UNIVERSIDADE FEDERAL DO PARANÁ

PATRICK BARROS TIBURCIO

SOLID-STATE FERMENTATION OF *THEOBROMA CACAO* POD HUSK USING *RHIZOPUS STOLONIFER* – PROSPECTION OF BIOMOLECULES

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

Orientadora: Prof (a). Dra Adriane Bianchi Pedroni Medeiros

Co-orientador: Prof. Dr. Luiz Alberto Junior Letti

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TERMO DE APROVAÇÃO

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Curitiba, 04 de Abril de 2017.

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RESUMO

O objetivo principal deste estudo foi avaliar de forma inédita o cultivo do fungo filamentoso *Rhizopus stolonifer* em fermentação no estado sólido em cascas do fruto de cacau (*Theobroma cacao)* obtidas do estado do Pará, região norte do Brasil. Na primeira etapa foi selecionada a cepa *Rhizopus stolonifer NRRL* 28169 de acordo com sua capacidade de produzir moléculas com atividade antioxidante e de colonizar a casca de cacau sem nenhum suplemento nutricional. Na sequência foi realizado um estudo que determinou o método de extração de compostos com capacidade antioxidante de forma prática e econômica utilizando uma solução de etanol em 50% (v/v). A casca de cacau já possui um enorme potencial biotecnológico, com inúmeros biocompostos apresentados pelas análises de cromatografia gasosa acoplada a espectrometria de massa. O processo de fermentação em estado sólido nos permite observar uma mudança na composição presente casca, gerando novas moléculas bioativas com potencial antioxidante, chegando a 7,09 ± 0,05 µM de equivalência em Trolox no teste DPPH e 5,170 ± 0,035 x 10^4 µM de equivalência em Trolox no teste ORAC. O aumento na concentração dos compostos fenólicos durante a fermentação fica evidenciado pela quantificação de fenóis totais alcançando um concentração de 114,40 mg em equivalência de ácido gálico.

Palavras-chave: Casca de cacau; *Rhizopus*; Fermentação em estado sólido; Atividade antioxidante; Ácido cinâmico.

ABSTRACT

The main objective of this study was to evaluate in an unprecedented way the cultivation of the filamentous fungus *Rhizopus stolonifer* in solid state fermentation in the cocoa pod husk from *Theobroma cacao* obtained from the state of Pará, northern region of Brazil. In the first step of this study the strain *Rhizopus stolonifer NRRL* 28169 was selected according to its capacity to produce molecules with antioxidant activity and to colonize the cocoa pod husk without any nutritional supplementation. Consequently, a study was carried out to determine the extraction method of compounds with antioxidant capacity in a practical, economical and manageable using a 50% (v / v) ethanol solution. The cocoa pod husk already has an enormous biotechnological potential, with numerous biocomposites presented by GC-MS and HPLC. The solid-state fermentation process allows us to observe a change in the present composition of cocoa pod husk, generating new bioactive molecules with antioxidant potential, reaching 7.09 ± 0.05 μM equivalence in Trolox in the DPPH assay and 5.170 ± 0.035 x 104 μM in Trolox equivalence in the ORAC assay. The increase in the concentration of the phenolic compounds in during the fermentation is evidenced by the quantification of total phenols reaching a concentration of 114.40 mg in gallic acid equivalence in TPC assay.

Keywords: Cocoa pod husk; *Rhizopus*; Solid-state fermentation; Antioxidant activity; Cinnamic acid.

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CHAPTER I (LITERATURE REVIEW)

1. INTRODUÇÃO

The *Theobroma cacao* agro-industry generates an enormous amount of waste, which in practice has no benefit, and is often used to feed animals, burned in boilers or even thrown in the soil to serve as a nutritional source to the soil of crops. The solid-state fermentation of these residues are low-cost, allowing the production of biomolecules of commercial interest. By definition, bioactive compounds are essential and non-essential nutritional constituents that occur in small quantities in food and generate benefits on human health (Biesalki 2009; Kris-Etherton 2002). These compounds have been the target of various studies evaluating its protective effect against cardiovascular disease (Hung 2000), anticancer activity (Cragg 2005), anti-inflammatory effects (Calixto 2003; Calixto 2004), among other biochemical activities.

1.2 BIOACTIVE COMPOUNDS

According to National Cancer Institute at the National Institutes of Health from United States, bioactive compounds may be found in small amounts in plants, fruits, vegetables, nuts, oils, whole grains and certain foods (NCI 2016). These compounds have important actions in human body, where several studies aim the prevention of cancer, heart disease, diabetics and others diseases (Rice-Evans 1997; Kris-Etherton *et al.* 2002; Heim 2002). Bioactive compounds are in most instance secondary metabolites, such as phenolic compounds, plant growth factors, alkaloids, antibiotics and mycotoxins (Martins *et al*. 2011).

1.3 PHENOLICS COMPOUNDS

Phenolic compounds are substances originating from the secondary metabolism of plants, mainly in fruits and vegetables, since they are essential for their growth and reproduction, acting as antipathogenic agents (Shahidi *et al*. 1995). In relation to chemical structure, phenols are compounds that stand out in their structure with one or more hydroxylic flavors, including their functional groups (Lee *et al*. 2005). The phenolic compounds are classified in three groups (figure 1).

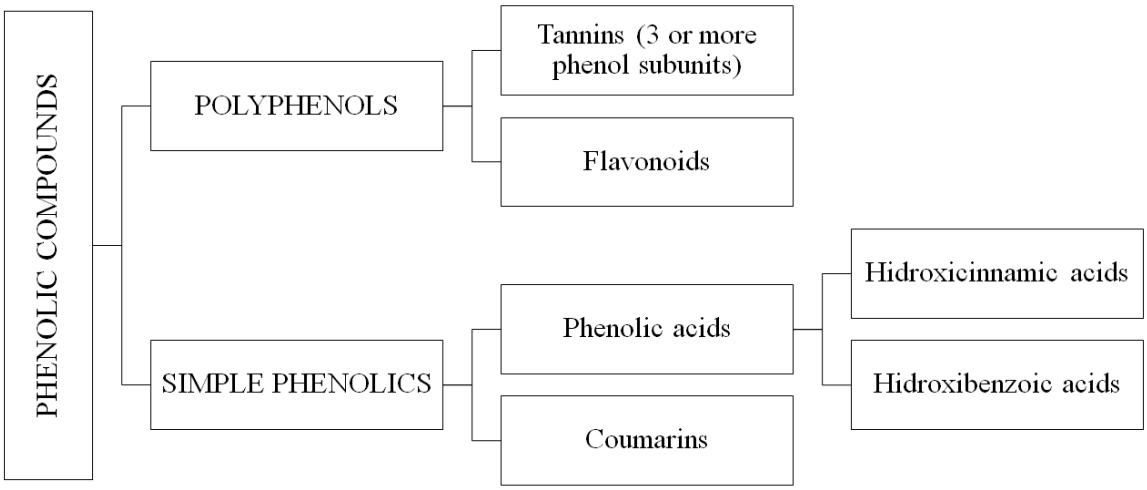

Figure 1. Phenolic compounds classification.

Tannins and flavonoids comprehend the polyphenols group, where tannins are formed by three or more phenol subunits playing a hole in plant protection against predation, and perhaps as pesticides (Chung *et al.* 1998). Flavonoids in general consist of two phenyl rings and heterocyclic ring can be classified into flavone, chalcone, flavonol, flavanone, isoflavone, flavanol and anthocynidin. These compounds are described and studied due to its biological activities as antioxidant, hepatoprotective, antibacterial, anti-inflammatory and antiviral (Heim 2012; Kumar and Pandey 2013). Coumarains, stillbenes and lignans has different characteristics. Coumarins consist of a large class of phenolic substances with pharmacological properties such as anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, anti-tubercular, anticonvulsant, anti-adipogenic, anti-hyperglycemic, antioxidant, and neuroprotective properties (Venugopala 2013).

In human feed, the main sources of phenolic compounds are cereals, fruits, vegetables and their derivatives, such as juices, beer and wine. In nature, fruits such as citrus, along with papaya, grape, cherry, pear and apple are those with higher amount of these substances (Pimentel *et al*. 2005). Since they encompass from simple molecules to the ones with high degree of polymerization, they can be found in plants in free form or linked to sugars and proteins (Bravo 1998).

There are a large number of studies involving the action of a compound, due to its beneficial properties to human health. The tannins, lignins, flavonoids, phenolic acids, simple phenols and coumarins are the most representative of the heterogeneous group they form. The chelation of metals, antioxidant activity and enzymatic inhibition are the mechanisms that are part of the biological activity of phenols (Su *et al*. 2007). The importance of phenolic compounds is not restricted only to pharmacological and medicinal areas, since these substances are also applied in the food industry because of dyes, arome and astringency in various foods (Soares 2002).

1.4 HIDROXICINNAMIC ACIDS

Hydroxycinnamic acids are a class compounds derivatives of Cinnamic acid (CA), which according to Chemical Abstractshe is also known as 3-Phenyl-2-propenoic acid, and it has the possibility of isomerism cis/trans. However, trans-cinnamic acid is more common in nature (figure 2). CA is an organic compound ($C_9H_8O_2$), which occurs in superior plants and it is found in essential oils, resins, balsams and oil of cinnamon. This compound is an important intermediate in the shikimate and phenylpropanoid biochemical pathways (Edreva 2005). Therefore, CA belongs to auxin group, vegetable hormone responsible for regulating growth, lignin formation and differentiation of cells (Niero 2010). It also originates flavonoids, which plays an important role in the plant defence against attack by microorganisms and insects (Thimann 1969).

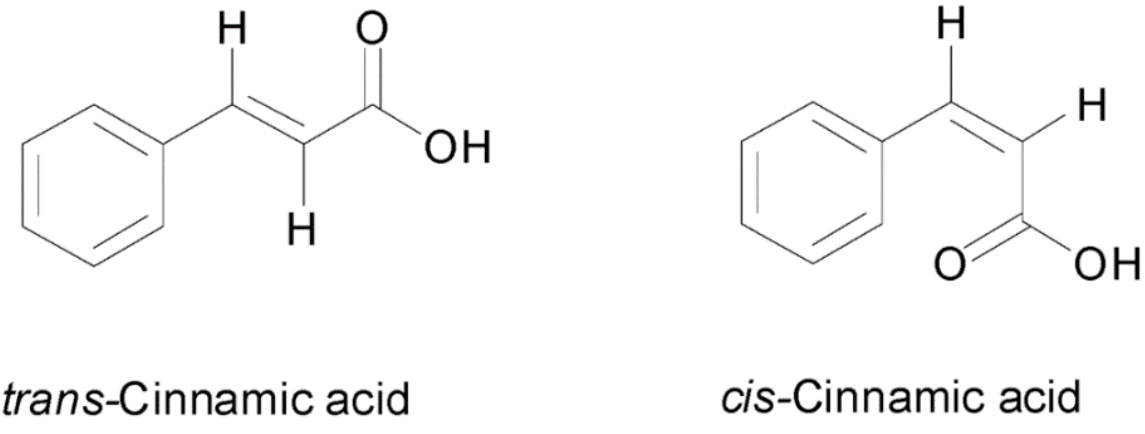

Figure 2. Chemical structure of trans and cis-cinnamic acid (Guzman 2014).

In a mini-review, Sova (2012) presents several antimicrobial and antioxidant activity of CA and its derivates (CAD). The potential of these substances are directly

related with the differentiation (addition and substitution of radicals) which occurs in innumerous biochemical pathways (figure 3).

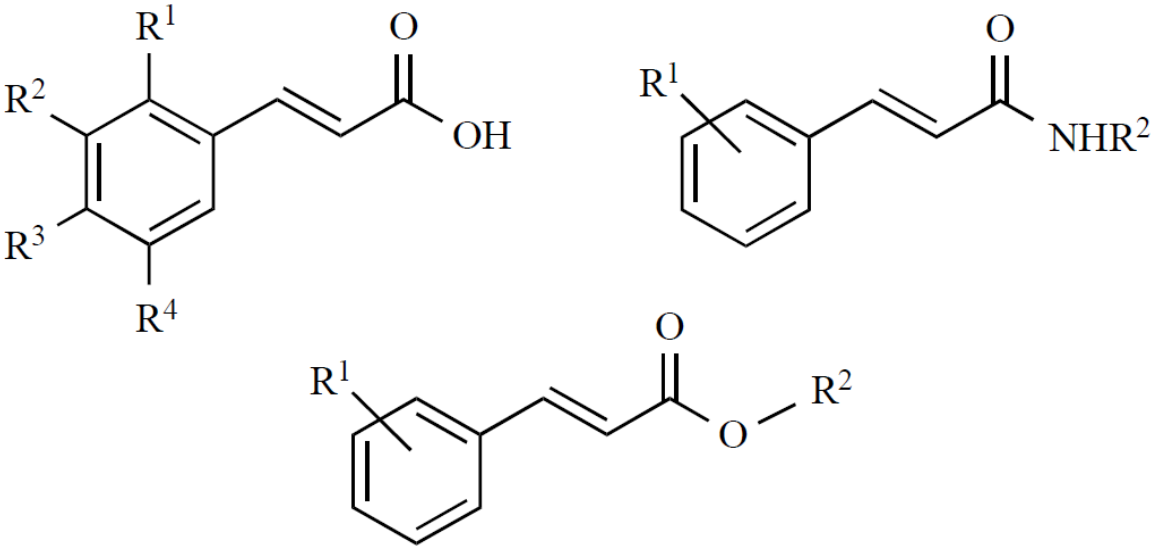

Figure 3. The possible recombination at Cinnamic acid structure by addition or substitution of radicals (R^n).

CA demonstrate efficacy when used in treatment against colon carcinoma cells (Caco-2) *in vitro* and against cells lines from lung, prostate, glioblastoma and melanoma cancer (Ekmekcioglu 1998; Liu 1995). Hafizur *et al* (2015), demonstrate that CA exerts anti-diabetic activity by improving glucose tolerance *in vivo* and stimulating insulin secretion *in vitro*.

Derivate compounds of CA as ρ-Coumaric acid, Caffeic acid, Ferulic acid and Sinapic acid have various functionalities as demonstrated in the table 1. Therefore, the production of CA and CAD using industrial and agro-wastes, such as cocoa pod husk (CPH) in Solid-satate fermentation (SSF) is a low cost and environmental-friendly process.

Table1. Application of Cinnamic acid and some of its derivatives

Compound	Application	Reference
Cinnamic acid	Anti-Diabetic Cancer's treatment	Hafizur *et al*. 2015. Liu 1995; Ekmekcioglu 1998.
ρ-Coumaric Acid	Antioxidant activity Modulator of glucose and lipid metabolism Preventive effects on lysosomal dysfunction and myocardial infarct size	Shaheen 2011. Yoon et al. 2013. Roy and Prince 2013.
Caffeic Acid	Antioxidant Activity	Nitzsche et al. 2004; Kang et al. 2009.
Ferulic Acid	Antioxidant Activity	Graf 1992.
Cinnamic Acid Esters	Antimicrobial Activity	Tawata et al. 1996; Narasimhan et al. 2004.
Cinnamic Acid Amides	Antimicrobial Activity	Narasimhan et al. 2004.

1.5 CINNAMIC ACID PRODUCTION

The CA can be obtained from plant extracts and its concentration in plant tissues is low, which results in low purity or low yields of the final products. Because of that, some alternative production methods are necessary to increase the final amount of the acid (Limem 2008). Thus, researchers sought other alternatives, such as chemical method of Perkin reaction and Knoevenagel condensation, that improve the yield of CA and CAD in relation to natural extraction realized in plants. Enzymatic production demonstrated a high yield percentage, but this method requires specifics enzymes immobilized which demand higher costs.

The production through fermentation processes using strains with the potential to produce these compounds or strains genetically modified for this purpose can make the production of CA and CAD commercially favorable in relation to the other processes using low-cost carbon and nitrogen sources.

1.5.1 Perkin reaction

Perkin reaction is the most frequently chemical method for the preparation of the cinnamic acid and its derivatives. This synthesis method of cinnamic acid is based in a reaction using benzaldehyde in acetic anhydride and anhydrous sodium acetate (figure 4). However, even being an easy method of production, there are some disadvantages in this reaction, such as the appearance of unwanted molecules and not suitable for electron donor substituent as it leads to low yield (Perkin 1868; Perkin 1877; Johnson 1942).

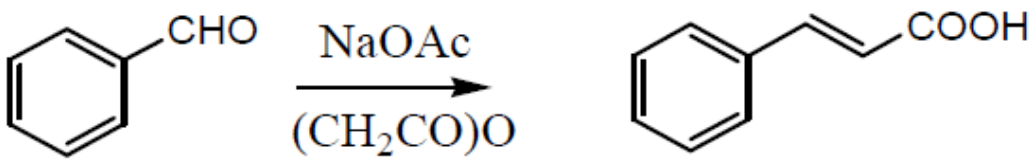

Figure 4. Perkin reaction scheme (Sharma 2011).

1.5.2 Knoevenagel condensation in water

Gupta and Wakhloo (2007) reported an environmental-friendly procedure for Knoevenagel condensation between aldehydes or ketones and malonic acid in the presence of tetra butyl ammonium bromide, K_2CO_3 and water under microwave irradiation.

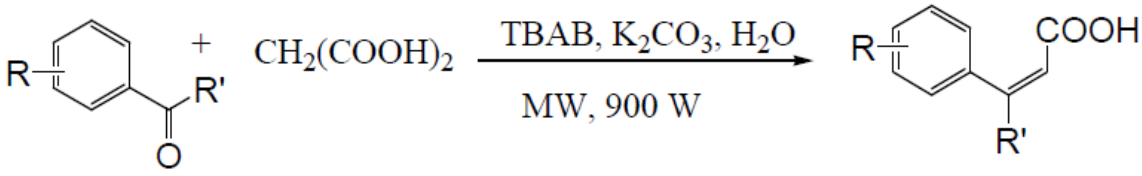

Figure 5. Cinnamic acid production by Knoevenagel condensation (Sharma 2011)

This process is simple, efficient and economical. However, the microwave irradiation requires a technical attention and equipament. Compared to the production by microorganisms, the production by this method becomes still expensive.

1.5.3 Enzymatic production

Lee at al. (2006) reported a CAD synthesis from an enzymatic method. They used ethanol and ferulic acid with Novozym 435 (*Candida antartica* lipase immobilized on acrylic resin) as a catalyst to produce ethyl ferulate. This method is efficient in shorter reaction time and better conversion to CAD. Guyot *et al*. (1997) used the same enzyme to produce esters between cinnamic, caffeic and ferulic acid and alcohols, with the aim to produce compounds with high antioxidant activity. The disadvantage of these methods is the loss of catalytic activity of enzyme due to the presence of ethanol.

1.5.4 Biochemical synthesis

In superior plants, phenylalanine (Phe) is a precursor of CA. It is due to a deamination of Phe by the phenylalanine-ammonia-lyase (PAL). Therefore, CA is an important precursor in metabolic pathways of higher plants for production of polyphenols compounds as shown in figure 6.

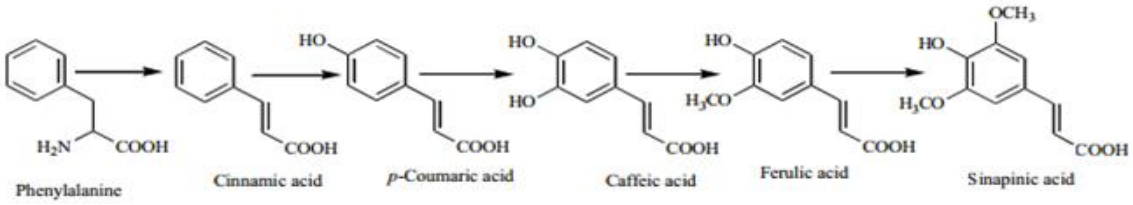

Figure 6. Cinnamic acid metabolic synthesis from the deamination of the amino acid phenylalanine by phenylalanine-ammonia-lyase (De 2011).

The first isolation of PAL was reported by Koukol and Conn in 1961. PAL was isolated from *Hordeum vulgare* like phenylalanine deaminase (Koukol and Conn 1961). Recent developments include novel purifications strategies which allowed the discovery of a cofactor, named 3,5-dihydro-5-methyldiene-4H-imidazol-4-one (D'Cunha 1996a; Calabrase 2004).

The PAL is widely found in many microorganisms (Vance 1975; Sikora 1982; Graf 1992; *Aydaş* 2013) and in fungi, it was demonstrated abundantly in *Rhodotorula* genus (Fritz 1976), as well in *Sporobolomyces roseus* and *Sporidiobolus pararoseus* (Watanabe 1992).

In superior plants, PAL uses L-Phe, but in microorganisms this metabolic pathway extents to L-Tyr due to the presence of Tyrosine-ammonia-lyase (TAL). Others common amino acids are not deaminated; such as D-Phe and D-Tyr, which do not serve as substrate and eventually, became competitive inhibitors of PAL (Ogata 1967; Hodgins 1971). Thus, in biotransformation of L-Phe in CA is essential its presence in solid or liquid medium, such as demonstrated by Bonvehí and Coll, where phenylalanine has an average of 0.45g ± 0.05 and L-Tyr has an average of 0.42g ± 0.04 for each 100g of CPH (Bonvehí 1999).

1.6 PRODUCTION BY MICRORGANISM

Microorganisms are used in bioprocess even before the knowledge of their existence. The production of breads, alcoholic beverages and fermented vegetables occurs in humanity history for centuries until the discovery of microorganisms. With the emergence of biotechnology, the processes are controlled and applied consciously. So that specific products are produced from selected strains

Webley (1955) made a study of mechanism of breakdown of ω-phenyl substituted fatty acids by *Nocardia opaca*, gram-positive bacteria. Acids such as phenyl propionic, phenylvaleric and phenylheptylic were converted to benzoic and cinnamic acid during the fermentation.

The biotechnological solutions to phenylpropanoids shyntesis is an alternative to large-scale production (Vargas-Tah and Gosset 2015). Some studies have presented strategies that include the generation of modified microbial strains with the capacity to synthesize CA (Sariaslani 2007). Vargas-Tah et al (2015), reported CA and *p*-hydroxycinnamic acid production. They used *E. coli* genetically modified with heterologous PAL/TAL gene from *Rhodotorula glutinis* and *Arabidopsis thaliana*. Furthermore, CA and CAD production by fermentation process could use innumerous carbon sources, i.e. fermentation process allows use of agro and industrial waste as carbon source for the strains used in process.

In so many studies, the use of genetic engineering of PAL/TAL genes in a host microorganism (table 2) reports a significant increase of production when compared to natural extraction and chemical synthesis. Nijikamp et al (2007a) reported a production of 799.5 mg/L of CA, using *Pseudomonas putida* S12 engineered strain and using glycerol as a carbon source.

Table 2. Microorganisms generally used as a host to increase CA production

Host microorganisms	CA (mg/L)	Reference
Escherichia coli	186.0	Vannelli et al (2007a)
	151.4	Vargas-Tah et al (2015)
Saccharomyces cerevisiae	-	Vannelli et al (2007a)
Pseudomonas putida	46.5	Nijikamp et al (2005)
	799.5	Nijikamp et al (2007)
Streptomyces lividans	460.0	Noda et al (2011)
	490.0	Noda et al (2012)
	-	Kawai et al (2013)
Rhizopus oligosporus NRRL 2710	124.7	Miyaoka (2012)

Vannelli et al (2007a) has not shown a production of CA using *S. cerevisiae* strain, but has reported a production of 31.8 mg/L of *p*-hydroxycinnamic acid. Such as, Kawai et al. (2013) reported a 500.0 mg/L of *p*-hydroxycinnamic acid production with *S lividans*. Several works using genetically modified microorganisms demonstrated significant differences when different carbon sources were utilized in medium culture.

1.7 RHIZOPUS

Fungi from genus *Rhizopus* are generally saprophytes and colonized decaying or dead organic material, such as leaves, husks and soil. The most described member of *Rhizopus* genus is *Rhizopus oryzae*. This fungus is used in tempeh, a dish from fermented soybeans.

Rhizopus has a diverse catabolism and anabolism of carbohydrates. Regardless of the substrate and condition, almost all sugar derivates are channeled through pyruvate. By a limited oxygen supply, pyruvate will be transformed into lactic acid or ethanol, depending on the *Rhizopus* strain. In presence of oxygen, the pyruvate will form acetyl-Coa and enter the tricarboxyl acid cycle that allows a highest biomass production. *Rhizopus* strains utilize two pentose sugars, xylose and arabinose in anaerobic conditions. The *Rhizopus* metabolism converting xylose to xylitol that was converting to xylulose, which is consumed by cells (Lennartsson and Taherzadeh 2014). Arabinose was converting in xylitol follow the same metabolic pathway (figure 7).

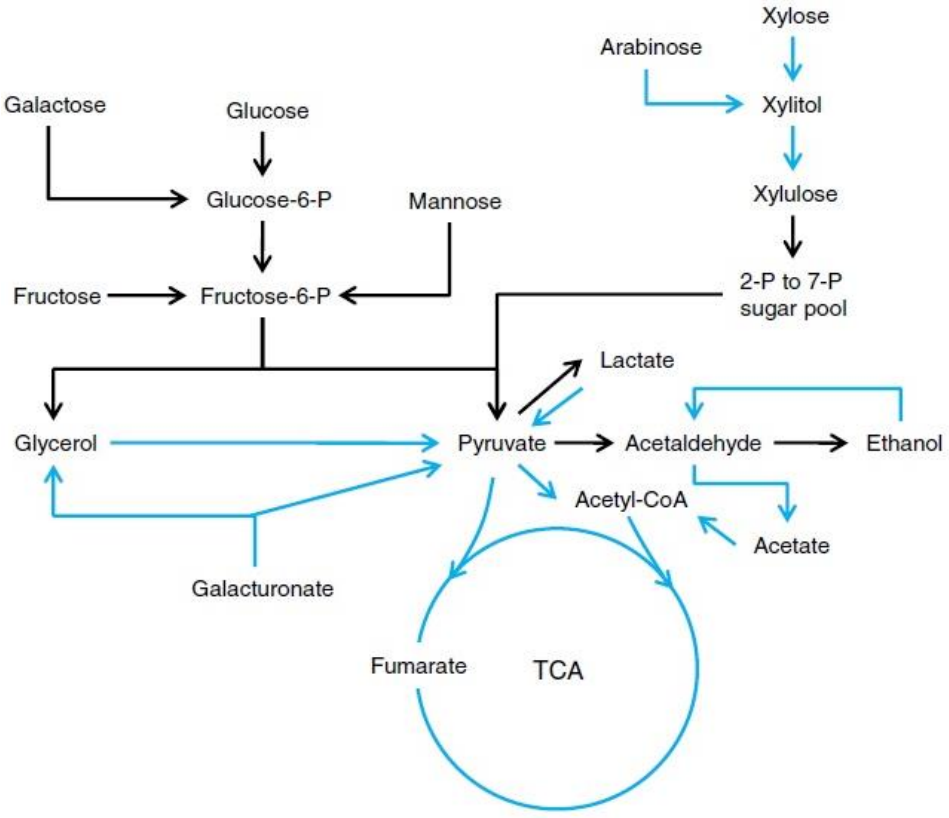

Figure 7. The metabolic pathways of *Rhizopus*. Reactions depending on or significantly induced by aerobic conditions have been marked as blue (Lennartsson and Taherzadeh 2014).

Considering multiple carbon sources, *Rhizopus* prefers hexoses, which is readily utilized even during anaerobic conditions. Hexose dimers, such as cellobiose, is also readily utilized, followed by the pentoses, assuming aerobic conditions. However, the adaptation of *Rhizopus* toward the more easily degradable polymers found in plant material and utilizations of galacturonic acid are to be expected. Galacturonic acid is split into pyruvate and L-glyceraldehyde, which is reduced to glycerol. This utilization seems to be strictly aerobic metabolism (Lennartsson and Taherzadeh 2014).

Then species have been divided into two subgroups, basing in lactic acid and fumaric acid production. So, the use of *Rhizopus* strains is related with its ability to utilize innumerous sources of starch and carbon (Lennartsson and Taherzadeh 2014). The *Rhizopus* is widely investigated due to its enzymes production, which turns this species able to catalyses pectin, which is present in many agro-wastes, including cocoa pod husk (*Lennartsson* 2014; Adomako 1972).

1.8 SOLID STATE FERMENTATION

Solid-state fermentation (SSF) is defined as a process in which microorganisms are grown on solid substrates in the absence of free water. Th solid base is generally a low cost source (Pandey 2003). Substrate as CPH is commonly used due to the physical and chemical features to produce many biocompounds. Gupte and Madamwar (1997) have demonstrated the production of enzymes as cellulases and β-glucosidase from lignocellulosic waste of several agro-industries lines and using fungi as biotransformation agent.

Rhizopus is a filamentous fungi genus has also been investigated such as treatment of industrial wastewater and production of animal feed (Lateef *et al.*2008). Soccol et al (1994), produced fumaric acid utilizing orange peels as substrate (Soccol 1994a; Soccol 1994b). In this way, cocoa pod husk can be used in SSF process as a nutritional source with low cost to be applied and so transform this environmental problem in a viable process.

Lateef et al. (2008) used CPH and others agro-waste as a substrate in a SSF utilizing a *Rhizopus stolonifer* strain to produce phenolic compounds with antioxidant activity and

to improve the nutritional qualities of this agro-wastes. This work has demonstrated the potential use of CPH as a nutritional source using *Rhizopus* strains in a SSF.

1.9 COCOA POD HUSK

Africa has 68% of total world production of *Thebroma cacao L.*, where the largest producers are Ivory Coast, Ghana, Nigeria and Cameroon. Asia and Oceania have together 17% of world production (main producers are Indonesia, Malaysia and Papua New Guinea). In America (15% of total world production), the production is concentrated mainly in the countries of Brazil, Ecuador and Colombia (World Cocoa Foundation 2015).

Around 80 to 90% of cocoa crops come from small family farms in the countries mentioned above. Cocoa pod husk (CPH) is a waste obtained after the removal of cocoa beans and thereabout for each ton of cocoa beans produced 10 t of CPH are generated which represents a serious challenge for waste management (Figueira and Janick 1993).

Syamsiro et al. (2012) demonstrated that CPH could be a source of renewable energy when used for biomass burning. As well as is considered by Osei et al. (1999) a valuable source of dietary fiber that combined with others qualities can transform CPH in an attractive nutrition source to complement animal feed (Adomako, 1972; Donkoh, 1991; Bonvehí,1999; Vriesmann, 2012). Even it is a sustainable alternative to this small family farms but there are economically better alternatives to manage this waste. Considering the biochemical composition of CPH, it may be a significant source for fermentation processes (Bello, 2011).

In nature, the decomposition of this waste occurs when it is exposed to physical, chemical and biological factors. Physical factors are generally represented by temperature and light exposition while moisture is an important chemical factor. Decomposer microorganisms generally represents biological factors, as bacteria and fungi. Bacteria have a lower power in case of decomposing plant residues, due to their difficulty to digest cellulose, hemicelluloses and lignin. Thus, fungi are the most common and capable of decomposing these complex structures, using them as a nutritional source. As demonstrated by Rahim et al. (2015), several fungi species colonize and decompose stems

of plant in cocoa cropping. So, a screening of biocompounds in this fungi species and then metabolic pathways can be better explored. Nevertheless, many microorganisms are known in the literature for their abilities to produce various metabolites of commercial interest using waste as nutritional source. The substrate is the most important condition in fermentation process, so is essential that required nutrients remain available for the microorganism, allowing both cell maintenance and the production of secondary metabolites of interest.

Bonvehí and Coll (1999) investigated samples of CPH from *Theobroma cacao L*. which grown in Ivory Coast, Nigeria, Cameroon, Colombia, Ecuador, Guinea and Brazil. The study showed that the amount of protein varies between 12.5 and 17.60g / 100g of CPH. The presence of various free amino acids was checked with an average content of 315mg / 100g of CPH. Lipid levels ranged between 1.8 and 3.0 / 100g of CPH. Glucose is the most commonly found sugar, with no significant traces of sucrose. The total free sugars and starch averaged 2.8g / 100g of CPH (Bonvehí 1999). Vriesmann et al. (2011) investigated the composition of CPH from crops in Brazilian Northeast and as known, minerals are key components in the microorganism cell. This study demonstrated that ions of Ca, Fe, K, Mg and Na are found in significant concentration (mg/100g of CPH) and ions of Cu, Mn, Se and Zn are found in trace concentrations (mg/ Kg of CPH). These components perform various functions in microorganisms, including metabolic and physiological functions.

CPH presents a composition rich in lignin, non-starch polysaccharides (cellulose, hemicelluloses and pectin), besides terpenoids (Crysoplenol), flavonoids (Kaempferol derivates, Rhamnetin), phenolic and carboxylic acids (Protocatechuic acid, salicylic acid, citric acid and tartaric acid) and some amino acids (glutamine, asparagines, serine and lysine) (Adomako 1972; Donkoh 1991; Osei 1999; Bonvehí 1999). Despite its high potential as a substrate in bioconversions, there are still few reports in the literature about its use for the production of bioactive compounds.

The secondary metabolite of higher plants, as well as *Theobroma cacao*, has numerous substances classified as bioactive compounds, among which may be highlighted antibiotics, polyphenolic compounds, growth factors, pigments and phytohormones (Martínez 2012). It is largely demonstrated on literature the antioxidant

activity of cocoa products (Sanbongi 1998), cocoa beans (Arlorio 2008) and waste (Azizah 1999; Martínez 2012). In many cases, these metabolites could be precursors of biochemical pathways of microorganisms that can come to produce other molecules with even more relevance.

CHAPTER II (RESEARCH RESULTS) – IMPROVEMENT IN ANTIOXIDANT ACTIVITY

1. INTRODUCTION

The utilization of agro-industries waste as a nutritional source in solid-state fermentation is an environmental friendly. Cocoa manufacturing generates million tons of residual waste, which can be used in fermentation processes. Cocoa pod husk is a residual biomass with potential to be applied in solid-state fermentation due to its physicochemical characteristics. *Rhizopus* is a genus of filamentous fungi with enormous applicability in solid-state fermentation and able to produce biocompounds with antioxidant activity.

Antioxidant compounds is an important factor against oxidation process, which promotes innumerous cell damages, increasing diseases risk. Therefore, agro-industries residual waste applied in solid-state fermentation using a *Rhizopus* strain can generate antioxidant compounds with medical and commercial application.

2. MAIN OBJECTIVES

The study proposes the increase of antioxidant activity and bioactive compounds production in the cocoa pod husk through the solid-state fermentation using *Rhizopus stolonifer*.

2.1 Secondary Objectives

- Physical-chemical characterization of the cocoa pod husk.
- Determination of an extraction method for cinnamic acid and its derivatives.
- Selection of the strain to be used in solid state.
- Dosage of total phenols and antioxidant activity before and during the fermentation process.
- Identification of the biocomposites produced by the fermentation.

3. MATERIALS AND METHODS

3.1 Raw Material

The cocoa pod husks were obtained from crops located in state of Pará, at northern region of Brazil. The raw material was dried in 60 ºC for 24 hours (figure 8). Subsequently, CPH were fragmented and separated into different granulometries, being used in this study the fraction between 0.84 – 2.0mm.

Figure 8. Cocoa pod husk dried at 60 °C after 24 hours. Source: the Author

3.2 Physicochemical Characterization

The total and reducing sugar was determined by 2-hydroxy-3,5-dinitrobenzooic acid reaction (DNS) (Miller 1959). Protein was determined by Bradford method (Bradford 1976). The ion chromatographic analysis was performed in a Metrohm CH-9101 system to determine the presence of cations and anions. The AquaLab CX-2 was used in the water activity analysis. The pH was measured in a mixture obtained by suspending 1g of sample in 10 mL of distilled water.

3.3 *Rhizopus* strain selection

All strains used in this study are from National Center for Agricultural Utilization Research (NRRL, Peoria, Illinois) and belong to the Department of Bioprocess Engineering and Biotechnology of the Federal University of Paraná as follow described:

Microorganism
R. oligosporus NRRL 3271
R. stolonifer NRRL 28169
R. oryzae NRRL 395
R. arrhizus NRRL 28425
R. arrhizus NRRL 2582
R.oryzae NRRL 3562
R.oryzae NRRL 28627
R. oligosporus NRRL 2710
R.oryzae NRRL 28168
R. delemar NRRL 34612
R.oligosporus NRRL 5905
R. circicans NRRL 1475
R. delemar NRRL 1472
R. arrhizus NRRL 16179
R. sp NRRL 25975

The strains were grown in PDA medium (Potato Dextrose Agar) for five days at 30 °C. The spores were collected with distilled water and this suspension was used to inoculate the submerged and solid-state fermentation throughout the study.

Selection of the strain was previously based on the production of compounds capable of absorbing free radicals in DPPH assay. For this, a submerged fermentation was carried out in YM medium during seven days, being carried out a comparative of the third and seventh day of fermentation. The pre-selected strains were submitted to solid-state fermentation, and the best strain to colonize the cocoa pod husk was chosen.

3.4 Submerged fermentation

The conditions of fermentation were based in the natural needs of *Rhizopus* strains, using the temperature maintained around 30 °C and pH 5.5 (Lennartsson and Taherzadeh, 2014). The submerged fermentation occurs in erlenmeyer flasks containing X ml of Yeast and Malt Broth (YM) describe in table 3. The flasks were incubated in a rotatory shaker at 100 rpm during 7 days. The inoculum size was 10^7 spores/ mL.

Table3. Nutritional composition of Yeast Malt Broth medium used in submerged fermentation.

Medium compound	$g.L^{-1}$
Glucose	10
Peptone bacteriological	5
Yeast extract	3
Malt extract powder, refined	3

3.5 Solid-state fermentation

The fermentation conditions were based in the natural needs of *Rhizopus* strains (Lennartsson and Taherzadeh 2014). Erlenmeyers with 5 g of CPH were inoculated with 5 ml of a spore suspension containing 10^7 spores/ml. The initial pH of the CPH was 5.5 ± 0.1. The moisture content was adjusted with distilled water at 50%. Therefore, the flasks were incubated at 30°C. The extraction of cocoa pod husk fermented (CPHF) occurs between 0 and 144h of fermentation. Experiments were done in triplicates.

3.6 Cinnamic Acid and Phenolic compounds extraction

All extractions were made with 5.0 g of CPH and 50 mL of solvent. The solvent solutions were prepared with ultrapure water. Thus, was used methanol (99.8%) and ethanol (99.8%) in initial concentrate and diluted in water at 50% (v/v). The experimental design analysis considered three independent variables at two levels: time (1 and 2 hours), temperature (25 and 35°C) and agitation (100 and 150 rpm). The purpose was to determinate how these factors influence the extraction of compounds and which of the solvents has the better response. Therefore, the response (dependent) variable used was

the radical scavenging of extracts in a DPPH assay. It was a complete 2^3 factorial design, so the number of assay was equal eight and made in triplicate (table 4).

Table 4. Experimental design 2^3 generate eight assays

Assay	Time (hours)	Temperature (°C)	Agitation (rpm)
1	-1(1h)	-1(25°)	-1(100)
2	1(2h)	-1(25°)	-1(100)
3	-1(1h)	1(35°)	-1(100)
4	1(2h)	1(35°)	-1(100)
5	-1(1h)	-1(25°)	1(150)
6	1(2h)	-1(25°)	1(150)
7	-1(1h)	1(35°)	1(150)
8	1(2h)	1(35°)	1(150)

The extraction of cinnamic acid and other phenolic compounds from CPH using ethanol aqueous solution was compared with the method proposed by Counet and Collin (2003) with some modifications made by Gonçalves (2016). Where 10 g of CPH were extracted three times with 50 ml of acidified acetone solution composed by acetone, water and acetic acid (70: 29.5: 0.5 v/v). The sample was agitated for 1 h at 25 °C. After each extraction, the suspension was filtered on Whatman paper. The solvent of combined filtrates were totally evaporated in vacuum kiln (MMM Vacucell) at 40 °C and suspended in ultrapure water.

3.7 Radical Scaveging Activity using DPPH Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity is known as a standard assay in antioxidant activity researches. In this study, the DPPH radical scavenging activity was measured according to the methodology presented by Blois (1958) with modifications. The reaction mixture consisted of 100 µl of CPH and CPHF extracts (diluted to 1:20 w/v) added to 1.4 mL of DPPH methanolic solution (100µM). Absorbance was reset with distilled water and the read at 517 nm after 30 min of reaction using a UV-VIS spectrophotometer SP-2000. To calculate the scavenging activity of samples was used a control, which consist in solvent of extracts in same reaction and applied in the following equation:

Radical scavenging activity (%)=[(Control Abs-Sample Abs)/Control Abs]x 100

Where the *Control Abs* is the absorbance of ethanol 50% (v/v) in DPPH solution and *Sample Abs* is the absorbance of samples CPH and CHPF of in DPPH solution, both after 30 min of reaction.

3.8 Total Phenolic Content (TPC)

TPC was measured by Folin-Ciocalteu method (Zheng 2001) modified by Gouveia and Castilho (2011). The extract was dissolved in methanol to yield a concentration (w/v) of 10 mg/mL. 50 µL aliquots were mixed with 1.25 mL of Folin-Ciocalteu reagent (diluted 1:10) and 1 mL of 7.5% sodium carbonate solution. After 30 min, at room temperature, the decrease in absorbance was measured at 765 nm. The result was expressed in milligram of gallic acid equivalent per gram of CPH/CPHF (mg GAE/ g).

3.9 Oxygen Radical Absorbance Capacity (ORAC) assay

The Oxygen Radical Absorbance Capacity (ORAC) method was performed on a TECAN InfiniteM200 multilayer equipment using fluorescein. The reaction was performed at 37 °C. The reaction occurred for 30 min and the absorbance was measured every minute, generating a decay curve of the absorbance value. The obtained values were then related to the trolox (Sigma-Aldrich) standard curve, providing an ORAC result in µM trolox. For calculations, the following equation was used:

$$\text{ORAC (μM TE)} = C^* K^* \frac{(AUCs - AUCb)}{(AUCt - AUCb)}$$

Where *C* is the trolox concentration (50μM), *K* is the sample dilution factor, AUC is the area below the fluorescence decay curve of the sample (*AUCs*), blank (*AUCb*) and trolox (*AUCt*) that is calculate by equation:

$$AUC = \left(0.5 + \frac{f5}{f0} + \ldots + \frac{fn+5}{f0}\right) * 5$$

Where $f0$ is the initial fluorescence and fn is the fluorescence at time n.

3.10 Volatile compounds determination by Gas chromatography coupled to Mass Spectrophotometry (GC-MS)

The analysis of volatile compounds from the CPH and CPHF was performed using a headspace vial coupled to a solid phase microextraction (SPME) fiber (5% Carboxen [CARB]/95% Polydimethylsiloxane [PDMS], df 75 μm, partially cross-linked, Supelco, St. Louis, MI, USA). For each determination, 1 g of sample was stored in a 20 mL HS vial. The flask was heated at 70 °C for 10 min without shaking, followed by 15 min of fiber exposure in COMBI-PAL system for balancing the volume within the vial. The compounds adsorbed by the fiber were desorbed into the gas chromatograph injection system gas phase (CGMS-gun TQ Series 8040 and 2010 Plus GC-MS Shimadzu, Tokyo, Japan) at 250 °C. The compounds were separated on a column 95% PDMS, 5% phenyl (30 m x 0.25 mm, 0.25 μm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard Enterprise, Palo Alto, CA, USA). Helium was used as carrier gas at a rate of 1.0 mL/min. Mass spectra were obtained by electron impact at 70 eV. The compounds were identified by comparison to the mass spectra from the library database (Nist'98 and Wiley7n) (Carvalho Neto *et al*. 2017). The same procedure were followed to perform the GC-MS analysis of cocoa pod husk and fermented cocoa pod husk extracts (ethanol:water and acidified acetone solution). The extracts were completely dried and resuspended in ethanol P.A HPLC grade. For each determination, 1 mL of sample extract was stored in a 20 mL HS vial. The flask was heated at 50 °C for 10 min without shaking, followed by 15 min of fiber exposure in COMBI-PAL system for balancing the volume within the vial. The fiber exposure has been reduced to 50 °C due to ethanol boiling temperature (78 °C) to avoid the saturation of fiber.

3.11 Phenolic Compound identification by High Performance Liquid Chromatography (HPLC)

Both extracts (ethanol aqueous and acetone acidified) were passed through a clean-up SPE cartridge (SPE-ED, Octadecyl C18-E, 55µm, 70A, 500mg / 3ml). The activation of the cartridge was made using 2 mL of methanol and 2 mL of water were eluted. Thus, 4 mL of each extract was totally evaporated in vacuum kiln (MMM Vacucell) at 40°C and resuspended in 2 mL of water. Therefore, the sample was homogenized in an ultrasonic bath (sonic-tech ultrasonic clearing / Schuster L-100) and added to the SPE cartridge. The elution of the compounds of interest was done with 2 mL of methanol: water (60: 40 v/v). The identification and quantification of the phenolic compounds were done using HPLC apparatus (Agilent Technologies 1260 Infinity Series; Agilent Technologies, Santa Clara CA, USA). The column used was Eclipse XDB-C18 (150 x 4.6 mm, 5 µm). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) acidified with 2.5% acetic acid, filtered in 0.22 µm membrane Gonçalves (2016).

3.12 Statistical analysis

The data were analyzed in a completely randomized design with three replicates. A Fisher's exact test was performed using Statistica 7.5 (StatSoft, Tulsa, OK). Level of significance was established in a two-sided P-value < 0.05.

4 RESULTS AND DISCUSSION

4.1 Physico-chemical composition of Cocoa Pod Husk

In this study, the granulometry between 0.80 and 2.00 mm of CPH were used to A_w, moisture and ash analysis, while the aqueous extracts of these fragments were used to measure the pH, protein and reducing sugar contents. The CPH were submitted to a hydrolysis with hydrochloric acid (37%) before the total sugar analysis. The anions were identified in aqueous extract by Ion analysis Metrohm CH-9101. These results can be observed in table 4.

Table 4. Physicochemical composition of CPH

Analysis	Value in CPH
pH	6.18 ± 0.01
A_w	0.283 ± 0.001
Ash	8.2%
Moisture	3.4%
	Value (mg/g)
Total sugar	173.47 ± 0.26
Reducing sugar	11.67 ± 0.15
Protein	0.117 ± 0.001
	Value (mg/g)
F^-	0.114 ± 0.009
Cl^-	0.318 ± 0.015
Br^-	0.198 ± 0.008
NO_2^-	0.359 ± 0.005
SO_4^{-2}	0.649 ± 0.029
PO_4^{-3}	0.335 ± 0.010
Na^+	0.022 ± 0.003
NH_4^+	0.080 ± 0.010
K^+	0.200 ± 0.006
Mg^+	0.440 ± 0.021
Ca^+	0.032 ± 0.009

CPH present a low free sugar and protein concentration when compared an ideal medium described for Lennattsson and Taherzadeh (2014) that is 30g.L^{-1} of glucose and 5 g.L^{-1} of yeast extract. However, the CPH has a concentration of minerals that supplied some *Rhizopus* needs. The mediums composition described by Lennattssin and Taherzadeh (2014) has a relation of needful compounds (annexes) that can be supplied by CPH composition tough in lower concentration than rich medium.

4.2 *Rhizopus* strain selection

Initially, the selection was based on the potential of *Rhizopus* strains to produce biomolecules with antioxidant activity without precursors or rich medium. Thus, the fermentation occurred in YM medium for 7 days (figure 9) with initial inoculum of 10^7 spores / mL of medium, being maintained at 30 °C and agitated at 100 rpm.

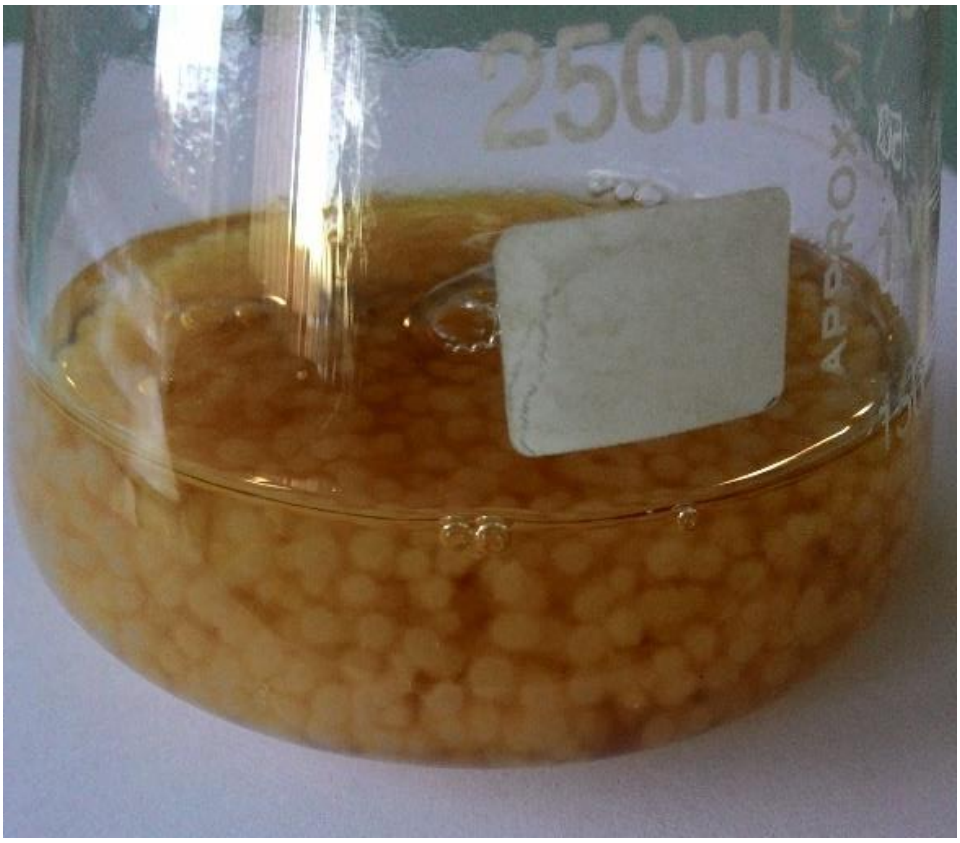

Figure 9. Submerged fermentation using *Rhizopus* strains in YM medium.

The selection was based in radical scavenging potential measured by DPPH assay (figure 10) directly from fermented broth.

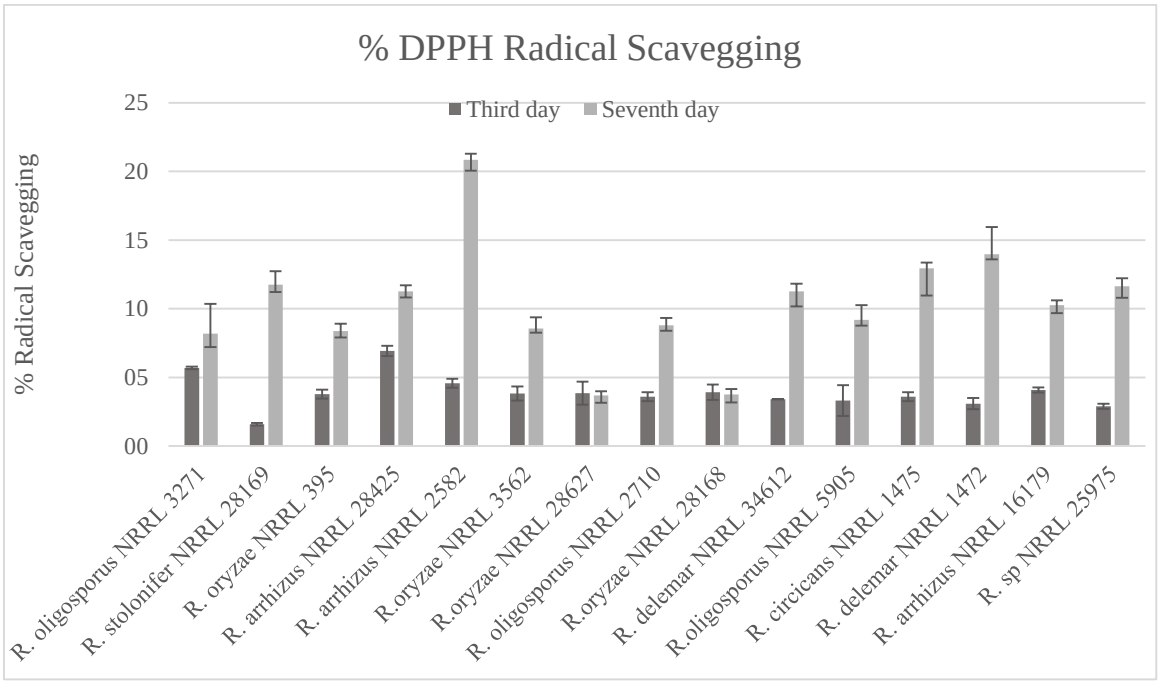

Figure 10. Antioxidant activity of fermented broths cultivated with different *Rhizopus* strains

These results showed that even without using any precursor the strains are capable of producing compounds with antioxidant properties. Based on this result, the strains: *R. stolonifer; R.arrhizus* NRRL 28425*; R. arrhizus* NRRL 2582*; R. delemar* NRRL 34612*; R. circicans* NRRL 1475*; R. delemar* NRRL 1472 and *R. sp* NRRL 25975 were applied in solid-state fermentation.

The strains selected were applied in solid-state fermentation of CPH, however each strain present a different response and not all of them showed a satisfactory colonization in CPH, presenting visible low filamentous growth. One of the possible reasons is the high concentration of bioactive compounds that can inhibit the metabolism of some strains or even a smaller production of enzymes capable of degrading pectin and cellulose present in the CPH. Thus, only the *R. stolonifer* showed a good growth in CPH and was used along of the study.

The solid-state fermentation was made at Erlenmeyer flasks, in which was add 5 grams of CPH. Therefore, all flasks were sterilized and the strains manipulated in asseptic conditions. The inoculum size used had 10^7 spores per gram of CPH and the strain *R. stolonifer* showed a good adaptability, able to grow on CPH in three days (figure 11).

Figure 11. Solid-state fermentation using *R. stolonifer* in CPH after 72 h.

4.3 Experimental design for extraction

This experimental design was initiated in order to investigate how the different independent variables (time, temperature and agitation) and their interactions influence the extraction of compounds with antioxidant activity. Different solvents were tested based on the extraction efficiency, the low cost and the simplicity of handling and the lowest hazard when compared to other reagents described in the literature. The following tables demonstrate the results of each assay into the experimental design proposed.

Table 5. Experimental design (2^3) with independent variables and experimental responses of the antioxidant activity (percentage) using methanol 99.8% as solvent.

	Independent variables			Experimental responses
Assay	Time (min)	Temperature (°C)	Agitation (rpm)	%AA
1	60	25	100	4.19
2	120	25	100	4.99
3	60	35	100	4.66
4	120	35	100	4.73
5	60	25	150	5.41
6	120	25	150	2.12
7	60	35	150	5.37
8	120	35	150	3.85

Table 6. Experimental design (2^3) with independent variables and experimental responses of the antioxidant activity (percentage) using methanol 50.0 % as solvent.

	Independent variables			Experimental responses
Assay	Time (min)	Temperature (°C)	Agitation (rpm)	%AA
1	60	25	100	7.80
2	120	25	100	8.72
3	60	35	100	6.27
4	120	35	100	4.90
5	60	25	150	5.59
6	120	25	150	4.84
7	60	35	150	6.44
8	120	35	150	6.82

Table 7. Experimental design (2^3) with independent variables and experimental responses of the antioxidant activity (percentage) using ethanol 99.8% as solvent.

	Independent variables			Experimental responses
Assay	Time (min)	Temperature (°C)	Agitation (rpm)	%AA
1	60	25	100	2.92
2	120	25	100	2.22
3	60	35	100	2.81
4	120	35	100	3.03
5	60	25	150	3.45
6	120	25	150	3.06
7	60	35	150	1.96
8	120	35	150	1.80

Table 8. Experimental design (2^3) with independent variables and experimental responses of the antioxidant activity (percentage) using ethanol 50.0% as solvent.

	Independent variables			Experimental responses
Assay	Time (min)	Temperature (°C)	Agitation (rpm)	%AA
1	60	25	100	25.27
2	120	25	100	15.32
3	60	35	100	16.74
4	120	35	100	38.58
5	60	25	150	15.70
6	120	25	150	48.64
7	60	35	150	33.54
8	120	35	150	47.12

According to the results presented in the tables, it is possible to observe that the activity found in the 50% ethanol extracts is superior to the other solvents. This is due to the fraction of compounds extracted at this concentration, which differ in polarity from the compounds of the other extracts. The DPPH assay is based on the scanning of free electrons, so the extractive compounds in ethanol 50% have a higher scanning capacity. However, the other fractions extracted may contain compounds of biological and pharmacological interest, but this was not the objective of this study.

In order to determinate the extraction parameters using ethanol 50%, the results of the assay was applied in a Pareto chart (figure 12) which not shown a significant result.

Even not being significant variables to optimize the extraction method these results indicate the importance of the time of exposition and the contact of cocoa pod husks with the solvents.

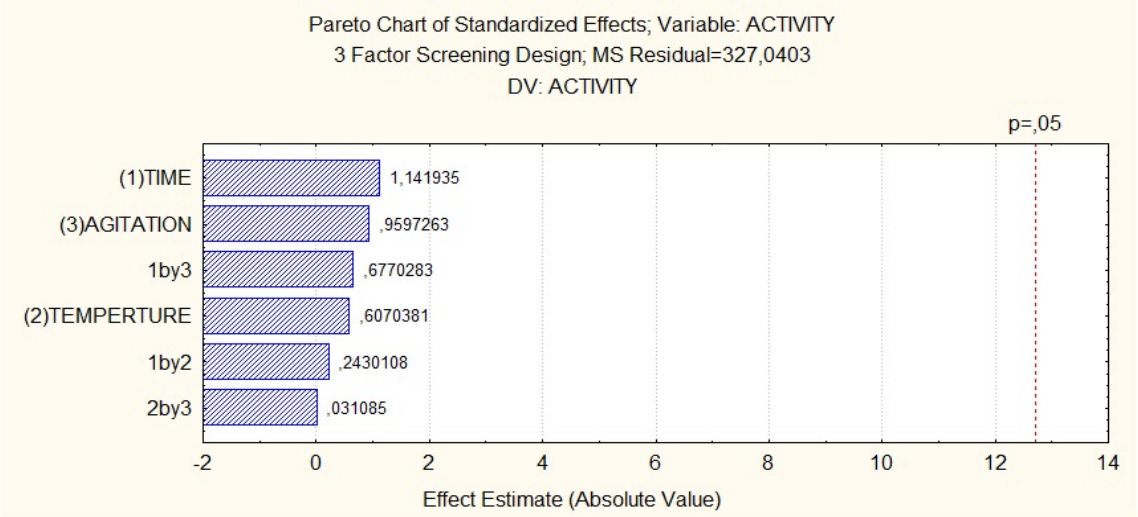

Figure 12. Pareto graphics of the dependent variable 'antioxidant activity'.

From the Pareto chart it is not possible to conclude that temperature, in the studied range is not a significant variable, such as its interactions with time and agitation, what reduces the cost of the process. However, the temperature of 35 °C was used due to higher antioxidant activity of the extracts when compare with the same process using 25 °C.

Based on the response surface (figure 13), it can be observed that the antioxidant activity value tend to increase according to interaction between time and agitation increases, but the spread of variables selected does not allow to visualized the optimum point. Moreover, in lab scale is difficult to extend the agitation above 150 rpm. Therefore, along the study the conditions of 150 rpm, for 2 hours at 35°C for all extraction performed with ethanol 50%.

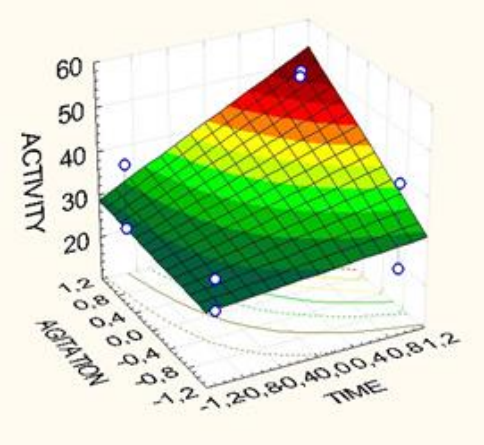

Figure 13. Response surface for the dependent variable ‘antioxidant activity’in relation to Agitation and Time of extraction.

4.4 Solid state-fermentation

Rhizopus are known by the pectin, cellulose and hemicellulose degradation due to enzymes production, which allowed this genus to colonize this kind of raw material. The pH slightly decreases indicating the production of compounds with acid characteristic. In the beginning of fermentation process, can be observed an increase in free reducing sugar content probably due to a breakdown of total sugars. After 96h of fermentation the consumption of reducing sugars became practically constant, but with a regular degradation of total sugar.

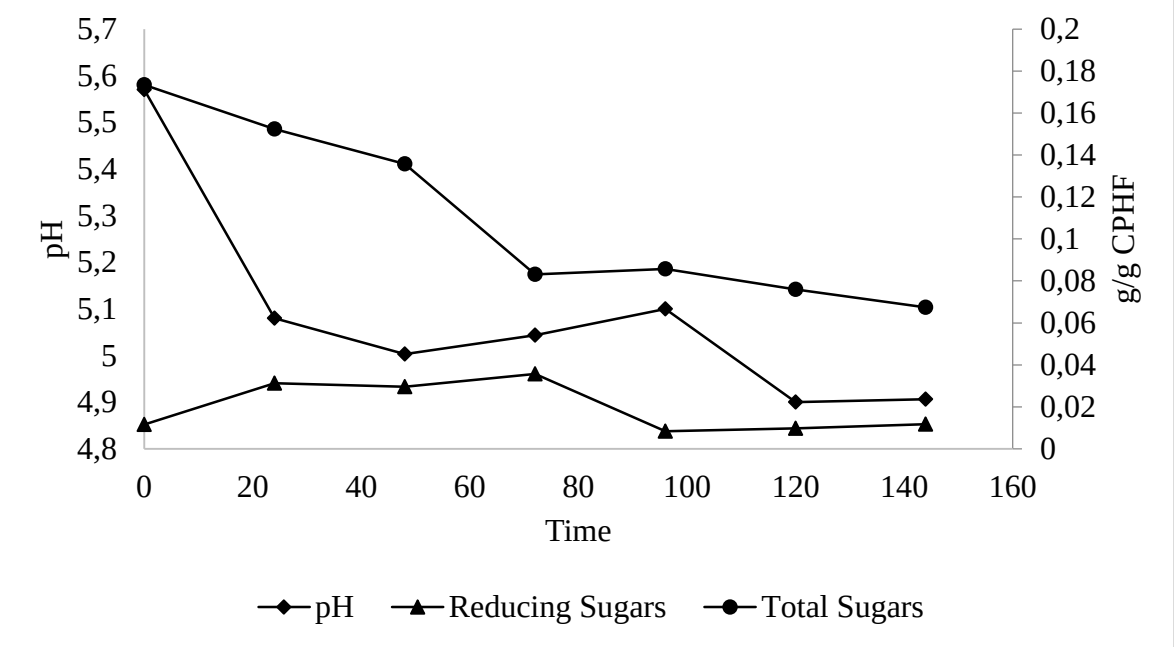

Figure 14. Total sugars, reducing sugars concentration and pH during the 144h of CPH solid-state fermentation by *R. stolonifer*.

At this point, the carbon source becomes a limiting factor for the production of phenolic compounds, as can be observed in the TPC (figure 15) and DPPH assay (figure 16).

4.5 Total Phenolic Content

The total phenolic content of the extracts from CPH and CPHF as determined by Folin-Ciocalteu method is reported in gallic acid equivalent (GAE). A standard curve of gallic acid was constructed($y = 0.0117x + 0.0343$; $R^2 = 0.9994$), where it is possible to

relate the total phenols present in extracts against gallic acid activity. The result obtained from the equation was applied in another equation to relate the GAE mass with initial mass of CPH and CPHF (figure 15).

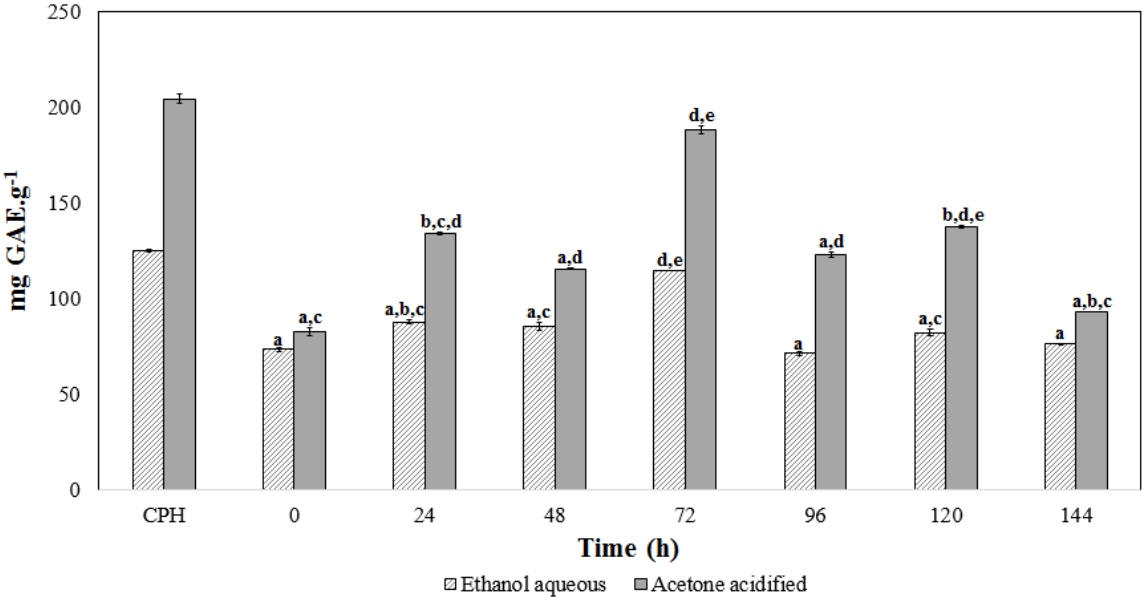

Figure 15. Total phenolic content of cocoa pod husk and between third and seventh day of fermentation.

Theobroma cacao L. is known and described in the literature by the rich composition of phenolic compounds. In this study, even the husk undergoing a drying process at 60 ° C and being subsequently stored at room temperature, 125.00 ± 1.26 and 204.48 ± 0.53 mg of GAE / g of dried CPH were found. But this concentration decrease to 73.17 ± 2.64 and 82.38 ± 0.85 after sterilization at 120 °C in autoclave for 20 min. Probably the high pressure and temperature of the autoclave can hydrolyze the phenolic compounds. During fermentation, an increase of these phenols can be observed in both extracts (figure 15) until the 72h, reaching 114.70 and 188.21 ± 0.63 mg GAE / g CPHF. It is an interesting result when compared with 69.0 mg GAE/ g fresh CPH from Côte d'Ivoire registered by Yapo *et al.* (2013). However, a decrease after the fourth day of fermentation of the concentration of these phenols was observed, reaching close to the initial concentration present at 0h of fermentation. The decrease of phenolic content could be related to decrease of carbon source as presented in figure 14. Therefore, these phenolic compounds can be used as carbon sources to *Rhizopus* or even used in esterification of cinnamic acid and its derivates. According to Narasimhan *et al*. (2004),

these compounds are able to form ester and amides that present antimicrobial activity, such as antifungal activity (Tawata 1996). Statistical analysis showed that despite the higher concentration obtained in the method using acetone acidified as solvent, there was no significant difference with the method obtained by the experimental design. Thus, it is justified the use of the ethanolic solution and of the parameters of extraction of phenolic compounds due to its lower cost in relation and its lower hazard.

4.6 Free Radical Scavenging Activity in DPPH assay

The free radical scavenging activity of CPH and CPHF extracts were tested through DPPH method. The principle of the method assay is that antioxidants react with the stable free radical. The reaction with α,α-diphenyl-β-picryhydrazyl convert it to α,α-diphenyl-β-picryhydrazine, so the deep violet color of solution has a discoloration that indicates the scavenging potentials of CPH and CPHF

In figure 16 is possible to observe the high antioxidant potential of the compounds that are already present in the CPH, reaching an activity of almost 85% in relation to the control. This result is similar to potential present by Yapo *et al*. (2013), where they obtained around 85% of antioxidant activity using a fresh cocoa pod husk from Ivory Coast.

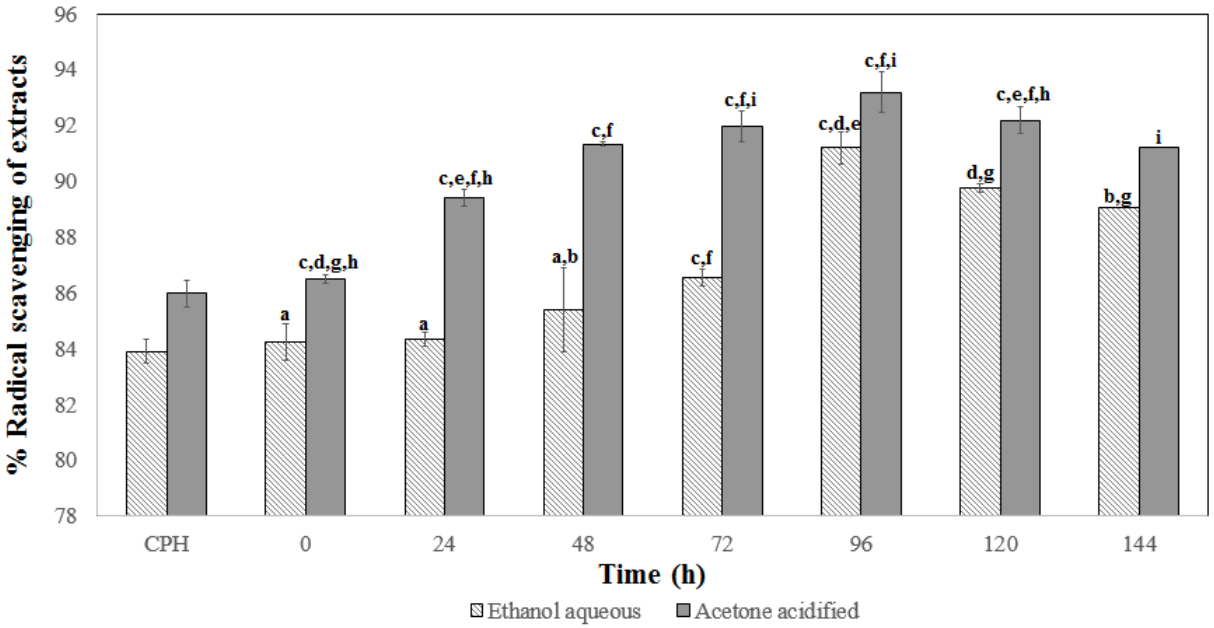

Figure 16. Antioxidant activity of CPH and respective days of fermentation

However, based on TPC this activity should be higher than CPHF. Esters and amides can be made, while it is known by antioxidant and antimicrobial potential (Narasimhan *et al*. 2004).

The activity measured with the extracts referring to 72h of fermentation did not have significant difference in relation to the extracts obtained with 96, 120 and 144 h. Thus, there may be a reduction in the fermentation time in 72 hours, which allows reducing the maintenance costs of the process in half. The analyzes also showed no significant difference between the methods, reinforcing the extraction efficiency using ethanol solution.

The activity equivalence measured by DPPH in µM of trolox (table 6) confirms the potential of compounds produced in fermentation. However, when compared directly in this assay, it is notable a significant difference between the methods. The polarity of solvents can influenced in the compounds extracted. So the compounds extracted by acetone acidified has more similarity with trolox kinetic of radical scavenging.

Table 6. Antioxidant activity equivalence in µM TE for each gram of CPH and CPHF.

Sample	DPPH (µM TE/ g sample)	
	Ethanol:Water (50:50 v/v)	Acetone:Water:Acetic acid (70:29,5:0,5 v/v)
CPH	6,52 ±0,03[a]	6,6 ±0,03[a]
0	6,55 ±0,05[a]	6,72 ±0,01[a]
24	6,56 ±0,05[a]	6,95 ±0,02[b]
48	6,64 ±0,08[a]	7,10 ±0,02[b]
72	6,73 ±0,09[a]	7,15 ±0,01[b]
96	7,09 ±0,05[a]	7,25 ±0,04[b]
120	6,98 ±0,01[a]	7,17 ±0,05[b]
144	6,93 ±0,09[a]	7,09 ±0,03[b]

In order to confirm the antioxidant potential of CPHF ethanol aqueous extract was realized a measured of trolox equivalence by ORAC assay, which has a different reaction mechanism.

4.7 Oxygen Radical Absorbance Capacity of Ethanol aqueous extract

During this assay, a decay occurs in the intensity of fluorescence that is monitored, generating a curve (figure 17).

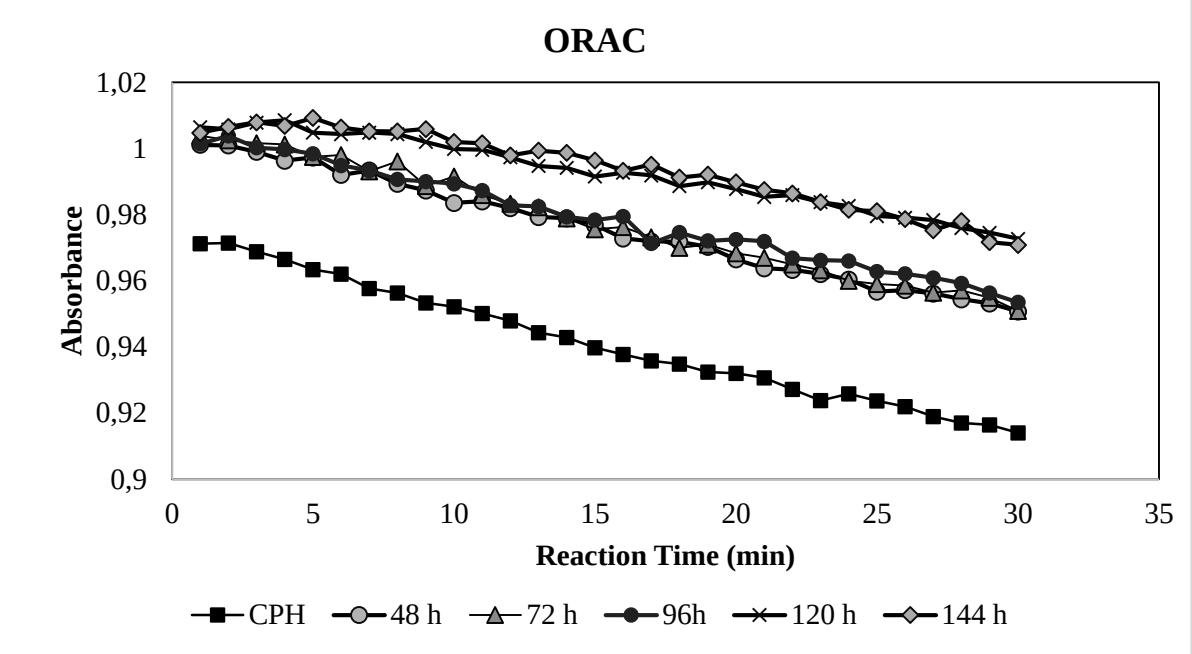

Figure 17. Curves of decaying fluorescein absorbance during 30 min assay in front of ethanol aqueous extract of CPH and CPHF after 48, 72, 96,120 and 144 h if fermentation.

This assay is extremely sensitive, so the samples were diluted (1: 500) prior to analysis to avoid any interference of antioxidant compounds in high concentration. The results of trolox equivalence are presented in table 7.

Table 7. Oxygen radical absorbance capacity (ORAC, trolox equivalent - TE) of extracts of the cocoa pod husk in the interval between the third and the seventh day of fermentation.

SAMPLE	ORAC (µM TE/ g sample)
CPH	$(4.770 \pm 0.028) \times 10^4$
48 h	$(5.030 \pm 0.048) \times 10^4$
72 h	$(5.040 \pm 0.045) \times 10^4$
96h	$(5.050 \pm 0.080) \times 10^4$
120 h	$(5.160 \pm 0.008) \times 10^4$
144 h	$(5.170 \pm 0.035) \times 10^4$

The results of ORAC method demonstrates that an increase in antioxidant capacity occurs with solid-state fermentation confirming the results obtained in DPPH assays. The CPH presented a value of (4.770 ± 0.028) x 10^4 μM TE / g of CPH reaching the end of the fermentation at (5.170 ± 0.035) x 10^4 μM TE / g of fermented. The ORAC shows a different result than the one demonstrated by the DPPH test, in which a decay of the antioxidant activity occurs after the fermentation. Although these tests focus their reactions on identifying the antioxidant capacity. The measurement of ORAC integrates both degree and time of antioxidant reaction. This method uses biologically relevant free radicals.

The sterilization process is aggressive to the phenolic compounds of cocoa pod husk, since its content is reduced after sterilization and measured at the initial time of fermentation (Figure 14). However the extracts of fermented cocoa pod husk present an increasing antioxidant activity, reaching a peak of 93.19% at 96h in DPPH with a 7.15 ± 0.01 μM TE / g CPHF and 5.170 ± 0.035 x 10^4 μM TE / g CPHF at 144h in ORAC assay.

4.8 Volatile compounds determined by Gas chromatography coupled to Mass spectrophotometry

CPH can be a rich source of studies of physicochemical characterization, as it is possible to see in table 8. During the process of sterilization and fermentation these compounds are modified, such as biotransformed by *R. stolonifer*. The GC-MS results demonstrated an innumerous volatile compounds which can be explored as products in new studies, such as the appearance of β-Hydroxybutyric acid after fermentation process. β-Hydroxybutyric is a precursor to polyester, which are used in biodegradable plastics (Doi *et al.* 1988). The β-Hydroxybutyric acid methyl ester appear in CPHF and to date no studies have been elucidate its potencial.

The volatile compounds characterization is important to identification of the possible flavors present in CPH and be produced by *Rhizopus stolonifer* such as rosefuran, α-Methylbenzyl alcohol and methoxy citronella present in CPHF (table 8). The modification of volatile composition also can be observed in ethanol aqueous and acetone acidified extracts. Dimethyl phithalate is present after fermentation process and it is used as an insect repellent for mosquitoes and flies, such as methoprene that is used as insecticide (Baikova 1999; Wijayaratne 2016).

"Cinnamic acid, 4-hydroxy-3-methoxy-, (5-hydroxy-2-hydroxymethyl-6-[2-(4-hydroxy-3-methoxyphenyl)ethoxy]-4-(6-methyl-3,4,5-trihydroxytetrahydropyran-2-yloxy)tetrahydropyran-3-yl) ester" was found in both extractions method even in CPH than in CPHF, but was not found a study that described his application or natural source. These volatiles compounds has a similarity relation with fragments deposited in database library. Therefore, can exist a variation on chemical final structure with the same atomic configuration, i.e., a low similarity value; increase the chances of inexactly the predicted compound.

Majority of the compounds found in GCMS results do not have elucidated functions in the literature, always being described as compounds present in essential oils extracted from plants. Some compounds may not be directly related to the metabolism of *Rhizopus stolonifer* and may have an origin with the breakdown of more complex compounds initially present in the CPH. The initiative of studies based on volatile compounds is still new and has only a qualitative character in search of new forms to identify potential new products to be explored in both CPH and CPHF.

Table 8. Volatile compounds identified in cocoa pod husk and cocoa pod husk fermentation in solid-state and compounds identified from acetone acidified and ethanolic extract by Gas Chromatography Coupled to Mass Spectrophotometry (GC-MS)

	Solid-state		**Acetone acidified extract**				**Ethanolic extract**			
COMPOUNDS	CPH	CPHF	CPH	CPHF 48h	CPHF 96h	CPHF 144h	CPH	CPHF 48h	CPHF 96h	CPHF 144h
ALKOHOL										
(+-)-5-Methyl-2-hexanol	-	+	-	-	-	-	-	-	-	-
(2S,4S)-(+)-Pentanediol	+	+	-	-	-	-	-	-	-	-
(3R,6R)-2,2,6-Trimethy,l-6-vinyltetrahydro-2H-pyran-3-ol	-	+	-	-	-	-	-	-	-	-
(R)-(-)-2-Methyl-2,4-pentanediol	-	-	-	-	-	+	+	-	+	+
[5-Hydroxymethyl)-1,3-dioxolan-4-yl]methanol	-	-	-	-	-	-	-	-	-	+
1,2-Pentanediol	-	+	-	-	-	-	-	-	+	-
11-Methyldodecanol	+	-	-	-	-	-	-	-	-	-
1-Hexanol	+	-	-	-	-	-	-	-	-	-
1-Isopropyl-1-butanol	-	+	-	-	-	-	-	-	-	-
1-Methoxy-2-butanol	+	+	-	-	-	-	-	-	-	-
1-Methoxy-3-(2-hydroxyethyl)nonane	-	-	-	-	-	-	+	-	-	-
1-Nonanol	+	-	-	-	-	-	-	-	-	-
1-Octen-3-ol	+	-	-	-	-	-	-	-	-	-

1-Phenoxypropan-2-ol	-	-	-	-	+	-	-	-	-	-
2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol	-	-	-	-	+	-	-	-	-	-
2,3-Butanediol	+	+	-	-	-	-	-	-	-	-
2,6-Dimethylcyclohexanol	+	-	-	-	-	-	-	-	-	-
2-Ethoxy-1-propanol	-	-	+	-	-	-	-	-	-	-
2-Ethyl-1-hexanol	+	-	-	-	-	-	-	-	-	-
2-Ethyl-3-pentanol	+	-	-	-	-	-	-	-	-	-
3,4-Dimethylpent-2-en-1-ol	-	+	-	-	-	-	-	-	-	-
3-Phenoxy-1-propanol	-	-	-	-	-	-	-	-	+	-
Epoxylinalol	+	+	-	-	-	-	-	-	-	-
Hexylene glycol	-	-	-	-	-	+	+	-	+	+
Linalool	+	-	-	-	-	-	-	-	-	-
n-Tridecanol	+	-	-	-	-	-	-	-	-	-
Phenylethyl Alcohol	+	+	-	-	-	-	-	-	-	-
trans-Linalool oxide	+	-	-	-	-	-	-	-	-	-
α-Hydroxytoluene	-	+	-	-	-	-	-	-	-	-
α-Methylbenzyl alcohol	-	+	-	-	-	-	-	-	-	-
α-Terpineol	+	-	-	-	-	-	-	-	-	-
ALDEHYDE										
(E,E)-2,4-Heptadienal	+	-	-	-	-	-	-	-	-	-
Heptanal	+	-	-	-	-	-	-	-	-	-

Methoxycitronellal	-	+	-	-	-	-	-	-	-	-
Nonanal	+	+	-	-	-	-	-	-	-	-
Octanal	+	+	-	-	-	-	-	-	-	-
Trans-2-Hexenal	+	-	-	-	-	-	-	-	-	-
trans-2-Octenal	+	-	-	-	-	-	-	-	-	-
α-Toluic aldehyde	+	+	-	-	-	-	-	-	-	-
β-Cyclocitral	+	-	-	-	-	-	-	-	-	-
KETONE										
n-Amyl methyl ketone	+	-	-	-	-	-	-	-	-	-
6,10-dimethyl-5,9-Undecadien-2-one	-	-	-	-	-	-	-	+	-	-
1-Phenylethanone	+	-	-	-	-	-	-	-	-	-
2-Dodecanone	+	-	-	-	-	-	-	-	-	-
Ethanone, 1,1'-(1,3-phenylene)bis-	-	-	-	-	-	-	-	-	+	+
Ethanone, 1,1'-(1,4-phenylene)bis-	-	-	-	-	-	-	+	-	-	-
Pyrrole-α-methyl ketone	+	+	-	-	-	-	-	-	-	-
Sulcatone	+	-	-	-	-	-	-	-	-	-
trans-3-Octen-2-one	+	-	-	-	-	-	-	-	-	-
trans-β-Ionone	-	-	-	-	+	-	-	-	-	-
β-Ionone	+	-	-	-	-	-	-	-	-	-
ESTER										
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	-	-	+	-	-	-	-	-	-	-

1,2-Dimethylpropyl acetate	-	-	-	-	-	-	-	-	-	-
1-Ethylpentyl acetate	-	+	-	-	-	-	-	-	-	-
2-(Heptyloxycarbonyl)benzoic acid	-	-	+	+	-	-	-	-	-	-
2-Propenoic acid, 2-methyl-, 1,2-ethanediylbis(oxy-2,1-ethanediyl) ester	-	-	+	+	-	-	-	-	-	-
Cinnamic acid, 4-hydroxy-3-methoxy-,(5-hydroxy-2-hydroxymethyl-6-[2-(4-hydroxy-3-methoxyphenyl)ethoxy]-4-(6-methyl-3,4,5-trihydroxytetrahydropyran-2-yloxy)tetrahydropyran-3-yl) ester	-	-	-	-	-	-	+	+	+	+
Dimethyl phthalate	-	-	-	-	-	-	+	-	+	-
Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester	-	-	-	-	-	-	+	-	-	-
Ethyl iso-allocholate	+	+	-	-	-	-	-	-	-	-
Fumaric acid, 2-isopropylphenyl dodec-2-en-1-yl ester	-	-	-	-	+	-	-	-	-	-
Methyl N-hydroxybenzenecarboximidoate	+	+	-	-	-	-	-	-	-	-
Methyl salicylate	+	+	-	-	-	-	-	-	-	-
Octacosanoic acid, methyl ester	-	-	-	+	-	-	-	-	-	-
Oxalic acid, bis(6-ethyloct-3-yl) ester	+	-	-	-	-	-	-	-	-	-
Oxime-, methoxy-phenyl-_	-	-	-	-	-	-	-	+	+	+
β-Hydroxybutyric acid methyl ester	-	+	-	-	-	-	-	-	-	-

ϒ-Hydroxybutyric acid cyclic ester		+	-	-	-	-	-	-	-	-	-
ORGANIC ACID											
10,12-Tricosadiynoic acid		-	+	-	-	-	-	-	-	-	-
2-(Heptyloxycarbonyl)benzoic acid		-	-	-	-	-	-	-	-	+	+
2-Propenoic acid, 2-methyl-, 3,3,5-trimethylcyclohexyl ester		-	-	-	-	-	-	+	+	-	-
Dl-Mevalonic acid lactone		-	+	-	-	-	-	-	-	-	-
Isovaleric acid		+	-	-	-	-	-	-	-	-	-
Palmitic acid		-	-	-	+	-	-	-	-	-	-
Tetradecanoic acid		-	-	-	+	-	-	-	-	-	-
HYDROCARBON											
(-)-Aristolene		-	+	-	-	-	-	-	-	-	-
1,2,4-Trimethylcyclopentane		-	+	-	-	-	-	-	-	-	-
13-Phenylpentacosane		-	+	-	-	-	-	-	-	-	-
1-Heptadecene		+	-	-	-	-	-	-	-	-	-
1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene		-	+	-	-	-	-	-	-	-	-
2,6,10,15-Tetramethylheptadecane		+	-	-	-	-	-	-	-	-	-
2-Cyclopropylidene-1,7,7-trimethylbicyclo[2.2.1]heptane		-	+	-	-	-	-	-	-	-	-
2-Ethylhexene		-	+	-	-	-	-	-	-	-	-
2-Methyl-n-hexacosane		+	-	-	-	-	-	-	-	-	-

D-Limonene	+	+	-	-	-	-	-	-	-	-
Eicosane	+	-	-	-	-	-	-	-	-	-
Heneicosane	+	-	-	-	-	-	-	-	-	-
Isoledene	+	-	-	-	-	-	-	-	-	-
Valerena-4,7(11)-diene	+	-	-	-	-	-	-	-	-	-
α-Copaene	+	+	-	-	-	-	-	-	-	-
ϒ-Cadinene	+	-	-	-	-	-	-	-	-	-
ϒ-Muurolene	+	-	-	-	-	-	-	-	-	-
AMINE										
Octanenitrile	+	-	-	-	-	-	-	-	-	-
1,4-Butanediamine	+	-	-	-	-	-	-	-	-	-
2-Hydrazino-2-imidazoline			-	+	-	-	-	-	-	-
Benzothiazole	-	-	-	-	-	-	+	-	+	+
AMIDE										
Caffeine	-	-	+	-	-	-	-	-	-	-
Caprolactam	-	-	-	-	-	-	-	-	-	+
ETER										
Rosefuran	-	+	-	-	-	-	-	-	-	-
β-Hydroxyethyl phenyl ether	-	-	-	-	-	-	-	-	+	-

4.9 Phenolic Compound identification by High Performance Liquid Chromatography (HPLC)

According to phenolic total content, the CPH has a higher concentration of phenolic compounds when compared to CPHF. However, the antioxidant activity of CPHF extracts is superior both in the DPPH and in the ORAC assays. This can be occurs due to the biotransformation of the initial compounds, such as hydrolysis of polyphenols in phenolic compounds.

The HPLC results is describe in table 9, where it is observed that the CPH has so many compounds when compared with aqueous ethanol and acidified acetone solution extracts at 96h of fermentation. In this study was used standards of trans-cinnamic acid, 3-hydroxycinnamic acid, caffeic acid, ferulic acid, quercetin and catequin (Sigma-Aldrich). However, only the Cinnamic acid was identified against standard compounds with a retention time equal to 28.093 min present in both the CPH extract and CPHF made with acetone acidified and using a standard curve (y=96,661x-76; R=0,998) was calculated in 6,03 and 5,54 mg/L, respectively. The most of compounds present in CPH are not present in CPHF, when present in both, after the fermentation occurs a decrease of the peak area that can be related to the decrease of the concentration. It is can be caused by sterilization process or even a biotransformation of these compounds in others compounds by *R. stolonifer*. Therefore, the compounds produce by solid-state fermentation presents in both extracts has a higher antioxidant activity than compounds presents in CPH

Table 9. Retention time of compounds presents in CPH and CPHF at 96h of fermentation in both extraction methods after SPE clean-up process.

Extract	Ethanol:Water (50:50 v/v)		Acetone:Water:Acetic acid (70:29.5:0.5 v/v)	
	CPH	CPHF	CPH	CPHF
	Area (mAU×s)		Area (mAU×s)	
Retention Time (min)				
1.334	244.46306	-	-	-
1.408	-	-	2206.13550	-
1.420	-	2443.87598	-	1466.96057
1.437	503.33347	-	-	-

1.531	-	-	152.82296	231.35809
1.58	-	412.07944	-	125.08664
1.707	-	5056.53223	1009.07235	2067.74927
1.746	74.39305	-	728.01343	-
1.830	34.85575	-	-	-
1.945	63.34652	-	-	-
2.127	-	-	-	32.20187
2.243	19.79626	-	-	-
2.309	-	-	-	11.37261
2.3663	-	-	38.15900	-
2.394	-	-	-	34.05267
2.431	-	74.02154	-	-
2.953	188.30139	-	-	123.55819
3.604	-	-	99.91131	-
3.969	45.28275	-	-	-
4.179	-	61.87481	-	-
4.245	-	-	-	11.12795
4.425	-	-	-	21.6044
4.573	36.89502	48.53430	-	-
5.282	15.76734	21.90223	39.41171	-
5.338	-	-	-	21.09547
5.942	119.09779	-	-	-
6.985	-	-	301.86176	-
7.562	52.06685	-	-	-
8.620	-	298.05585	-	-
8.771	-	-	-	375.09547
8.8586	-	-	34.05828	-
9.892	208.26181	-	-	-
15.313	37.77657	-	-	-
16.394	69.32378	-	-	-
21.084	97.26589	-	-	-
22.272	-	-	79.00801	-
22.935	30.14432	-	-	-
22.696	-	-	-	32.18713
22.981	-	-	4024.37964	-
23.267	68.06731	-	-	-
23.352	-	84.79358	-	101.42841
23.504	-	-	46.17573	-
23.652	1473.19385	-	-	-
24.077	-	-	142.71317	-
24.286	-	79.30058	-	92.61911
24.442	121.23961	-	50.16321	-
25.601	-	-	91.89872	96.71447
24.722	-	-	65.64679	-
24.805	-	-	-	37.57733
25.023	-	-	373.31488	-
25.104	-	26.96553	-	-
25.262	100.5453	-	-	30.72475
25.411	-	-	110.32256	-

25.601	-	-	-	96.71447
25.721	-	-	572.70020	-
25.927	-	-	-	70.17031
26.014	166.98349	-	-	18.18190
26.146	-	-	402.85895	-
26.359	-	-	-	28.73689
26.435	-	-	583.01288	-
26.576	-	-	-	30.11361
26.677	-	-	582.60028	-
26.839	-	-	-	14.01515
27.019	-	-	138.60832	-
27.553	3713.57568	1252.47327	7775.25684	3861.25244
27.745	-	531.24652	-	-
28.093	-	-	659.12817	611.24097
28.131	637.80133	368.84344	-	-

The solvents used in the study were able to extract different compounds due to their different polarities as are showed above. Nevertheless, the some compounds detected in aqueous ethanol extract have retention times closely to acidified acetone extract. It occurs in trans-cinnamic acid, which appear in 28.093 in acidified acetone but in aqueous ethanol should appear at 28.131. The solvents can be interfere on compounds retention time and these interference can not be elucidate to this date. In this case an alternative study is the use of liquid chromatography couple to mass spectrophotometry in order to elucidate through the chemical structures if there are differents compounds in close time retention.

5 CONCLUSION

Rhizopus stolonifer when cultivated in a solid-state fermentation of cocoa pod husk has potential to produce compounds with antioxidant activity. Physico-chemical characterization of cocoa pod husk showed its potential as substrate and as a source of bioactive compounds. The extraction method proposed utilizing aqueous ethanol solution of 50% (v/v) was effective as the comparative method, with the exception of having extracted different compounds due to its different polarities.

Dosage of total phenolic content confirms the rich composition of phenolic compounds present in cocoa pod husk extracts, and the production of phenolic compounds by *R. stolonifer* during the solid-state fermentation. The solid-state fermentation improves the antioxidant activity present in CPH. Although, the identification of these biocompounds are essential to conclude how commercial relevance this process own. Studies to improve the conditions of pre-treatment, fermentation and extraction are needful to exploit the cocoa pod husk as much as possible. These improvement and optimization of fermentation conditions will allow the use of other strains.

Future studies to improve the conditions of fermentation are needful to exploit the cocoa pod husk as much as possible. These improvement and optimization of fermentation conditions will allow the use of other strains such as *R. Arrhizus* and *R. delemar*, which presented a great potential in antioxidant compounds production.

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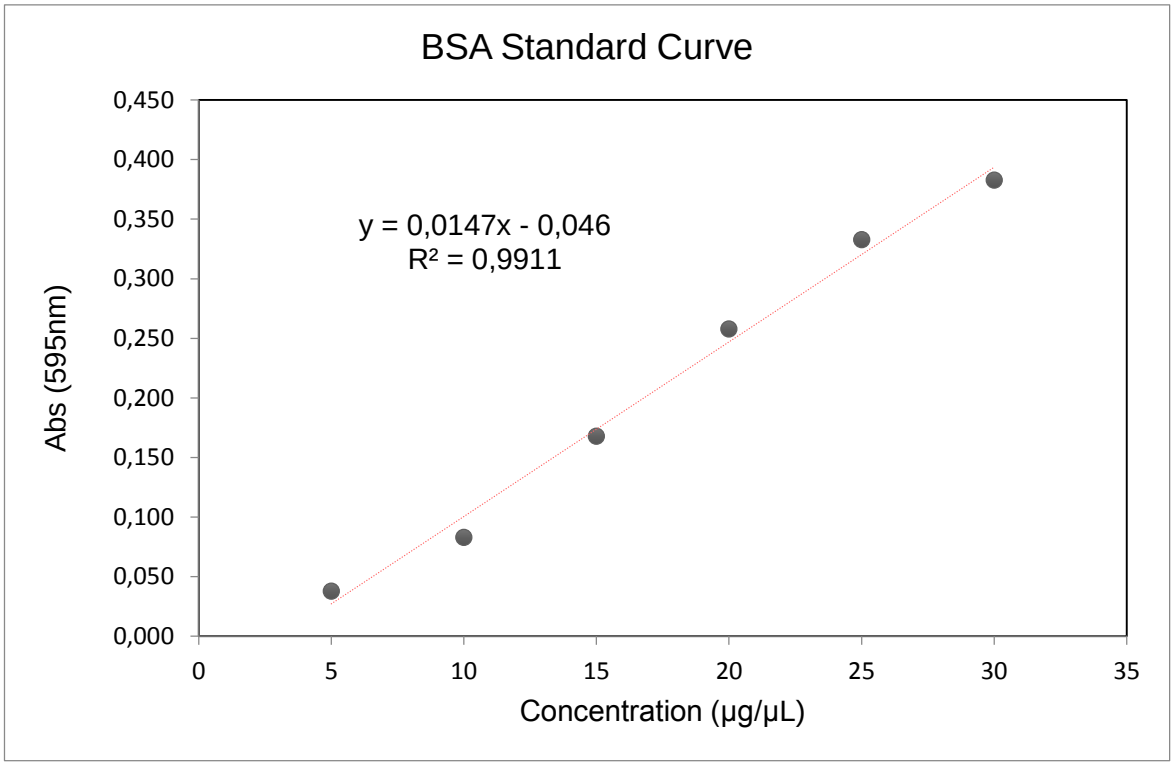
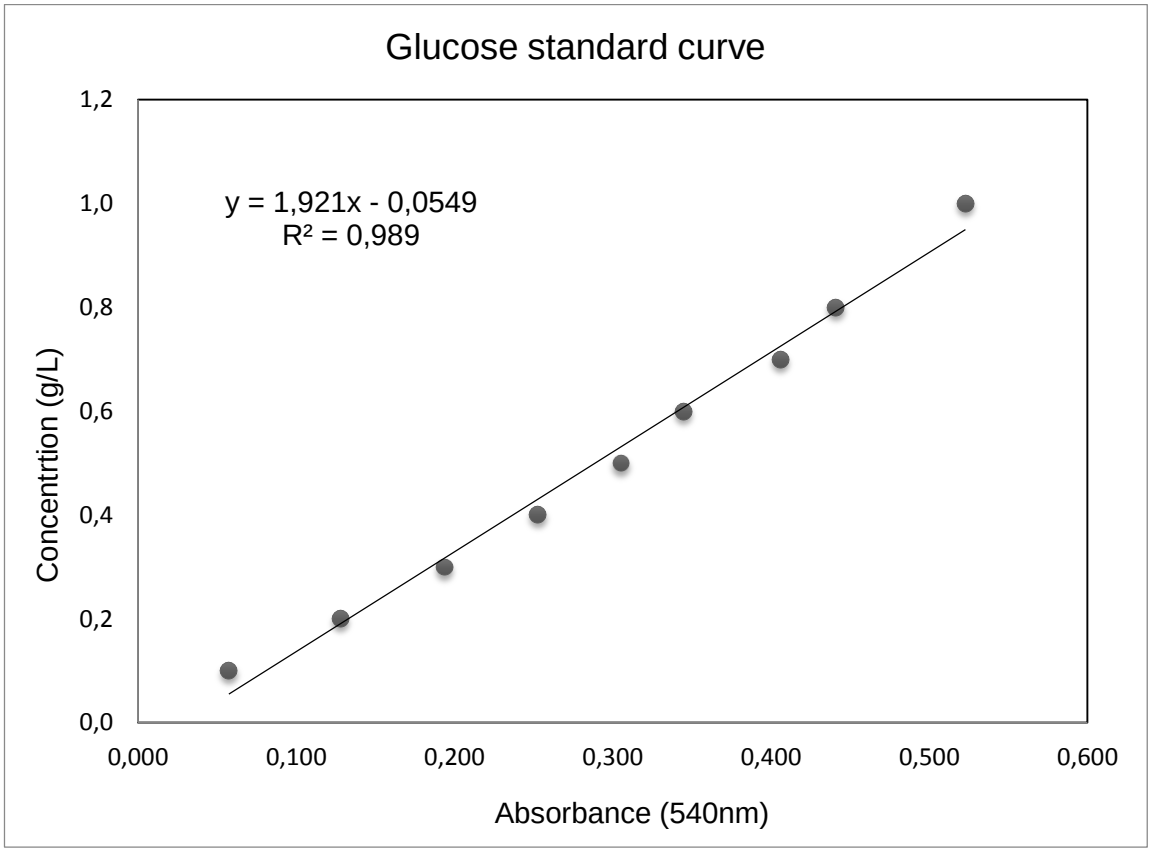
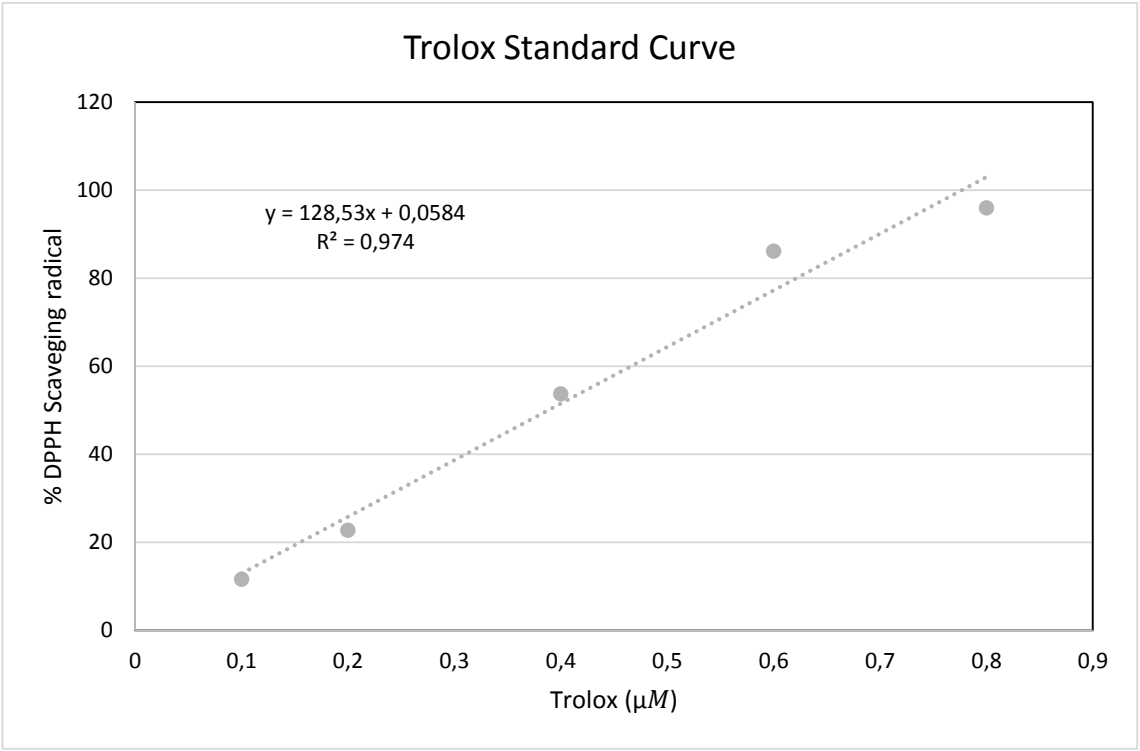
7 ANNEXES

Table 10 . Composition of basal and rich medium for *Rhizopus* cultivation present by Lennattsson and Taherzadeh (2014).

Compound	**Compound Concentration ($g.L^{-1}$)**
Basal médium	
D-glucose	30
$(NH_4)_2SO_4$	7.5
KH_2PO_4	3.0
$MgSO_4.7H_2O$	0.5
Trace metals	10 $ml.L^{-1}$
Rich medium	
D-glucose	30
Yeast extract	5
$(NH_4)_2SO_4$	7.5
KH_2PO_4	3.5
$CaCl_2.2H_2O$	1
$MgSO_4.7H_2O$	0.75
Trace metals	10 $ml.L^{-1}$
Trace metal solution	
EDTA ($C10H14N2Na2O_8.2H_2O$)	3.0
$CaCl_2.2H_2O$	0.90
$ZnSO_4.7H_2O$	0.90
$FeSO_4.7H_2O$	0.60
H_3BO_3	0.20
$MnCl_2.4H_2O$	0.19
$Na_2MoO_4.2H_2O$	0.080
$CoCl_2.2H_2O$	0.060
$CuSO_4.5H_2O$	0.060
Kl	0.020


BSA Standard Curve
y = 0,0147x - 0,046
R² = 0,9911
Abs (595nm)
Concentration (µg/µL)
0,450
0,400
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
0
5
10
15
20
25
30
35


Glucose standard curve
y = 1,921x - 0,0549
R² = 0,989
Concentrtion (g/L)
Absorbance (540nm)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0,000
0,100
0,200
0,300
0,400
0,500
0,600


Trolox Standard Curve
y = 128,53x + 0,0584
R² = 0,974
% DPPH Scaveging radical
Trolox (μM)
0
20
40
60
80
100
120
0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9

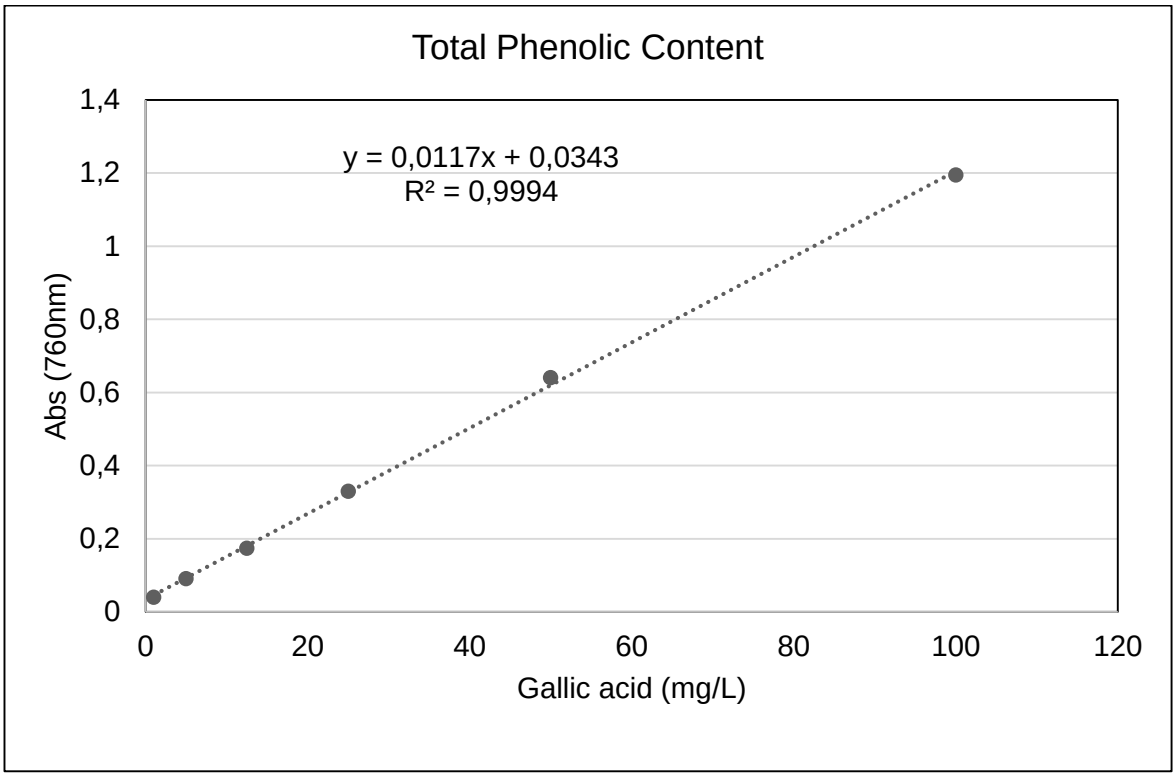

Total Phenolic Content
y = 0,0117x + 0,0343
R² = 0,9994
Abs (760nm)
Gallic acid (mg/L)
0
0,2
0,4
0,6
0,8
1
1,2
1,4
0
20
40
60
80
100
120

Table 11 . Volatile compounds identified in cocoa pod husk and cocoa pod husk after 96h of fermentation by Gas Chromatography Coupled to Mass Spectrophotometry (GC-MS).

		CPH			CPHF	
Volatile compound		Mol Wt	Similarity (%)	Fragments	Similarity (%)	Fragments2
(-)-1,2,2.alpha.,3,3,4,6,7,8,8.alpha.-decahydro-2.alpha.,7,8-trimethylacenaphthylene	C15H24	204	88	41;79;91;93;105;119;133;145;147;163;175;189	83	41;79;91;93;105;119;133;145;147;163;164;175;189
(-)-Aristolene	C15H24	204	-	-	77	55;69;79;91;93;105;119;133;161;189
(+-)-5-Methyl-2-hexanol	C7H16O	116	-	-	93	45;55;70;83;101;115
(2S,4S)-(+)-Pentanediol	C5H12O2	104	87	45;55;71;89	95	45;55;63;71;89
(3R,6R)-2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol	C10H18O2	170	-	-	87	-
(E,E)-2,4-Heptadienal	C7H10O	110	86	56;67;81;91;95;110	-	-
1,2,4-Trimethylcyclopentane	C8H16	112	-	-	84	55;70;83;112
1,2-Benzenedicarboxylic acid, dibutyl ester	C16H22O4	278	81	57;81;121;135;149;167	-	-
1,2-Dimethylpropyl acetate	C7H14O2	130	86	0	-	-
1,2-Pentanediol	C5H12O2	104	-	-	89	43;55;61;73;87;103
1,4-Butanediamine	C4H12N2	88	75	43;59;70;88	-	-
10,12-Tricosadiynoic acid	C23H38O2	346	-	-	75	43;55;57;91;93;105;119;133;145;161;189
11-Methyldodecanol	C13H28O	200	88	55;57;82;97;111;125;154	-	-
13-Phenylpentacosane	C31H56	428	-	-	79	43;57;71;91;92;105;119;133;147;161;189

1-Ethylpentyl acetate	C9H18O2	158	-	-	82	43;59;69
1-Heptadecene	C17H34	238	88	43;57;69;84;97;111;125;140;182 -		-
1-Hexanol	C6H14O	102	95	31;43;56;69;84;101	-	-
1-Isopropyl-1-butanol	C7H16O	116	-	-	86	43;55;57;73;97
1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene	C15H24	204	-	-	82	41;55;69;91;93;105;117;119;134;147;161;176;189
1-Methoxy-2-butanol	C5H12O2	104	88	31;45;55;59;71;75	91	31;45;55;59;71;75
1-Nonanol	C9H20O	144	90	56;70;83;97;111	-	-
1-Octen-3-ol	C8H16O	128	90	43;55;57;72;81;85;99; 110	-	-
1-Phenylethanone	C8H8O	120	87	43;77;91;105;120	-	-
2,3-Butanediol	C4H10O2	90	-	-	95	45;57;72;75;90
2,6,10,15-Tetramethylheptadecane	C21H44	296	91	43;55;57;71;81;85;99;109;113;127; 141;169;183		- -
2,6-Dimethylcyclohexanol	C8H16O	128	86	55;58;71;81;85;95;109;110;128 -		-
2-Acetoxypentane	C7H14O2	130	87	-	-	-
2-Cyclopropylidene-1,7,7-trimethylbicyclo[2.2.1]heptane	C13H20	176	-	-	77	55;69;79;91;93;105;119;133;161
2-Dodecanone	C12H24O	184	85	43;55;58;71;85;109;113	-	-
2-Ethyl-1-hexanol	C8H18O	130	89	41;55;57;70;82;83;98	-	-
2-Ethyl-3-pentanol	C7H16O	116	86	31;45;59;115	-	-
2-Ethylhexene	C8H16	112	-	-	85	55;70;83;112
2-Methyl-n-hexacosane	C27H56	380	85	43;55;57;71;85;99;113;127;141		-
3,4-Dimethylpent-2-en-1-ol	C7H14O	114	-	-	80	43;57;71

3-Hydroxy-2,3-dihydromaltol	C6H8O4	144	-	-	78	43;55;101;115;144
3-Isopropyl-1-pentanol	C8H18O	130	94	41;55;57;69;84;97	-	-
3-Methyl-4-heptanol	C8H18O	130	-	-	86	43;55;57;73;
3-Methyl-4-penten-1-ol	C6H12O	100	90	41;55;57;67;82;98	-	-
4-Hydroxybutanoic acid	C4H8O3	104	87	42;55;56;81;86	-	-
4-Methyl-5-hexen-2-ol	C7H14O	114	86	45;55;70;81;85;96	-	-
5-Methyl-1-hexanol	C7H16O	116	80	43;55;53;70;83	-	-
5-methyl-2-(1-methylethyl)-Cyclohexanol	C10H20O	156	-	-	86	55;57;71;81;83;95;109;123;138;155
5-Methyl-2-hexanol	C7H16O	116	-	-	93	45;55;70;83;101;115
9-methylheptadecane	C18H38	254	81	43;55;57;71;85;99;127;140	-	-
Acetic acid, isopropoxy-, isopropyl ester Isopropyl isopropoxyacetate	C8H16O3	160	-	-	82	31;43;55;59;75;88;102;117;131
Benzaldehyde	C7H6O	106	93	39;51;63;73;77;85	88	41;55;77;91;92;105;119;133;145;147;161;175
Cis-2-Decen-1-ol	C10H20O	156	85	57;81;110	-	-
Cis-Geranylacetone	C13H22O	194	90	43;55;69;83;93;107;111;125;136;151;161;169;176;189;194	-	-
Cis-Linalool oxide	C10H18O2	170	83	43;55;59;81;109;111;137;155	83	43;55;59;94;109;111;137;155
D-(-)-Pantolactone	C6H10O3	130	84	43;57;71	-	-
Dimethoxane	C8H14O4	174	-	-	81	43;55;61;71;87;105;113;131
D-Limonene	C10H16	136	86	41;53;68;79;91;93;107;115;121;136;137	86	53;68;79;93;107;121;136
Dl-Mevalonic acid lactone	C6H10O3	130	-	-	80	43;71
Eicosane	C20H42	282	90	43;55;57;71;85;99;109;113;127;		

				141;155;169;183	-	-
Epoxylinalol	C10H18O2	170	86	43;55;59;68;94;119;137;155	87	43;55;59;68;94;137;155
Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	C13H22O4	242	96	43;55;59;94;109;111;139;155 -		-
Ethyl iso-allocholate	C26H44O5	436	81	43;55;57;69;81;83;111;121;149;171	74	43;55;57;69;81;95;111
Heneicosane	C21H44	296	90	43;55;57;71;82;85;99;109;113;127;141;155;169;183	-	-
Heptanal	C7H14O	114	89	55;57;70;81;86;96	-	-
Isoledene	C15H24	204	87	41;57;69;81;91;105;117;119;133;145;147;161;175;161;175;189;200	-	-
Isovaleric acid	C5H10O2	102	75		-	-
Linalool	C10H18O	154	93	43;55;57;71;80;93;111;121;136 -		-
Methoxycitronellal	C11H22O2	186	-	-	80	55;73;111
Methyl N-hydroxybenzenecarboximidoate	C8H9NO2	151	76	55;77;105;117;133;137;151	79	55;77;105;117;133;137;151
Methyl salicylate	C8H8O3	152	87	39;65;81;92;109;120;137;152	79	39;65;81;91;92;120;152
n-Amyl methyl ketone	C7H14O	114	90	32;43;55;58;71;81;85;99;114 -		-
Nonanal	C9H18O	142	94	41;55;57;70;82;83;98;109;114;124;141	94	41;57;70;82;98;109;114;124;141
n-Tridecanol	C13H28O	200	91	55;57;69;83;97;111;125;154	-	-
Octanal	C8H16O	128	84	41;55;69;81;84	84	43;55;69;81;84;110

Octanenitrile	C8H15N	125	83	41;57;82;110	-	-
Oxalic acid, bis(6-ethyloct-3-yl) ester	C22H42O4	370	84	43;55;57;71;81;85;99;111;141-		-
Phenylethyl Alcohol	C8H10O	122	97	39;51;63;65;77;91;92;122	97	31;39;51;63;65;77;91;92;103;122
Pyrrole-α-methyl ketone	C6H7NO	109	-	-	80	39;66;91;94;109
Rosefuran	C10H14O	150	-	-	78	91;107;135;137;150
Sulcatone	C8H14O	126	85	43;55;69;83;93;108;111;126		-
Sulfurous acid, decyl 2-ethylhexyl ester	C18H38O3S	334	90	43;55;57;71;85;99;141		-
Tetracosane, 1-iodo-	C24H49I	464	90	43;55;57;71;85;99;113;127;141;155;169;197		-
Trans-2-Hexenal	C6H10O	98	88	41;55;57;69;83	-	-
trans-2-Octenal	C8H14O	126	83	41;55;57;70;83	-	-
trans-3-Octen-2-one	C8H14O	126	80	43;55;56;69;83;97;108;111;126-		-
trans-Linalool oxide (furanoid)	C10H18O2	170	95	43;55;59;94;109;111;137;155-		-
Valerena-4,7(11)-diene	C15H24	204	85	41;55;67;79;91;93;105;117;119;133;145;147;161;164;175;189;191	-	-
α-Copaene	C15H24	204	94	41;55;69;105;117;119;133;147;161;175;189	93	41;55;65;69;91;105;119;133;147;161;189
α-Hydroxytoluene	C7H8O	108	-	-	95	39;51;79;91;108
α-Methylbenzyl alcohol	C8H10O	122	-	-	85	43;51;79;107;122
α-Terpineol	C10H18O	154	84	43;59;67;81;91;93;107;121;136;139-		-

α-Toluic aldehyde	C8H8O	120	91	51;63;65;77;91;92;120	91	39;51;63;65;77;91;92;120
β-Cyclocitral	C10H16O	152	83	55;67;81;91;95;109;110;123;134;137;152		-
β-Hydroxybutyric acid methyl ester	C5H10O3	118	-	-	75	43;55;74;87;103
β-Ionone	C13H20O	192	78	43;55;135;177;192	-	-
ϒ-Cadinene	C15H24	204	84	41;55;69;81;91;105;117;119;134;161;176;189;191;200	-	-
ϒ-Hydroxybutyric acid cyclic ester	C4H6O2	86	87	42;55;56;81;86	-	-
ϒ-Muurolene	C15H24	204	86	41;79;93;105;119;133;161;189	-	-

Table 9. Volatile compounds identified in ethanol aqueous (50% v/v) extract from cocoa pod husk and cocoa pod husk after 48, 96 and 144h of fermentation by Gas Chromatography Coupled to Mass Spectrophotometry (GC-MS).

Volatile compound		Mol Wt	Similarity (%)				
			CPH	CPHF 48h	CPHF 96th	CPHF 144th	Fragments
(R)-(-)-2-Methyl-2,4-pentanediol	C6H14O2	118	86	-	89	80	31;43;59;67;85;103
[5-Hydroxymethyl)-1,3-dioxolan-4-yl]methanol	C5H10O4	164	-	-	-	73	31;45;55;61;75;103;133
1-Methoxy-3-(2-hydroxyethyl)nonane	C12H26O2	248	71	-	-	-	55;69;83;95;109
1-Phenoxypropan-2-ol	C9H12O2	152	-	-	71	-	51;77;91;94;108;119;137;152
3-Phenoxy-1-propanol	C9H12O2	152	-	-	72	-	45;51;59;77;91;94;108;133

2-(Heptyloxycarbonyl)benzoic acid	C15H20O4	264	-	-	80	79	
2-Propenoic acid, 2-methyl-, 3,3,5-trimethylcyclohexyl ester	C13H22O2	304	71	79	-	-	55;69;83;95;109;110;124
6,10-dimethyl-5,9-Undecadien-2-one	C13H22O	196	-	85	-	-	43;69;93;107;111;125;136;137;151;161;189
Benzothiazole	C7H5NS	135	78	-	82	74	38;45;54;63;69;82;91;108;135;138
Caprolactam	C6H11NO	113	-	-	-	75	55;67;85;113
Cinnamic acid, 4-hydroxy-3-methoxy-, (5-hydroxy-2-hydroxymethyl-6-[2-(4-hydroxy-3-methoxyphenyl)ethoxy]-4-(6-methyl-3,4,5-trihydroxytetrahydropyran-2-yloxy)tetrahydropyran-3-yl) ester	C31H40O15	652	70	72	71	71	43;55;105;111;121;137;177
Dimethyl phthalate	C10H10O4	194	79	-	85	85	50;77;92;104;133;163;164;194
Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester	C19H34O6	358	70	-	-	-	45;55;73;74;97;149
β-Hydroxyethyl phenyl ether	C8H10O2	138	-	-	87	-	51;66;77;91;94;107;119;138
Ethanone, 1,1'-(1,3-phenylene)bis-	C10H10O2	162	-	-	93	88	43;50;91;104;119;147;162
Ethanone, 1,1'-(1,4-phenylene)bis-	C10H10O2	162	91	-	-	88	43;50;65;77;91;92;104;118;119;133;147;162
Ethyl iso-allocholate	C26H44O5	436	72	70	75	75	43;55;69;81;91;95;107;111;119
Ethyl iso-allocholate	C26H44O5	436	-	-	-	-	55;57;81;95;111;121;179
Hexylene glycol	C6H14O2	118	87	-	90	80	31;43;59;67;85;103;115
Oxime-, methoxy-phenyl-_	C8H9NO2	151	-	83	82	76	31;77;105;133;151

Table 10. Volatile compounds identified in acetone : water : acetic acid (70 : 29,5 : 0,5 % v/v) extract from cocoa pod husk and cocoa pod husk after 48, 96 and 144h of fermentation by Gas Chromatography Coupled to Mass Spectrophotometry (GC-MS).

Volatile compound		Mol wt	Similary (%)				Fragments
			CPH	CPHF 48h	CPHF 96h	CPHF 144h	
2-Ethoxy-1-propanol	C5H12O2	104	80	-	-	-	31;45;55
(1R,2R,8S,8Ar)-8-hydroxy-1-(2-hydroxyethyl)-1,2,5,5-tetramethyl-cis-decalin	C16H30O2	254	-	-	71	-	57;69;81;83;109;177;191
(R)-(-)-2-Methyl-2,4-pentanediol	C6H14O2	118	-	-	-	87	
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	C16H22O4	278	83	-	-	-	41;57;76;104;121;149;167;189
2-(Heptyloxycarbonyl)benzoic acid	C15H20O4	264	83	84	-	-	41;76;83;93;104;149
2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol	C14H24O4	256	-	-	72	-	43;55;69;81;95;109;149;165;193
2,4a,8,8-Tetramethyldecahydrocyclopropa[d]naphthalene	C15H26	206	-	-	71	-	57;69;81;109;177;189;191
2-Dodecen-1-yl(-)succinic anhydride	C16H26O3	266	-	-	80	-	41;55;69;81;83;109;123
2-Hydrazino-2-imidazoline	C3H8N4	100	-	76	-	-	43;57;100
2-Propenoic acid, 2-methyl-, 1,2-ethanediylbis(oxy-2,1-ethanediyl) ester	C14H22O6	286	92	-	-	-	41;69;86;100;113;143;172
2-Propenoic acid, 2-methyl-, 1,2-ethanediylbis(oxy-2,1-ethanediyl) ester	C14H22O6	286	-	94	-	-	31;41;69;82;86;100;113;129;143;156;172
1,8,8-trimethyl-3-[2-(4-morpholinyl)ethyl]-3-Azabicyclo[3.2.1]octane-2,4-dione, 1,8,8-trimethyl-3-[2-(4-morpholinyl)ethyl]-	C16H26N2O3	294	-	80	-	-	100;101;113

3-Hydrazinocarbonylmethoxy-benzoic acid, hydrazide	C9H12N4O3	224	-	-	-	58	
Benzothiazole	C7H5NS	135	80	-	86	77	1
Caffeine	C8H10N4O2	194	71	-	-	-	55;82;83;109;110;165;194
Cinnamic acid, 4-hydroxy-3-methoxy-, (5-hydroxy-2-hydroxymethyl-6-[2-(4-hydroxy-3-methoxyphenyl)ethoxy]-4-(6-methyl-3,4,5-trihydroxytetrahydropyran-2-yloxy)tetrahydropyran-3-yl) ester	C31H40O15	652	66	69	-	71	43;55;105;111;121;137;177
Cis-1-Chloro-9-octadecene	C18H35Cl	286	-	-	85	-	43;55;69;83;97;109;111;125;147
Decanal	C10H20O	156	-	-	-	72	
Dimethyl phthalate	C10H10O4	194	-	-	-	79	39;50;64;74;77;92;104;111;120;149;163;194
Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester	C19H34O6	358	-	76	-	-	
2-phenoxy-ethanol	C8H10O2	138	-	-	90	-	1
1,1'-(1,3-phenylene)bis-ethanone	C10H10O2	162	87	-	92	87	1
(2-methoxyethoxy)-ethene	C5H10O2	102	78	-	-	-	31;45;55
Ethyl iso-allocholate	C26H44O5	436	76	-	82	70	1
Fumaric acid, 2-isopropylphenyl dodec-2-en-1-yl ester	C25H36O4	400	-	-	70	-	43;111;147;189
Hexylene glycol	C6H14O2	118	-	-	-	88	
Lanosta-7,9(11)-dien-18-oic acid, 22,25-epoxy-3,17,20-trihydroxy-, .gamma.-lactone, (3.beta.)-	C30H44O5	484	-	-	72	-	55;109;111;135;173
Methoprene	C19H34O3	310	-	-	-	70	
Morpholine, 4-[3-(4-fluoro-3-nitrophenylsulfonyl)propyl]- $$ 4-(3-[(4-Fluoro-3-nitrophenyl)sulfonyl]propyl)morpholine	C13H17FN2O5S	332	-	80	-	-	100;101

n-Hexadecanoic acid	C16H32O2	256	-	89	-	-	1
Octacosanoic acid, methyl ester	C29H58O2	438	-	83	-	-	0
Tetradecanoic acid	C14H28O2	228	-	88	-	-	43;55;60;73;87;97;115;125; 129;143;157;171;185;199
trans-β-Ionone	C13H20O	192	-	-	75	-	43;69;119;135;159;177;192