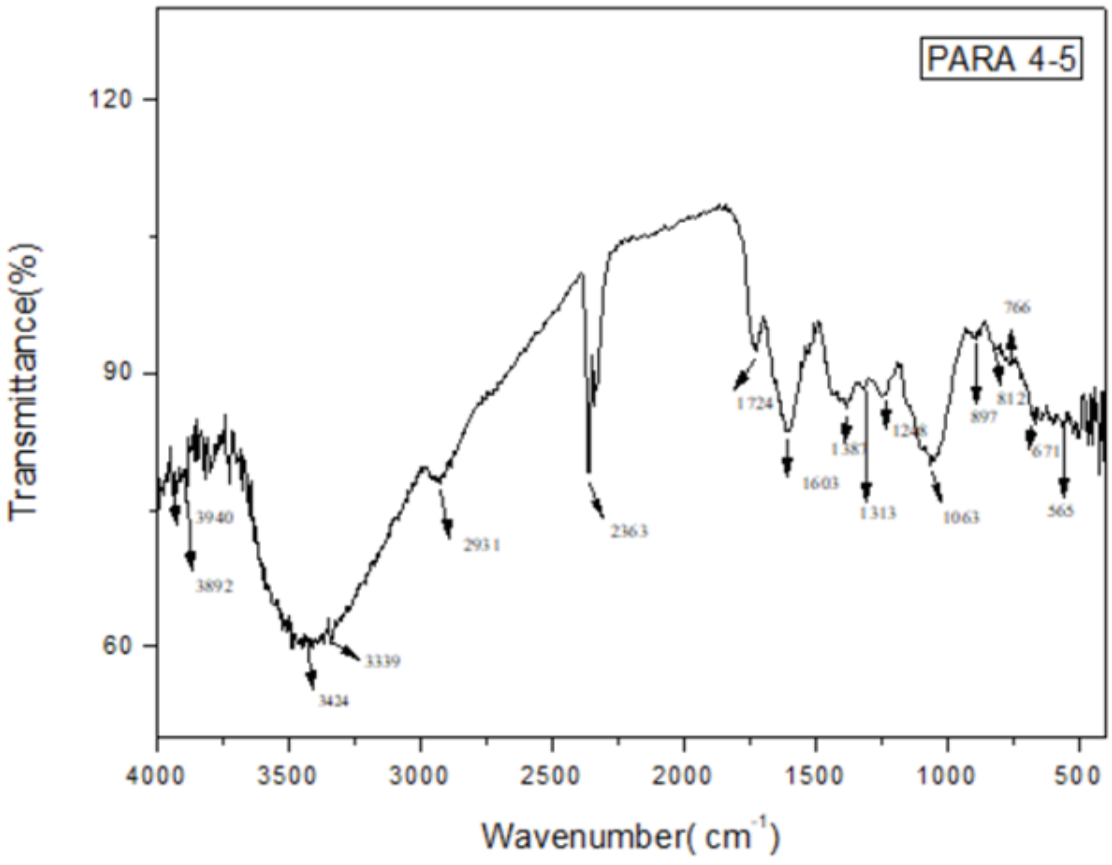
5.2.1.1. Functional groups by Fourier-Transform Infrared spectroscopy (FTIR) of CPH fermented with *Pleurotus* strains

The spectral features of fermented products of CPH with *Pleurotus* strains (PL9, PL20, PL22, PL22E, PL23, PL24, PL31, PL50, PL3501 y PL3824) were obtained by FTIR analysis and are shown in Figure 19.

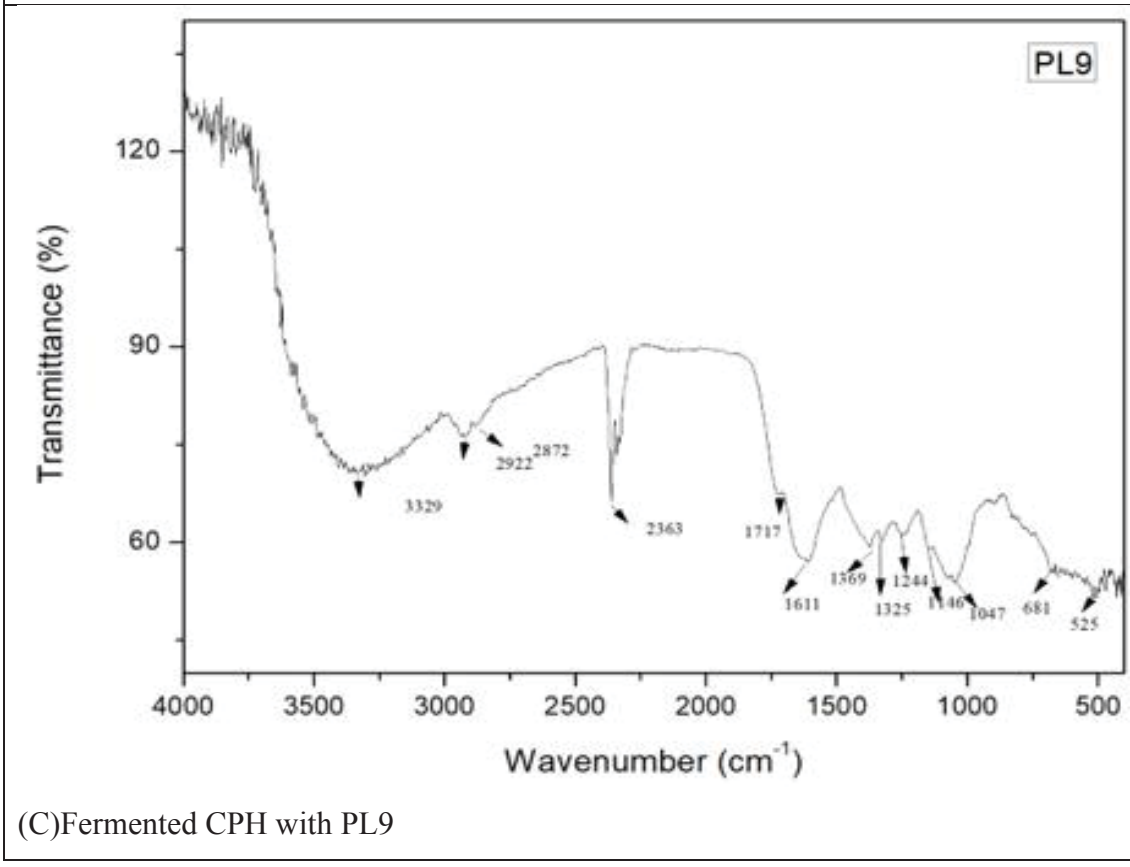
FIGURE 19 - FTIR OF FERMENTED PRODUCTS FROM CPH AND *Pleurotus* STRAINS.



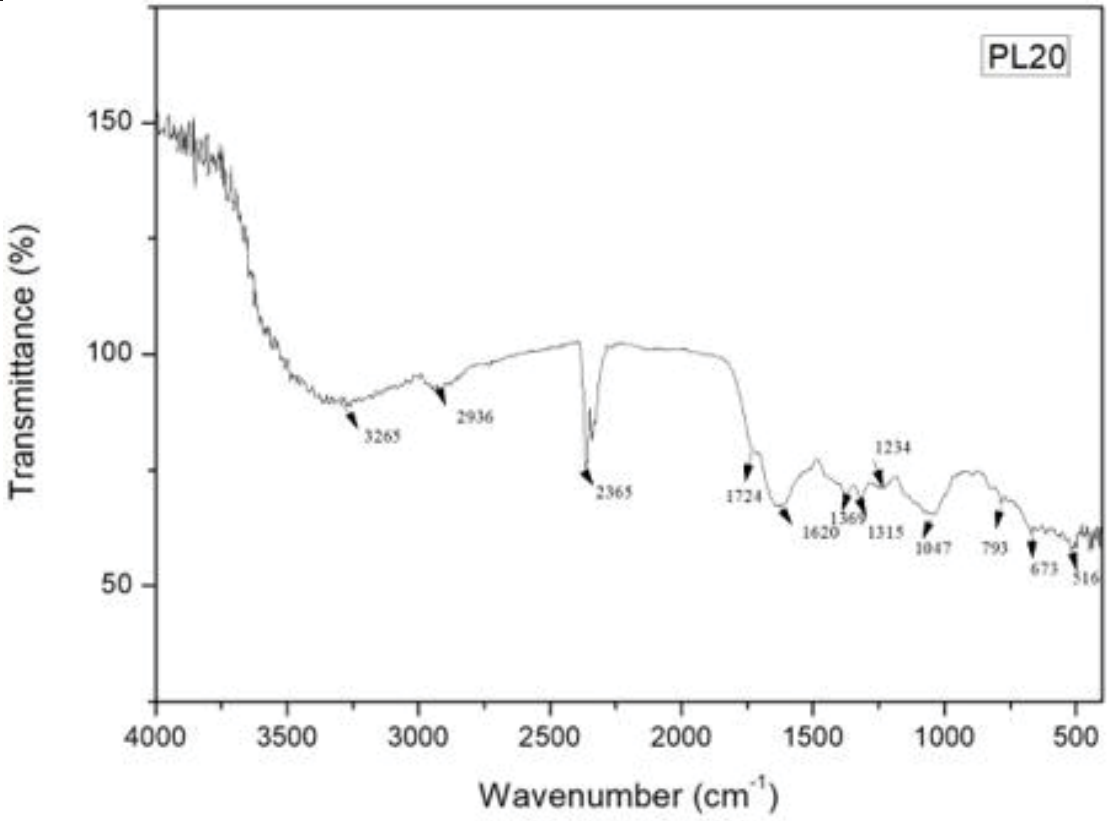
(A)PARÁ Cocoa Pod Husk



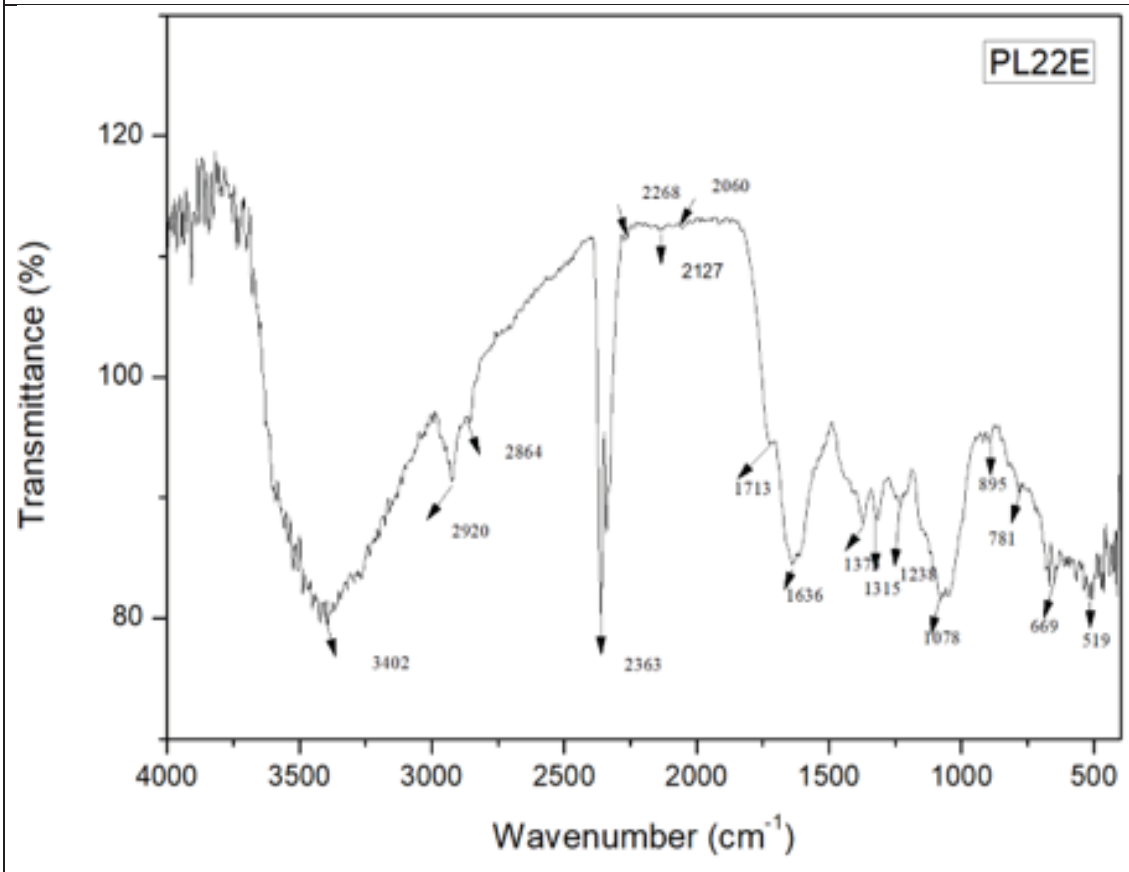
(B)PARÁ (4-5) Cocoa Pod Husk



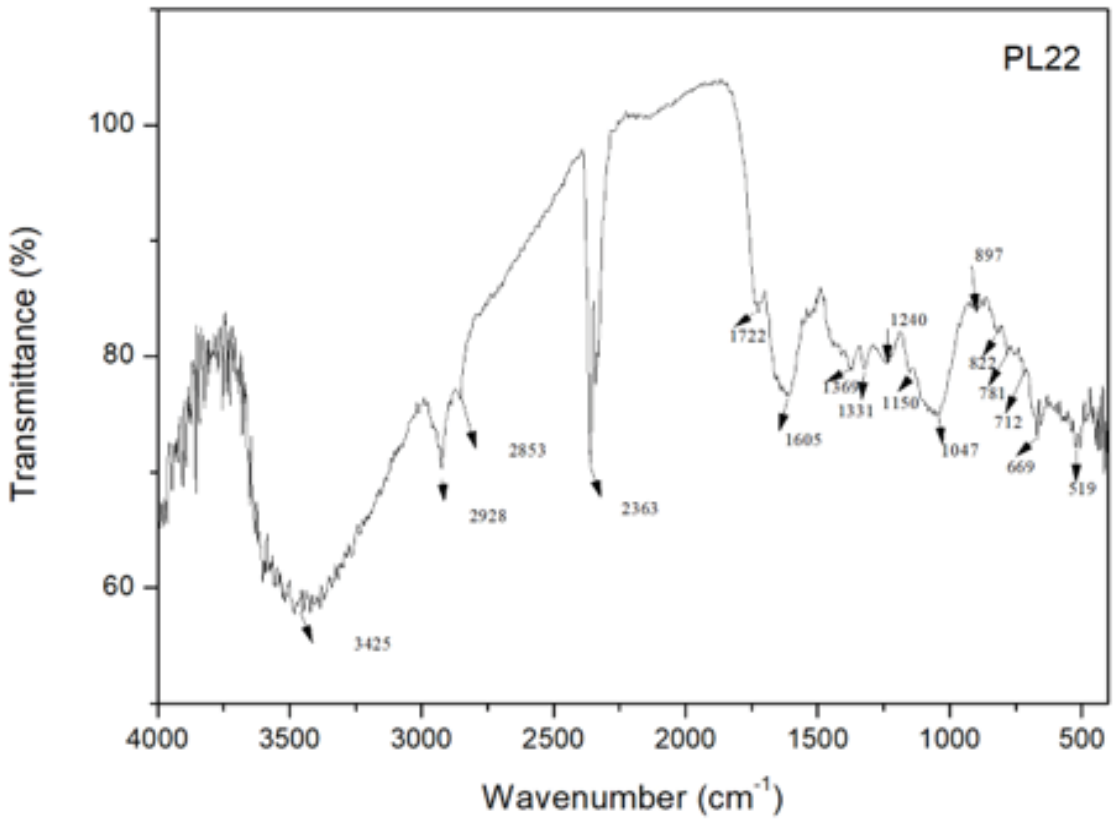
(C)Fermented CPH with PL9



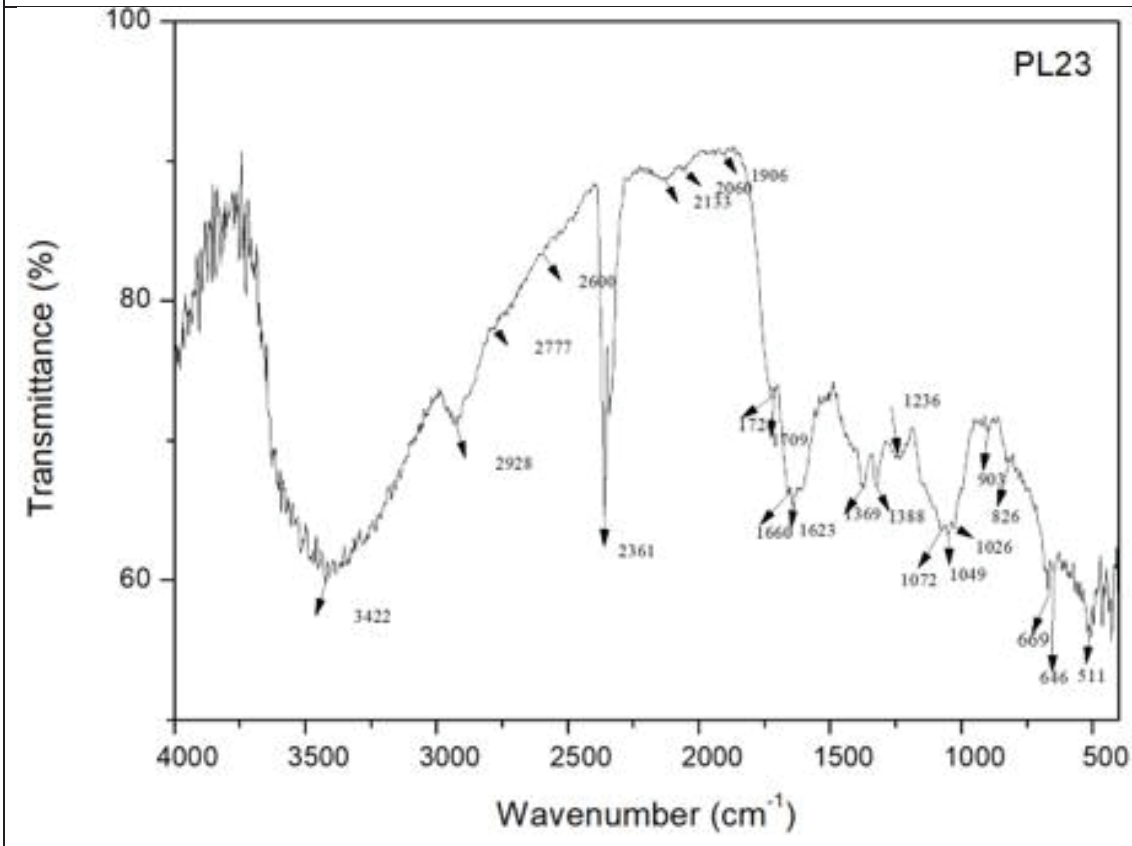
(D) Fermented CPH with PL20



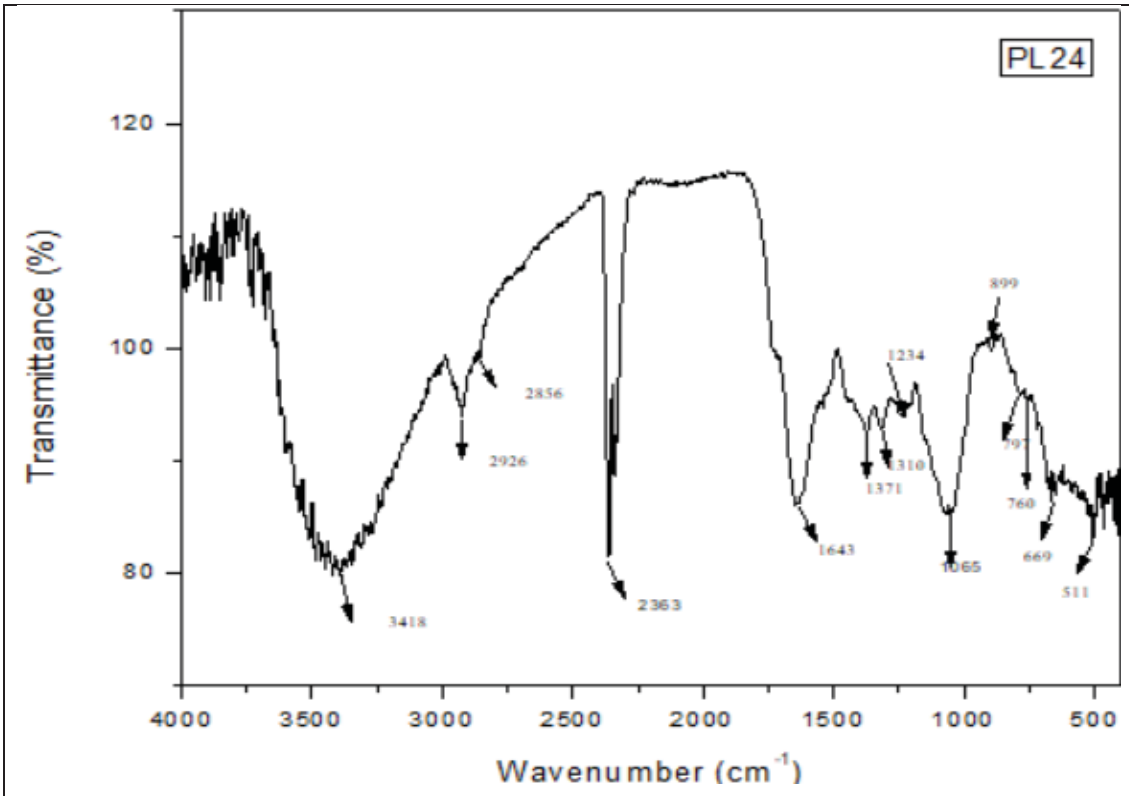
(E) Fermented CPH with PL22E



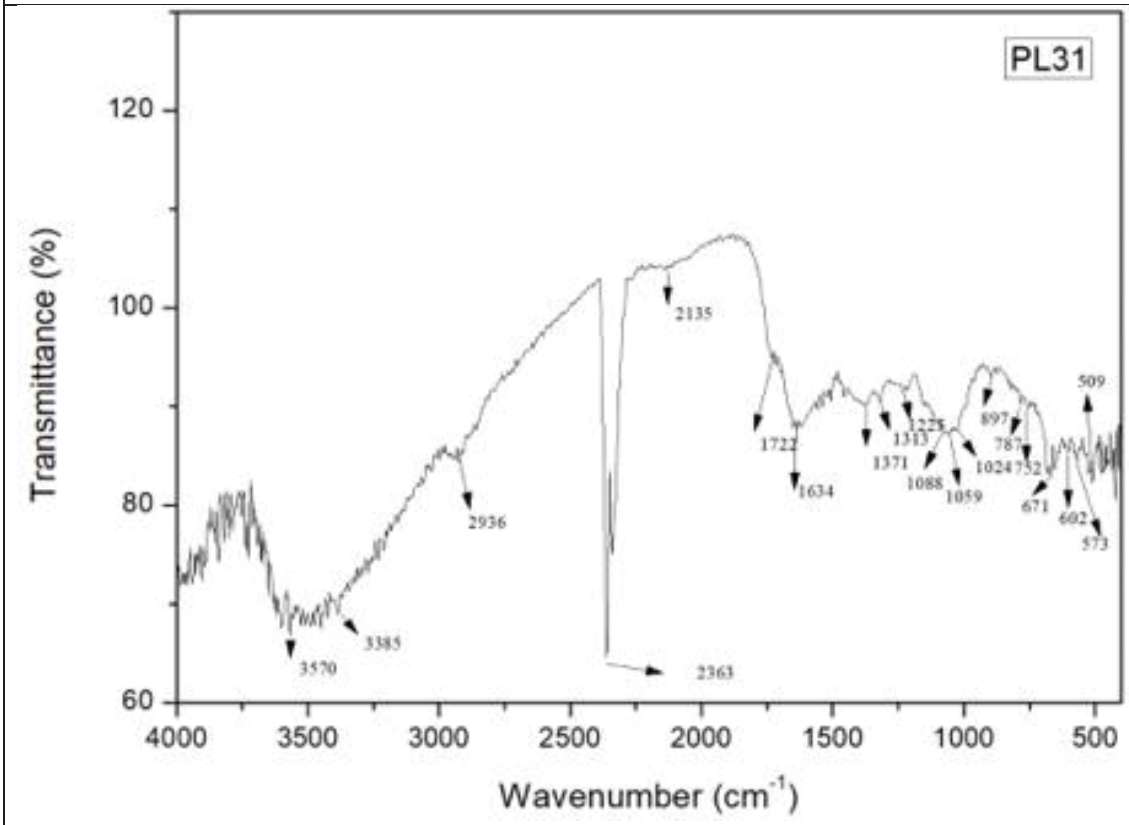
(F) Fermented CPH with PL22



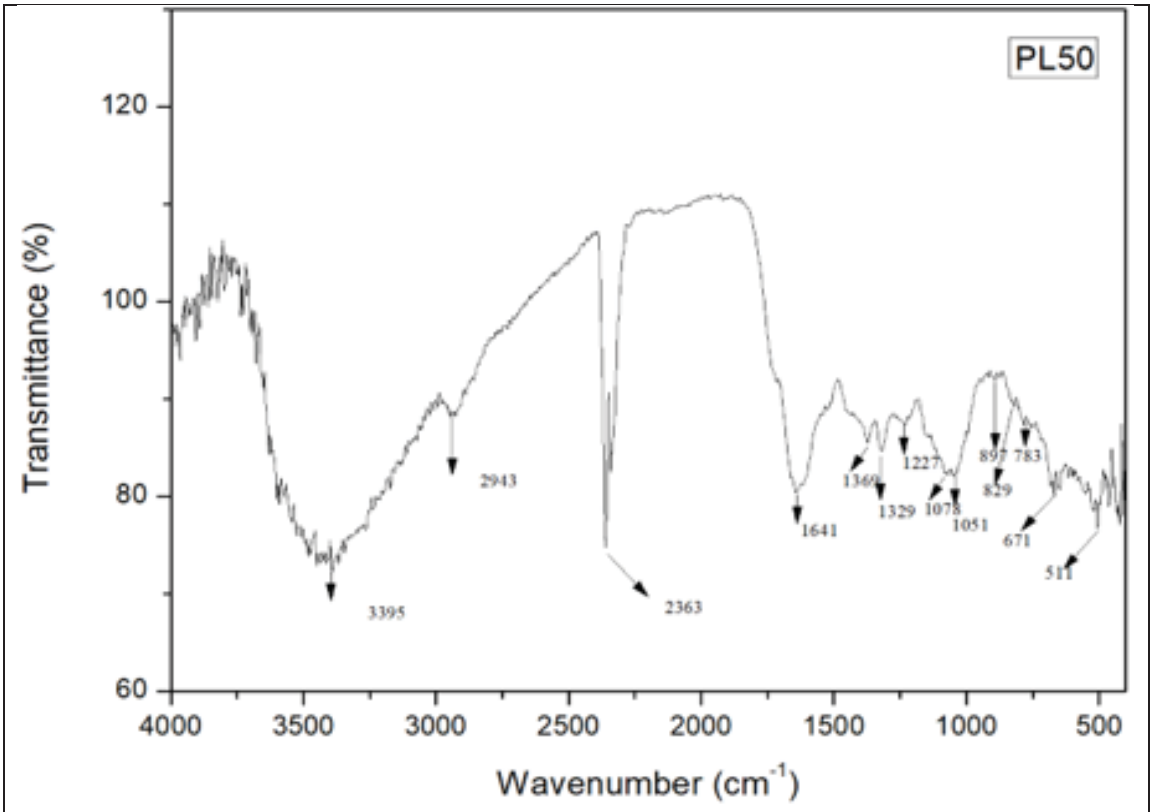
(G) Fermented CPH with PL23



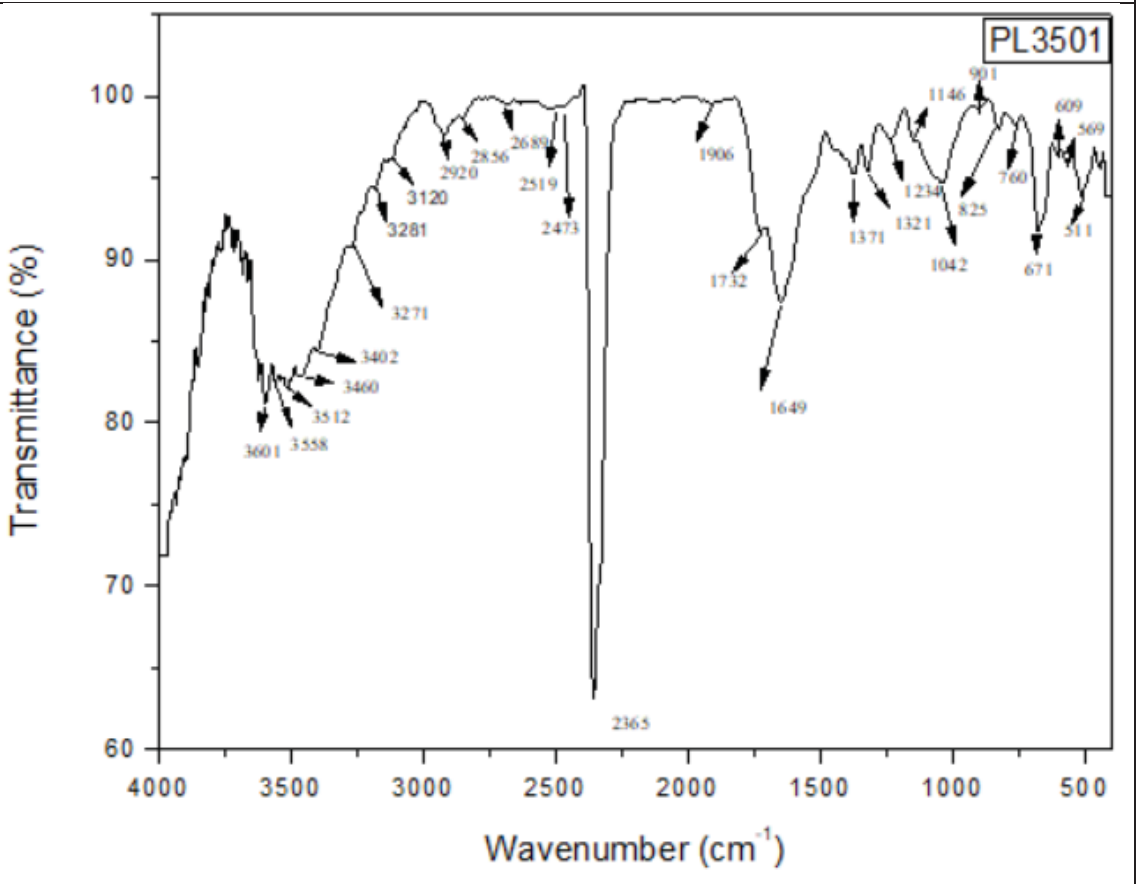
(H) Fermented CPH with PL24



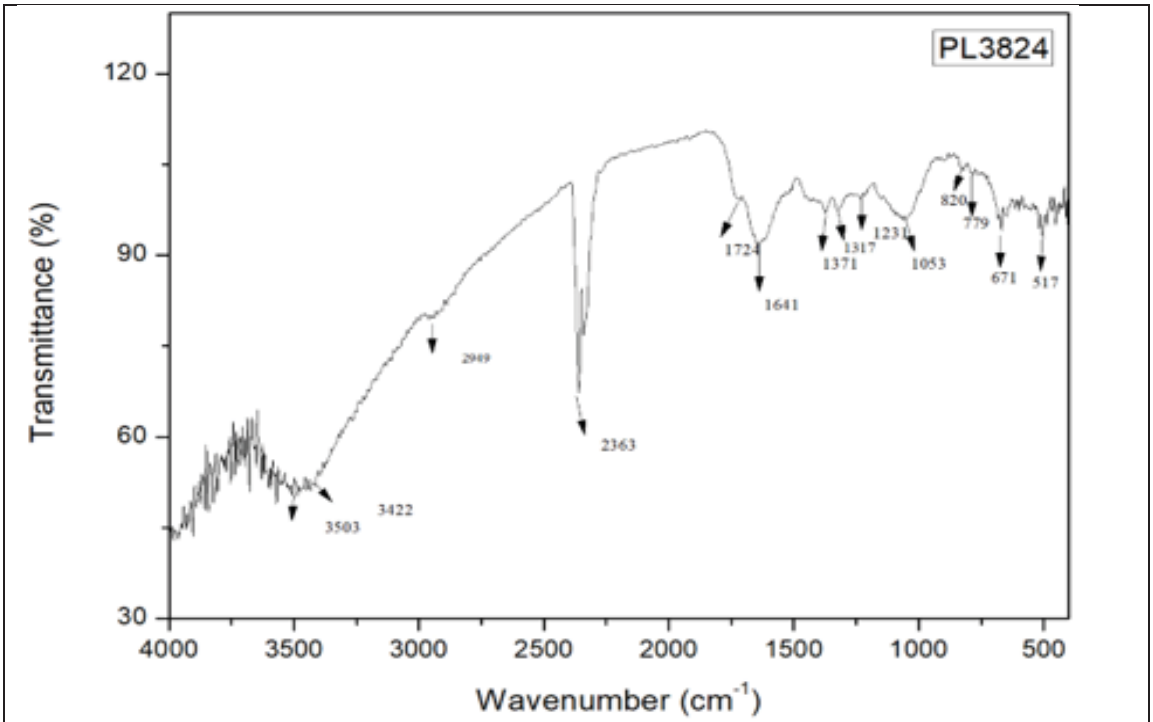
(I) Fermented CPH with PL31



(J) Fermented CPH with PL50



(K) Fermented CPH with PL3501



(L) Fermented CPH with PL3824

Source: the author (2018).

In Figure 19, the region 2400 a 2200 cm^{-1} corresponds to CO_2, (A) Pará, the main functional groups are observed at the wavenumber of 3562-3320 cm^{-1} corresponding to phenolic groups (attributed to the stretch of O-H) and 3200-2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds). For aromatic groups it is in the band 1645-1544 cm^{-1} (attributed to C-C stretch of aromatic ring); In the band 1410-1310 cm^{-1} corresponds to the tertiary alcohol, for the band 1085-1030 cm^{-1} is the saturated primary alcohol (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(B) Pará 4-5, which corresponds to the CPH after extraction with LPG, the main functional groups observed are in the band region of 3562-3320 cm^{-1} that corresponds to the group of phenols (Attributed to the stretch of O-H); for a wavelength of 1745-1715 cm^{-1} corresponds saturated aliphatic ketone, with respect to a wavelength between 1440-1325 cm^{-1} is found the saturated aliphatics, between a wavelength of 1085-1030 cm^{-1} corresponds to saturated primary alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007) .

(C)PL9, the main functional groups are observed at the wavelength of 3562-3320 cm^{-1} corresponding to the groups of the phenols (attribed to the stretch of O-H) and 3200-2500 cm^{-1}

(attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds); for a wavelength of 1745-1715 cm^{-1} corresponds saturated aliphatic ketone, with respect to a wavelength between 1440-1325 cm^{-1} is found the saturated aliphatics, for the band region between 1350-1200 cm^{-1} are the primary and secondary alcohols, for the wavelength of 1085-1030 cm^{-1} is the saturated primary alcohol (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(D) PL20, the main groups are observed in the wavelength of 3300-3150 cm^{-1} corresponds to Oxime (result of condensation of hydroxylamine with an aldehyde or ketone), for the band region of 3200-2500 cm^{-1} corresponds to the phenolic group (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), for wavelength 1625-1590 cm^{-1} corresponds to the aromatics group (benzene and aromatics), for the band of 1085-1030 cm^{-1} are the saturated primary alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(E) PL22E, in the functional groups observed in the wavelength between 3562-3322 cm^{-1} corresponds to the phenolic groups (attributed to the stretch of O-H) and also for 3200-2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), in the wavelength of 1655-1635 cm^{-1} of β-keto ester (oenolic form), for the region of band between 1085-1030 cm^{-1} are the saturated primary alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(F) PL22, the main functional groups are observed at the wavenumber of 3562-3320 cm^{-1} corresponding to phenolic groups (attributed to the stretch of O-H) and 3200-2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), for the wavelength 1615-1575 cm^{-1} are the pyridine and its derivatives, in the wavelength 1085-1030 cm^{-1} are the saturated primary alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(G) PL23, in the functional groups observed in the band region of 3562-3322 cm^{-1} corresponds to phenolic groups (attributed to the stretch of O-H) and 3200-2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), for 1660-1625 cm^{-1} are nitrates, for the wavelength between 1390-1360 cm^{-1} are RCH_2-NO_2 and R_2CH-NO_2 (ring), for the band region between 1085-1030 cm^{-1} are saturated primary alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(H) PL24, the main functional groups are observed at the wavenumber of 3562-3320 cm^{-1} corresponding to phenolic groups (attributed to the stretch of O-H) and 3200-2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), for the band region 1645-1544 cm^{-1} to the aromatic group (attributed to C-C stretch of aromatic), for the wavelength 1390-1360 cm^{-1} belong RCH_2-NO_2 and R_2CH-NO_2 (ring), in the band region 1410-1310 cm^{-1} are the tertiary alcohols, in 1085-1030 cm^{-1} are the primary saturated alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(I) PL31, in the functional groups observed at the wavelength of 3590-3200 cm^{-1} are the compounds R-OH, in the band region of 3562-3322 cm^{-1} corresponds to phenolic groups (attributed to the stretch of O-H) and 3200-2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), for the wavelength between 1410-1544 cm^{-1} are the tertiary alcohols, for the band region of 1125-1085 cm^{-1} are the secondary saturated alcohols, for the wavelength of 1085-1030 cm^{-1} are the primary saturated alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(J) PL50, the main functional groups are observed that at the wavenumber of 3562-3320 cm^{-1} corresponding to phenolic groups (attributed to the stretch of O-H) and 3200-2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), for aromatic groups it is in the band 1645-1544 cm^{-1} (attributed to C-C stretch of aromatic ring), for the wavelength of 1085-1030 cm^{-1} are the primary saturated alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(K) PL3501, in the functional groups observed at the wavelength of 3620-3590 cm^{-1} are the compounds Ar-OH (phenols diluted in solution), in the band region of 3562-3322 cm^{-1} corresponds to phenolic groups (attributed to the stretch of O-H) and 3200-2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), in the wavelength of 1745-1715 cm^{-1} corresponds saturated aliphatic ketone (diluted solution of little polar solvent), in the region of band between 1410-1310 cm^{-1} are the tertiary alcohols, in the region of band 1085-1030 cm^{-1} belong to the primary saturated alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

(L) PL3824, the main functional groups are observed that at the wavenumber of 3562-3320 cm^{-1} corresponding to phenolic groups (attributed to the stretch of O-H) and 3200-2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), for aromatic groups it is in the band 1645-1544 cm^{-1} (attributed to C-C stretch of aromatic ring),

for the wavelength of 1085-1030 cm^{-1} are the primary saturated alcohols (BATISTA *et al*., 2016; SILVERSTEIN; WEBSTER; KIEMLE, 2005; BARBOSA, 2007).

According to the results obtained, which have been more frequently present in CPH and in products fermented with *Pleurotus* strains, there have been mainly three stripes:

For the wavelength 3562 - 3322 cm corresponding to phenolic groups (attributed to the O-H section), according to Rodriguez *et al*. (1979) for the study of the region 4000 to 3000 cm^{-1} of the ternary solutions phenol benzene tetrachloride, shows the appearance of the band ʊ OH of the free OH vibrator, which defines an isobiestic point, where the total absorvance of the sample does not change before a chemical reaction or physical change, where the absorvance remains stable before a probable physical or chemical change.

For the wavelength 3200 – 2500 cm^{-1} (attributed to phenolic groups in the ortho position with intramolecular hydrogen bonds), corresponds to the functional group of carboxylic acid OH stretch.

For the wavelength of 1085 - 1030 cm^{-1} belong to the primary saturated alcohols, and comprises the deformation of the structures of CH-O and C-C, due to the degradation of *Pleurotus* with respect to CPH (GLORIA *et al.,* 2016).

5.2.1.2. Determination of sugars, alcohols and acids by HPLC of cocoa pod husk and CPH fermented by different strains of *Pleurotus*

Solvents are classified as non-polar and polar (aproticos and proticos), these can be differentiated from each other, by their physical properties as: entalpia of vaporization, dielectric constant, refractive index, boiling point, among others (MOITY *et al*., 2014); also by the interrelation between solute and solvent, as occurs in non-specific interactions (Vander Waals Forces and ionic/dipolar forces) and specific interactions (hydrogen bond donor and/or hydrogen bond acceptor, electron pair donor/acceptor and solvo-phobic interactions) (REICHARDT; WELTON, 2011; MOITY *et al.*, 2014). As well as between the existing interrelation of the degree of polarity and solubilization of the solute with the solvent (REICHARDT; WELTON, 2011).

In the determination of alcohols, sugars and acids by using HPLC, three solvents (water, absolute ethanol and LPG) were used to extract components of the Para cocoa pod husk fermented with different strains of *Pleurotus* (PL9, PL20, PL22, PL22E, PL23, PL24, PL31,

PL50, PL3501 and PL3824), and the following results were obtained for the different fermented ones:

In the Table 29 results of the extraction with water of organic compounds of CPH and fermented CPH identified with HPLC are presented.

TABLE 29 - ORGANIC COMPOUNDS OF CPH AND FERMENTED CPH IDENTIFIED BY HPLC EXTRACTED WITH WATER.

Fermented	Citric acid	Glucose	Succinic acid	Propionic acid	Fucose	Methanol
Product	g/100g of extract					
PL9	1.63					
PL20						
PL22			0.11			
PL22E						0.29
PL23						
PL24						
PL31						
PL50	0.59					
PL3501		0.86				
PL3824				0.64	0.1	

Fermented products in white space, values not detected.
Source: the author (2018).

In Table 29, water was used as solvent for the extraction of organic compounds (sugars, alcohols and acids), where citric acid was extracted being higher in the fermented product PL9 than PL50; in this case, the citric acid extracted in the samples is less than that reported by Schneider et al. (2014) which was from 6.1 to 38.3 g of citric acid/100g of extract. In the product fermented with PL3501 was extracted small amount of glucose, which failed to be fermented in the process, unlike other fermented products, with respect to the glucose obtained was somewhat less than 1.1 g of glucose/100g of extract (BONVEHI; JORDÂ, 1998). For the product fermented with PL22 succinic acid was obtained and for PL22E methanol was obtained; for the fermentation with PL3824 propionic acid and fucose were extracted, small quantities of these compounds were extracted by the interaction of solute (fermented products) and polar solvent (water).

In the Table 30, results of the extraction with ethanol of organic compounds of CPH and fermented CPH identified with HPLC are presented.

TABLE 30 - ORGANIC COMPOUNDS OF CPH AND FERMENTED CPH IDENTIFIED BY HPLC EXTRACTED WITH ETHANOL.

Fermented Product	Citric acid	Glucose	Succinic acid	Lactic acid	Acetic acid	Propionic acid	Xylose	Fucose
	g/100g of extract							
PL9	1	1.336				0.208		
PL20	0.142	0.046				0.145		0.369
PL22	0.208	0.049	0.03		0.157	0.132		0.08
PL22E	0.791	0.783		0.044		0.115		
PL23	0.256	0.033	0.024			0.1		
PL24	0.265	0.065				0.119		
PL31		3.877		0.755				
PL50	0.01	0.019						0.001
PL3501	0.009	0.012					0.005	
PL3824					0.57	0.38		

Fermented products in white space, values not detected.
Source: the author (2018).

In Table 30, absolute ethanol was used to extract fermented compounds (sugars, alcohols and acids); this is due to the polarity of the solvent and the solubility of the solute, between them there must be interaction to be able to extract and be extracted; the citric acid values (PL9, PL20, PL22, PL22E, PL23, PL24, PL50 and PL3501) obtained are lower than those reported by Schneider et al. (2014) that was from 6.1 to 38.3 g / 100 g of extract; with respect to obtaining glucose there are values that exceed those reported by (BONVEHI; JORDÂ, 1998), this happens with fermented products with the exception of PL3824. Succinic acid has been obtained for fermented PL22 and PL23 products; lactic acid has also been obtained in fermented PL22E and PL31 products. As well as propionic acid in the fermented products (except for PL31, PL50 and PL3501), xylose (PL3501) and Fucose (PL20, PL22 and PL50) were found.

In the Table 31, results of the extraction with LPG of organic compounds of CPH and fermented CPH identified with HPLC.

TABLE 31 - ORGANIC COMPOUNDS OF CPH AND FERMENTED CPH IDENTIFIED BY HPLC EXTRACTED WITH LPG.

Fermented Product	Citric acid	Glucose	Fructose	Succinic acid	Lactic acid	Acetic acid	Propionic acid	Xylose	Fucose	Methanol
	g/100g of extract									
PL9										
PL20				0.016		0.057	0.181	0.076	0.073	
PL22		2.404		0.015	0.038		0.108		0.141	
PL22E						0.104	0.121	0.1		
PL23				0.036		0.04	0.07			
PL24		4.975			0.038		0.089	0.634	0.074	
PL31	0.146	0.115	0.338							0.607
PL50	0.01						0.006	0.096	0.093	
PL3501				0.018		0.199			0.075	
PL3824	9.802				2.067	0.694				

Fermented products in white space, values not detected.
Source: the author (2018).

In Table 31, for the extraction of sugars, alcohols and acids, LPG was used as solvent, which is apolar, unlike polar water and ethanol. For citric acid, the result of PL3824 is within that reported by Schneider *et al*. (2014), and in the extraction of glucose has given data higher than those reported by Bonvehi and Jordâ (1998) in PL22 and PL24, with respect to fructose in PL31 the result is lower than that reported by Bonvehi and Jordâ (1998)) of 0.56 g / 100 of extract.

5.2.1.3. Determination of antioxidants, phenolic compounds and alkaloids by HPLC of cocoa pod husk and CPH fermented by different strains of *Pleurotus*

Basidiomycetes are macro-fungi that produce a wide variety of natural products with great pharmaceutical and industrial value (from components with antitumor and immunological activity to antimicrobial, antifungal agents, antivirals, cytostatics, enzymes, growth regulators and aromas) (BRIZUELA *et al*., 1998).

a) Results of the determination of antioxidants and phenolic compounds

In the determination of antioxidants and phenolic compounds by the use of HPLC, absolute ethanol was used to extract the components of the cocoa pod husk fermented with different strains of *Pleurotus* (PL9, PL20, PL22, PL22E, PL23, PL24, PL31, PL50, PL3501 and PL3824), and the following results were obtained for the different fermented products ones:

With this method the following results were obtained for the fermented products:

- For PL9 was found 5.*50* (mg/100 g extract) of quercetin, this bioactive compound has important action as antioxidant, anti-inflammatory, antithrombotic and anti-cancer (JIN *et al*., 2004).
- For PL22 was obtained 4.47 (mg/100 g extract) of dihydrokaempferol, being an important biological precursor of flavonoids (phenolic compounds); dihydroquercetin and dihydrokaempferol are effective precursors of kaenompferol, quercetin and cyanidine, as well as Immediate precursors for catechins (ZAPROMETOV; GRISEBACH, 1973).
- PL22E yielded 2.39 (mg/100 g extract) of catechin which is below the values obtained in cocoa powder, these values were 45.6 and 168.1 mg/100 g (SHUMOW; BODOR, 2011); catechin is a secondary metabolite distributed ubiquitously in plants (KINGHORN *et al*., 2012) its bioactive compound is beneficial to human health because it plays the role of antioxidant (KODAMA *et al*., 2010), antibacterial (RAWDKUEN *et al*., 2012), anti-aging (SAUL *et al*., 2009), liver protector (SHAW *et al*., 1982).
- 12.39 (mg/100 g extract) was obtained from trans-cinnamic acid for PL9, it can be used in fragrances for perfumes and in flavors for processed foods (GARBE, 2012).
- PL50 yielded 9.62 (mg/100 g extract) of epicatechin, 6.62 (mg/100 g extract) of Dihydrokaempferol and 1.69 (mg/100 g extract) of catechin; this epicatechin value obtained is below that found in cocoa powder, these values were 25.4 and 331.2 (mg / 100 g) (SHUMOW; BODOR, 2011).
- For PL3501 was obtained 1.6 (mg/100 g extract) of catechin which is below the values obtained in cocoa powder, these values were 45.6 and 168.1 (mg/100 g) (SHUMOW; BODOR, 2011); this flavonoid is also cardioprotective (EL-AZIZ *et al.*, 2012), renoprotective (CHENNASAMUDRAM *et al*., 2012) and neuroprotective (LIANG *et al.,* 2014, RUAN *et al*., 2011).

b) Results of the determination of antioxidants and alkaloids

In the Table 32, antioxidants and alkaloids found in the cocoa pod husk extracted by the methods of maceration and soxhlet were presented.

TABLE 32 - ANTIOXIDANTS AND ALKALOIDS IN THE COCOA POD HUSK OF BAHÍA AND PARÁ.

Cocoa Pod Husk	Theobromine*	Theophylline**	Quercetin**	Rutin*
	(mg/100g extract)			
Bahía	1.067	12.195	4.182	
Pará	0.572	5.228	5.228	5.015

*Maceration method, **Soxhlet method.
Source: the autor (2018).

In the Table 32, it is shown that theobromine values of 1.067 (mg/100g extract, Bahía CPH) and 0.572 (mg/100g extract, Pará CPH) were found, with the value of Bahia CPH being similar and the value of Pará CPH being less than 1.06 mg of theobromine /100g of extract reported by Bonvehí and Jordàb (1998). For theophylline values of 12.195 (mg/100g extract, CPH Bahia) and 5.228 (mg/100g extract, CPH Pará) were found; theobromine and theophylline are alkaloids found in plants and beverages, and are frequently used as smooth muscle relaxants, (MEYER *et al*., 1996; SRDJENOVIC *et al*., 2008).

The values obtained from quercetin were 4.182 (mg/100g extract, Bahía CPH) and 5.228 (mg/100g extract, Pará CPH). According to studies quercetin may help men with chronic prostatitis, also to reduce the ailment of interstitial cystitis in women and men; quercetin has probable positive effects in combating or helping to prevent cancer (D'MELLO *et al*., 2011; HÄKKINEN *et al*., 1999; SMITH *et al*., 2016). This flavonol (antioxidant) has important reactions in improvement and prevention of coronary ailments and diseases, (HÄKKINEN *et al*., 1999; SMITH *et al*., 2016).

The value obtained from rutin was 5.015 (mg/100g extract) for Para CPH, Rutin is a flavonoid with high antioxidant power, it has pharmacological effects (vasoconstrictive, spasmolytic, antiviral, antitumor activity, positive inotropic among others) (KREFT *et al.*, 2002).

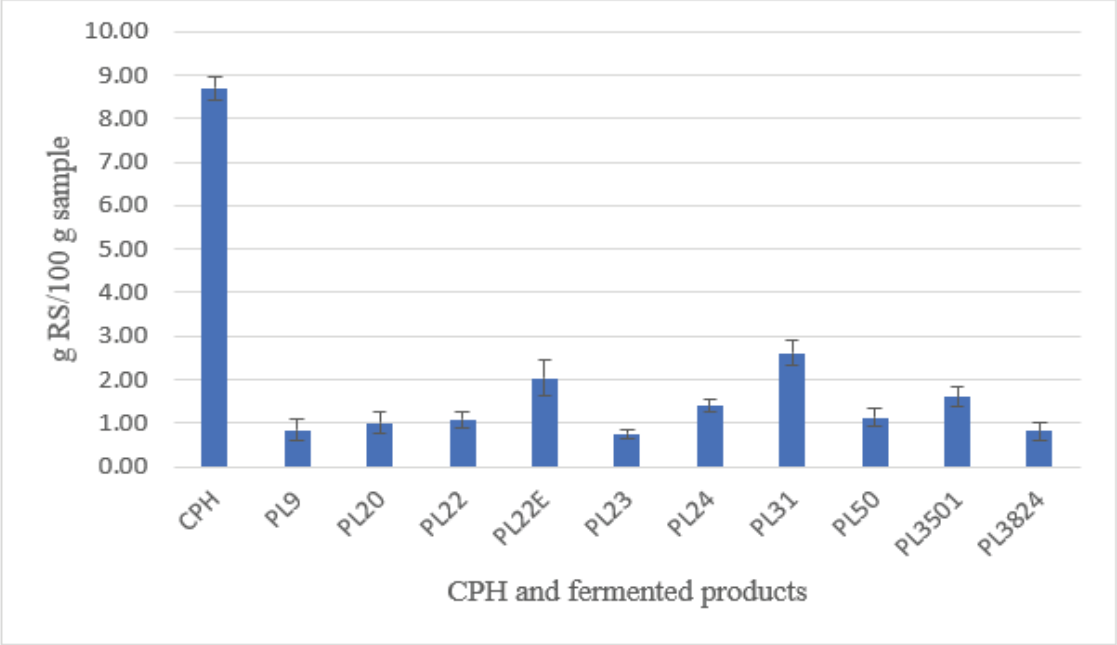
5.2.2. Selection N° 1 of *Pleurotus* strains

The selection of the best strain of *Pleurotus* that acts in the fermentation of solid state in the cocoa pod husk for 30 days, the following criteria were considered basically: the consumption of reducing sugar, the values of the degradation of the total content of polyphenols and its antioxidant activity, as well as the morphological growth during fermentation (in dry matter).

a) Reducing sugar

The Figure 20 is presented, in where the comparison of the consumption of reducing sugars between the fermented products is visualized.

FIGURE 20 - COMPARISON OF REDUCING SUGARS OF COCOA POD HUSK AND CPH FERMENTED BY DIFFERENT STRAINS OF *Pleurotus* (IN DRY MATTER).



R.S.: Reducing sugar

C.P.H.: Cocoa Pod Husk

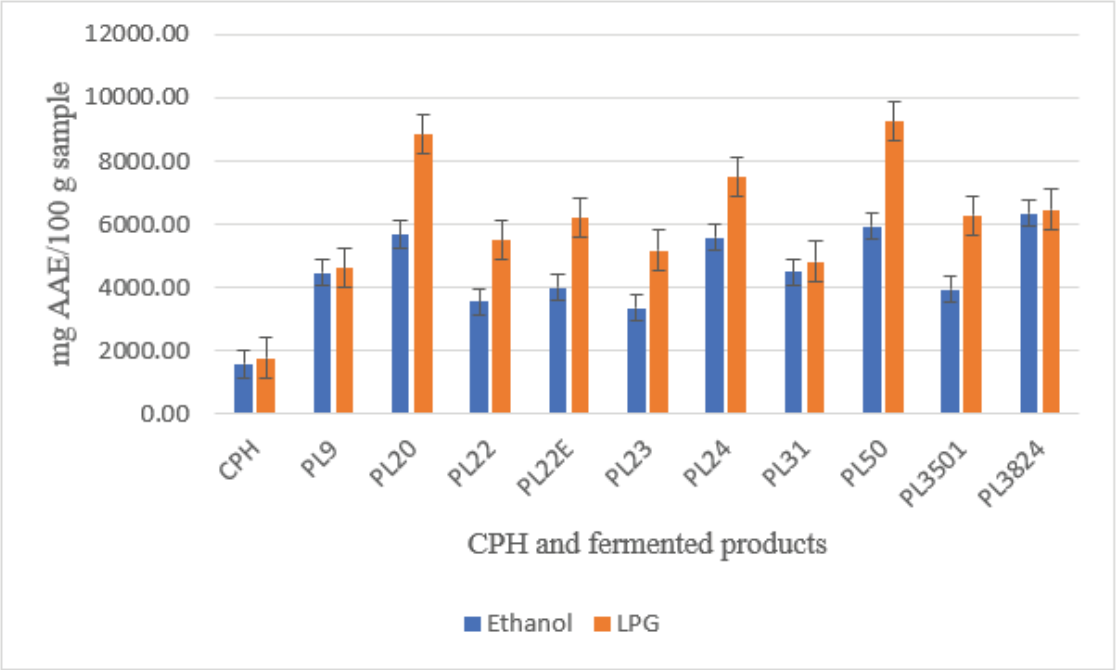
Source: the author (2018).

Ten coded strains of *Pleurotus* were evaluated in cocoa husks from the state of Pará, and compared with the unfermented cocoa husk. The sugar content was determined in each solid fermentation, and the strains that were consumed the most were PL9, PL20, PL22, PL23 and PL3824. The fungi begin their growth after inoculation; therefore, they require a carbon source found in the available sugars of the substrate, which is generally 1 to 2% by dry weight, and then they continue with the polysaccharides and lignin that are part of the cellular wall (URBEN, 2017).

b) Antioxidant activity

Figure 21 shows the results obtained in relation to the degradation of the antioxidant activity of the extracts of the fermented, extracted with LPG and absolute ethanol, in the fermentation process.

FIGURE 21 - COMPARISON OF THE DEGRADATION OF ANTIOXIDANT ACTIVITY OF FERMENTED COCOA POD HUSK EXTRACTS EXTRACTED WITH LPG AND ABSOLUTE ETHANOL (IN DRY MATTER).



A.A.E.: Ascorbic Acid Equivalent

C.P.H.: Cocoa Pod Husk

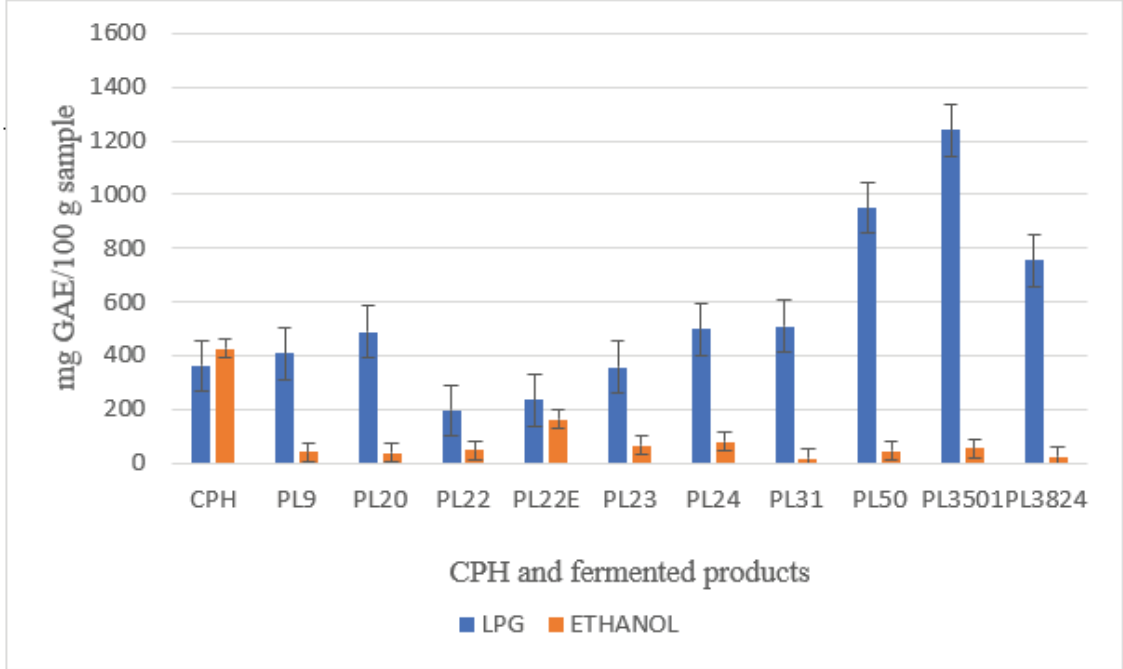
Source: the author (2018).

The antioxidant activity was analyzed in the fermentation process of *Pleurotus* and cocoa husk, extracted with LPG and absolute ethanol, the fermented product being extracted with LPG with higher values of antioxidant activity in PL9, PL24, PL31, and PL3824; the antioxidant capacity values for fermented products extracted with absolute ethanol that stood out the most were PL20, PL24, PL50 and Pl3824. According to Othman et al. (2007), found no correlation between the antioxidant activity and the total polyphenol content, and indicated that a high capacity of inhibition in the DPPH radical could not be due to phenolic compounds in the cocoa extracts.

c) Total polyphenols content

In the Figure 22, shows the results obtained in relation to the total polyphenols of the extracts in the fermented samples, extracted with LPG and absolute ethanol are shown.

FIGURE 22 - COMPARISON OF THE DEGRADATION OF THE TOTAL POLYPHENOLS OF THE EXTRACTS OF FERMENTED COCOA, EXTRACTED WITH LPG AND ABSOLUTE ETHANOL (IN DRY MATTER).



G.A.E.: Gallic Acid Equivalent

C.P.H.: Cocoa Pod Husk

Source: the author (2018).

The total polyphenols degraded in the fermentation process of *Pleurotus* fermented products with cocoa pod husk were analyzed, the extract was extracted with LPG and absolute ethanol, the fermented products that had more total polyphenols with higher values were extracted with LPG in PL20, PL24, PL31, PL50, PL3501 and PL3824; while for the products extracted with absolute ethanol, the one with the highest quantity of total polyphenols was for the cocoa pod husk. According to Hagerman et al. (1998), that by condensing and oxidizing simple total polyphenols into complex tannins, the content of total polyphenols can be reduced and the antioxidant capacity increased.

d) Morphological growth of the *Pleurotus* strains during fermentation

Through the use of photographs, the morphological growth of the *Pleurotus* strains in the cocoa pod husk during fermentation in the solid state is visualized. In the Figure 23, the fermented products of the cocoa pod husk (CPH) with the strains of Pleurotus studied are shown.

FIGURE 23 - FERMENTED PRODUCTS OF COCOA POD HUSK IN SOLID STATE.

PL 9

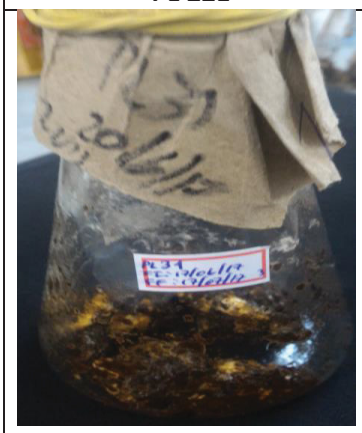
PL 20

PL 22

PL 22E

PL 23

PL 24



PL 31

PL 50

PL 3501

PL 3824

The strains that had better and faster growth of mycelium on the cocoa pod husk were PL 9, PL 20, PL 22E, PL 23, PL 50.

e) Summary of selection results 1

In the Table 33 shows the results obtained from total polyphenols, antioxidant activity, remaining reducing sugars and morphological (qualitative) growth in the fermented cocoa pod husk by different strains. It is observed that LPG extraction resulted in higher concentration of total polyphenols and compounds with antioxidant activity than ethanol extraction (in dry matter).

TABLE 33 - SUMMARY OF EVALUATION INDICATORS TO CHOOSE THE BEST WORKING STRAINS IN THE FERMENTED COCOA POD HUSK.

	Total polyphenols content		Antioxidant Act. (VCEAC)		Reducing sugar	Morphological
FERMENTED	(mg GAE/100 g sample)		(mg AAE/100 g sample)		(g RS/100g sample)	growth*
PRODUCTS	LPG	Ethanol	LPG	Ethanol	Water	
PL9	407.34	40.63	4617.4	4463.60	0.84	(++++)
PL20	487.88	38.05	8839.29	5685.71	1.00	(+++++)
PL22	194.71	47.39	5500.8	3560.39	1.08	(++++)
PL22E	233.94	162.54	6205.48	4002.34	2.04	(+++++)
PL23	356.06	65.57	5187.33	3360.42	0.75	(++++)
PL24	497.00	78.69	7497.48	5587.40	1.40	(+++)
PL31	508.33	15.84	4835.74	4495.87	2.62	(++)
PL50	950.05	44.56	9255.96	5945.54	1.13	(+++)
PL3501	1238.97	53.55	6276.11	3948.72	1.61	(+)
PL3824	752.98	22.34	6472.55	6345.25	0.81	(+)

* Morphological growth (visual observation): () lower growth to higher growth (+++++)
GAE: Gallic Acid Equivalent
AAE: Ascorbic Acid Equivalent
RS: Reducing sugar
VCEAC: Vitamin C equivalent antioxidant capacity

Source: the author (2018).

In Table 33, the best results were selected obtaining the strains PL9, PL20, PL22, PL22E, PL23, PL24 and PL50 to continue with the present study; according to the values obtained in the process of fermentation in the degradation of the total polyphenols were PL3501, PL24, PL50, PL22E, PL23 among others; as well as for the antioxidant activity were PL50, PL20, PL3824, PL24, PL22E among others. For the remaining reducing sugars in the ferments that have the highest values are PL31, PL22E, PL3501, PL24, PL50 among others.

Morphological growth was assessed by visual observation (through photographs) during the fermentation process. The best results were obtained with PL9, PL20, PL22E, PL23 and PL50.

Regarding the first selection, it should be noted that 7 CPH fermented with Pleurotus strains had the best results among the 10 products evaluated. For example, for PL22E with respect to total polyphenols it had a good degrading action of 233.94 and 162.54 mg GAE/100 g (for LPG and ethanol extraction) compared to other results; for the case of antioxidant activity it has high values of 6205.48 and 4002.34 mg GAE/100 g (for LPG and ethanol extraction), which does not necessarily have to have a correlation between antioxidant activity and total polyphenols (not all antioxidants are polyphenols); in reducing sugars, 1.08 g RS/100 g were obtained having a reserve of reducing sugar to be used as a carbon source for their metabolic processes, and had a very good performance in their morphological growth.

5.3. PECTIN CONTENT OF THE COCOA POD HUSK OF PARÁ AND THE EVALUATION OF THE ENZYMATIC ACTIVITY OF PECTINASE (POLYGALACTURONASE)

The ability of the polygalacturonase enzyme to degrade pectins will depend on the percentage of pectin methylation, the size of the methylated chain, and the position of the substitute in the galacturonic acid residue and the affinity with the active site (BONNIN *et al*., 2003).

a)pH

After having selected the 7 fermented products of the Pará cocoa pod husk and the *Pleurotus* strains (PL9, PL20, PL22, PL22E, PL22E, PL22E, PL23, PL24 and PL50), the fermentation was carried out in solid state. The evolution of the pH in the fermentation process of the different *Pleurotus* strains is presented in Table 34.

TABLE 34 - EVOLUTION OF THE pH IN THE FERMENTATIVE PROCESS OF *Pleurotus* STRAINS.

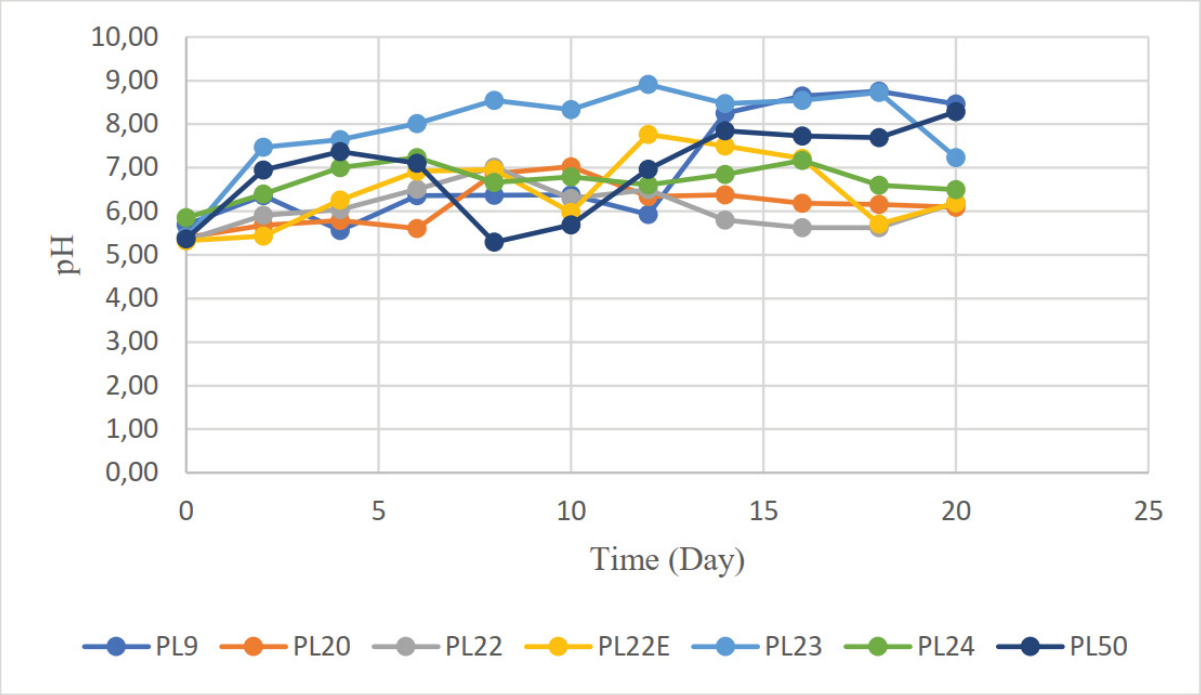
Day	pH						
	PL9	**PL20**	**PL22**	**PL22E**	**PL23**	**PL24**	**PL50**
0	5.69 ± 0.045	5.40 ± 0.014	5.34 ± 0.007	5.33 ± 0.191	5.40 ± 0.141	5.85 ± 0.071	5.37 ± 0.021
2	6.36 ± 0.601	5.68 ± 0.184	5.91 ± 0.396	5.43 ± 0.198	7.47 ± 0.184	6.40 ± 0.120	6.95 ± 0.354
4	5.56 ± 0.255	5.79 ± 0.120	6.04 ± 0.870	6.26 ± 0.198	7.65 ± 0.219	7.01 ± 0.035	7.37 ± 0.453
6	6.36 ± 0.318	5.61 ± 0.007	6.51 ± 0.219	6.92 ± 0.049	8.02 ± 0.573	7.24 ± 0.955	7.11 ± 0.629
8	6.37 ± 0.099	6.86 ± 0.160	7.02 ± 0.488	6.96 ± 0.007	8.55 ± 0.156	6.67 ± 0.304	5.29 ± 0.057
10	6.38 ± 0.198	7.03 ± 0.761	6.30 ± 0.035	5.98 ± 0.276	8.33 ± 0.311	6.79 ± 0.438	5.69 ± 0.141
12	5.93 ± 0.057	6.35 ± 0.134	6.50 ± 0.332	7.77 ± 0.049	8.91 ± 0.325	6.61 ± 0.078	6.97 ± 0.191
14	8.25 ± 0.311	6.38 ± 0.198	5.80 ± 0.049	7.50 ± 0.516	8.48 ± 0.049	6.85 ± 0.651	7.85 ± 0.141
16	8.65 ± 0.247	6.18 ± 0.042	5.62 ± 0.170	7.22 ± 0.389	8.55 ± 0.148	7.17 ± 0.856	7.73 ± 0.255
18	8.76 ± 0.148	6.16 ± 0.049	5.65 ± 0.354	5.71 ± 0.028	8.73 ± 0.085	6.60 ± 0.240	7.70 ± 0.955
20	8.47 ± 0.092	6.10 ± 0.106	6.20 ± 0.354	6.20 ± 0.431	7.24 ± 0.346	6.50 ± 0.071	8.29 ± 0.120

Source: the author (2018).

In the Table 34, it is observed that the fermentation processes start with an average pH of 5,48 on day zero, in most cases there are variations in pH along the days; the fermentation of PL9 starts with a pH of 5.69 ± 0.045, has variations on the days of fermentation and ends with a pH of 8.47 ± 0.092; as well as in the other fermented products, there is a variation of the pH in the metabolic process through the days, conserving its own characteristics of the fluctuation of the pH in each one of them. This variation in pH is due to the metabolical process given in the fermentation process, where substrate is consumed and metabolites are produced by chemical and enzymatic reactions, being the pH an important variable in this process as well as other factors (temperature, humidity, water activity, availability and concentration of the substrate, aeration, particle size, incubation time, among others) in solid fermentation (THOMAS *et al.,* 2013); the change in pH affects the ionization status of the different components of the system, and how proteins have several ionizable sites that affect enzyme activity (JOSHI *et al*., 2015).

In the Figure 24, it is shown the variation of the pH in the fermentative process of the different strains of *Pleurotus* in the course of the days analyzed.

FIGURE 24 - VARIATION OF pH IN THE FERMENTATION PROCESS.



Source: the author (2018).

In the Figure 24, the variation of pH is observed, it affects the enzymatic activity that interferes in the degree of ionization of the constituent amino acids of the protein and can cause denaturation (VITOLO *et al*.,2015). For optimal solid fermentation for some *Pleurotus* species, the pH should be between 5.5 - 7.0 (PINEDA-INSUASTI *et al*., 2014); although the values of some samples are above those stated values, it may condition the metabolical process they are subjected to in fermentation and to the differente *Pleurotus* species.

The enzyme polygalacturonase (pectinase) has activity and acts at both acid and alkaline levels (JOSHI *et al*., 2015); also the selectivity and the yield of the enzyme decrease at pH above 7. A pH of 5 it has high specificity and decreases under very acidic and alkaline conditions that can denature the structure of the enzyme (AMID *et al.,* 2016).

b)Reducing sugar

In the Table 35, the process of consumption of reducing sugar in the various fermentative processes of the *Pleurotus* strains is presented (in dry matter).

TABLE 35 - CONSUMPTION OF REDUCING SUGAR IN THE FERMENTATIVE PROCESS OF THE *Pleurotus* STRAINS.

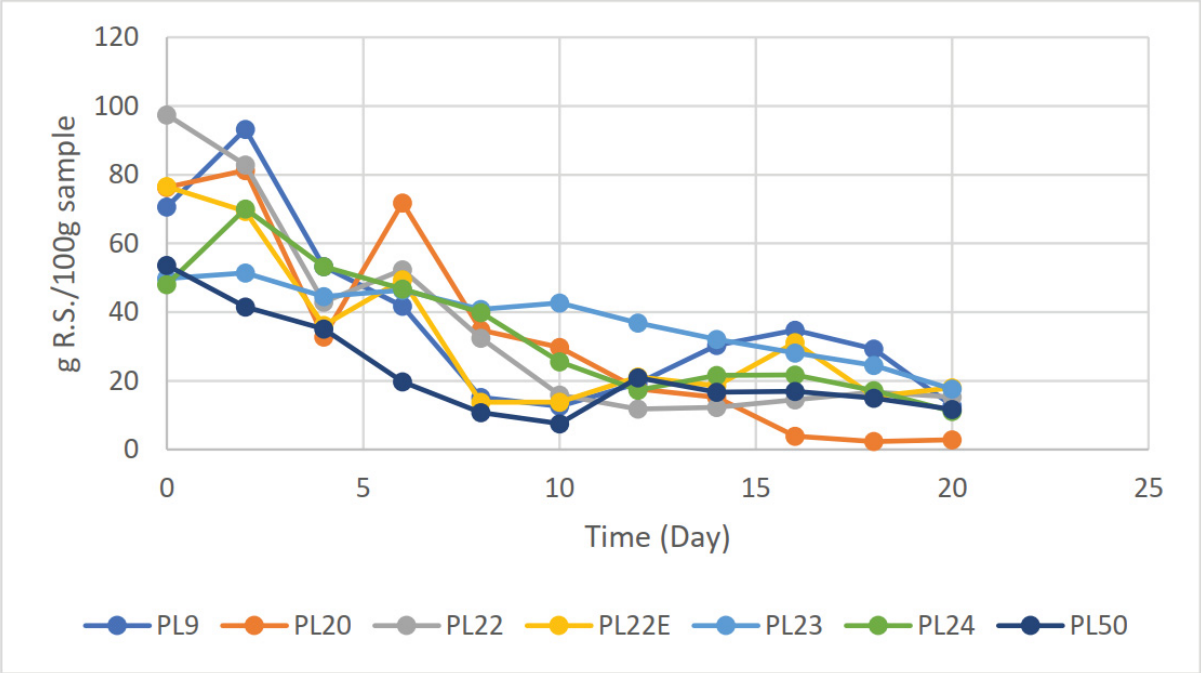
Day	Fermented products (g R.S./100 g sample)						
	PL9	**PL20**	**PL22**	**PL22E**	**PL23**	**PL24**	**PL50**
0	70.65 ± 0.599	76.30 ± 2.360	97.45 ± 0.632	76.52 ± 0.629	49.73 ± 0.431	48.01 ± 2.320	53.67 ± 0.787
2	93.24 ± 1.122	81.27 ± 3.447	82.88 ± 1.323	69.39 ± 1.139	51.40 ± 2.424	70.08 ± 4.270	41.52 ± 1.119
4	53.26 ± 0.970	32.72 ± 3.408	42.91 ± 1.981	36.10 ± 3.760	44.51 ± 4.835	53.32 ± 6.141	35.04 ± 2.098
6	41.72 ± 2.401	71.84 ± 3.396	52.28 ± 0.452	49.48 ± 5.125	46.37 ± 0.447	46.78 ± 5.286	19.76 ± 1.650
8	15.17 ± 1.444	34.71 ± 4.857	32.37 ± 1.026	13.77 ± 1.632	40.79 ± 3.685	39.87 ± 2.394	10.77 ± 2.173
10	12.57 ± 0.401	29.73 ± 2.969	15.83 ± 5.308	13.81 ± 0.660	42.63 ± 6.592	25.57 ± 2.136	7.54 ± 2.795
12	19.21 ± 1.285	17.58 ± 0.000	11.77 ± 0.650	21.07 ± 5.147	36.90 ± 0.787	17.25 ± 1.029	20.88 ± 1.084
14	30.31 ± 1.292	15.15 ± 1.553	12.23 ± 0.281	18.56 ± 5.162	32.07 ± 3.034	21.63 ± 4.515	16.69 ± 0.868
16	34.74 ± 2.415	3.83 ± 2.380	14.51 ± 0.788	31.13 ± 0.467	28.11 ± 1.224	21.75 ± 2.092	16.93 ± 0.486
18	29.27 ± 1.496	2.31 ± 0.455	16.65 ± 0.534	15.40 ± 1.140	24.48 ± 1.653	17.18 ± 0.967	14.94 ± 0.487
20	12.77 ± 0.657	2.88 ± 0.273	15.35 ± 1.679	18.02 ± 1.126	17.58 ± 0.757	11.08 ± 0.454	11.72 ± 0.655

Source: the author (2018).

In the Table 35, the consumption of reducing sugars is visualized along in the fermentative processes, the consumption begins in the day zero and from the course of the days the amount of initial sugars is reducing. They are a source of carbon that *Pleurotus* needs to carry out its fermentative activity; the fungi are heterotrophic that require an external source of carbon for their growth (GALVAGNO; FORCHIASSIN, 2010), being for the *Pleurotus* a source of nutrient.s Agroindustrial residues like the cocoa pod husk are lignocellulosic materials and are degraded by this genus of fungus (PINEDA-INSUASTI *et al.*, 2014).

In the Figure 25, the consumption of reducing sugars by *Pleorotus* is shown, from day 0 to day 20.

FIGURE 25 - CONSUMPTION OF REDUCING SUGARS IN THE FERMENTATION PROCESS.



Source: the author (2018).

In the Figure 25, *Pleurotus* performs the consumption of CPH sugars as an energy source, and to metabolize in other products that happen from day zero to day 20; its degradation and its biotransformation into reducing sugars for consumption in the enzymatic kinetic process (QI *et al*., 2011).

c)Pectinase activity

In the Table 36, the enzymatic activity of polygaracturonase in the fermentative process is presented during the 20 days (in dry matter).

TABLE 36 - EVOLUTION OF THE PECTINASE (POLYGALACTURONASE) ACTIVITY IN THE FERMENTATIVE PROCESS OF THE *Pleurotus* STRAINS.

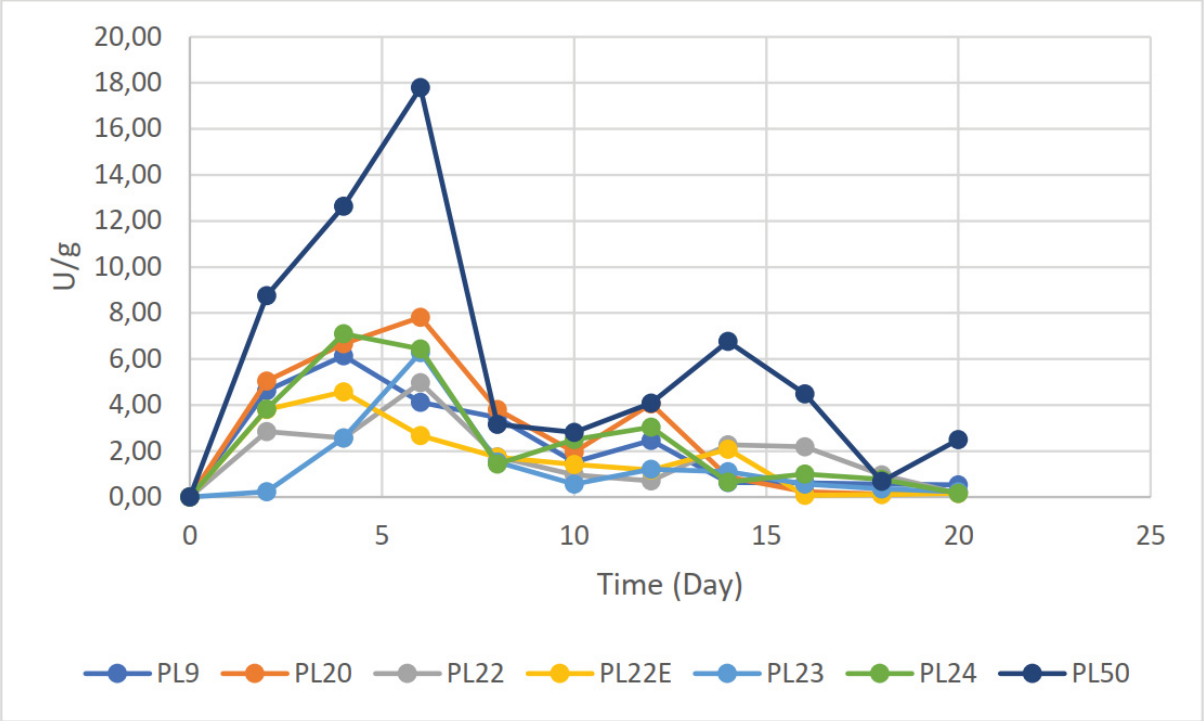
Day	Fermented products (U/g)						
	PL9	**PL20**	**PL22**	**PL22E**	**PL23**	**PL24**	**PL50**
0	7.37 ± 1.901	0.00 ± 0.000	31.81 ± 3.487	12.15 ± 0.899	3.28 ± 1.698	10.76 ± 3.486	14.15 ± 1.297
2	26.28 ± 3.008	29.69 ± 0.935	18.23 ± 1.366	19.03 ± 1.942	1.43 ± 1.008	25.28 ± 0.991	48.61 ± 3.963
4	**36.07 ± 2.783**	40.85 ± 4.254	18.51 ± 3.594	**26.75 ± 1.047**	18.54 ± 2.438	**49.11 ± 6.012**	73.90 ± 2.707
6	25.34 ± 1.682	**49.30 ± 4.219**	**30.66 ± 1.224**	17.11 ± 0.702	**45.10 ± 0.735**	42.79 ± 2.752	**112.52 ± 3.145**
8	21.85 ± 5.238	24.61 ± 4.552	12.91 ± 0.423	9.76 ± 0.509	10.60 ± 0.717	10.01 ± 3.830	20.39 ± 4.389
10	9.74 ± 4.493	14.20 ± 0.741	6.90 ± 0.556	8.61 ± 1.079	4.19 ± 2.792	15.70 ± 4.613	18.14 ± 3.029
12	16.03 ± 5.131	26.94 ± 1.815	4.24 ± 0.903	6.89 ± 0.645	8.24 ± 5.793	22.47 ± 1.219	26.81 ± 4.419
14	4.13 ± 2.004	6.25 ± 1.113	14.54 ± 0.663	13.30 ± 1.054	8.23 ± 1.625	3.98 ± 1.993	45.01 ± 1.147
16	4.06 ± 1.349	1.62 ± 1.069	12.38 ± 0.783	0.48 ± 0.367	4.23 ± 1.666	6.21 ± 0.633	29.80 ± 3.145
18	3.79 ± 1.344	0.83 ± 0.631	7.01 ± 4.087	0.76 ± 0.416	2.65 ± 1.167	5.78 ± 0.574	4.60 ± 1.565
20	3.50 ± 1.611	1.19 ± 0.917	1.01 ± 0.627	1.02 ± 0.824	1.49 ± 0.677	1.02 ± 0.826	16.53 ± 5.616

Source: the author (2018).

According to Table 36, the fermented products that presented the lowest productivity were for PL22 with 5.11 U/g.d (pH of 6.51 and on the sixth day) and the one with the highest productivity corresponded to PL50 with 18.75 U/g.d (pH of 7.11 and on the sixth day).

In the Figure 26, it is shown the development of the enzymatic activity of the polygaracturonase in the fermentative process.

FIGURE 26 - ENZYMATIC ACTIVITY OF PECTINASE (POLYGALACTURONASE) IN THE FERMENTATIVE PROCESS.



Source: the author (2018).

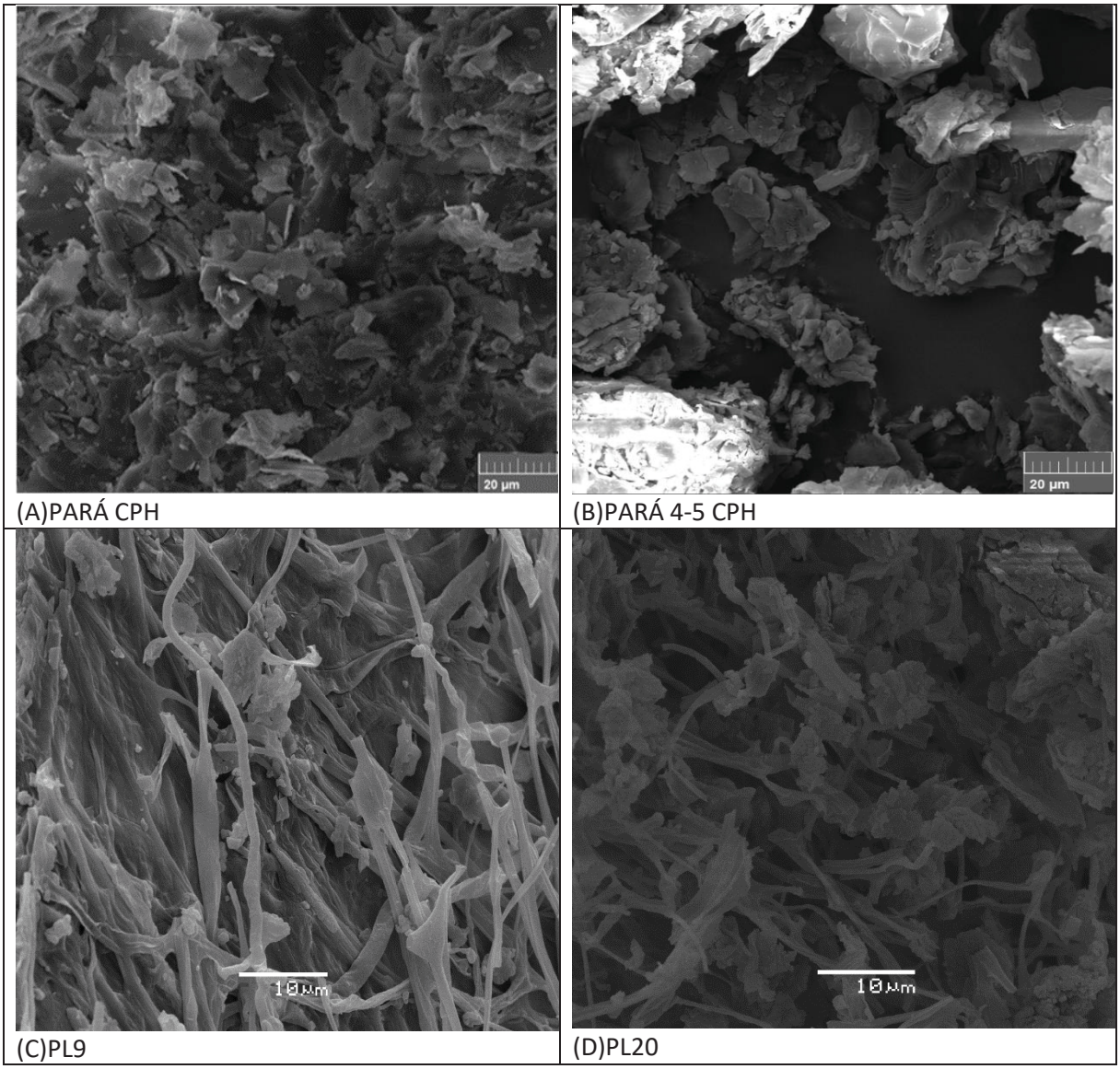
In the Figure 26, the enzymatic activity of polygalacturonase is observed along the days, where the maximum enzymatic activity in the fermented was 112.52 ± 3.145 U/g (6 days) for PL50, the variation of pHs was 5.56 ± 0.255 to 8.02 ± 0.573 and the amount of sugar used was 15.28 g R.S./100 g in the the fermentation varied between 4 to 6 days. The amount of polygalacturonase obtained in this research was lower than that obtained by the fungus *Moniliella sp*. (26 U/g) and greater with respect to *Penicillium sp.* (12 U/g) (MARTIN *et al*., 2004). The effect that pH has on the culture medium for the production of endo polygalacturonase (pectinase) by the fungus is very important, for pH values of 6 to 8 good results were obtained (BANU *et al*., 2010).

5.4. DEGRADATION OF LIGNIN

5.4.1. Morphology by scanning electronic microscopy (SEM) in Pará CPH and fermented CPH

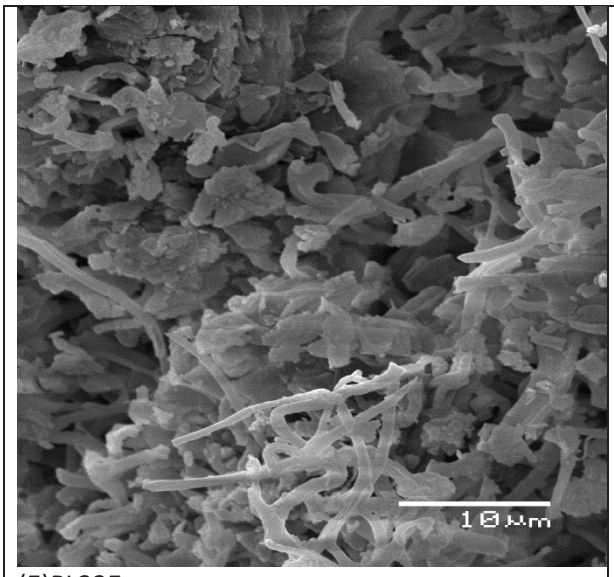
The biodegradation of the cocoa pod husk of Pará by the strains of *Pleurotus*, is due to a series of enzymes that act and compounds of low extracellular molar mass, because lignocellulosic materials must be depolymerized to simpler compounds that can be transported from the cell wall towards the metabolism of fungi (FERRAZ, 2010). Figure 27 shows the electron microscopy photographs of the CPH and its fermentations with the *Pleurotus* strains, where the morphological structure is visualized.

FIGURE 27- PARÁ CPH AND FERMENTED CPH TO 2000X BY ELECTRONIC MICROSCOPIC SEM.

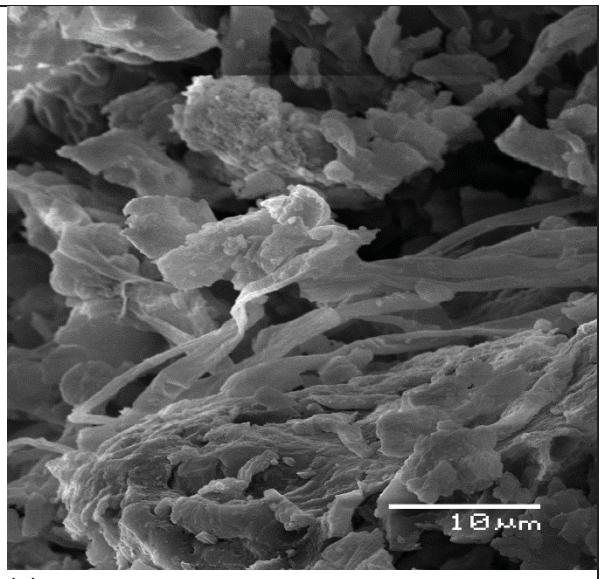


(A)PARÁ CPH (B)PARÁ 4-5 CPH

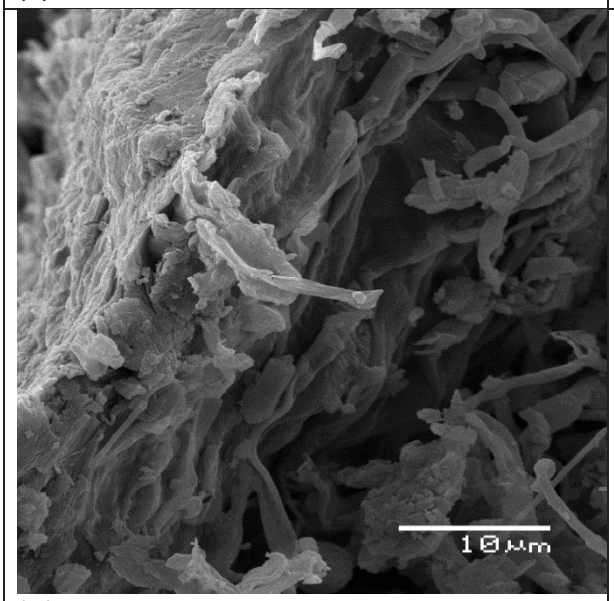
(C)PL9 (D)PL20



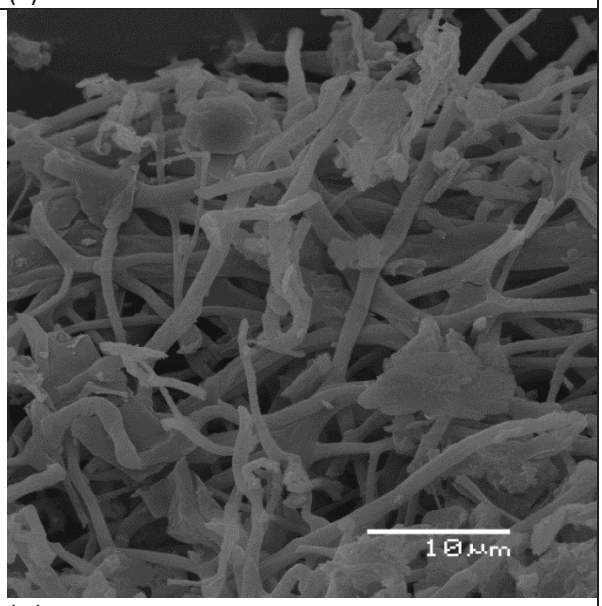
(E)PL22E



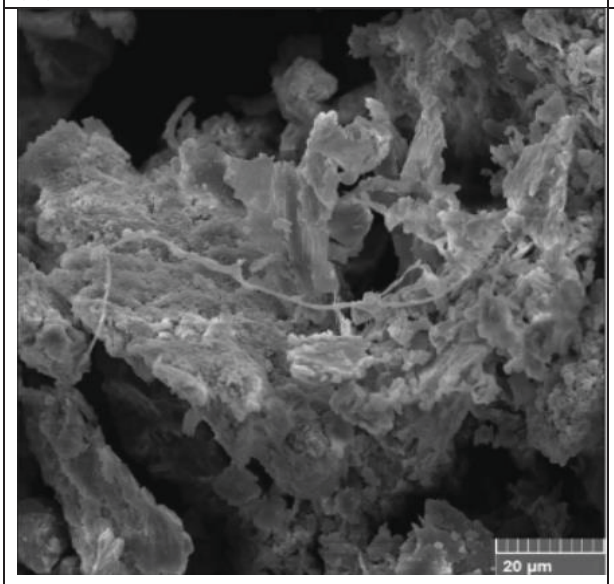
(F)PL22



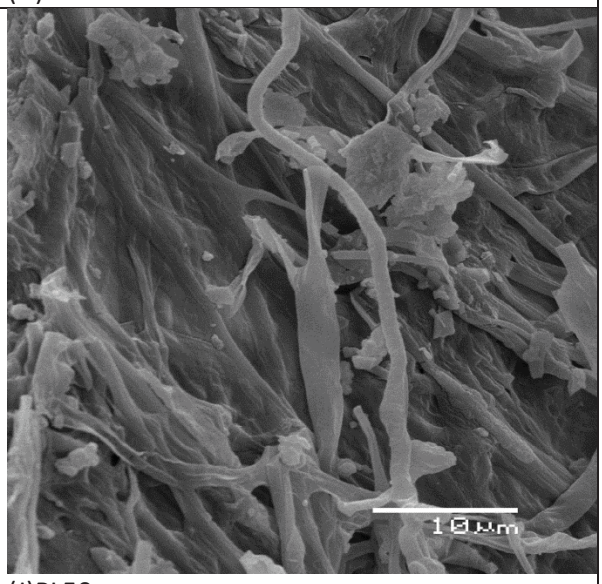
(G)PL23



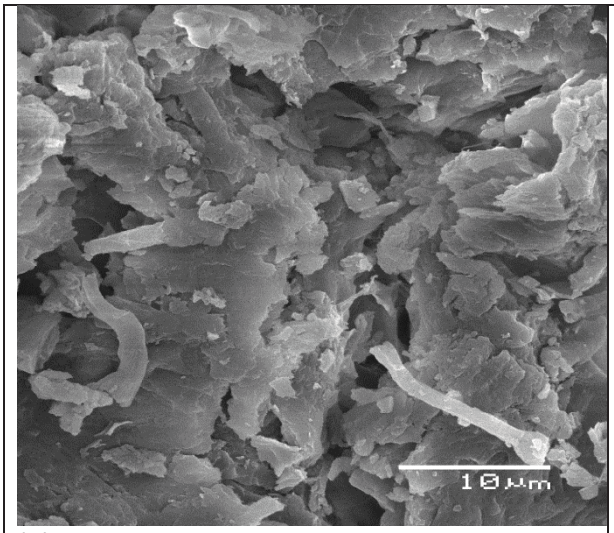
(H)PL24



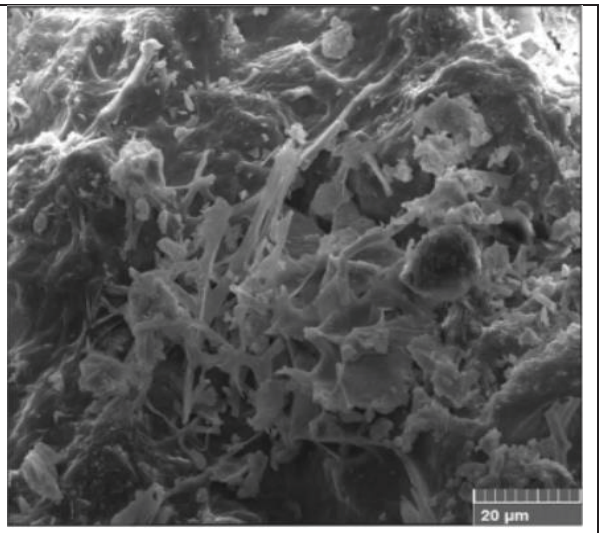
(I)PL31



(J)PL50



(K)PL3501



(L)PL3824

Source: the author (2018).

In Figure 27, the SEM photographs are observed (A) for Pará CPH (which has only suffered deterioration due to mechanical effect), (B) in Para 4-5 it is the CPH after undergoing the extraction by LPG without having fermented, this has already been explained previously it serves to compare with fermented ones; in other cases, there has been solid fermentation with *Pleurotus* for 30 days.

(C) For the fermented PL9, it is clearly observed that the lignocellulosic enzymes (one of them being Laccase) of the fungus, has efficiently degraded the lignocellulosic material, presenting a structure with possession of the hyphae of the fungus on the surface of the CPH.

(D) In the PL20, the contudence of the degradation of the enzymes on the lignocellulosic material is visualized in comparison with Pará CPH that has not undergone fermentation, like PL9, the hyphae of the fungi are consuming the substrate where they produced extracellular metabolites that degraded the cell wall.

(E) For the PL22E, where the degradation has been lower than in the previous the result of the enzymatic lysis is observed on the substrate, and the hyphae of the fungus are present in this activity.

(F) In PL22, as in the previous cases, the degradation of lignocellulosic material is due to the presence of enzymes that deteriorate and degrade CPH.

(G) For PL23, it has suffered degradation of the CPH cell wall, a process of incrustation has also been observed in the cell wall, progressively and irregularly degrading.

(H) In the PL24, there is degradation not in the measure of the first fermented observed, like the PL23, there is also a process of flaking on the fermented surface. In that photograph, the hyphae and fibers, which are clearer than fibers, can be seen.

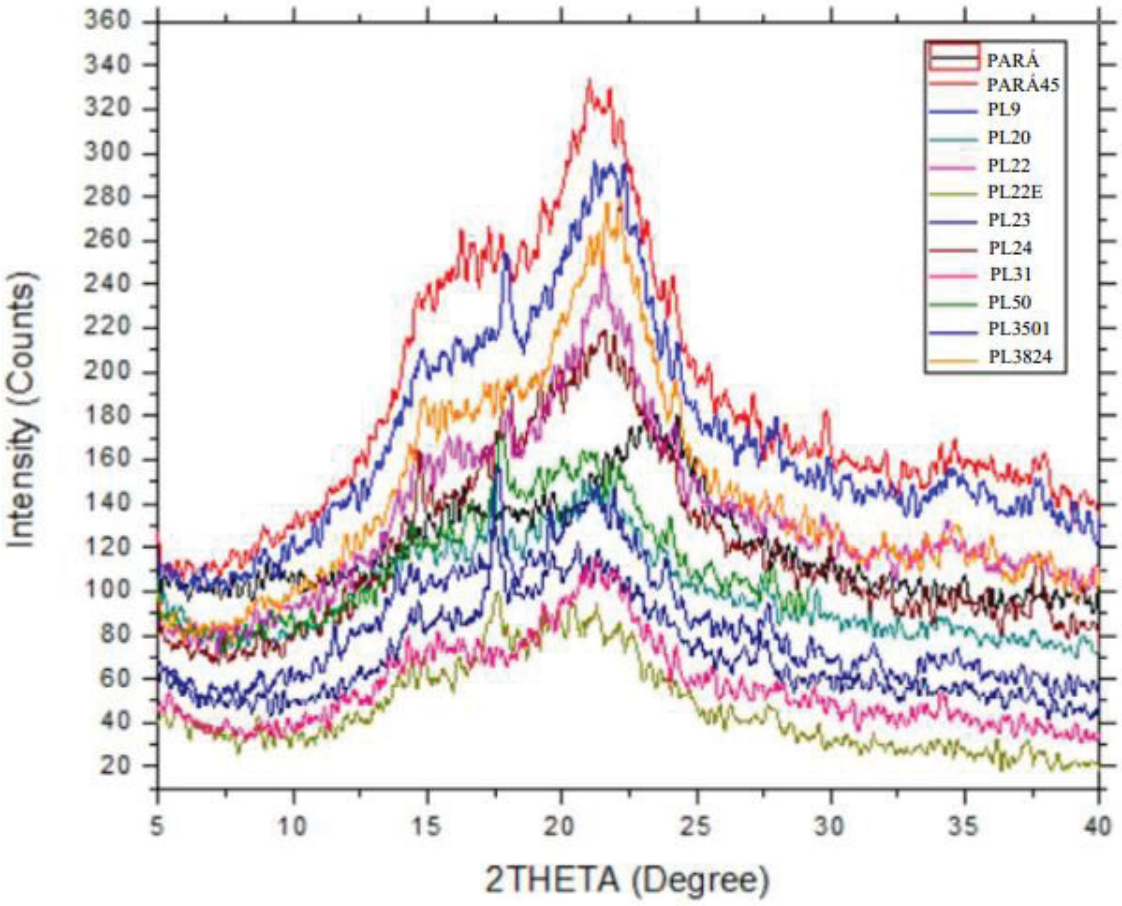
(I) For the PL31, the degradation of the lignocelulosic material of CPH is observed, the hyphae of the fungus through its enzymes and metabolites biologically degrade the cell wall of the CPH.
(J) In the PL50, degradation of the CPH cell wall exists, but it is still necessary to degrade part of the lignocellulosic material, a compact structure between the fungus and the CPH is observed.
(K) For the PL3501, the compaction of the fermentation is observed, where there is a degradation by desquamation of the cell wall of the CPH and it is progressive.
(L) In PL3824, there is a process of degradation of the lignocellulosic material of CPH and erosion of the cell wall, where a solid conjunction between fungal hyphae with agroindustrial residue is observed.

The *Pleurotus* degrade the lignocelulosic material of the CPH, this occurs through the penetration of its hyphae in the lumen of the plant cells, as can be seen in Figure 26, rooted hyphae will produce a variety of extracellular metabolites that will be responsible for degrading the cell wall of plants (FERRAZ, 2010). The lignin component of the cell wall of vegetables is highly resistant to chemical and biological degradation, which gives it the mechanical strength of wood (MARTÍNEZ *et al*., 2005). For its degradation lignocellulosic fungi are needed that secrete a mixture of metabolites and oxidative enzymes, which allow the delignification of the lignocellulosic complex (GOMES; BOSCOLO; DA SILVA, 2014). There are two main situations of degradation of the plant cell by fungi, the first one refers to the cell wall flaking (lumen-lamella media), resulting in the progressive and irregular decrease of the tenor of the cell wall, the other way of degradation is removal of lignin and hemicellulose in the cell wall (FERRAZ, 2010).

5.4.2. Degree of cristalinity by X ray diffraction (XRD) of the Pará CPH and CPH fermented with *Pleurotus* strains

This analysis was carried out in order to evaluate the changes in the chemical structure of the cocoa pod husk, it was possible to evaluate the change in the crystallinity of the cocoa pod husk after fermentation with the different strains of *Pleurotus*. The Figure 28 of the diaphragtograms (XRD) of the CPH of Pará and CPH fermented with *Pleurotus* strains is presented:

FIGURE 28 - XRD OF THE PARÁ CPH AND CPH FERMENTED WITH *Pleurotus* STRAINS.



Source: the author (2018).

In the Figure 28, the CPH diaphragms and their fermented products with different strains of *Pleurotus* can be seen, with 2 well defined zones (crystalline and amorphous) with reference to the cellulose biopolymer, the crystalline zone which is the maximum peak intensity between the angles 2θ of 22° and 23° for type I cellulose or between the angles 2θ of 20° to 22° for type II cellulose. For the amorphous zone, the minimum peak intensity is highlighted between the angles 2θ from 15° to 17° for type I cellulose or between the angles 2θ from 12° and 13° for type II cellulose (BORYSIAK; DOCZEKALSKA, 2005; TORRES JARAMILLO *et al*., 2017).

For CPH, it can be observed that the intensity of the highest peak of the crystalline zone is not very pronounced and in the amorphous zone it is wide; in Pará 45 CPH that has been extracted compounds with portable extrator, it is observed that the intensity of the highest peak of the crystalline zone is the one that stands out more and its amorphous zone also its intensity of peak is high; for PL22E, both its peak intensities in the crystalline and amorphous zone are low, that in comparison of the samples that have not been fermented, one can observe the

change that the CPH has undergone by the physical and chemical effect in Para 45 and the degradation by the fungus in PL22E.

In the Table 37, the crystallinity index (CI) and the type of cellulose that most stands out in the diaphragtograms in Pará CPH and fermented CPH with Pleurotus strains are presented:

TABLE 37 - CRISTALLINITY INDEX OF PARÁ CPH AND FERMENTED CPH WITH *Pleurotus* STRAINS.

	PARA	**PARA 45**	**PL9**	**PL20**	**PL22**	**PL22E**	**PL23**	**PL24**	**PL31**	**PL50**	**PL3501**	**PL3824**
Cristallinity Index	27.78	59.57	52.29	60.87	33.92	43.12	52.50	30.23	57.69	22.46	43.04	36.26
Type of cellulose	I	II	II	II	II	II	II	I	II	I	II	I

Souce: the author (2018).

In the Table 37, the crystallinity index was 27.78 for Para CPH, which means that the amorphous zone predominates over the crystalline zone and also in the crystalline zone the most outstanding is cellulose of type I, this value obtained is close to 24.16 CI reported by Torres (2016). In Para 45 CPH, the IC it was 59.57 where the crystalline zone predominates over the amorphous zone and cellulose type II predominates, it has undergone modification with respect to the CPH without extraction. In contrast, for PL22E the IC was 43.12 where there is a slight predominance of the amorphous zone over the crystalline zone and its cellulose is of type II, where the *Pleurotus* strain PL22 has had a good job in degrading the components of the fermented CPH.

In the other cases of fermented CPH the degradation of the CPH and its compounds can be observed, some of them highlighting the amorphous zone as PL50 whose IC was 22.46 and others highlighting more the crystalline zone as PL20 whose IC was 60.87. Also type I cellulose (Pará CPH), is transformed into most of the CPH fermented into type II cellulose, due to a polymorphic transformation (BORYSIAK; DOCZEKALSKA, 2005; ZUGENMAIER, 2008).

5.4.3. Degradation of the total polyphenols content in the fermentative process of *Pleurotus* strains

Table 38 shows the degradation of the total polyphenol in the fermentation process of the CPH with the *Pleurotus* strains (in dry matter).

TABLE 38 - DEGRADATION OF THE TOTAL POLYPHENOLS CONTENTS IN THE FERMENTATIVE PROCESS OF *Pleurotus* STRAINS.

Day	Fermented products (g GAE/100 g of sample)						
	PL9	**PL20**	**PL22**	**PL22E**	**PL23**	**PL24**	**PL50**
0	10.39 ± 1.257	6.46 ± 0.323	12.35 ± 0.342	11.38 ± 0.918	5.91 ± 1.831	9.76 ± 1.835	17.54 ± 1.986
2	4.02 ± 0.600	4.02 ± 0.709	6.44 ± 0.259	4.28 ± 0.156	5.46 ± 0.759	6.70 ± 0.575	10.54 ± 0.236
4	1.85 ± 0.308	0.37 ± 0.447	3.56 ± 0.301	2.49 ± 0.088	2.37 ± 1.351	3.41 ± 1.016	1.59 ± 0.487
6	3.13 ± 0.768	0.32 ± 0.064	1.25 ± 0.659	1.09 ± 0.118	2.75 ± 0.317	1.93 ± 0.914	2.50 ± 0.227
8	0.27 ± 0.082	0.50 ± 0.269	0.81 ± 0.486	0.60 ± 0.047	1.21 ± 0.521	0.60 ± 0.259	2.90 ± 0.324
10	0.58 ± 0.144	1.04 ± 0.139	0.19 ± 0.104	0.27 ± 0.074	3.42 ± 1.426	1.55 ± 1.229	0.24 ± 0.112
12	1.89 ± 0.281	0.39 ± 0.114	0.24 ± 0.101	0.03 ± 0.033	1.61 ± 1.254	1.19 ± 1.178	0.70 ± 0.409
14	1.00 ± 0.112	0.45 ± 0.99	0.01 ± 0.000	0.02 ± 0.019	1.44 ± 0.138	0.73 ± 0.267	0.44 ± 0.162
16	3.72 ± 0.305	0.12 ± 0.148	0.24 ± 0.031	0.72 ± 0.185	1.32 ± 0.720	0.32 ± 0.045	1.60 ± 0.190
18	0.13 ± 0.146	0.09 ± 0.074	0.17 ± 0.041	0.10 ± 0.087	1.51 ± 0.927	0.63 ± 0.255	0.33 ± 0.395
20	0.01 ± 0.000	0.02 ± 0.021	0.43 ± 0.160	0.46 ± 0.286	1.72 ± 0.131	0.72 ± 0.231	0.08 ± 0.135

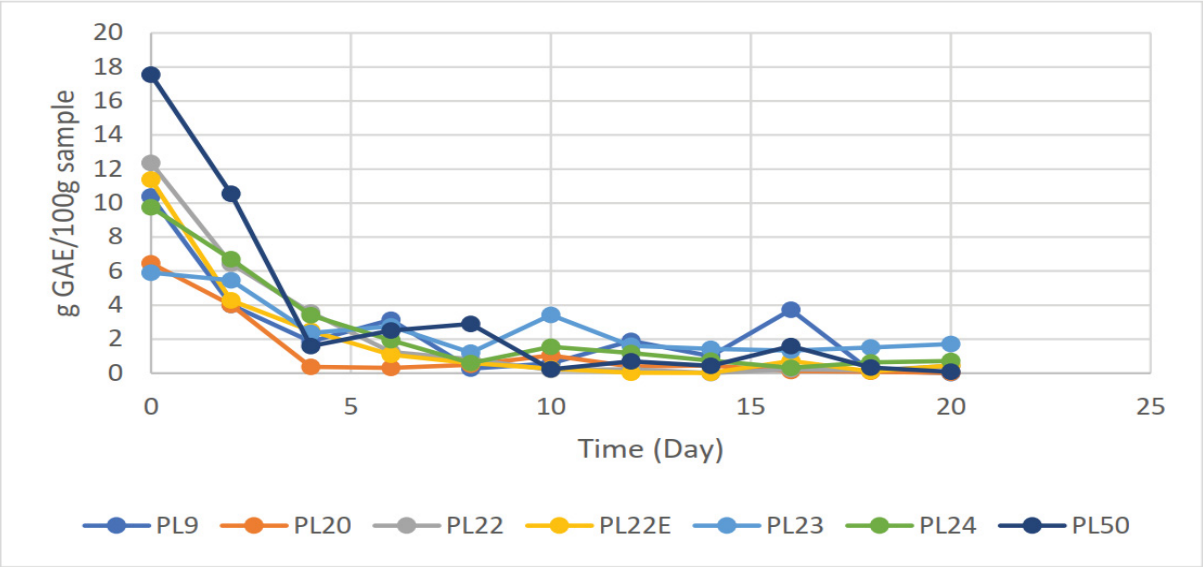
Source: the autor (2018).

Table 38 shows the values of the degradation of the total polyphenols of the fermentation from day 0 to 20, where the polyphenols content has been decreasing day by day by the action of *Pleurotus* and production of laccase. This enzyme catalyzes oxidation reaction with the polyphenols and aromatic groups present in the fermentation (CORREA *et al*., 2017).

The white rot fungi are frequently being used to produce Laccases enzyme (GALHAUP *et al*., 2002; DIAZ *et al*.,2014). The presence of aromatic and phenolic substances (such as ferulic acid, 2,5 xylidine, p-anisidine and veratrile alcohol) stimulate the activity of white rot fungi (TYCHANOWICZ *et al*., 2006); the laccases are glycosylated polyphenols oxidases having 4 copper ions per molecule, that catalyze the oxidation of a wide variety of phenolic compounds and aromatic amines by reducing 4 electrons from the oxygen to water (LEONOWICZ *et al*., 2001; ZHU *et al*., 2016).

In Figure 29, the degradation of the polyphenols during the fermentative process by the strains of *Pleurotus* with CPH is presented.

FIGURE 29 - EVOLUTION OF THE DEGRADATION OF THE TOTAL POLYPHENOLS CONTENTS IN THE FERMENTATIVE PROCESS.



Source: the author (2018).

In this Figure 29, it is visualized how the polyphenols have degraded mainly by the action of the Laccase. On day 4 the values of the total polyphenols abruptly dropped until reaching almost zero, could be explained by the reaction affinity of the laccase by this substrate for its oxidation.

5.4.4. Development of the Laccase activity in the fermentative process of *Pleurotus* strains

In the Table 39, the development of the enzymatic activity of Laccase in the fermentative process of *Pleuorotus* strains with CPH is shown (in dry matter).

TABLE 39 - EVOLUTION OF THE LACCASE ACTIVITY IN THE FERMENTATIVE PROCESS OF THE *Pleurotus* STRAINS.

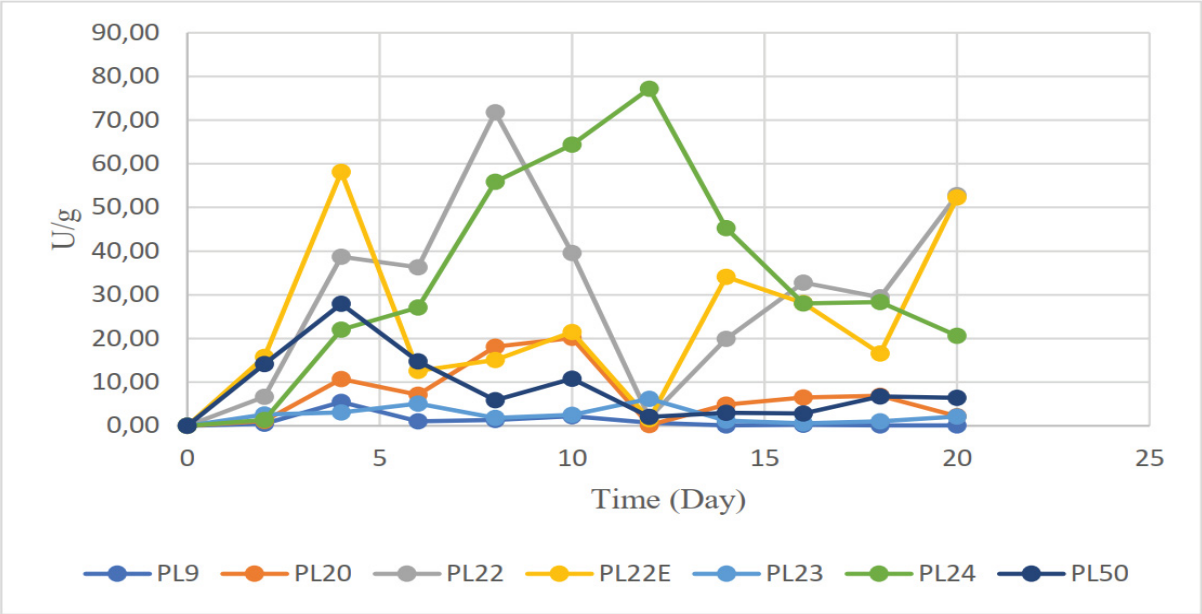
Day	Fermented products (U/g)						
	PL9	**PL20**	**PL22**	**PL22E**	**PL23**	**PL24**	**PL50**
0	1.50 ± 1.350	0.00 ± 0.000	5.33 ± 2.870	0.87 ± 0.668	4.11 ± 1.824	5.34 ± 2.110	12.54 ± 2.305
2	2.78 ± 1.782	6.39 ± 2.029	42.35 ± 2.052	78.77 ± 3.869	16.33 ± 0.881	8.84 ± 3.692	78.49 ± 4.941
4	**35.02 ± 4.522**	65.66 ± 4.034	279.54 ± 2.610	**339.80 ± 3.679**	21.97 ± 5.334	152.46 ± 3.599	**163.18 ± 1.518**
6	2.30 ± 2.512	44.95 ± 5.742	224.01 ± 2.688	80.41 ± 3.063	36.59 ± 3.633	179.89 ± 6.234	93.23 ± 4.094
8	8.63 ± 2.458	116.80 ± 4.641	**528.20 ± 4.790**	85.38 ± 2.787	12.90 ± 1.728	388.42 ± 5.942	37.72 ± 1.832
10	14.05 ± 2.062	**145.37 ± 6.158**	284.01 ± 4.903	131.22 ± 3.018	18.36 ± 5.919	405.95 ± 5.319	69.80 ± 1.970
12	4.56 ± 1.667	0.92 ± 0.589	11.52 ± 4.242	8.61 ± 3.747	**42.47 ± 4.825**	**569.49 ± 2.044**	13.23 ± 0.559
14	0.46 ± 0.122	34.83 ± 4.688	126.88 ± 3.061	217.73 ± 4.194	8.85 ± 4.585	279.70 ± 5.002	19.71 ± 3.779
16	1.68 ± 2.040	43.60 ± 3.219	185.22 ± 3.023	179.60 ± 5.913	4.02 ± 5.968	172.91 ± 1.154	18.76 ± 2.357
18	0.66 ± 0.177	42.30 ± 2.120	214.74 ± 2.496	119.92 ± 2.493	7.71 ± 4.863	209.85 ± 4.409	44.67 ± 1.117
20	0.51 ± 0.078	13.27 ± 1.251	323.06 ± 2.606	323.79 ± 1.551	14.09 ± 2.389	127.80 ± 4.485	42.38 ± 2.465

Source: the author (2018).

In Table 33 and Table 39, the maximum enzymatic activity of Laccase can be observed with respect to the time elapsed and the pH. For example, the lowest productivity corresponds to PL23 with 3.539 U/g.d with a pH of 8.91 ± 0.325, and the highest productivity corresponds to PL22E with 84.95 U/g.d and pH of 6.26 ± 0.198; as it can be observed greater productivity was achieved with a pH close to the optimum and smaller productivity was achieved at alkaline pH close to 9, not suitable for the optimal growth of the fungus. The pH is not the only important factor that influences the development of *Pleurotus,* the source of carbon and nitrogen, temperature, aeration, luminosity, among others are also important.

In Figure 30, the development of enzymatic activity of laccase in the fermentative process is shown.

FIGURE 30 - LACCASE ENZYMATIC ACTIVITY IN THE FERMENTATION PROCESS.



Source: the author (2018).

In Figure 30, the enzymatic activity of Laccase is observed with respect to the fermentation days, where the maximum enzymatic activity was 569.49 ± 2.044 U/g (12 days) for PL24, the variation of pH was 5.56 ± 0.255 to 8.91 ± 0.325 and the fermentation time varied between 4 to 12 days. The values obtained in this investigation, with respect to the enzymatic activity of Laccase were greater in relation to Elisashvili *et al*. (2006) of 39 U/g whose medium was supplemented with Xylan and *Pleurotus dryinus* was used in a submerged fermentation. Laccases enzymes are secreted as multiple isoenzymes by white-rot fungi that are under specific nutritional conditions (carbon and nitrogen ratio, composition of the medium, pH, temperature, amount of aeration), (SANJEEVIRAYAR *et al*., 2015; NIKU-PAAVOLA *et al*., 1990). There is a relationship between Laccase with respect to antioxidant activity, total polyphenol content

and sugar consumption, because polyphenols are antioxidants and are a component of lignin, the latter is a substrate par excellence of the enzyme laccase also, the fungus needs a carbon source to nourish itself and finds it in reducing sugars. Laccase catalyzes the oxidation of phenolic compounds and aromatic amines (SANJEEVIRAYAR *et al*., 2015).

5.5. SELECTION N° 2 OF *Pleurotus* STRAIN

For the selection of the best strain of *Pleurotus* for fermentation in solid state in the cocoa pod husk for 20 days, the following criteria were considered basically: the consumption of reducing sugar, the values of the degradation of the total content of polyphenols, the maximum enzyme activity of lacasse (productivity) and pectinase (activity).

The values presented in the following Table 40 were obtained from the Table 35 (Reducing sugar), Table 36 (Pectinase activity), Table 38 (Total polyphenols) and Table 39 (Lacasse activity); maximum productivity is the result of dividing the maximum enzymatic activity per time in days. The following Table 40 is presented (in dry matter).

TABLE 40 - SUMMARY OF EVALUATION INDICATORS TO CHOOSE THE BEST WORKING STRAIN TO FERMENT COCOA POD HUSK.

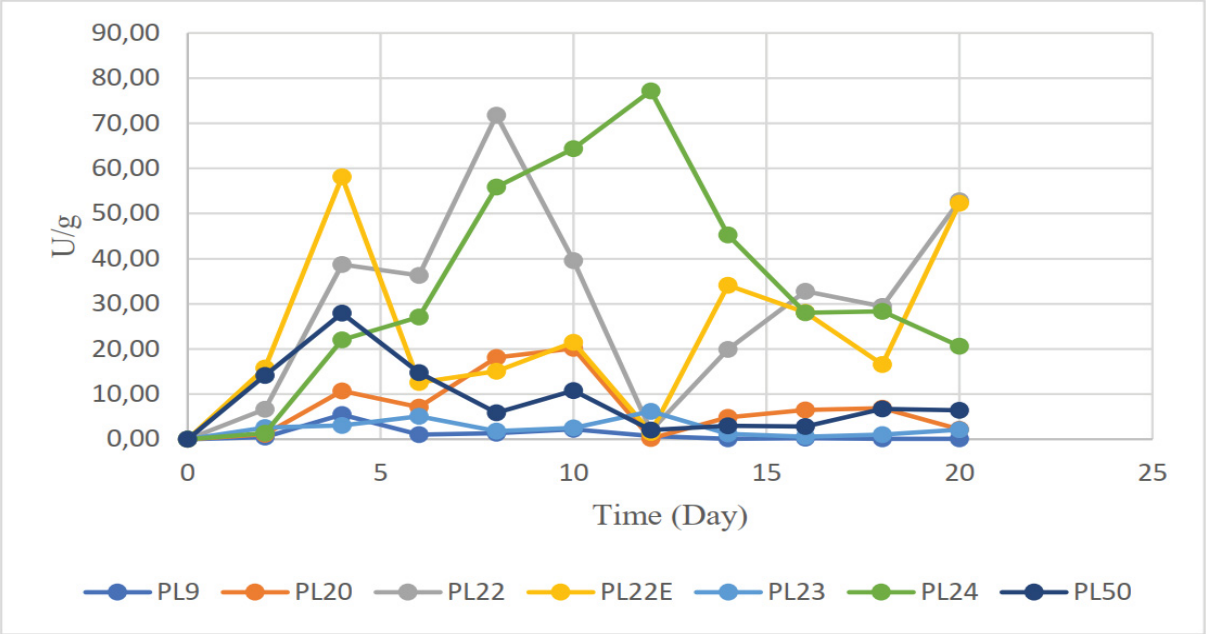
Fermented Product	Total polyphenols (mg GAE/100g)*	Laccase Activity (U/g)	Laccase Productivity (U/g.d)	Pectinase Activity (U/g)	Pectinase Productivity (U/g.d)	Reducing sugar (g /100g) *
PL9	0.01	35.02 (4d)	8.8	36.07 (4d)	9.02	12.77
PL20	0.02	145.37 (10d)	14.5	49.30 (6d)	8.22	2.88
PL22	0.43	528.2 (8d)	66.0	30.66 (6d)	5.11	15.35
PL22E	0.46	339.8 (4d)	85.0	26.75 (4d)	6.69	18.02
PL23	1.72	42.47 (12d)	3.5	45.10 (6d)	7.52	17.58
PL24	0.72	569.49 (12d)	47.5	49.11 (4d)	12.28	11.08
PL50	0.08	163.18 (4d)	40.8	112.52 (6d)	18.75	11.72

*Degradation on day 20, d: day.

Source: the author (2018).

The Figure 31 shows the comparison of *Pleurotus* strains according to the results obtained.

FIGURE 31 - COMPARISON OF *Pleurotus* STRAINS WITH RESPECT TO EVALUATION INDICATORS.



Source: the author (2018).

According to the Table 40 and Figure 31, it is observed that the fermented product with the best results in the total polyphenol content was PL23, followed by PL24, **PL22E**, PL22, PL50, PL20 and PL9; in reducing sugars it was **PL22E** that obtained the best results, followed by PL23, PL22, PL9, PL24, PL50 and PL20; for the best productivity of the laccase activity was **PL22E**, followed by PL22, PL50, PL24, PL20, PL9 and PL23; for the best productivity in the Pectinase activity was PL50, PL24, PL9, PL20, **PL22E**, PL23 and PL22.

Analyzing the results, the best strain that stood out over the other *Pleurotus* strains was the PL22E strain that corresponds to *Pleurotus ostreatus* of Embrapa (Brazil).

5.6. ANALYSIS OF SOLID-STATE FERMENTATION (SSF) AND SEMI SOLID-STATE FERMENTATION (SSSF) IN OF THE PARA CPH FERMENTED WITH PL22E STRAIN

As previously determined, the best results were obtained with PL22E strain corresponding to *Pleurotus ostreatus*. A comparison has been performed with Pará CPH and the PL22E strain in Solid State Fermentation and Semi Solid-State Fermentation. The strain PL22E (*Pleurotus spp*.), from studies conducted by Dr. Karp from UFPR, identified this species as *Pleurotus ostreatus*.

a) Moisture (%)

The following Table 41 shows the humidity of the fermentation of Para CPH during the Solid-State Fermentation process, carried out during 8 days.

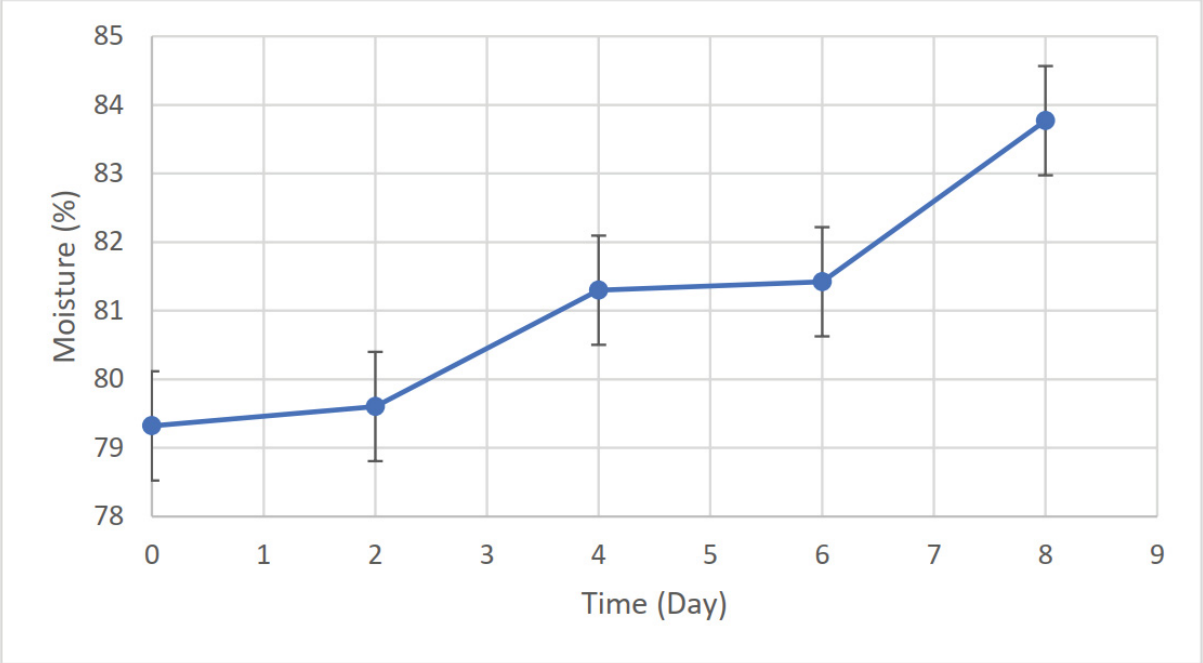
TABLE 41 - MOISTURE OF THE PARÁ CPH DURING FERMENTATION IN SOLID STATE WITH PL22E STRAIN.

Day	Moisture (%)
0	79.321 ± 0.868
2	79.604 ± 1.136
4	81.299 ± 0.578
6	81.423 ± 0.239
8	83.771 ± 0.857

Source: the author (2018).

As can be seen in the Table 41, the moisture of CPH has increased slightly throughout the 8 days of the Solid-State Fermentation process. This can be seen in the following Figure 32.

FIGURE 32 - MOISTURE IN THE SOLID-STATE FERMENTATION OF PARÁ CPH WITH PL22E STRAIN.



Source: the autor (2018).

It is observed in the Table 41 and Figure 32, the increase in moisture from day 0 to day 8 was 5.31%, that is 79.321% reached 83.771% in the eighth day, which favors the growth and development of the *Pleurotus ostreatus* fungus in the CPH because it is within its humidity conditions of 60 - 97 %RH (BELLETTINI *et al*., 2006).

b) pH

In the Table 42, the pH during SSF and SSSF of the cocoa pod husk with *Pleurotus ostreatus* is presented.

TABLE 42 - THE pH OF PARÁ CPH DURING SSF AND SSSF WITH PL22E STRAIN.

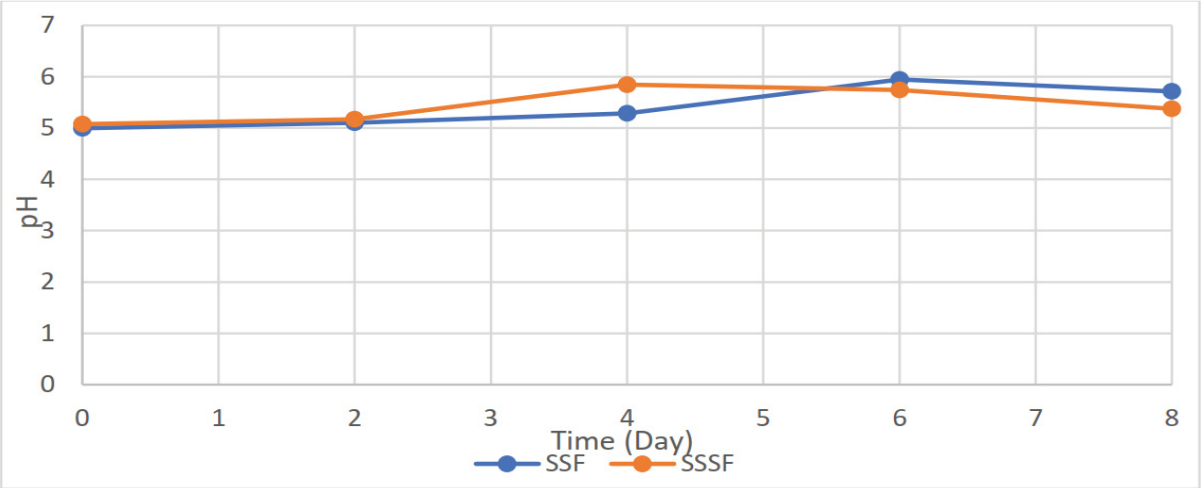
Day	Solid State Fermentation	Semi-Solid State Fermentation
		Ph
0	5.00 ± 0.035	5.08 ± 0.023
2	5.10 ± 0.015	5.17 ± 0.040
4	5.29 ± 0.032	5.85 ± 0.657
6	5.95 ± 0.523	5.74 ± 0.954
8	5.71 ± 0.202	5.38 ± 0.344

Source: the autor (2018).

As can be seen in the Table 42, the pH values have been increasing until day 6 in SSF and 4 in SSSF and then have been slightly decreasing. These pH values in both types of fermentations are below that reported by Belletini *et al*. (2006) in the range of 6.5 to 7.5 for optimal growth of *Pleurotus sp*; It will also depend on the type of substrate used and the fermentation conditions and type of microorganism used, which can vary the pH value shown and the performance in fermentation.

Figure 33 shows the pH variation in the 2 types of fermentations (SSF and SSSF).

FIGURE 33 - DEVELOPMENT OF pH OF PARÀ CPH DURING SSF AND SSSF WITH PL22E STRAIN.



In the Figure 33, the development of the pH in SSF and SSSF during the 8 days is presented, there is similarity throughout the process (except in the fourth day), being the values between 5 and less than 6 in both fermentations**.** According to Urvan (2004) (cited by VITOLA,

2018), the optimum pH for mycelial growth is in the range of 4.0 to 7.0 and for the formation of basidiomes it is between 3.5 and 5.0.

c)Reducing sugar

Table 43 shows the reducing sugar present in CPH with PL22E strain during the SSF and SSSF, along the 8 days of the process. (in dry matter).

TABLE 43 - REDUCING SUGAR OF PARÀ CPH DURING SSF AND SSSF WITH PL22E STRAIN.

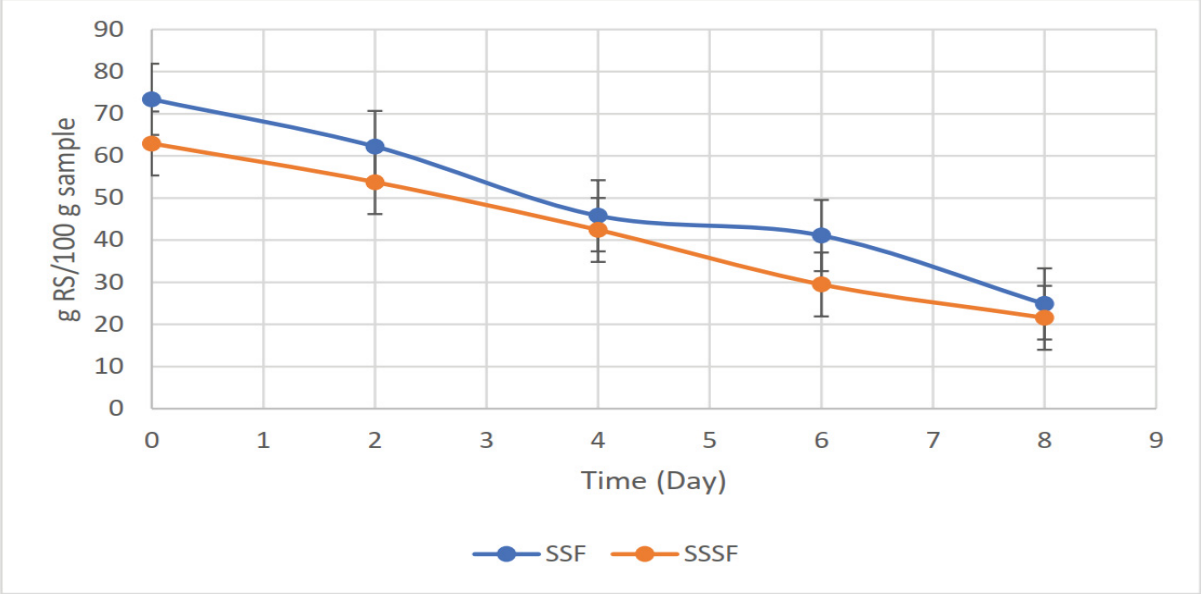
Day	Solid State Fermentation	Semi-Solid State Fermentation
	Reducing sugar (g/100 g)	
0	73.431 ± 3.264	62.949 ± 4.179
2	62.234 ± 7.302	53.781 ± 1.726
4	45.787 ± 4.616	42.425 ± 0.610
6	41.096 ± 1.205	29.489 ± 2.211
8	24.868 ± 3.373	21.573 ± 1.832

Source: the author (2018).

In the Table 43, the consumption of reducing sugars of the medium by *Pleurotus ostreatus* in the fermentative process of SSF and SSSF is observed. The consumption of reducing sugars goes down in the course of the days by the metabolic necessities required for the production of basidiomes (BANO *et al*., 1988).

The Figure 34 below shows the trend of consumption of reducing sugars in SSF and SSSF by *Pleurotus ostreatus*.

FIGURE 34 - CONSUMPTION OF REDUCING SUGARS IN SSF AND SSSF BY *Pleurotus ostreatus*.



Source: the author (2018).

As it is observed in the Figure 34, the tendency of the consumption of reducing sugars in both types of fermentations during the 8 days is practically linear. These reducing sugars come from CPH or have been released from more complex carbohydrates and served as source of energy for the strain PL22E.

d) Antioxidant activity

The following Table 44 shows the Antioxidant activity (VCEAC) of the fermentation of Para CPH during the Solid-State Fermentation and Semi Solid-State Fermentation, carried out during 8 days (in dry matter).

TABLE 44 - THE ANTIOXIDANT ACTIVITY OF PARÀ CPH DURING SSF AND SSSF WITH PL22E STRAIN.

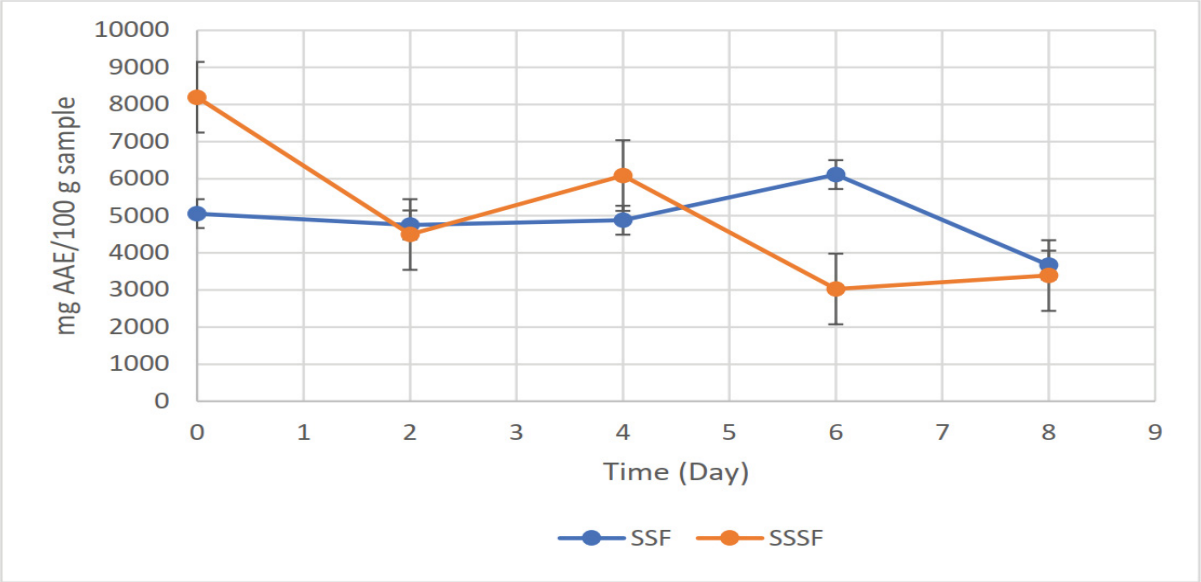
Day	Solid State Fermentation	Semi-Solid State Fermentation
	Antioxidant activity VCEAC (mg AAE/100 g)	
0	5058.945 ± 20.4	8196.828 ± 34.0
2	4755.329 ± 26.8	4495.4464 ± 23.3
4	4880.594 ± 16.2	6084.990 ± 18.3
6	6111.194 ± 6.4	3026.283 ± 20.5
8	3670.206 ± 17.8	3388.905 ± 17.7

Source: the author (2018).

In the Table 44, after inoculation of the strain PL22E into the CPH, it is observed the decrease of the antioxidant activity when passing the days of the SSF and SSSF, this can be due to the use of these bioactive compounds in the fungal nutrition. The hyphae of fungi produce enzymes and organic acids that are mixed with the substrate in order to degrade cellulose, starch, sugars, proteins and other compounds that serve as a source of energy for growth (URBEN, 2017).

In the Figure 35, the evolution of the antioxidant activity (VCEAC) in the fermentative processes SSF and SSSF, during the 8 days is presented.

FIGURE 35 - THE EVOLUTION OF THE ANTIOXIDANT ACTIVITY IN THE FERMENTATIVE PROCESS.



Source: the author (2018).

According to Summa *et al*. (2008), DPPH radical capacity is affected by other compounds such as methylxanthines, flavonoids and pigments, in addition to variety, post-harvest, extraction methods, solvents and extract concentration. The Figure 35 shows the decrease in antioxidant activity, which is not homogeneous throughout the fermentation process.

e) Total polyphenols content

The Table 45 shows the total polyphenol content in the SSF and SSSF of CPH and PL22E strain (in dry matter).

TABLE 45 - TOTAL POLYPHENOLS OF PARÀ CPH DURING SSF AND SSSF WITH PL22E STRAIN.

Day	Solid State Fermentation	Semi-Solid State Fermentation
	Total polyphenols (mg GAE/100g)	
0	4.454 ± 0.785	40.107 ± 5.452
2	4.733 ± 0.640	21.519 ± 1.226
4	1.723 ± 0.859	24.513 ± 5.313
6	1.188 ± 0.421	20.459 ± 3.278
8	0.705 ± 0.212	11.075 ± 1.350

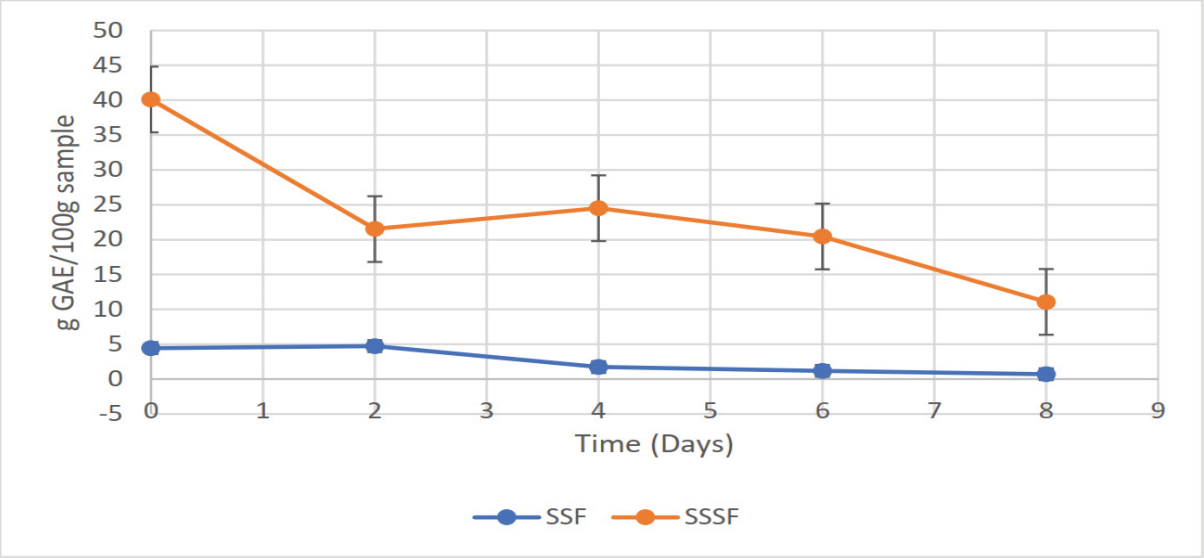
Source: the author (2018).

In the Table 45, the decrease of the total polyphenol values in both types of fermentations during the fermentation process is observed; this is due to the degradation of the polyphenols catalyzed by enzymes secreted by the myceliums of the *Pleurotus* fungus during fermentation. The enzymatic complex formed by laccase and manganese peroxidases is responsible for

catalyzing the oxidation of lignin structures, creating free radicals that cause disintegration of the macromolecule (BALDRIAN, 2006; HOFRITER, 2002).

Figure 36 shows the degradation of total polyphenols in SSF and SSSF.

FIGURE 36 - THE DEGRADATION OF THE TOTAL POLYPHENOLS CONTENTS IN THE FERMENTATIVE PROCESS.



Source: the author (2018).

In the Table 45 and Figure 36, it is observed that the values of total polyphenols in the SSSF are much higher than those obtained in the SSF, therefore, the effects of agitation, oxygenation and the liquid medium have mainly favored the obtaining of these results. In both cases, the fermentation processes SSF and SSSF from day zero to day eight decrease the total polyphenol values of the fermented samples.

f) Laccase activity

In the Table 46, the development of laccase activity in the SSF and SSSF processes is presented during the 8 days (in dry matter).

TABLE 46 - LACCASE ACTIVITY OF PARÀ CPH DURING SSF AND SSSF WITH PL22E STRAIN.

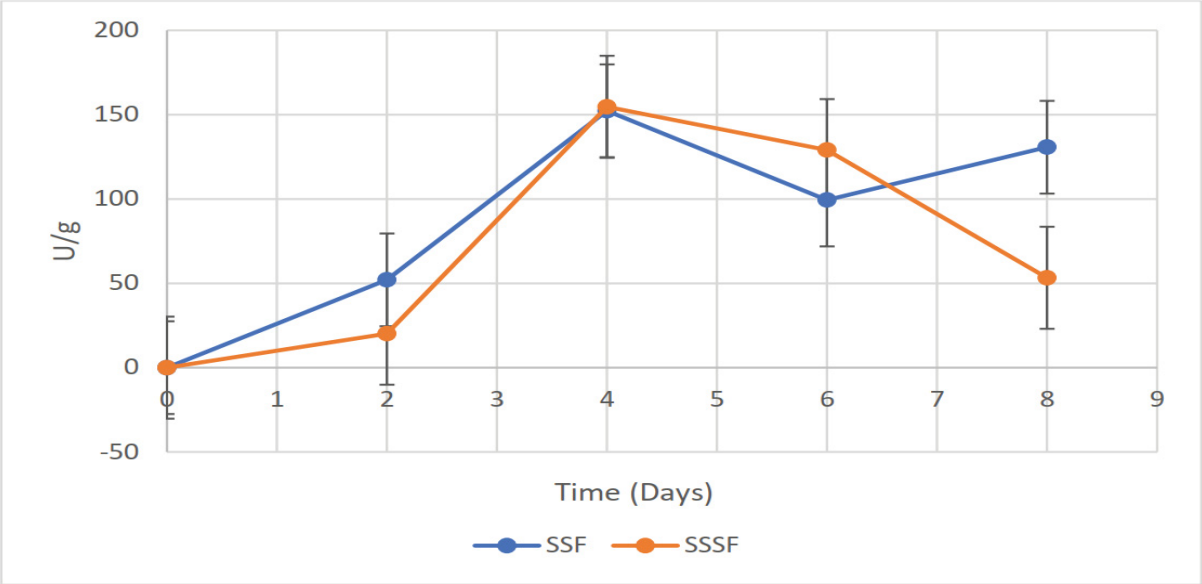
Day	Solid State Fermentation	Semi-Solid State Fermentation
	Laccase activity (U/g)	
0	0.000 ± 0.000	0.000 ± 0.000
2	52.057 ± 7.437	20.142 ± 1.951
4	152.317 ± 8.356	154.678 ± 10.250
6	99.404 ± 2.283	128.999 ± 2.469
8	130.715 ± 9.092	53.295 ± 4.174

Source: the author (2018).

In the Table 46 shows, the lacase activity of the two types of fermentation (SSF and SSSF), reaches its maximum activity of this enzyme on the fourth day, and then the values decrease during the fermentation process, due to the interaction of the lacase in the components of the substrate and the manifestation of its enzymatic activity over time.

In Figure 37, the activity of laccase in the SSF and SSSF is shown.

FIGURE 37 - LACCASE ACTIVITY IN THE SSF AND SSSF.



Source: the author (2018).

In the Table 46 and Figure 37, it is observed that the maximum enzymatic activity in the two fermentations is the fourth day, being the productivity similar in both cases of 38.08 U/(g.d) for SSF and 38.67 U/(g.d) for SSSF, at a pH of 5.29 ± 0.032 and 5.85 ± 0.657, respectively. Comparing the maximum enzymatic activity of laccase obtained in SSF and SSSF with other researches, the result was greater; Karp et al. (2015) obtained 151.6 U/g of laccase activity in the fourth day using inductor (Ferulic acid) in the SSF of cane bagasse with *Pleurotus ostreatus;* for Iandolo et al. (2011) in the SSF of *Pleurotus ostreatus* and *Trametes versicolor* obtained 36 U/g of maximum lacase activity; according to Mishra and Kumar (2007), in the SSF with use of agroindustrial residues and supplemented with Cyanobacterial biomass, 65.42 U/g of lacase activity (*Pleurotus ostreatus*) was obtained.

g) Pectinase activity

In the Table 47, the development of Pectinase (polygalacturonase) activity in the SSF and SSSF process is presented during the 8 days (in dry matter).

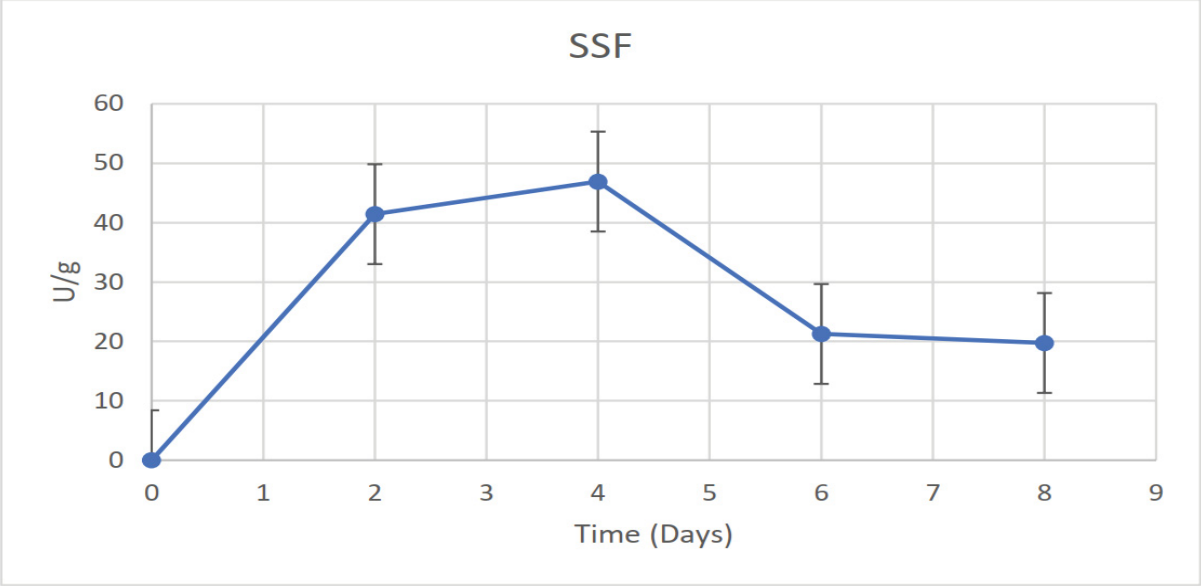
TABLE 47 - THE PECTINASE ACTIVITY OF PARÀ CPH DURING SSF AND SSSF WITH PL22E STRAIN.

Day	Solid State Fermentation	Semi-Solid State Fermentation
	Pectinase activity (U/g)	
0	0.000 ± 0.000	
2	41.426 ± 5.252	Does
4	46.914 ± 2.612	not
6	21.260 ± 1.710	register
8	19.738 ± 6.810	

Source: the author (2018).

In the Table 47, the enzymatic activity of pectinase in SSF and SSSF is presented during the 8 days, it is observed that there is pectinase activity in Solid State Fermentation and its maximum productivity at the fourth day is 2.19 $U.g^{-1}.d^{-1}$ (pH de 5.85 ± 0.657), and no data of enzymatic activity were recorded in the Semi Solid State Fermentation. Figure 38 is presents on the pectinase activity in the fermentation process.

FIGURE 38 - PECTINASE ACTIVITY IN THE FERMENTATIVE PROCESS.



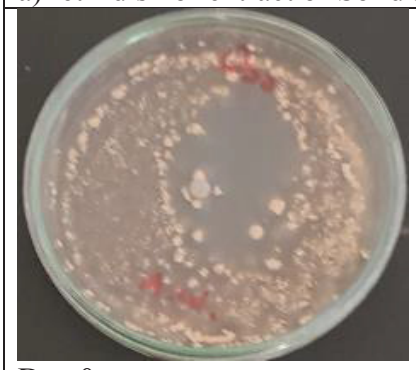
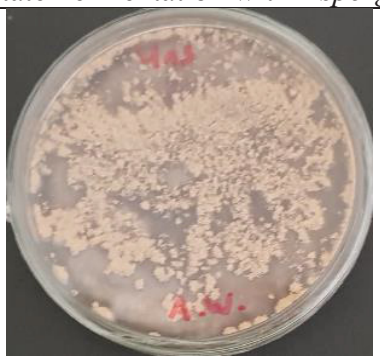
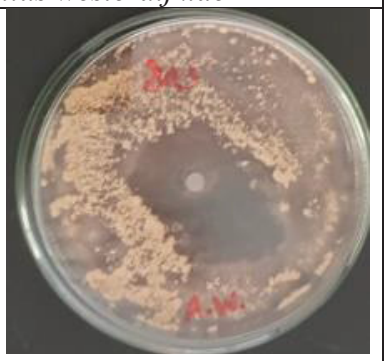
Source: the author (2018).

In the Table 47 and Figure 38, it is observed that the maximum activity of pectinase (polygalacturonase) was 8.77 ± 0.481 U/g (day 4) with a pH of 5.85 ± 0.657. According to Shivakumar and Nand (1995), the maximum polygalacturonase activity was 4.74 U/mL from a consortium of microorganisms with agro-industrial residues; the maximum activity of polygalacturonase of *Penicillium* was 12 U/g and of the fungus *Moniliella sp*. was 26 U/g (MARTIN *et al*., 2004).

5.7. ANTIFUNGAL ACTIVITY

In the Figure 39, the antifungal activity of PL22E extracts carried out in SSF (extract extracted with absolute ethanol) and SSSF (extract obtained from the aqueous medium) in the period of 0, 4 and 8 days is presented; their activity against *Aspergillus westerdijkiae* spores was measured.

FIGURE 39 - ANTIFUNGAL ACTIVITY OF THE PL22E EXTRACT OF SSF AND SSSF WITH RESPECT TO *Aspergillus westerdijkiae* SPORES.

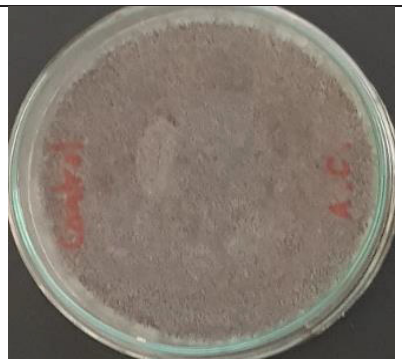
<table>
<tr><td></td><td> 
Control petri dish</td><td></td></tr>
<tr><td colspan="3">a)Petri dish of extract of Solid State Fermentation with Aspergillus westerdijkiae</td></tr>
<tr><td> 
Day 0</td><td> 
Day 4</td><td> 
Day 8</td></tr>
<tr><td colspan="3">b)Petri dish of extract of Semi Solid State Fermentation with Aspergillus westerdijkiae</td></tr>
<tr><td> 
Day 0</td><td> 
Day 4</td><td> 
Day 8</td></tr>
</table>

Source: the author (2018).

There is antifungal effect of *Pleurotus* spp. extract with respect to *Aspergillus westerdijkiae* spores, where best results were obtained with the extract in SSF (extract extracted with absolute ethanol) and on day 8 of fermentation. In the SSSF (extract obtained from the aqueous medium) there is some similarity between the antifungal results, standing out in 4 and 8 days of fermentation.

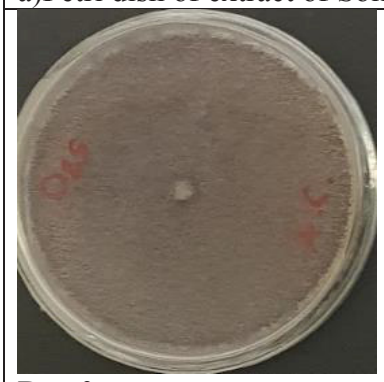
In the Figure 40, the antifungal activity of *Pleurotus ostreatus* extracts carried out in SSF (extract extracted with absolute ethanol) and SSSF (extract obtained from the aqueous medium) in the period of 0, 4 and 6 days is presented; their activity against *Aspergillus carbonarius* spores was measured.

FIGURE 40 - ANTIFUNGAL ACTIVITY OF THE PL22E EXTRACT OF SSF AND SSSF WITH RESPECT TO *Aspergillus carbonarius* SPORES**.**

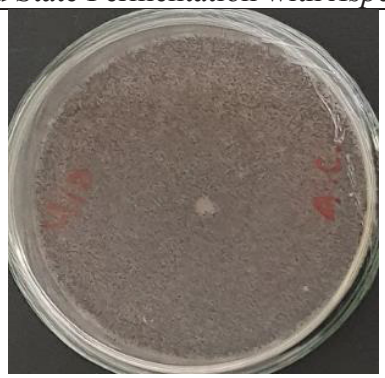


Control petri dish

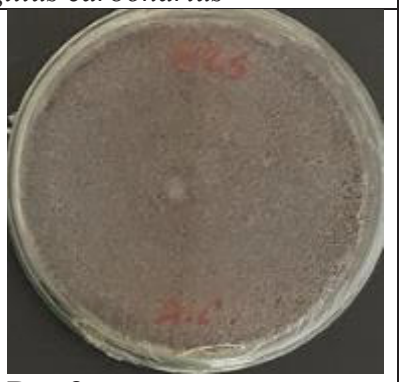
a)Petri dish of extract of Solid State Fermentation with *Aspergillus carbonarius*



Day 0

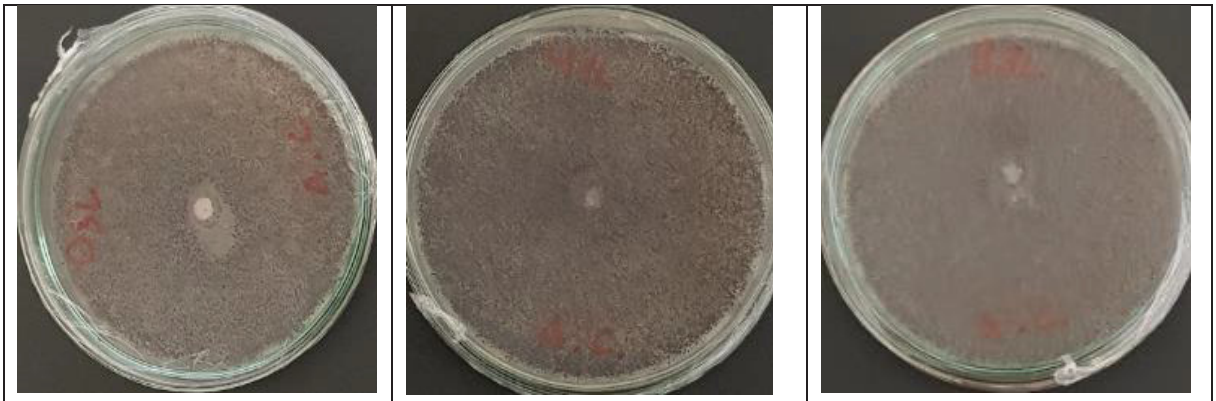


Day 4



Day 8

b)Petri dish of extract of Semi Solid State Fermentation with *Aspergillus carbonarius*



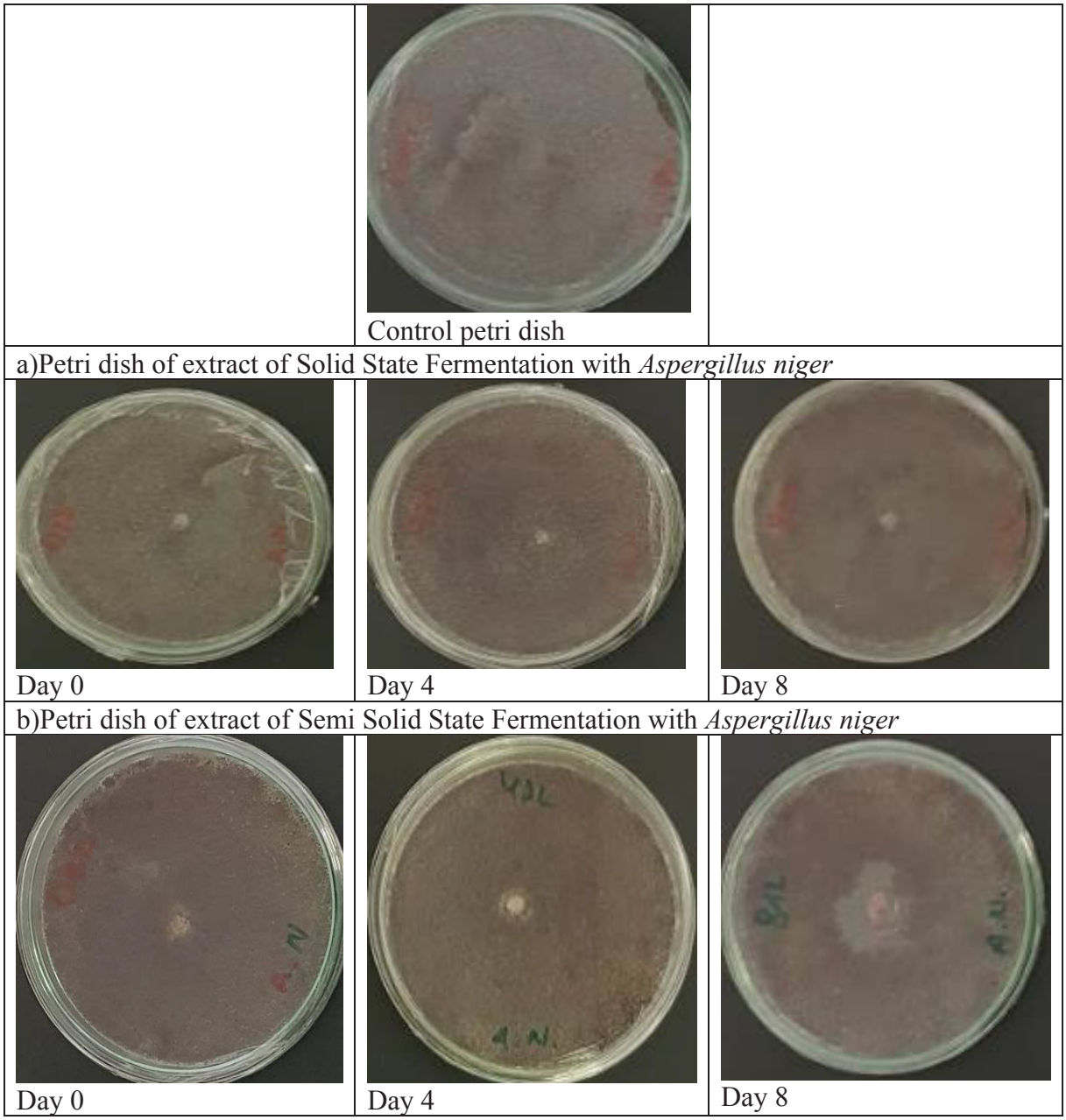
Day 0 | Day 4 | Day 8

Source: the author (2018).

With respect to this Figure 40, there is little antifungal effect of *Pleurotus ostreatus* extract with respect to *Aspergillus carbonarius* spores, where best results were obtained with the extract in SSF (extract extracted with absolute ethanol) and on day 8 of fermentation. In the SSSF (extract obtained from the aqueous medium) there is some similarity between the antifungal results, standing out in 4 and 8 days of fermentation. The best results of antifungal activity were found with the SSSF and there was parity between the analyzed days of fermentation. For the SSF there was less antifungal activity than the other fermentation.

In the Figure 41, the antifungal activity of PL22E extracts carried out in SSF (extract extracted with absolute ethanol) and SSSF (extract obtained from the aqueous medium) in the period of 0, 4 and 6 days is presented; their activity against *Aspergillus niger* spores was measured.

FIGURE 41 - ANTIFUNGAL ACTIVITY OF THE PL22E EXTRACT OF SSF AND SSSF WITH RESPECT TO *Aspergillus niger* SPORES.



Source: the author (2018).

In the Figure 41, it is observed that there is minimum antifungal effect of *Pleurotus ostreatus* extract with respect to *Aspergillus niger* spores extracts obtained, SSSF (extract obtained from the aqueous medium) had better antifungal results than SSF (extract extracted with absolute ethanol), the day that had the highest antifungal activity was the 8th day of fermentation.

The antifungal effect of the extract obtained by *Pleurotus ostreatus* against spores of *Aspergillus westerdijkiae, Aspergillus carbonarius* and *Aspergillus niger* in SSF and SSSF is presented in the Table 48.

TABLE 48 - ANTIFUNGAL EFFECTS OF *Pleurotus ostreatus* EXTRACT AGAINST THE SPORES OF THREE SPECIES OF *Aspergillus.*

Pleurotus ostreatus.	**Characteristics**	**Days of incubation**		
Extract		**0**	**4**	**8**
	SSF:			
	(mm)	8	13	25
With spores	Inhibition (%)	9.26	14.07	27.78
of *Aspegillus*	Concentration (g/mL)	0.1	0.1	0.1
Westerdijkiae	**SSSF:**			
	(mm)	10	12	12
	Inhibition (%)	11.48	12.96	12.96
	Concentration (g/mL)	0.05	0.05	0.05
	SSF:			
	(mm)	6	6	7
With spores	Inhibition (%)	7.04	7.04	7.41
of *Aspegillus*	Concentration (g/mL)	0.1	0.1	0.1
Carbonarius	**SSSF:**			
	(mm)	9	8	8
	Inhibition (%)	9.63	8.52	8.89
	Concentration (g/mL)	0.05	0.05	0.05
	SSF:			
	(mm)	2	3	3
With spores	Inhibition (%)	2.59	2.96	3.7
of *Aspegillus*	Concentration (g/mL)	0.1	0.1	0.1
Niger	**SSSF:**			
	(mm)	6	7	12
	Inhibition (%)	6.67	7.78	12.96
	Concentration (g/mL)	0.05	0.05	0.05

mm= Diameter of fungal inhibition zones in millimeters.

SSF (Solid State Fermentation).

SSSF (Semi Solid-State Fermentation).

Source: the author (2018).

In the Table 48, it is observed that despite the low values of diameter of inhibition of the fungi and of the percentage of inhibition (colony diameter of mycelium from a control petri plate x 100/colony diameter of mycelium from a test petri plate), there one of some presence of antifungal activity. The concentration of SSF extracts is twice the SSSF (check extraction methods), the best results were given in SSSF, which differs in its fermentation characteristics

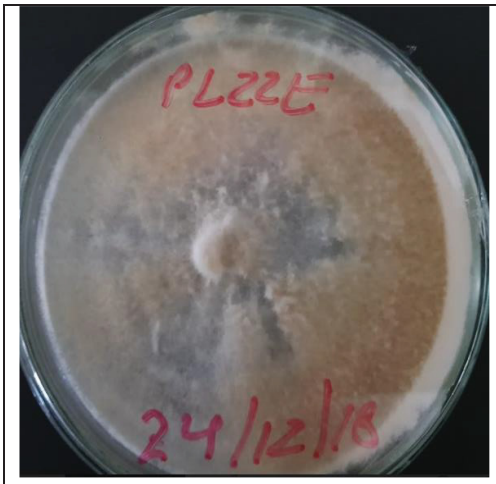
such as growth medium, agitation, luminosity, is in a moving state (dynamic) as opposed to SSF (static state).

The presence of certain phenolic compounds such as thymol, γ-terpinene, p-Cymene, limonene gives certain antimicrobial characteristics to the extract (DORMAN; DEANS, 2000; VARDAR-UNLU *et al*., 2003). The loss of phenolic compounds and antioxidants can reflect the decrease or absence of antimicrobial activitysince along fermentation phenolic compound are degraded and other secondary metabolites are also produced. Other bioactive components may also be responsible for giving it antimicrobial and antifungal properties; as mentioned by Lull et al. (2005), lipids, proteins, polysaccharides, phenols, tripertenes, can fulfill that antimicrobial or antifungal function.

5.8. ANTAGONISM

In the Figure 42, the Petri dishes of control and the interactions, between *Pleurotus* sp. (*P. ostreatus*) and *Moniliophthora perniciosa* are shown.

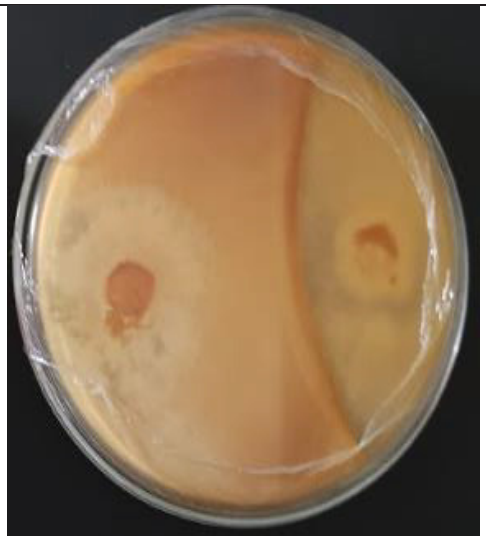
FIGURE 42 - ANTAGONISM AND PROTECTION MECHANISM OF *Pleurotus* spp. WITH *Moniliophthora perniciosa.*



(a) *Pleurotus* sp. (control)

(b) Moniliophthora perniciosa (control)

(c)PL22E vs. M.P. (front side)



(d)PL22E vs. M.P. (Reverse side)

(e) PL22E confronts M.P. in agar

(f) M.P. confronts PL22E in agar

Source: the author (2018).

In the Figure 42, the petri dish (a) and (b) is observed, where the control fungi are shown, the *Pleurotus ostreatus* had a faster growth than *Moniliophthora perniciosa.* Both are basidiomycetes that fulfill different role in nature; while the first is an edible fungus and has nutraceutical properties healthy for the human being, the other is a pathogen that attacks and destroys mainly cocoa crops (ALMEIDA *et al*., 2017; BELLETTINI *et al*., 2016). In the case of *Pleurotus ostreatus* the incubation at 28 ± 2 °C was 8 days (the mycelium petri dish was full) and for *Moniliophthora perniciosa* it was 18 days (the mycelium petri dish was not covered).

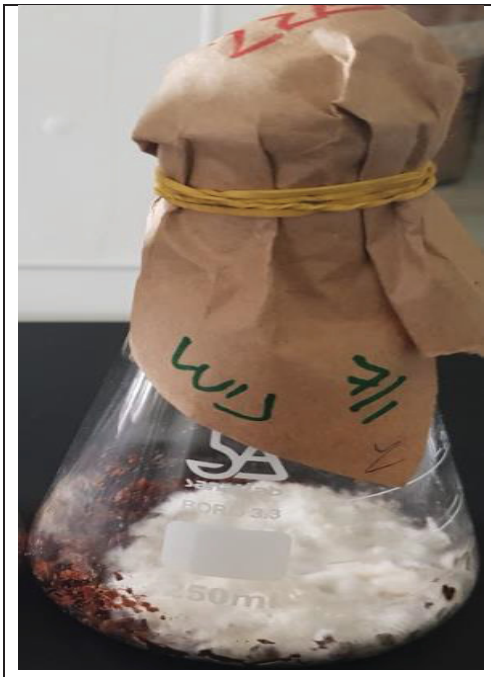
In (c) and (d), the antagonism of *Pleurotus ostreatus* and *Moniliophthora perniciosa* is observed, the dark beige zone corresponds to the mycelium of the first mentioned fungus that are approximately 2/3 of the observed petri dish and the other third (white zone) corresponds

to the mycelium of the other fungus. Competition is the most frequent cause of interaction between white rot fungi (BODDY, 2000; HISCOX *et al*., 2018). It is also possible to visualize a common zone between the mycelia of both fungi (0.6 cm of common area, approximately) when they were incubated on the stove for 22 days at 28 ± 2 °C. This interaction could induce the production of metabolites and cause changes in the metabolic pathways in response to antagonistic interaction; interesting changes could occur in certain metabolites (LUO *et al*., 2017).

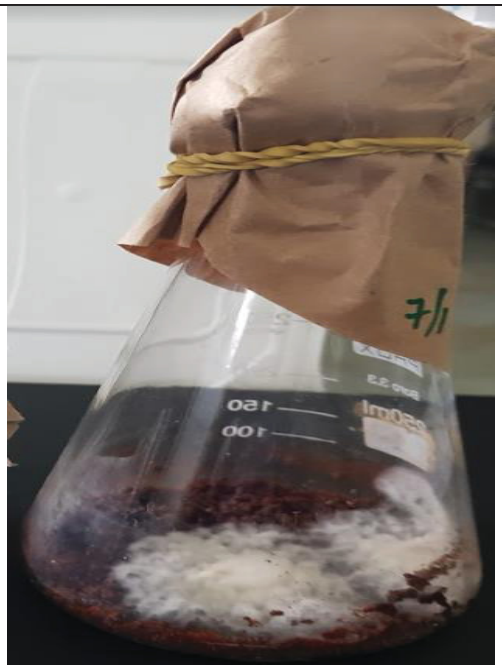
In (e) and (f), it is shown the confrontation between 2 different species of fungi that belong to the same division; in (e) *Pleurotus ostreatus* has gained advantage over *Moniliophthora perniciosa.* This secreted a small milky cream exudate; in (f) the encounter between these fungi continues, in this case Moniliophthora perniciosa tries to have more presence in the environment than *Pleurotus ostreatus*, but the latter that secretes a creamy exudate that limits the advance of the Moniliophtora perniciosa; these fungi were incubated in the stove at 28 ± 2 °C during 31 days. The different species of fungi found in the same environment, the action they develop occurs between the myceliums to defend or compete for the resources of the environment, which are always accompanied by the secretion of extracellular metabolites and changes in mycelial morphology. (BODDY, 2000; HEILMANN-CLAUSEN; BODDY, 2005; LUO *et al.,* 2017).

In the Figure 43, the growth of *Pleurotus ostreatus* and *Moniliophthora perniciosa* in CPH in SSF is presented, as well as the growth of pellets in liquid medium.

FIGURE 43 - GROWTH OF *Pleurotus ostreatus* AND *Moniliophthora perniciosa* IN CPH AND IN LIQUID MEDIUM.

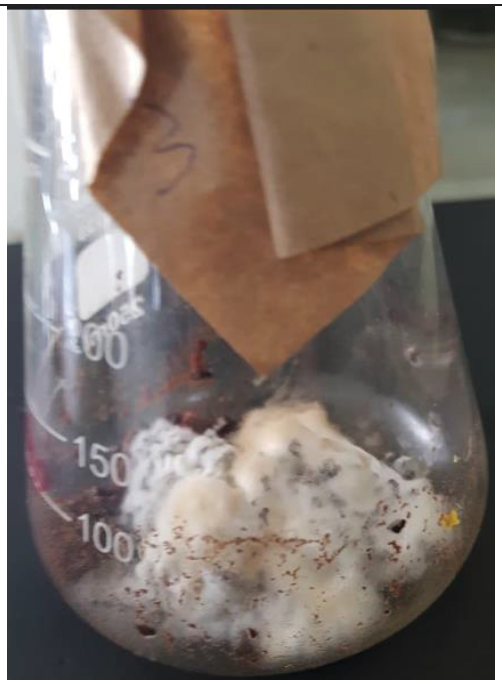


(a) *Pleurotus ostreatus* (control)



(b) *Moniliophthora perniciosa* (control)

(c) PL22E confronts M.P. in CPH



(d) M.P. confronts PL22E in CPH

(e)Pellets of *Pleurotus ostreatus.* (PL22E) (f)Pellets of *Moniliophthora perniciosa* (M.P.)

Source: the author (2018).

In this Figure 43, it is shown in (a) and (b) the growth in SSF of *Pleurotus ostreatus* and *Moniliophthora perniciosa* in CPH. The first fungus developed in its growth faster than its competitor, after 18 days of fermentation at 28 ± 2 °C.

In (c) and (d), the antagonism and interaction is presented in the CPH between *Pleurotus ostreatus* and *Moniliophthora perniciosa*, where each one of them comprises a certain area of the substrate for its growth and development, frontier can be observed in (c); in (d) the two fungi were mixed and both require CPH nutrients. These fungi were incubated for 18 days at 28 ± °C; metabolomics is being applied more frequently in order to identify the metabolites produced by fungi that are a consequence of stress (LUO *et al*., 2017).

In (e) and (f), the growth and development of pellets of *Pleurotus ostreatus* and *Moniliophthora perniciosa* in liquid medium is presented. While the growth of the pellets of *Pleurotus ostreatus* formed a great mass without definite form, *Moniliophthora perniciosa* formed small and homogeneous pellets. These fungi were incubated in the shaker at 120 rpm at 28 °C for 22 days.

There was antagonistic activity between the two fungi, with predominance of *Pleurotus ostreatus* against *Moniliophthora perniciosa*. Taking advantage of these characteristics, natural biological products from *Pleurotus ostreatus* to control *Moniliophthora perniciosa* (witches' broom) in the fields of cocoa production could be investigated.

6. CONCLUSIONS

In the characterization of the compositional analysis of the cocoa pod husk of Bahia and Pará, the one that gave better results was the Pará CPH. There were differences in chemical composition between untreated cocoa pod husk from Bahia and Pará.

There was a decrease in the content of reducing sugars, proteins, antioxidant activity and total polyphenols after fermentation with *Pleurotus*.

FTIR spectra showed differences in functional groups for cocoa pod husk from Bahia and Pará, also among the samples of CPH fermented with *Pleurotus* strains.

X-ray Difractograms showed that lignolytic fungi degrade cocoa pod husk, using nutrients and components available.

The extraction process with LPG in 4 cycles was effective for Total Phenols and better results for cocoa pod husk of Pará were obtained.

Extractives in LPG showed higher antioxidant activity (VCEAC).

HPLC identified sugars, phenols, alkaloids and organic acids in the fermented and peel in small quantities. GC-MS qualitatively identified lipids, organic acids and phenolic compounds in the cocoa pod husk of Parà and Bahia.

The *Pleurotus* strain PL22E was selected for its best development best results were obtained by semi-solid fermentation than by the solid-state fermentation.

In the cocoa pod husk of Bahia and Para, there were significant differences in the extraction of pectin, favoring the CPH of Pará; the pectin used as a substrate for pectinase, where the *Pleurotus* strains had enzymatic activity of pectinase. The PL22E was submitted to the fermentation in solid state and to the semi-solid fermentation, presenting results of activity in SSF and not in SSSF.

The lignin content in the cocoa pod husk of Bahia and Para was statistically similar, when the Pará CPH was subjected to solid state fermentation, the electron microscopy showed the degradation of the CPH plant wall by the hyphae of *Pleurotus*. This could be confirmed through XRD where there was change in the crystalline and amorphous zone of the CPH fermented with *Pleurotus;* also, through the degradation of polyphenols in enzymatic kinetics, a decrease of

total polyphenol values was observed during fermentation and the lacase activity of *Pleurotus* was observed fulfilling its role in the fermentative process.

Antioxidant activity was obtained from the cocoa pod husks as well as from fermented extracts of the CPH, and phenolic and alkaloid compounds with antioxidant activity were identified by HPLC.

The extract of *Pleurotus* spp. has little antifungal activity with respect to the spores of *Aspergillus westerdijkiae, Aspergillus carbonarius a*nd *Aspergillus niger*. *Pleurotus ostreatus* was also subjected to antogonic activity test against *Moniliophthora perniciousa*, with *Pleurotus ostreatus* showing significant.

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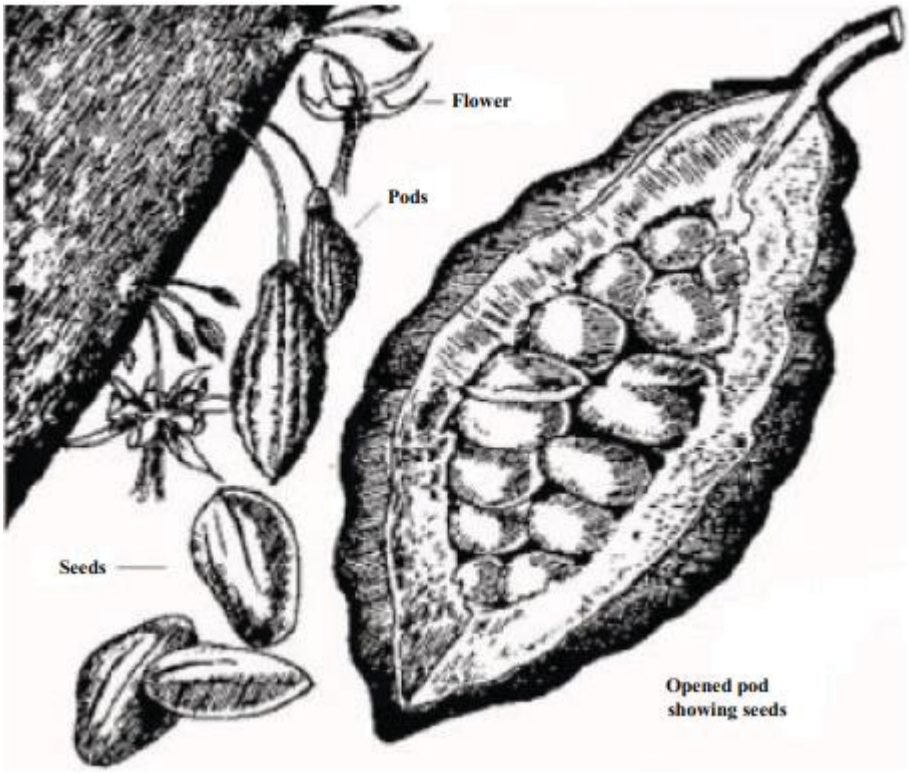
ANEXO 1 - FRUIT OF COCOA



Source: https://www.agrotechnomarket.com/2011/08/systematics-anatomy-and-morphology-of.html

ANEXO 2 - SPECIES OF *Pleurotus*, ACCEPTED ITS SCIENTIFIC NAME

Accepted scientific name
Pleurotus abieticola R.H. Petersen & K.W. Hughes, 1997
Pleurotus abscondens (Peck) Sacc., 1887
Pleurotus achilleae Velen., 1927
Pleurotus agaves Dennis, 1970
Pleurotus albidus (Berk.) Pegler, 1983
Pleurotus albipes Beauseign., 1926
Pleurotus allochrous (Pers.) Sacc. & Traverso, 1911
Pleurotus alocasiae Corner, 1981
Pleurotus alveolus Velen., 1927
Pleurotus anas Overeem, 1927
Pleurotus anastomosans Rick, 1930
Pleurotus angustatus (Berk. & Broome) Sacc., 1887
Pleurotus arbuticola Pilát, 1935
Pleurotus armeniacus Corner, 1981
Pleurotus arrhenioides Henn. & E. Nyman, 1899
Pleurotus aureovillosus Corner, 1981
Pleurotus australis Sacc., 1891
Pleurotus badius (Murrill) Murrill, 1916
Pleurotus bajocalifornicus Esteve-Rav., G. Moreno & N. Ayala, 1993
Pleurotus berberidicola (Speg.) Sacc., 1891
Pleurotus bipindiensis Henn., 1901
Pleurotus bourdotii (Quél.) Sacc. & Traverso, 1911
Pleurotus caespitosoterrestris (Henn.) Pilát, 1935
Pleurotus caldwellii A. Mackay, 1905
Pleurotus calyptratus (Lindblad ex Fr.) Sacc., 1887
Pleurotus carolinus Coker, 1944
Pleurotus cavarae Bres., 1920
Pleurotus chrysorrhizus Corner, 1981
Pleurotus cinerascens Velen., 1927
Pleurotus citrinopileatus Singer, 1942
Pleurotus colae Massee, 1912
Pleurotus colensoi Berk. ex Massee, 1899
Pleurotus columbinus Quél., 1881
Pleurotus compactis Herp., 1912
Pleurotus concha (Hoffm.) Sacc. & Traverso, 1910
Pleurotus convivarum Dunal & Delile, 1901
Pleurotus cornucopiae (Paulet) Rolland, 1910
Pleurotus craspedius (Fr.) Gillet, 1876
Pleurotus cretaceus Massee, 1899
Pleurotus cucullatus (Bres.) Bres., 1920
Pleurotus cyatheae S. Ito & S. Imai, 1939

Pleurotus cyatheicola Corner, 1981

Pleurotus cystidifer Velen., 1927

Pleurotus cystidiosus O.K. Mill., 1969

Pleurotus decipiens Corner, 1981

Pleurotus densifolius Murrill, 1912

Pleurotus diabasicus Velen., 1920

Pleurotus diffractus Pilát, 1941

Pleurotus djamor (Rumph. ex Fr.) Boedijn, 1959

Pleurotus dracaenae Torrend, 1909

Pleurotus dryinus (Pers.) P. Kumm., 1871

Pleurotus elegantissimus Speg., 1922

Pleurotus eleuterophyllus (Lév.) Sacc. & Trotter, 1912

Pleurotus eous (Berk.) Sacc., 1887

Pleurotus epilobii Velen., 1930

Pleurotus eremita Maire, 1931

Pleurotus eryngii (DC.) Quél., 1872

Pleurotus eugeniae (Earle) Sacc. & Traverso, 1911

Pleurotus euosmus (Berk.) Sacc., 1887

Pleurotus fagineus Velen., 1927

Pleurotus favoloides Singer, 1989

Pleurotus ferulaginis Zervakis, Venturella & Cattarossi, 2014

Pleurotus flabellatus Sacc., 1887

Pleurotus floridanus Singer, 1948

Pleurotus fuligineocinereus (Britzelm.) Sacc. & Trotter, 1912

Pleurotus fuscosquamulosus D.A. Reid & Eicker, 1998

Pleurotus geesterani Singer, 1962

Pleurotus gelatinosus Petch, 1924

Pleurotus giganteus (Berk.) Karun. & K.D. Hyde, 2011

Pleurotus guaraniticus Speg., 1922

Pleurotus gussonei (Scalia) Bres., 1920

Pleurotus gypseus Velen., 1920

Pleurotus herbarum Velen., 1927

Pleurotus heteropus (Speg.) Speg., 1887

Pleurotus hollandianus Sumst., 1906

Pleurotus hortensis Velen., 1926

Pleurotus hyacinthus Corner, 1981

Pleurotus hygrophanus (Earle) Sacc. & Traverso, 1911

Pleurotus ilgazicus Pilát, 1932

Pleurotus imbricatus (Earle) Sacc. & Traverso, 1911

Pleurotus immersus Velen., 1939

Pleurotus importatus Henn., 1897

Pleurotus incarnatus Hongo, 1973

Pleurotus inconspicuus Massee, 1892

Pleurotus inornatus Speg., 1919

Pleurotus insidiosus (Sacc.) Y.S. Chang & Kantvilas, 1993

Pleurotus juniperi Velen., 1927

Pleurotus kabulensis (Singer) Batyrova, 1985

Pleurotus komarnitzkyi Vassilkov, 1961

Pleurotus kotlabae Pilát, 1953

Pleurotus kudrnae Velen., 1927

Pleurotus lampas (Berk.) Sacc., 1887

Pleurotus lampyrinus Pat., 1915

Pleurotus langei Pilát, 1935

Pleurotus laricinus Velen., 1947

Pleurotus lazoi Donoso, 1981

Pleurotus lichenicola Speg., 1889

Pleurotus lilaceilentus Corner, 1981

Pleurotus lindquistii Singer, 1960

Pleurotus lobatus Henn. & E. Nyman, 1899

Pleurotus luctuosus Corner, 1981

Pleurotus luminosus Beeli, 1922

Pleurotus luteoalbus Beeli, 1928

Pleurotus luteosaturatus (Malençon) P.-A. Moreau, 2009

Pleurotus macilentus Massee, 1901

Pleurotus magnificus Rick, 1906

Pleurotus mallecanus Cleland, 1933

Pleurotus malleeanus Cleland, 1933

Pleurotus membranaceus Massee, 1901

Pleurotus mexicanus Guzmán, 1974

Pleurotus meyeri-herrmannii Henn., 1900

Pleurotus michailowskojensis (Henn.) Pilát, 1935

Pleurotus microleucus Singer, 1978

Pleurotus microspermus Speg., 1889

Pleurotus minor Sosin, 1960

Pleurotus minutoniger Lloyd, 1925

Pleurotus musae Corner, 1981

Pleurotus mutabilis Killerm., 1933

Pleurotus neapolitanus (Pers.) Singer, 1943

Pleurotus nebrodensis (Inzenga) Quél., 1886

Pleurotus nemecii Pilát, 1932

Pleurotus nepalensis Corner, 1955

Pleurotus novae-zelandiae (Berk.) Sacc., 1887

Pleurotus olivascens Corner, 1981

Pleurotus omnivagus Corner, 1981

Pleurotus opuntiae (Durieu & Lév.) Sacc., 1887

Pleurotus oregonensis (Murrill) Murrill, 1912

Pleurotus orizabensis (Murrill) Sacc. & Trotter, 1925

Pleurotus ostreatoroseus Singer, 1961

Pleurotus ostreatus (Jacq.) P. Kumm., 1871

Pleurotus palmicola Beeli, 1938

Pleurotus parsonsiae G. Stev., 1964

Pleurotus penangensis Corner, 1981

Pleurotus pinsitiformis Pilát, 1935

Pleurotus platypus Sacc., 1891

Pleurotus polyphemus (Cooke & Massee) Sacc., 1891

Pleurotus pometi (Fr.) Quél., 1872

Pleurotus pop-ivanensis Pilát, 1935

Pleurotus populinus O. Hilber & O.K. Mill., 1993

Pleurotus populneus Velen., 1931

Pleurotus problematicus Corner, 1981

Pleurotus proselyta E.H.L. Krause, 1928

Pleurotus pseudosepticus Hruby, 1930

Pleurotus pseudotremens Pilát, 1935

Pleurotus pubescens Peck, 1891

Pleurotus pulchellus S. Imai, 1939

Pleurotus pulmonarius (Fr.) Quél., 1872

Pleurotus purpureo-olivaceus (G. Stev.) Segedin, P.K. Buchanan & J.P. Wilkie, 1995

Pleurotus pusillus Speg., 1909

Pleurotus puttemansii Henn., 1908

Pleurotus radicosus Pat., 1917

Pleurotus ramosii Bres., 1926

Pleurotus rattenburyi Segedin, 1984

Pleurotus resinaceus Bres., 1920

Pleurotus rickii Bres., 1920

Pleurotus romellianus Pilát, 1935

Pleurotus rosarum Velen., 1927

Pleurotus rubi Velen., 1939

Pleurotus sambucinus Velen., 1920

Pleurotus samoensis Henn., 1896

Pleurotus sarasinii Henn., 1899

Pleurotus schwabeanus Henn., 1897

Pleurotus serotinoides (Peck) Sacc., 1887

Pleurotus similis Peck, 1901

Pleurotus smithii Guzmán, 1975

Pleurotus soyauxii Henn., 1891

Pleurotus spadiceus P. Karst., 1904

Pleurotus squamuliformis Velen., 1939

Pleurotus staringii Oudem., 1881

Pleurotus stella Pat., 1915

Pleurotus suballiaceus (Murrill) Murrill, 1943

Pleurotus subareolatus Peck, 1887

Pleurotus subbarbatulus (Murrill) Sacc. & Trotter, 1925

Pleurotus subglaber (Lloyd) Singer, 1951

Pleurotus subhaedinus (Murrill) Murrill, 1916

Pleurotus submembranaceus (Berk.) Pegler, 1988

Pleurotus submitis Speg., 1889

Pleurotus submutilus Speg., 1889

Pleurotus subostreatus Cleland & Cheel, 1919

Pleurotus subsapidus Murrill, 1912

Pleurotus subsepticus Henn., 1908

Pleurotus subulatus Henn. & E. Nyman, 1900

Pleurotus subviolaceus Corner, 1981

Pleurotus sulciceps (Cooke & Massee) Sacc., 1891

Pleurotus sutherlandii Singer, 1952

Pleurotus tahitensis Pat., 1906

Pleurotus terrestris Peck, 1907

Pleurotus thuidii Velen., 1927

Pleurotus tjibodensis Henn., 1900

Pleurotus togoensis Henn., 1897

Pleurotus tomentosulus (Peck) Sacc., 1891

Pleurotus tremelliformis (Murrill) Murrill, 1916

Pleurotus tubarius (Pat.) Pegler, 1983

Pleurotus umbonatus Peck, 1905

Pleurotus velatus Segedin, P.K. Buchanan & J.P. Wilkie, 1995

Pleurotus venosus Killerm., 1933

Pleurotus viaticus Velen., 1920

Pleurotus violaceocinerascens Henn., 1901

Pleurotus viscidulus (Berk. & Broome) Cleland, 1934

Pleurotus viscidus Harmaja, 1978

Pleurotus viticola (Murrill) Murrill, 1940

Pleurotus yuccae Maire, 1919

Pleurotus zimmermannii (Eichelb.) Sacc. & Trotter, 1912

Source: Roskov *et al*. (2017).