# DEGRADATION OF CAFFEINE IN COFFEE PULP BY SOLID-STATE FERMENTATION

By

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# Abstract

Coffee pulp is the most abundant solid waste in coffee processing plants. Its reutilization could be a challenge due to the presence of compounds such as caffeine. In the current study the solid-state fermentation was performed as a method to reduce caffeine present in coffee pulp. An optimization of the fermentation is proposed based on a parametric study, in which a caffeine degradation of about 53.5% was achieved on unground coffee pulp and 80% humidity content. Also, a preliminary study for the scaling-up of the process was performed. A compositional and structural study of the pulp before and after degradation was made to identify changes on the pulp and determine future uses of it, such as animal feed.



Keywords: coffee pulp, solid state fermentation, scale-up

## Resumen

La pulpa de café es el desperdicio sólido más abundante en plantas procesadoras de café. Dada la presencia de compuestos tóxicos, como la cafeína, su reutilización supone un reto. En este estudio se utilizó la fermentación en estado sólido como un método para reducir la cafeína en la pulpa de café. La optimización de esta fermentación se propone basada en un estudio paramétrico, donde se logró una degradación de cafeína de cerca de un 53.5% manteniendo la pulpa en su tamaño natural y con un 80% de contenido de humedad. El escalamiento del proceso de fermentación fue llevado a cabo como un estudio preliminar para escalas mayores. Un estudio composicional y estructural de la pulpa antes y después de la degradación fue realizado con el fin de identificar cambios en la pulpa y proponer usos futuros para esta, entre estos, su utilización como alimento para animales.



Palabras claves: pulpa de café, fermentación en estado sólido, escalado

# Dedication

*This work is primarily dedicated to God, through whom we live, move and exist... who gave me the strength and knowledge. To all my family for believe in me, to dad and mom for their efforts and help. To my love, my husband, Gustavo, who has supported and followed me in every decision I ever made. But above all, I dedicate this work to my gift from heaven, my motor, my son Gael Felipe, who drives me want to be a better person every day, for who I would not hesitate to give my life if necessary and whom I love with all the strength of my heart.*

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I want to share this success with all my family for their support, efforts and love. With dad and mom for every day they devoted to my affairs, sometimes forgetting of them. With the love of my life, Gustavo, by making sacrifices for me and follow me on each of my decisions. And especially with my beautiful son, Gael Felipe, who is my motivation in difficult times. God bless you all with all spiritual blessings.

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# 1. Introduction

## 1.1. Motivation

Coffee production is one of the current major agricultural activities in Puerto Rico. According to the Department of Agriculture of Puerto Rico, for the year 2010 there were produced about 5.5 thousand tons (approximately 120,000 quintals) of coffee in Puerto Rico and on 2012 was about 5.8 thousand tons (source: http://academic.uprm.edu/mmonroig/id58.htm). The Food and Agriculture Organization of the United Nations (FAO) estimated that for 2009, the world produced was about 7.9 million tons of coffee and for 2013, according to the International Coffee Organization (ICO), the global coffee production was estimated to be over 8.7 million tons (Bonilla-Hermosa et al., 2014). As a result of high production of coffee, a large amount of solid waste is formed.

Commercial coffee beans are produced from coffee cherries, 6% of which generate the coffee powder whereas the remaining 94% constitute the by-products, namely, coffee husk, coffee pulp (CP), wastewater, etc. (Dash & Gummadi, 2006). This suggests that from the total production of coffee of Puerto Rico in 2012, 5.5 thousand tons of coffee ended as waste and approximately 8.2 million tons worldwide on 2013. Because coffee growers place their greatest efforts on the production of the grain for sale, it takes away the attention to the waste problem.

Due to the presence of organic material, the coffee processing byproducts are highly pollutant residues and demand great quantities of oxygen to degrade. In addition, caffeine, tannins and polyphenols present in these materials confer a toxic nature to them (Mussatto et al., 2011). Some of the pollution problems that the inappropriate coffee waste disposal can cause are damage to and unnecessary use of land, spreading of diseases and contamination of groundwater

due to infiltration of contaminated water (Mauskar et al., 2007). At the same time, this yields on damage to aquatic ecosystems. On the other hand, discharges to the environment cause severe contamination and environmental pollution problems due to their toxic nature, and, on the other hand, burning or incineration results in the production of carbon dioxide (the green house gas effect) (Machado et al., 2012). This suggests a need to find the proper way to dispose or utilize these solid wastes. Coffee processing byproducts are also a potential source of compounds with functional properties (Esquivel & Jiménez, 2012).

The non-utilization of this by-products results in loss of resources which can serve as raw material for other purposes (Roussos et al., 1993). Some of the uses that have been considered over the years are composting, animal feed creation, production of organic fertilizers, production of single-cell proteins, biogas production, and grow of edible mushrooms on it to produce human food (Orozco et al., 2008). Another studies suggest its utilization for citric and gibberellic acid, vermicomposting, enzymes and secondary metabolites, ethanol production, dyes production, bioactive compounds, dietary fiber, anthocyanins, food products and aroma compound, production of particle board, fuels, activated carbon and biosorbents (Murthy & Madhava Naidu, 2012). Application of agroindustrial residues in bioprocesses at one hand provides alternative substrates and on the other side helps to solve problems which otherwise their disposal may cause (Pandey et al., 2000). Therefore, the conversion or bioconversion of the aforementioned by-products can be a viable and sustainable alternative for the reutilization of coffee processing solid waste.

## 1.2. Literature Review

### 1.2.1. Coffee processing and by-products formation and composition

After being harvested, the fruit of the coffee is carried to a coffee production plant for

processing and obtaining the commercial product known as ground coffee. The fruit processing consists, roughly of, de-pulping, drying, roasting and grinding coffee beans. During this process, certain wastes are produced. The process shown in Figure 1 corresponds to one way of making the coffee bean, which is the wet process and its by-product is the coffee pulp (CP). When the dry process is used, the by-product formed is the coffee husk. The wet process is the most widely used in Puerto Rico.

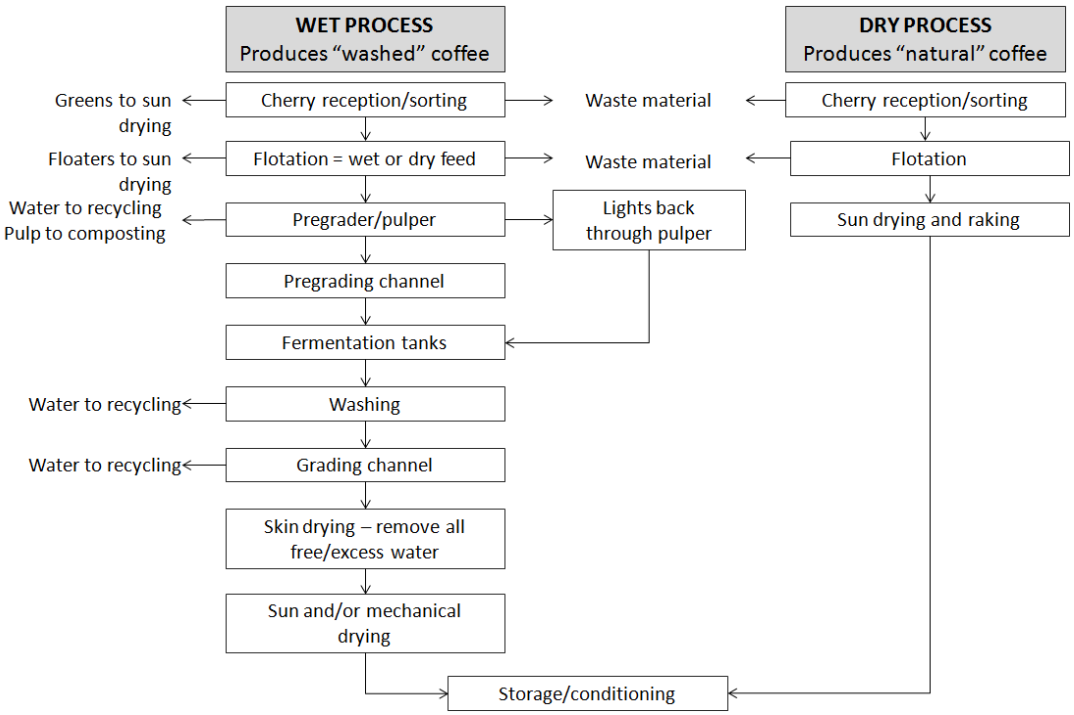


**Figure 1.** Coffee processing methods. Source: http://www.coffee-ota.org/grafico_en.htm.

The coffee fruit consists of a smooth tough outer skin or pericarp. The pericarp covers the soft yellowish, fibrous and sweet pulp or outer mesocarp. This is followed by a translucent, colorless, thin, viscous, and highly hydrated layer of mucilage. Then, there is a thin endocarp yellowish in color, also called parchment. Finally, the silverskin covers each hemisphere of the coffee bean (Figure 2) (Esquivel & Jiménez, 2012). The pulp correspond to 40% of the coffee cherry (Roussos et al., 1993) it is the major solid product generated after removal of the seed

from the coffee berry (Gutiérrez-Sánchez et al., 2012) and the most abundant by-product of coffee production. It is estimated in the literature that for every 2 tons of coffee produced, 1 ton of coffee pulp is obtained (Murthy & Madhava Naidu, 2012). In the production season of 1989-1990, coffee pulp produced worldwide was 2.8 million tons (Hakil et al., 1998).

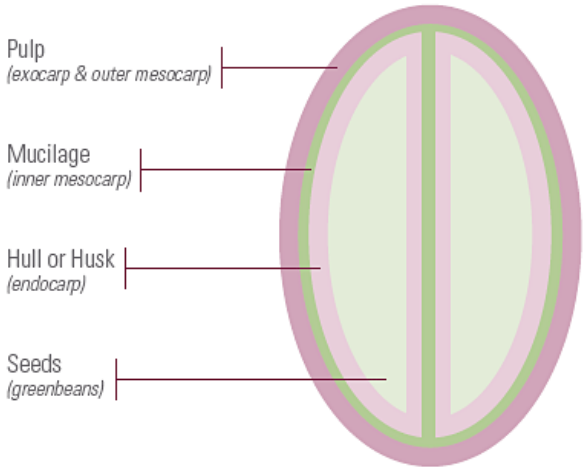


**Figure 2.** Coffee fruit morphology (Source: http://www.prioriskincare.com/coffeeberry-story/.)

As mentioned before, coffee pulp has potential to be used for composting, animal feed production, production of organic fertilizers, production of single-cell proteins, biogas production, and production of edible mushrooms, among other uses (Pandey et al., 2000; Ulloa Rojas et al., 2003). Up to now, most progress has been achieved in the use of coffee production byproducts for industrial processes other than food industry such as energy production (Kondamudi et al., 2008), adsorption of compounds (Oliveira et al.,2008), and manufacturing of industrial products such as particle boards, ethanol, gibberellic acid and α-amylase (Machado et al., 2002). As a source of lignocellulosic biomass, coffee pulp may be a feedstock in various chemical and biochemical processes for the production of fuels and chemicals of added value (Shenoy et al., 2011). Although coffee pulp is essentially rich in carbohydrates, proteins and minerals (Braham & Bressani, 1979; Porres et al., 1993; Roussos et al., 1994; Preethu et al.,

2007; Orozco et al., 2008; Murthy & Madhava Naidu, 2012; Menezes et al., 2013; López et al., 2013) almost all the pulp produced each year is discarded as a solid waste product and is considered the most abundant pollutant material in lakes and rivers located near coffee processing sites (Hakil et al., 1998). Also its high sugar content collaborate with the infestation of sites with insects (Porres et al., 1993).

Some of the main chemical components of coffee pulp have been investigated by many researchers. For the purpose of this research, an excerpt of these analyses has been compiled in Table 1. The amounts of each component are presented as a percentage of mass; however, as shown in the third column of Table 1, many researchers have reported these values and not necessarily agree with each other. The discrepancies in the composition may be due to differences in the processing mode and efficiency, variety of crops and growing conditions such as soil type, among others (Pandey et al., 2000). That is the main reason for which the sum of the values is not equivalent to one hundred percent.

The presence of carbohydrates, protein and fiber makes coffee pulp a particularly good candidate for animal feed. It has already been used for these purposes as dried coffee grounds can be easily incorporated into animal feed rations. Nevertheless, the energy costs associated to drying the pulp is a concern (Xu et al., 2007). Also, coffee pulp could be an agricultural byproduct with a great potential to reduce oxidative stress in animals physiological stages due to its high antioxidant capacity (Rios et al., 2014). It is comparable to corn in total protein and superior to it in calcium and phosphorous content (Gutiérrez-Sánchez et al., 2012). However, caffeine content and the presence of other by-products (such as, lignin, tannins and polyphenols) make it difficult, and its apparent digestibility seems to decrease as it concentration on the ration

increase (Xu et al., 2007). Also, its high moisture content poses problems of disposal due to putrefaction (Murthy & Madhava Naidu, 2012).

**Table 1.** Chemical composition of coffee pulp

| Component | Mass % | References |
|---|---|---|
| **Moisture** | 76.7 | Braham & Bressani, 1979 |
| **Caffeine** | 1.3 | Braham & Bressani, 1979; Porres et al., 1993 |
| **Carbohydrates** | 50 | Orozco et al., 2008; López et al., 2013 |
| **Protein** | 2.1[a] 8.25[b] 10[c,d] | [a]Braham & Bressani, 1979; [b]Roussos et al., 1994; [c]Orozco et al., 2008; [d]López et al., 2013 |
| **Fat** | 2.5 | Orozco et al., 2008; Menezes et al., 2013; López et al., 2013 |
| **Lignin** | 38.6[e] | [e]Preethu et al., 2007 |
| **Fibers** | 3.4[a] 18[c,d] | [a]Braham & Bressani, 1979; [c]Orozco et al., 2008; [g]López et al., 2013 |

Caffeine, chemically named 1,3,7-Trimethylxanthine (see Figure 3), is a commercially important alkaloid which belongs to the family of purine alkaloids synthesized by plants (Dash & Gummadi, 2006). This alkaloid is found in more than 60 plant species (Tagliari et al., 2003). It is considered an anti-nutritional element because of it adverse effects on the physiological system. It might be responsible for the decreases in nitrogen retention and also may cause nervousness and the increases in free fatty acids (Braham & Bressani, 1979). Caffeine has other adverse effects in animals such as, low feed intake (Brand et al., 2002) and effects on palatability of food. At the same time it interferes with nutrient availability and absorption in the gastrointestinal tract (Mazzafera, 2002) and nitrogen retention (Pandey et al., 2000). Also, the presence of caffeine in coffee pulp makes it more difficult to be used as compost.

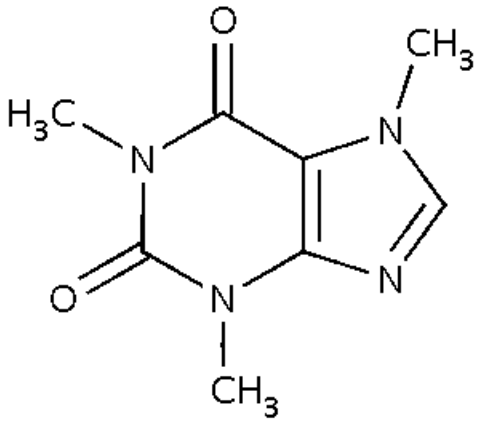


**Figure 3.** Caffeine chemical structure (Source: http://www.caffeine.com/).

Farmers use nitrogen-rich coffee pulp to produce compost or vermicompost which are used to fertilize coffee plantations and thus replace artificial fertilizers (Folmer, 2014). However, presence of caffeine in soil can affect soil fertility and inhibits seeds germination and growth of seedlings (Dash & Gummadi, 2006), it even causes inhibition of coffee seeds generation (Mazzafera et al., 1996), which, hampers its use as fertilizer (Braham & Bressani, 1979). Also, coffee pulp consumption might affect the ionic balance in the tissues, due to potassium consumption, which might have negative effects in animal performances. It also has been associated with inflammation of the extremities and skin ulcers (Braham & Bressani, 1979).

For all of these reasons mentioned above a preliminary treatment of coffee pulp it is recommended before being used for any other purpose. Decaffeination is an alternative to detoxify the material. Previous studies have confirmed that toxic materials can be minimized by hot water pretreatment, microbial degradation and aerobic fermentation (Kurtzman & Schwimmer, 1972; Murthy & Madhava Naidu, 2012; Nayak et al., 2013; Rios et al., 2014). Caffeine degradation techniques are discussed in the next subsection, among which solid state fermentation will be discussed in detail. Solid-state fermentation is an amenable strategy due to

its relatively low-cost technology for the upgrading of amylaceous materials (Penaloza et al., 1985). The chemical composition of the pulp makes it a potential substrate to carry out this type of fermentation (López et al., 2013).

### 1.2.2. Caffeine biodegradation mechanisms

Over the years, chemical methods have been used to degrade the caffeine present in coffee waste, meaning, coffee pulp and coffee husk. Chemical methods commonly used are solvent extraction and supercritical fluid extraction (Gummadi & Santhosh, 2010). These methods have the disadvantage of using toxic solvents and not to discriminate between the compounds removed when processing the by-products. However, biological processes for caffeine detoxification alleviate some of the drawbacks of chemical methods. These bioprocesses involve the use of microorganisms, mainly fungi and bacteria, which have shown to grow in the presence of caffeine. The ability to grow in the presence of caffeine has been related to their capacity to degrade the compound (Mazzafera et al., 1996). In solid-state fermentation processes filamentous fungi are the most widely used (about 50%) (López et al., 2013). *Aspergillus sp.* and *Penicillium sp.* are the fungal species that have been mostly identified as degraders of caffeine and the most commonly used bacterial microorganisms belong to the *Pseudomonas* (Mazzafera, 2002) and *Serratia* (Mazzafera et al., 1996) genus. However, microbial and enzymatic caffeine degradation are considered more suitable than conventional (chemical) methods because of avoidance of toxic solvents and specificity (Gummadi & Santhosh, 2010).

Here we will describe the most common mechanisms of caffeine biodegradation mentioned in literature. It is important to note that the degradation pathway of caffeine will depend on the microorganism carrying out the process; therefore, different pathways had been proposed. Nevertheless, many authors conclude that biodegradation involving fungi follows a

demethylation pathway. Figure 4 shows the pathway proposed by Tagliari et al. (2003) for fungal biodegradtation. Caffeine is converted into xanthine by caffeine, theophylline and 3-methilxanthine demethylase. Another step is suggested where, xanthine dehydrogenase converts xanthine into uric acid (Gutiérrez-Sánchez et al., 2012).

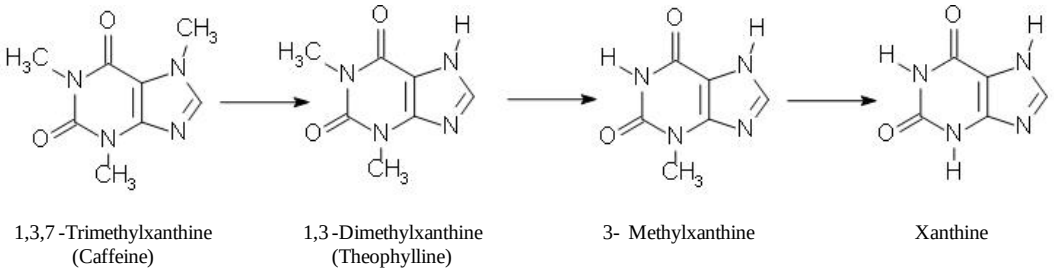


**Figure 4.** Caffeine degradation pathway by fungi (Source: Tagliari et al., 2003).

In the case of bacteria, alternative pathways have been described when using it to degrade caffeine (Yu et al., 2009). Figure 5 present two of these pathways. In both pathways the ending product is uric acid. In Figure 5(i) caffeine degradation is illustrated, where paraxanthine and theobromine are two possible intermediate steps of the degradation, in which demethylation takes place. Xanthine is eventually metabolized into uric acid. The majority of uric acid dissolves in blood and travels to the kidneys, where it exits through the urine. Figure 5(ii) begins with theophylline and is further converted into xanthine, which is oxidized to uric acid in the same way as Figure 5(i).

### 1.2.3. Solid-state fermentation in fungal bioprocesses

One of the methods used to biologically degrade compounds of interest, such as caffeine, is the solid-state fermentation. It has been frequently used for the biological detoxification of coffee pulp using fungal strains (Pandey et al., 2000). Solid-state fermentation is a fermentation process which involves a solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support growth and metabolism of the

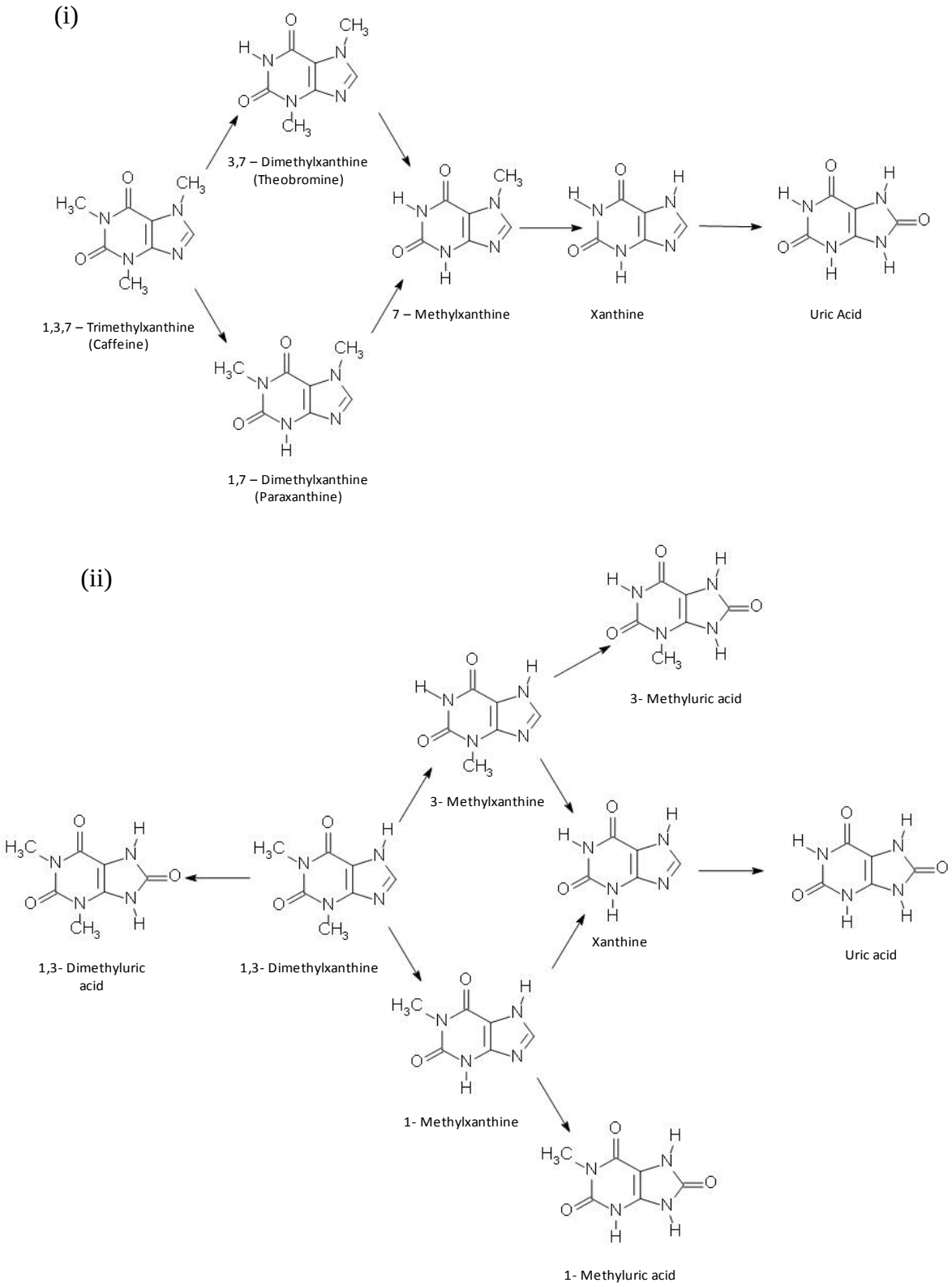


**Figure 5.** Caffeine degradation pathway by bacteria (Source: Yu et al., 2009). (i) Theobromine pathway, (ii) Theophylline pathway

microorganism (Pandey, 2003). It is a valuable alternative for the reuse of agricultural and agroindustrial residues, since it can be used for the production and/or extraction of compounds like enzymes, flavors, pigments, organic acids, among others, additionally, this technique does not require the use of chemical solvents, being more environmentally friendly (Machado et al., 2012). As a relatively low-cost technology it can be appropriate for the upgrading of amylaceous materials as a protein source in animal feeding (Penaloza et al., 1985). Some of the limiting factors of solid-state fermentation are temperature and moisture content (Durand, 2003), but there are other important aspects that have to be taken into account, such as, pH (Gummadi & Sucharita, 2009), aeration, bed properties, water activity with solid media, and nature of the solid employed (Singhania et al., 2009). This technique can be employed to detoxify coffee pulp in order to get a product with less caffeine content that can be employed for other purposes (Orozco et al., 2008).

The scaling up of the solid-state-fermentors is complicated and challenging due to the factors mentioned above. During solid-state fermentation in pilot and industrial scale reactors, temperature and concentration gradients are observed along the substrate bed, also the substrate size and shape might play an important role (Nava et al., 2011). Durand (2003) discussed some of these factors:

- Above some critical quantity of substrate, the heat removal becomes difficult to solve and restricts the design strategies available. The solid medium becomes compacted or creates air channeling, shrinkage, etc. All these factors affect heat and mass transfer, and is attributed to poor aeration
- The properties of the microorganism with respect to its resistance to mechanical stirring, its oxygen requirement and temperature range. When the mycelium hyphae do not have

septa, they can be destroyed by a mechanical stirring. Thus, the culture layer will be thin to allow heat removal which automatically orientates to a category of reactor

- The nature of the substrate and the need to pre-treat it or not, appropriate procedures for the inoculation, the sterility or the level of contamination acceptable for the process and the application
- The economy of the country where the process is developed especially with respect to the labor cost. Indeed some technologies need more manpower than others
- Handling poses different problems such as the ease of filling, emptying and cleaning the reactor.

Figure 6 presents a picture and schematic of a cylindrical fermentor such as the one used by Durand (2003). It consists of a stirred horizontal bioreactor composed by an air-inlet (1), temperature probes (2), water jacket (3), paddles (4), air-outlet (5), agitation motor (6), reactor (7), medium (8) and agitation shaft (9).

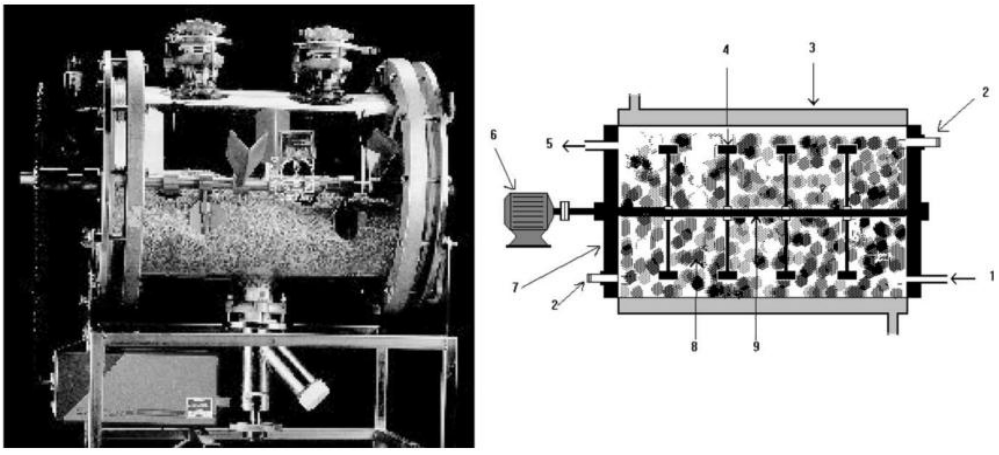


**Figure 6.** Horizontal fermentor picture and schematic (Source: Durand, 2003).

A relatively new technique that has been developed is comprised of trays. This design was first tested in the laboratory and then implemented on a larger scale; it is registered under the name of Platotex® (Adelin et al., 2011). It consists (see Figure 7) of three principal components:

a cylindrical 500L tank (1) that accepts a frame (4) supporting some cultivation plates (2 and 3) and mounted with a cover (5) that fits over the tank. A glass porthole (6) allows visual monitoring of the cultures, while individual screw holes (7) serve as ports for inoculation, inoculation medium is contained in 1-L bottles (10). Rotation is provided by a shaft (8) and a motor (9), different types of blades are fixed to the shaft, Teflon blades (11), brush blades (12) and perforated blades (13). Based on the above study, a tray system for the biodegradation of caffeine in coffee pulp is proposed as a base to be used in the current study.

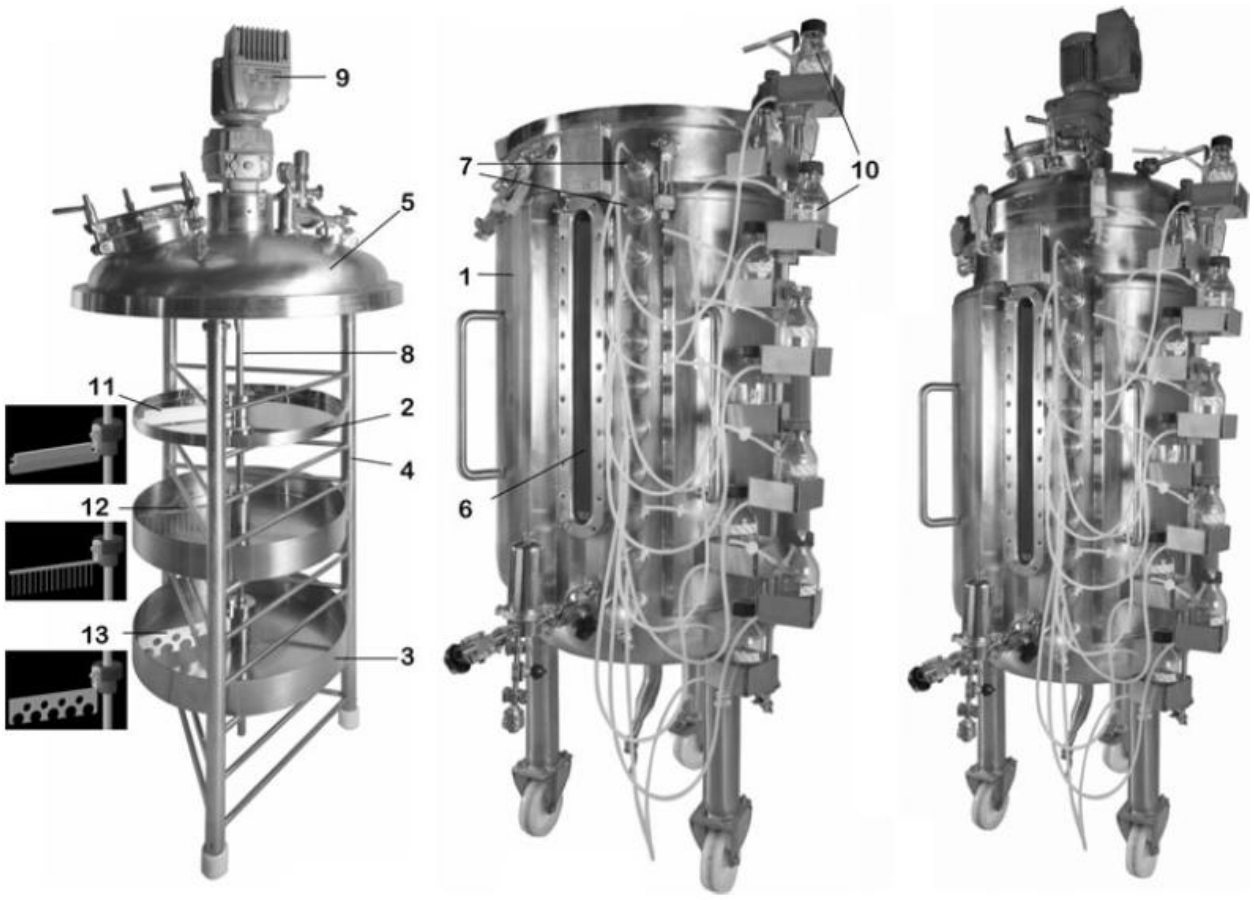


**Figure 7**. Platotex® array for solid-state fermentation. (Adelin et al., 2011)

## 1.3. Objectives

### 1.3.1. Primary objective

As discussed before, caffeine detoxification can be achieved by biological methods. According to Dash & Gummadi (2006) biological decaffeination provides an attractive alternative to the conventional methods of decaffeination. This research intends to achieve biological degradation of caffeine to get a detoxified product that can be utilized for purposes, such as animal feed, compost, among others. Filamentous fungi have been selected to conduct this study given their documented ability to degrade or convert recalcitrant compounds such as lignin (Tien & Kirk, 1983; Hammel et al., 1986; Srebotnik et al., 1994; Hammel, 1996; Hammel, 1997; Hammel & Cullen, 2008). Their filamentous growth mode is favorable for the solid-state conditions that were explored in the proposed project.

Previous work from our laboratory, performed by Sierra-Gómez (2012), identified *Alternaria alternata* from a screening of various species of fungi able to detoxify caffeine and it was proposed to be used in our study as the fungal to carry out the biodegradation of CP. A parametric assessment of solid-state fermentation was performed. Factors like coffee pulp particle size and humidity were inspected in terms of fungal growth and caffeine degradation. The conversion of caffeine was determined using an HPLC protocol. In addition, the structure of the pulp was analyzed for changes due to biodegradation processes. Finally, a scale-up study was performed in order to degrade a larger amount of the coffee pulp and compare the results with the analytical scale. This study is based mainly on the humidity percent and coffee pulp particle size. The research problem was addressed in collaboration with the local coffee farm, Hacienda Café San Pedro (Jayuya, P.R.), to which we would like to provide a solution for the trouble of waste management in their coffee-processing operation.

### 1.3.2. Specific objectives

#### *1.3.2.1. Parametric assessment of solid-state fermentation of coffee pulp for caffeine degradation*

According to factors that affect the fungal fermentation process, water content and particle size were selected as parameters to study with respect to microbial caffeine reduction in coffee pulp. This project aims to biodegrade the caffeine present in coffee pulp. Solid-state fermentation is the option that was used. Based on literature solid-state fermentation yields a decaffeinated product which can be used for other purposes (Roussos et al., 1993). Preliminary fermentation experiments with the coffee pulp were previously done in a submerged solid-state fermentation set-up using 83% humidity. This preliminary study intended to extrapolate the conditions of liquid-state fermentations using pure caffeine as substrate in which detoxification was observed. However, according to the literature, filamentous fungi degradative machinery is more likely to be expressed optimally in a solid-state environment where the moisture content is lower. This is the case with lignocelluloses degradation (Tengerdy & Szakacs, 2003). Therefore, for the current study the moisture content was set to less than what has been studied before, which used a submerged-type solid fermentation. The caffeine concentration present in the pulp was quantified using HPLC against a calibration curve using a pure caffeine standard.

#### *1.3.2.2. Structural and compositional analysis of biodegraded coffee pulp*

Caffeine detoxification of coffee pulp may cause changes in its structure and composition, such as, changes in the content of total and organic carbon, total and organic nitrogen, cellulose and lignin. The aim is to identify if these changes occur in the pulp. A comparative study of fresh and degraded coffee pulp samples was performed. The services of the University of Wisconsin Soil and Plant Analysis Laboratory were used to assess total carbon

(organic and inorganic), total nitrogen (organic and inorganic), elemental and heavy metal analyses and cellulose and lignin content. Fresh and degraded coffee pulps were provided to the analytical laboratory and their results were compared. Physical (structural) changes in the coffee pulp due to caffeine biodegradation, as a function of fungal growth, was determined using scanning electron microscopy (SEM).

### *1.3.2.3. Solid-state fermentation scale-up design*

According to Durand (2003), some of the aspects that make the scale-up challenging is the high heat generation and the heterogeneity that presents the large-scale system. This project set the basis for a scale-up. The adaptation of the laboratory-scale flask fermentor to a stationary tray system of a size of 13in x 9in is proposed (see Figure 8). The proposed operation should be easy to implement by the local coffee farms. Findings from objective 1.3.2.1 were used to propose a reactor.

**Figure 8.** Scale-up tray configuration.

# 2. Materials and Methodology

## 2.1. Submerged solid-state fermentation of coffee pulp for caffeine degradation

### 2.1.1. Inoculum preparation

The fungus used was *Alternaria alternata*. These microorganisms are inoculated and maintained in malt agar. Malt agar medium consists of (in g/L): malt extract (20) and agar (15) from BD Bacto Company. Once grown, it is induced on Coffee Pulp Extract (CPE) agar. CPE medium is prepared as follows (in g/L): an infusion, prepared with Fresh Coffee Pulp (40); as described by Gutierrez-Sanchez et al., 2003. In addition, sucrose (2.0) from Fisher Scientific Products, $KH_2PO_4$ (1.3) from Fisher Scientific Products, $Na_2HPO_4$ (0.19) from Fisher Scientific Products, $MgSO_4 \bullet 7H_2O$ (0.15) from Sigma Aldrich Company and $CaCl_2$ (0.2) from Sigma Aldrich Company are dissolved in the coffee infusion. The pH was adjusted to 5.6. Agar (20 g) was added. The medium was sterilized at 121ºC for 30 min and incubated at 25°C. In the coffee pulp extract the microorganism was allowed to grow for at least six days or until the agar surface on the Petri dish is almost completely covered by the microorganism.

### 2.1.2. Coffee pulp preparation

Coffee pulp was obtained from Hacienda Café San Pedro in Jayuya, Puerto Rico. It was stored in plastic storage bags on the refrigerator at approximately 4°C until it was time to be used for the fermentation. When fermentation was to be prepared, coffee pulp was completely dried at approximately 100°C until constant weight is achieved. Once it was dry, coffee pulp was milled to a size of 0.85-1.41mm.

### 2.1.3. Fermentation

Once microorganisms show optimal growth in CPE (approximately 6 days of growth), it was liquefied and mixed with a solution of salts and traces (20mL/L), as with a small amount of sucrose (2g/L) from Fisher Scientific Products (Gokulakrishnan et al., 2007 and Kurtzman & Schwimmer, 1972) to form a medium. Salts and traces solution was prepared as follows (in g/L): KCl (26) from Fisher Scientific Products, $MgSO_4 \cdot 7H_2O$ (26) from Sigma Aldrich Company and trace solution (10mL/L). The trace solution consists of (in g/L): $CuSO_4 \cdot 5H_2O$ (0.4) from Sigma Aldrich, $MnSO_4$ (0.8) from Sigma Aldrich, $Na_2MoO_4$ (0.8) from Sigma Aldrich Company and $ZnSO_4$ (8) from Sigma Aldrich. Four inoculated Petri dishes were added to each liter of medium.

Erlenmeyer flasks of 250mL were filled with 2g of dried coffee pulp and sterilized in the autoclave. Ten milliliters of the solution were added to each Erlenmeyer flask. All experiments were performed in six replicates. Samples were taken the days 0, 3, 6, 9, 12 and 20 starting from the beginning of the experiment. Control samples (no fungi) were made the first and last day of experiment. If any fungi growth is observed during the period of time on the control flasks, it will be an indicator of contamination of the experiment.

### 2.1.4. Sample processing and analysis

Biodegraded pulp was processed as follows. Seven milliliters of hot water (85°C) were added to each Erlenmeyer flask, the flask was agitated for 15 minutes at 250 rpm. The agitated sample was filtered using a Hirsch funnel and Whatman#2 filter paper (weighted accurately); an additional seven milliliters of hot water were added to rinse the empty flasks. Solid samples were dried and weighed and liquid sample will be processed as described below.

SPE cartridges packed with a C18-functionalized resin (Grace Davison Discovery Science, Deerfield, IL) were used to concentrate the samples. For use and conditioning of the cartridges, the manufacturer instructions were followed.

A High Performance Liquid Chromatography was used. It is composed of: Binary HPLC pump, (model Waters 1525), autosampler (Waters, Model 717 plus), Ultraviolet detector (Waters, Model 2487), Column Symmetry Shield $^{TM}$ $RP_{18}$ 5 μm (4.6x250mm) at room temperature. The software used for the analysis is Breeze 2 provided by Waters Company. An isocratic method was applied in which mobile phase consist of methanol: water (30:70). Methanol HPLC grade was purchased from Sigma-Aldrich (St. Louis, Mo). Mobile phase water was acidified with 0.1% trifluoroacetic acid from Sigma-Aldrich (St. Louis, Mo). Detection wavelength was set at 254 nm. Twenty microliters of sample solutions were injected in triplicate. Results were compared with a standard calibration curve made with different concentrations of caffeine.

### 2.1.5. Statistical analysis

This experiment was conducted two times. Sample analysis was performed in triplicate. The summarized data is presented as average values ± standard deviation of the mean.

## 2.2. Solid state fermentation of coffee pulp for caffeine degradation

The methodology used to prepare the samples and parameters measurements are the same as presented in sections 2.1.1 to 2.1.5. The only differences between these studies are that it was used 5g of coffee pulp and 20mL of medium were added to each Erlenmeyer flask. Medium was prepared as described in section 2.1.3 with the exception that 8 inoculated Petri dishes were added per liter of medium. This experiment was conducted two times.

## 2.3. Structural and compositional analysis of coffee pulp

### 2.3.1. Structural Analysis

Changes in the structure of the pulp due to the degradation of caffeine were examined using a Scanning Electron Microscope (SEM) (Model JEOL 5410LV). To accomplish this objective, the assistance of the Microscopy Center of the Department of Biology of the University of Puerto Rico at Mayagüez was requested. In order to accomplish the analysis a gold coating was applied to the coffee pulp samples using a gold coater instrument (Model EMS550 Sputter Coater). The SEM was operated at 20kV.

### 2.3.2. Compositional analysis

A comparative study of fresh and degraded coffee pulp samples was performed. Analysis consists of total carbon (organic and inorganic), total nitrogen (organic and inorganic), elemental and heavy metal analyses and lignin concentration. The services of the University of Wisconsin Soil and Plant Analysis Laboratory were requested.

#### *2.3.2.1. Analysis of major, minor and trace elements in plant tissue samples with ICP-OES and ICP-MS*

Half gram of dried coffee pulp sample and five (5) milliliters of concentrated nitric acid are added to a fifty (50) milliliter-Folin digestion tube. The mixture is heated to 120-130°C for 14-16 hours and is then treated with hydrogen peroxide. After digestion, the sample is diluted to fifty (50) milliliters. This solution is analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) for major and minor components, and further diluted and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) for minor and trace components.

Sample was then prepared for ICP-OES as follows: set eight (8) milliliters autosampler tubes in the ICP-OES sample racks. Transfer three (3) milliliters of each sample from Folin

digestion tubes into autosampler tubes adding three (3) milliliters of 2% nitric acid. Once samples are prepared the ICP-OES analysis was performed.

ICP-MS procedure also begins with sample preparation in which fourteen (14) milliliter Falcon tubes were set with five (5) milliliters of samples and five (5) milliliters of 2% nitric acid. Once samples are prepared and well mixed the ICP-MS analysis was performed.

#### *2.3.2.2. Acid detergent lignin (ADL)*

To accomplish lignin analysis an acidified quaternary detergent solution is used to dissolve cell solubles, hemicelluloses and soluble minerals leaving a residue of cellulose, lignin, and heat damaged protein and a portion of cell wall protein and minerals (ash). ADL is determined gravimetrically as the residue remaining upon ignition after 72% $H_2SO_4$ treatment.

## 2.4. Solid-state fermentation scale-up design

### 2.4.1. Sample preparation

The procedure and methodology presented in sections 2.1.1 to 2.1.3 was used here too. But, for the fermentation process, eight inoculated Petri dishes were added for each liter of medium. Metal trays (cooking pans) of a size of 13in x 9in ($117in^2$) were filled with 100g of dried coffee pulp and sterilized in the autoclave. 400mL of the solution were added to each pan. Six cooking pans metal trays were placed on the incubator. Two cooking pans were processed the days 0, 9 and 20 starting from the beginning of the experiment.

### 2.4.2. Sample processing and analysis

140 milliliters of hot water (85°C) were added to each tray for the biodegraded pulp analysis. The tray was agitated for 15 minutes at 250 rpm. The agitated sample was filtered using

a Hirsch funnel and Whatman#2 filter paper (weighted accurately); additional 140 milliliters of hot water will be added to rinse the empty pans. Solid samples was dried and weighed to obtain dry weight profile and liquid sample will be processed as described below. The subsequent analysis was performed as described on section 2.1.4.

### 2.4.3. Statistical analysis

This experiment was conducted one time. Sample analysis was performed in triplicate. The summarized data was presented on appendix section as average values ± standard deviation of the mean.

# 3. Results and Discussion

## 3.1. Solid-state fermentation of coffee pulp for caffeine degradation

The experiment used as a baseline to determine whether caffeine degradation is possible was described in section 2.1. On this experiment two grams of coffee pulp with a size of 0.85mm-1.41mm were inoculated with a solution of *Alternaria alternata*, salts and sucrose. Ten milliliters of this solution were used for each Erlenmeyer flask. Due to the amount of liquid present in this experiment, fermentation was classified as submerged. Fermentation was allowed to run for twenty days; and analysis were performed for six days corresponding to day 0, 3, 6, 9, 12 and 20. The results of this effort are shown in Figure 9.

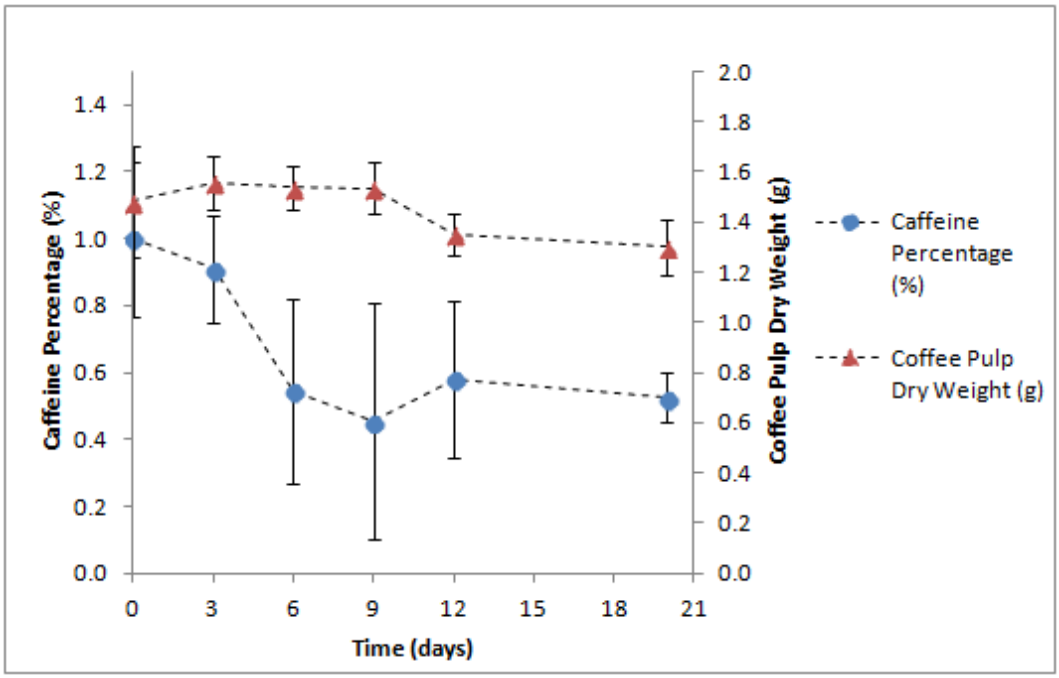


**Figure 9**. Submerged solid-state fermentation of coffee pulp by *Alternaria alternata*. Percent of caffeine was determined by HPLC and is shown in circle. The coffee pulp dry weight is shown in triangle. The coffee pulp dry weight is shown in triangle. The vertical bars correspond to the standard deviation of each data point. This is a representative experiment that was conducted two times, where n=6 according to methodology described in section 2.1.

Fermentation curves have a particular shape that was consistent in all experiments. First, there is a marked reduction in the content of free caffeine during the first 3-6 days of fermentation where lower values were observed and practically continued after that period. As fungal biomass increases there is an apparent increase in the amount of caffeine that hypothetically could be due to the release of caffeine contained in the solid as the fungus grows and degrades the material. At the end, an average caffeine reduction of 47.5% was achieved by day twenty.

An optimization of the parameters of fermentation humidity and size of coffee pulp was made. The main reason for the optimization lies on the reality that the coffee production process generates a great amount of waste and that the greatest efforts of the farms are directed to the production of ground coffee. Two experiments were designed for this purpose. In the first experiment, fungal growth of *Alternaria alternata* compared to the amount of water present in an Erlenmeyer flask was evaluated. Five grams of pulp were placed in a series of flasks to which were added ten, fifteen, twenty and twenty-five milliliters of medium and inoculum. These medium amounts correspond to 67%, 75%, 80% and 83% of humidity, respectively. Six flasks were set for each condition. Data were obtained qualitatively, namely it was only evaluated visibly if fungal growth was possible. Figure 10 shows the described assembly and conditions.

As mentioned in the last paragraph *Alternaria alternata* was inoculated in five grams of dried coffee pulp at different humidity conditions. Each flask contained five grams of pulp, and ten milliliters (Figure 10 (a) and (e)) was added to the first group, to the second group were added fifteen milliliters (Figure 10 (b) and (f)), while to the third and fourth group of flasks twenty (Figure 10(c) and (g)) and twenty five (Figure 10(d) and (h)) milliliters of solution of salts and fungi were added to achieve 67%, 75%, 80% and 83% humidity, respectively. As seen

on Figure 10 for day 50, fungal growth was only observed for 80% and 83% humidity, where 83% humidity corresponds to the base experiment previously called as submerged solid-state fermentation.

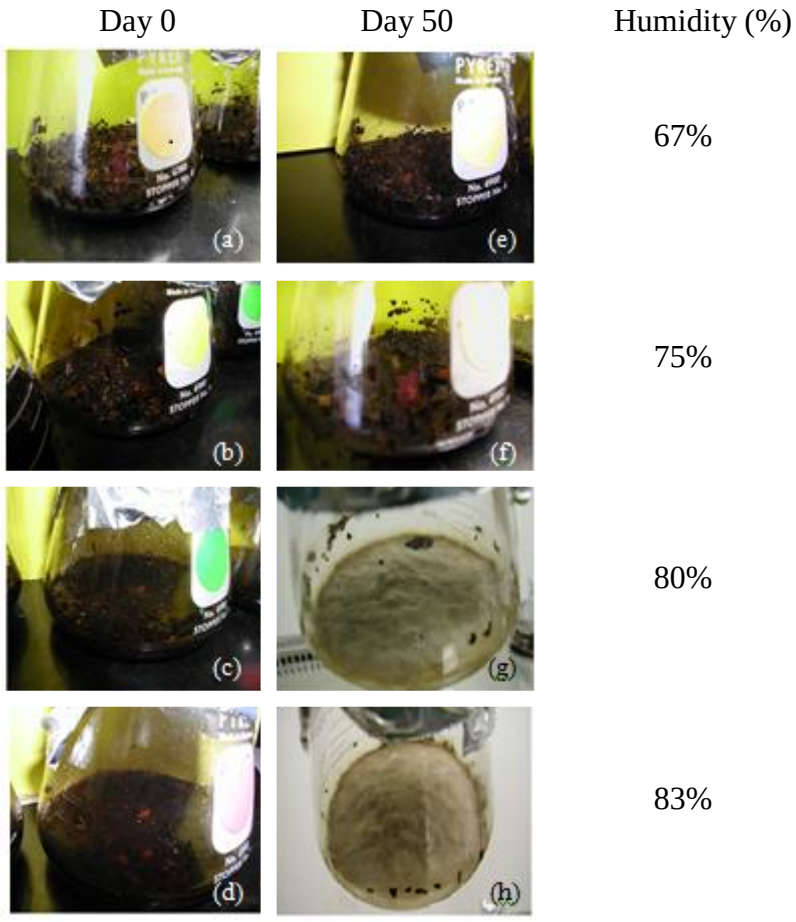


**Figure 10.** Different humidity conditions tested. (a) and (e) contains 67% humidity. (b) and (f) are set to 75% humidity. (c) and (g) are set to 80% humidity. And (d) and (h) contains 83% humidity. Pictures (a) to (d) represents day 0 for different humidity conditions. Pictures (e) to (h) represents growth profile for day 50 at different humidity conditions.

After determining the minimum moisture content that can be used based on fungal growth, a fermentation experiment was set to quantify caffeine degradation. Fermentation was carried out on three different dried coffee pulp sizes: pulverized, ground (0.85-1.41mm) and unground. For each coffee pulp size, five grams of coffee pulp were inoculated with *Alternaria alternata*, twenty milliliters of a solution of salts, sucrose and fungi (as described on materials

and methods section). An appropriate fungal growth was not achieved on pulverized coffee pulp, for this reason the analysis of caffeine was not carried out. For ground coffee pulp growth occurred uniformly on the material by day three. Total fermentation time was twenty days. Sampling was made on days 0, 3, 6, 9, 12 and 20 for caffeine concentration and coffee pulp dry weight.

Figure 11 shows the caffeine concentration profile and coffee pulp dry weight for each sampling day on 0.85-1.41mm sized coffee pulp. Again, fermentation curves follow the behavior seen before in Figure 10. A marked reduction in the content of free caffeine is observed during the first 3-6 days of fermentation where a minimum and then, as fungal biomass increases, in which there is an apparent increase in the amount of caffeine. Hypothetically, this could be due

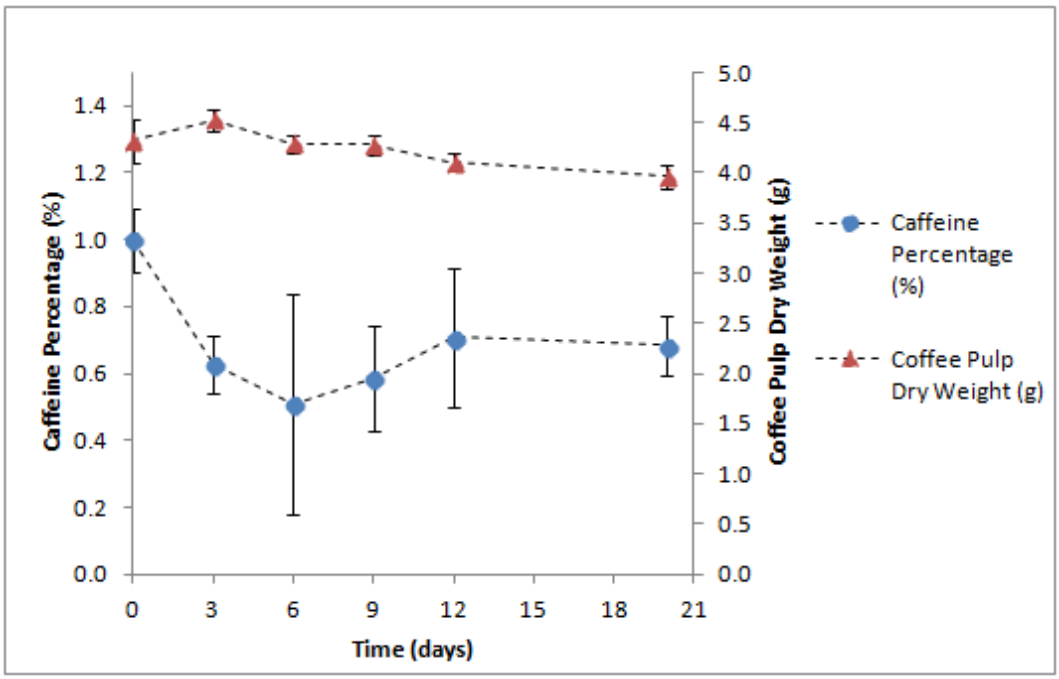


**Figure 11.** Solid-state fermentation of ground coffee pulp with *Alternaria alternata*. Percent of caffeine was determined by HPLC and is shown in circle. The coffee pulp dry weight is shown in triangle. The coffee pulp dry weight is shown in triangle. The vertical bars correspond to the standard deviation of each data point. This is a representative experiment that was conducted two times where n=6 according to methodology described in section 2.2

to the release of caffeine kidnapped in the solid as the fungus grows and degrades the material. Overall, a decrease on the caffeine content of an average of 31.5% was observed by day twenty.

In Figure 12 a comparison of caffeine concentration in terms of percentage are shown for the submerged solid-state experiment and the solid-state fermentation experiment. Although the two curves are statistically indistinct for the majority of the fermentation there is an interesting difference early in the fermentation around day 3 in which the solid state fermentation seems to be more efficient. However, degradation of caffeine can be observed in the two assemblies and a reduction of an average of 47.5% was obtained in the presence of more liquid while an average of 31.5% can be seen in the other experiment

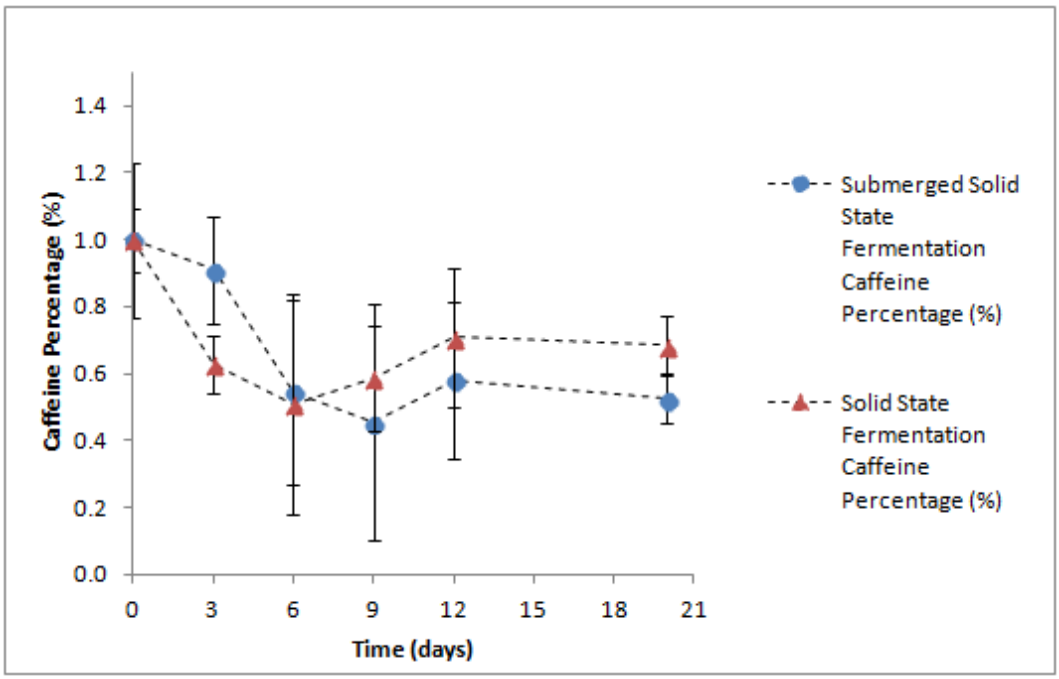


**Figure 12.** Caffeine on submerged solid-state fermentation versus solid-state fermentation. Percent of caffeine was determined by HPLC. Caffeine percentage on submerged solid-state are shown in circle and on solid-state fermentation are shown triangle. The coffee pulp dry weight is shown in triangle. The vertical bars correspond to the standard deviation of each data point. This is a representative experiment where n=6 according to methodology described in section 2.1 and 2.2.

The second set of experiments consisted in determining which is the best particle size to undergo caffeine degradation. Grind and pulverized coffee pulp fermentation was also performed using whole (not ground) coffee pulp as substrate to inoculate *Alternaria alternata*. Same conditions were used, but caffeine concentration and coffee pulp dry weight sampling was made on days 0, 6, 12 and 20 (once per week). Uniform growth was achieved by day 6. This time, fermentation curve does not follow exactly the same pattern. Anyhow, a rapid reduction in the content caffeine is observed during the first days and then the reductions become slower. Finally, the average caffeine reduction of about 53.5% was observed as shown on Figure 13. For details on these configurations refer to materials and methodology section.

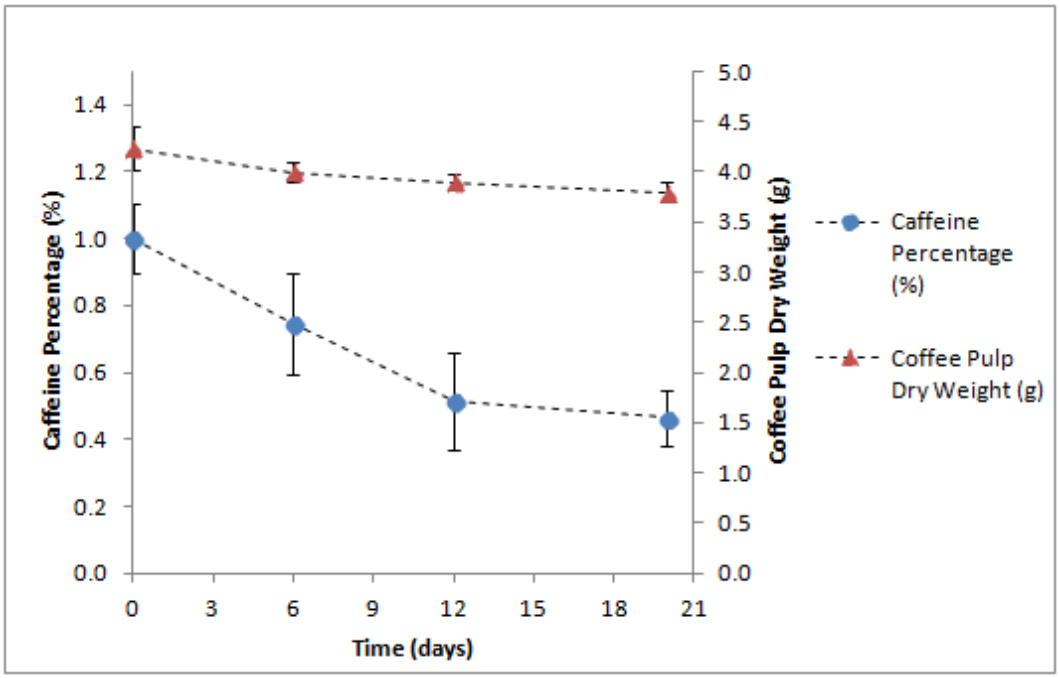


**Figure 13.** Solid-state fermentation on unground coffee pulp. Percent of caffeine was determined by HPLC and is shown in circle. The coffee pulp dry weight is shown in triangle. The coffee pulp dry weight is shown in triangle. The vertical bars correspond to the standard deviation of each data point. This is a representative experiment where n=6 according to methodology described in section 2.2.

Figure 14 shows a comparison of the caffeine concentration profile on fermentors with coffee pulp of two different sizes. As stated earlier, the curve corresponding to the profile of un-milled pulp is not as seen in previous graphs. This difference should be related to the size of the pulp. When the pulp is un-milled the caffeine associated to the solid is more difficult to access that why the caffeine reduction curve is linear and descending. In the case of the ground pulp caffeine concentration is transient because the fungus has improved access to solid. An average of 31.5% of decaffeination was observed on ground coffee pulp. As a reminder, ground coffee pulp represents a size of between 0.85 to 1.41 millimeters. In the case of unground coffee pulp, the decaffeination reached an average of 53.5%. The mayor finding of this experiment is precisely that it is not necessary to grind coffee pulp. Decaffeination can be achieved without grinding process, which represents an economical advantage for coffee producers.

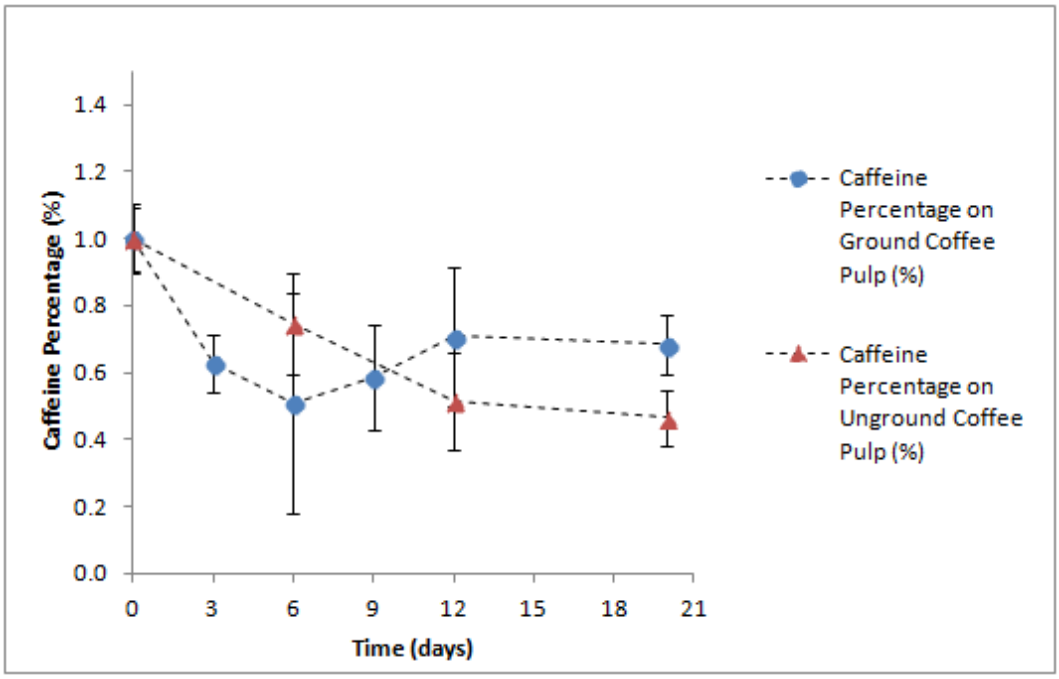


**Figure 14.** Coffee pulp size comparison. Percent of caffeine was determined by HPLC. The solid-state fermentation with a coffee pulp particle size of 0.85-1.41mm is shown in circle. The solid-state fermentation carried out with unground coffee pulp is shown in triangle. The coffee pulp dry weight is shown in triangle. The vertical bars correspond to the standard deviation of each data point. This is a representative experiment where n=6 according to methodology described in section 2.2

To summarize, the chart shown in Figure 15 illustrates the three conditions examined in terms of reduction of caffeine. It can be seen that the decaffeination is possible in these three conditions. For fermentation in submerged solid-state (83% humidity) and where the pulp was ground, it was possible to reduce the caffeine 47.5% in twenty days. For the pulp of the same size and less moisture content (80%) decaffeination was lower, 31.5% for twenty days of fermentation.

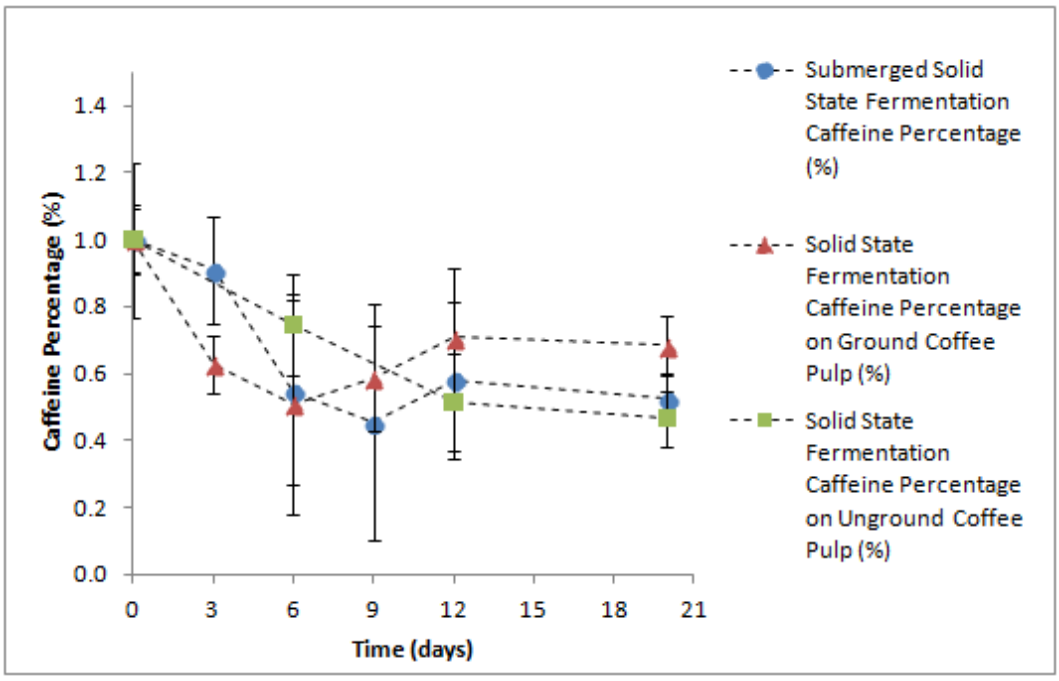


**Figure 15.** Comparison of analyzed conditions. Percent of caffeine was determined by HPLC. The submerged solid-state fermentation with a coffee pulp particle size of 0.85-1.41mm is shown in blue, the solid-state fermentation carried out with a coffee pulp particle size of 0.85-1.41mm is shown in red and the solid-state fermentation with unground coffee pulp is shown in green. The coffee pulp dry weight is shown in triangle. The vertical bars correspond to the standard deviation of each data point. This is a representative experiment where n=6 according to methodology described in section 2.

Finally, for the un-milled pulp with the same amount of medium than the previous (80% moisture) the decaffeination in twenty days was 53.5%. In statistical terms and based on the error bars, these values can be considered indistinct. However, whether the magnitude of the

degradation on un-ground coffee pulp is greater or equal to ground coffee pulp it is an important finding that the pulp does not have to be ground to be decaffeinated. This represents a great advantage in terms of the economy of the farms since that they can process this waste with less effort and less investment in water and electricity.

## 3.2. Solid-state fermentation scale-up design

Based on the laboratory scale experiment (previous section), the following experiment is performed as a preamble to the decaffeination of greater amounts of pulp at a time. It is done as a proof of concept to demonstrate that caffeine degradation can be achieved at a greater scale. Figure 16 shows how the scale-up assembly was made; also it can be observed fungal growth on coffee pulp. The tray dimensions are 13in x 9 in. It consisted of one hundred grams of unground coffee pulp inoculated with a solution of fungi (*Alternaria alternata*), sucrose and salts (as described in the materials and methodology section).


**Figure 16.** Scale-up setting. (a) Without fungi (b) Inoculated with *Alternaria alternata* at day 20

Analysis was conducted for twenty days; sampling days were day 0, 9 and 20. Figure 17 shows the caffeine percentage and coffee pulp dry weight profile during the analysis days. A relative caffeine reduction of an average of 41.2% was achieved by day twenty.

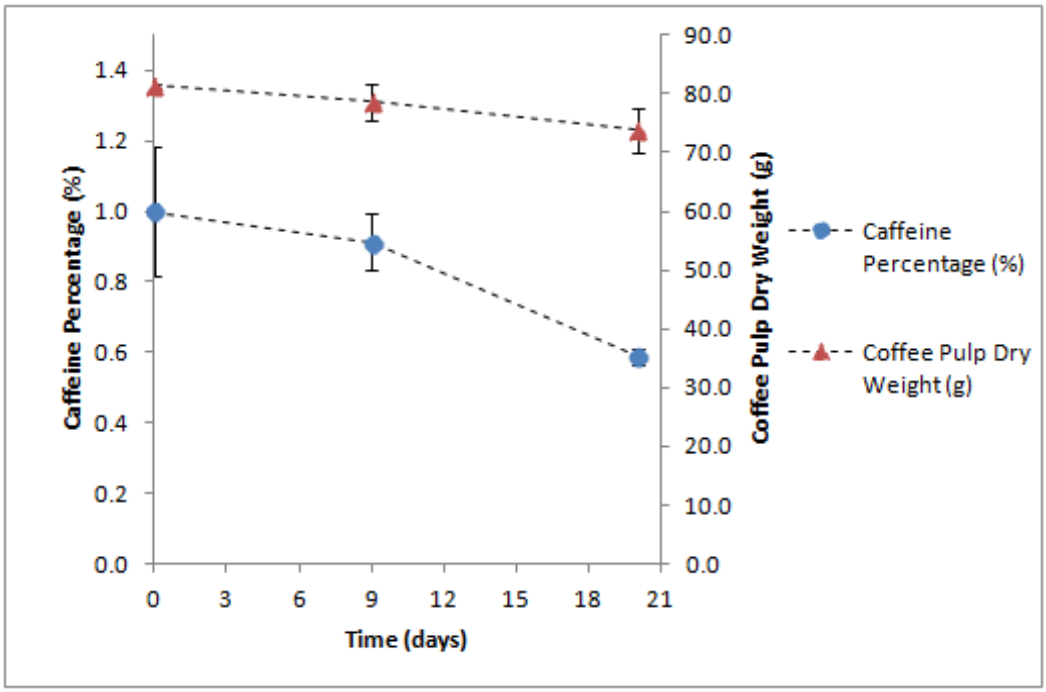


**Figure 17.** Scale-up caffeine percentage and dry weight analysis of coffee pulp. Percent of caffeine was determined by HPLC and is shown in circle. The coffee pulp dry weight is shown in triangle. The vertical bars correspond to the standard deviation of each data point. This is a representative experiment where n=3 according to methodology described in section 2.4

## 3.3. Structural and compositional analysis of coffee pulp

### 3.3.1. Structural Analysis

Figure 18 shows the growth of fungi at different days following inoculation. In panel (a), the pulp is fresh which it just had gone through the drying process. Panel (b) shows the pulp newly inoculated with the fungus *Alternaria alternata*. The pulp at day zero was previously

autoclaved and sterilized. If compared it can be seen the changes caused majorly by the process of sterilization and the presence of the inoculum. Panels (c) and (d) show the growth of the fungus in coffee pulp for 3 and 34 days of being inoculated. It is evident from the picture that there is a denser mycelium in the 34-day sample than on the third day. This is indicative of a progressive fermentation.

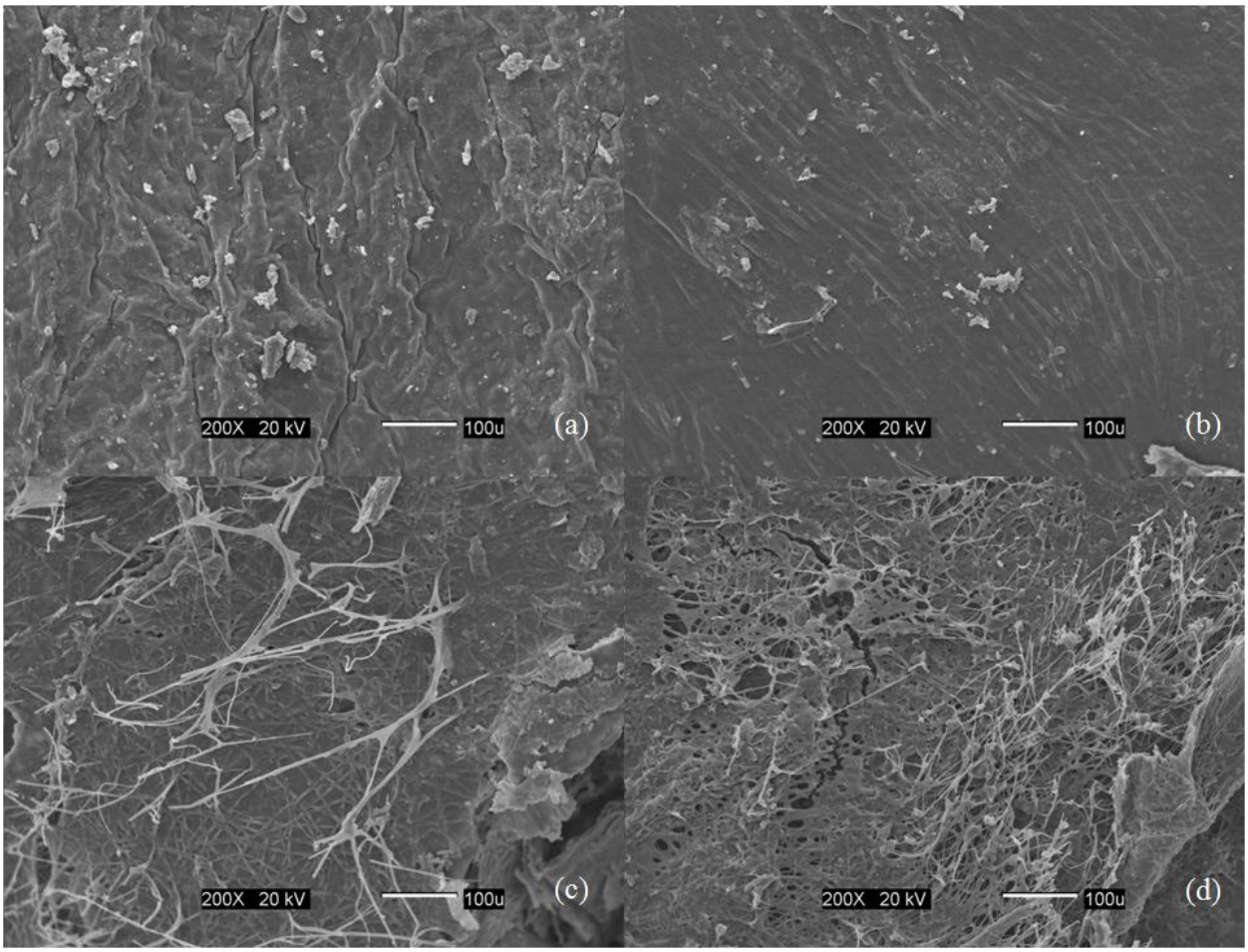


**Figure 18.** SEM micrographies taken from coffee pulp on different conditions. (a) fresh coffee pulp (no fungus) (b) coffee pulp at day 0 of analysis (inoculated with *Alternaria alternata*) (c) coffee pulp at day 3 of analysis (inoculated with *Alternaria alternata*) (d) coffee pulp at day 34 of being inoculated with *Alternaria alternata*

### 3.3.2. Compositional Analysis

#### *3.3.2.1. Elemental Analysis: Total Minerals and Heavy Metals*

Between the alternatives to reuse the degraded coffee pulp, the option of incorporating the pulp to animal feed is one that seems to have more potential. That is why we have used the compositional analysis to evaluate this option in terms of the nutritional requirements of animals such as cattle, pigs and poultry. But first a comparison between fresh dried coffee pulp and coffee pulp that have pass through a process of twenty days of fermentation, in Figure 19 the comparison corresponds to laboratory scale.

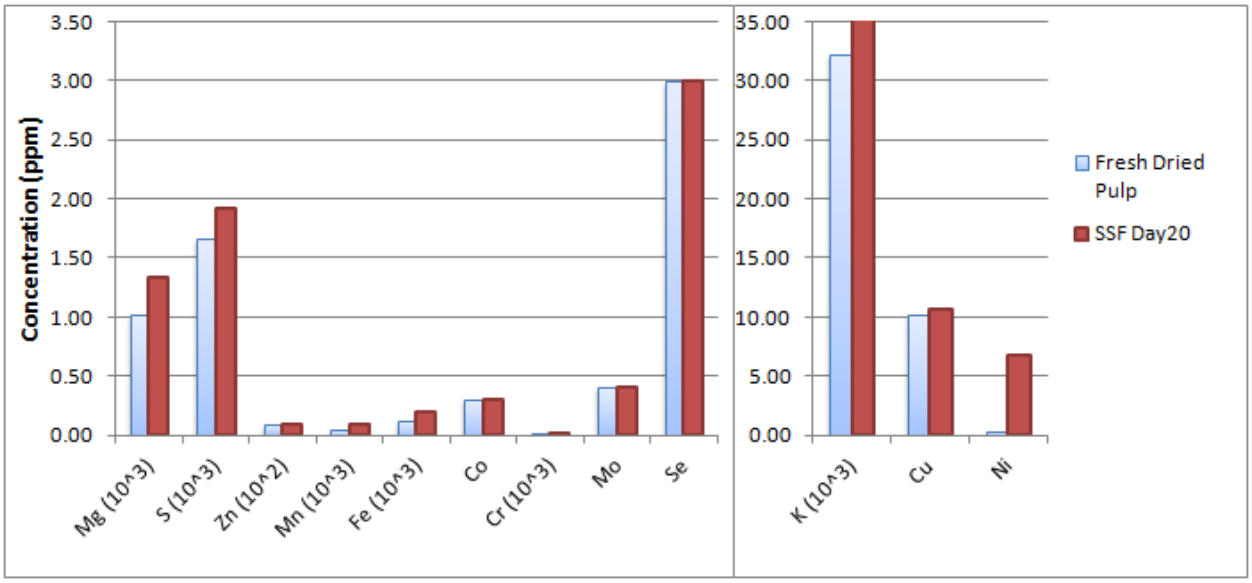


**Figure 19.** Comparison between fresh dried coffee pulp and day 20 of solid-state fermentation laboratory scale. Fresh coffee pulp shown in pale blue. Fermented pulp shown in dark red. Analysis was conducted by University of Wisconsin Soil and Plant Analysis Laboratory.

Figure 20 is made from the data of fresh dried coffee pulp and coffee pulp fermented on our scaled-up reactor previously described. The minerals and heavy metals shown are based on the nutritional requirements of cattle. These requirements were obtained from the Merck Veterinary Manual and compared in terms of concentration.

The Merck Veterinary Manual was consulted to assess the feasibility of the use of coffee pulp as animal feed. In the case of pigs and poultry some minerals, such as: potassium, manganese, iron and copper, among others appear to be greater than nutritional requirements and the maximum tolerable levels of these species. Even though any negative effects from over-supplementing diets are generally minimal except in case of extreme imbalance. However, special attention should be given to phosphorus because it is considered a potential environmental pollution and over-supplementing this mineral to animals can lead on higher concentrations of it through the excretory system.

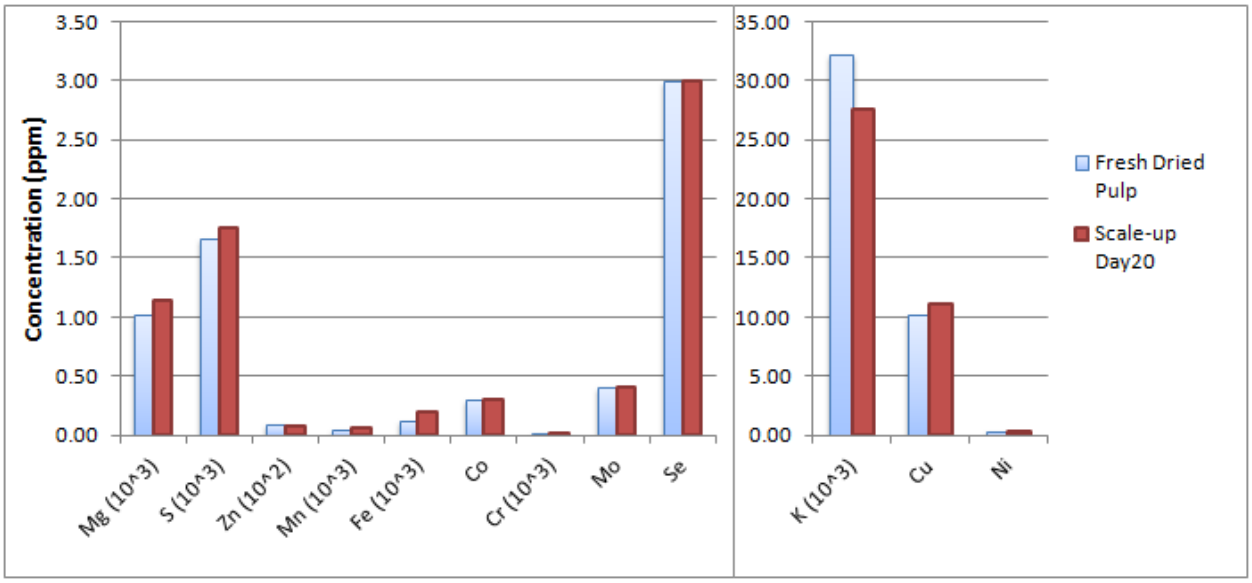


**Figure 20.** Comparison between fresh dried coffee pulp and day 20 of solid-state fermentation for scaled up reactor. Fresh coffee pulp shown in pale blue. Fermented pulp shown in dark red. Analysis conducted by University of Wisconsin Soil and Plant Analysis Laboratory.

In terms of cattle the Merck Manual remarks that mature pasture, crop residues or forage crops harvested in a manner that results in shattering, leaching or spoilage may be so reduced in nutritive value that they are suitable only in a maintenance ration for adult cattle. However, minerals concentration of coffee pulp does not exceed maximum tolerable levels on most of the studied minerals.

The following graphs show a comparison of the pulp with the maximum tolerable nutritional levels of cattle. Figure 21 compares fresh dried coffee pulp with cattle nutritional requirements. In this case, it can be seen that the analyzed parameters meet the highest concentrations prescribed for cattle, with the exception of selenium and potassium. Selenium is toxic, the maximum tolerable level is 2 ppm and the toxic level about 8 ppm. In cattle with chronic poisoning symptoms as a loss of vitality, drowsiness, emaciation can be observed, whereas if there is acute poisoning death of the animal can happen due to inhibition of some enzymes especially dehydrogenases (Villanueva, 2011). However, in our case, coffee pulp does not exceed 3 ppm. It is recommended dietary levels of aluminum, arsenic and copper to reduce the absorption or increase excretion of selenium. On the other hand, ingestion of moderate excess of potassium may result in imbalances, predisposing disorders gained in using magnesium and calcium. Metabolic diseases that occur in this situation, in the case of dairy cows and breeding are: grass tetany and milk fever; this occurs when there is lack of magnesium or calcium, respectively, in the animal's metabolism (Mufarrege, 2004). To equilibrate the potassium excess it is recommended to supplement the feed with anionic salts.

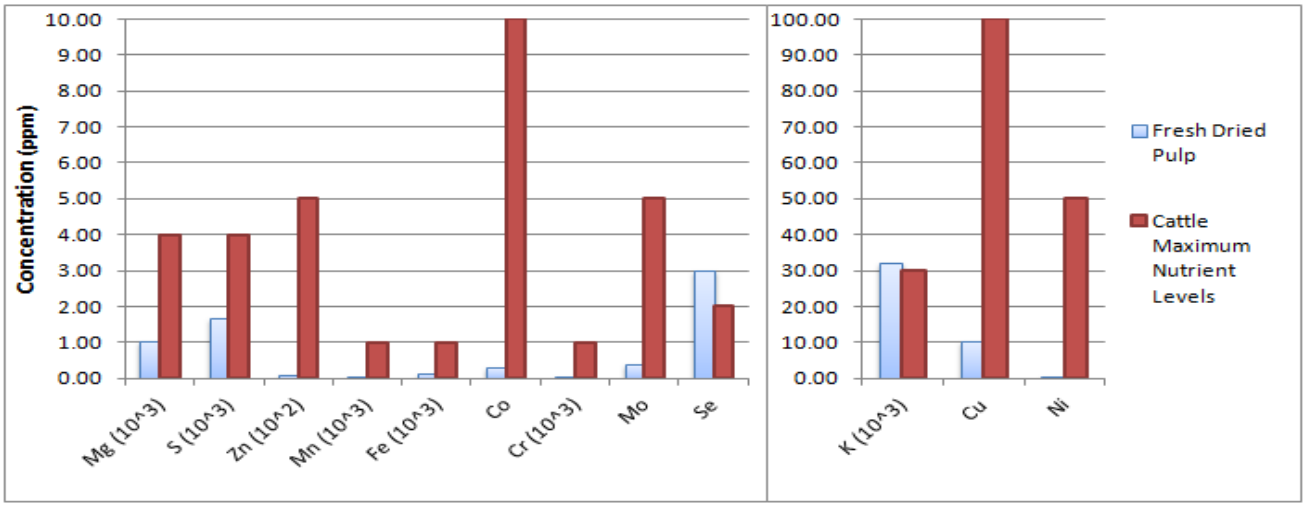


**Figure 21.** Nutritional requirements of cattle compared to fresh dried coffee pulp. Fresh pulp is shown in pale blue. Maximum nutrient levels are shown in dark red. Analysis conducted by University of Wisconsin Soil and Plant Analysis Laboratory.

Figures 22 and 23 show the compositional state of the pulp after undergoing the solid-state fermentation to degrade the caffeine present in the material. It can be observed the effect of fermentation and the presence of the fungus. Once again, selenium and magnesium does not meet the criteria, but it does not imply a substantial difference, that is why the modification of diet is recommended adding compounds such as arsenic, aluminum, copper and anionic salts to balance the concentrations of selenium and potassium.

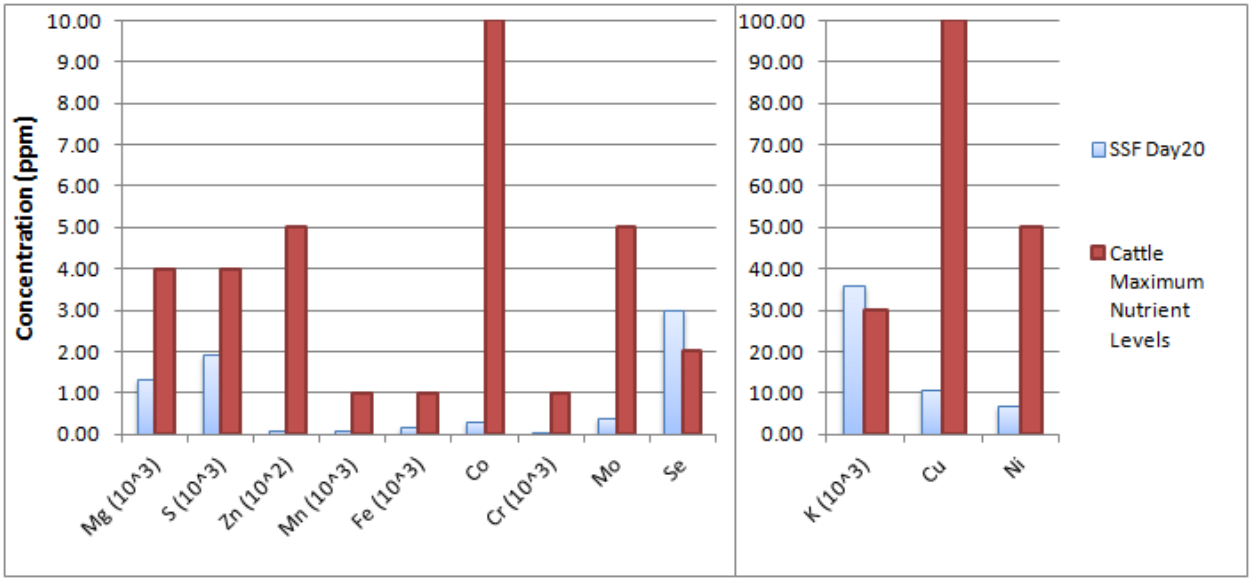


**Figure 22.** Cattle maximum nutrient levels versus solid-state fermentation at day 20. Analysis conducted by University of Wisconsin Soil and Plant Analysis Laboratory.

Figure 23 compares fermented coffee pulp in a scale-up reactor with cattle maximum tolerable levels. In this case only selenium overpasses the maximum tolerable limits by 1 ppm. Since it is not an exorbitant difference, the toxic concentration is about 8 ppm, this coffee pulp seems to be safe as animal feed, howsoever a modification on the diet is recommended adding compounds such as arsenic, aluminum and copper to balance the concentrations of selenium in cattle's organism.

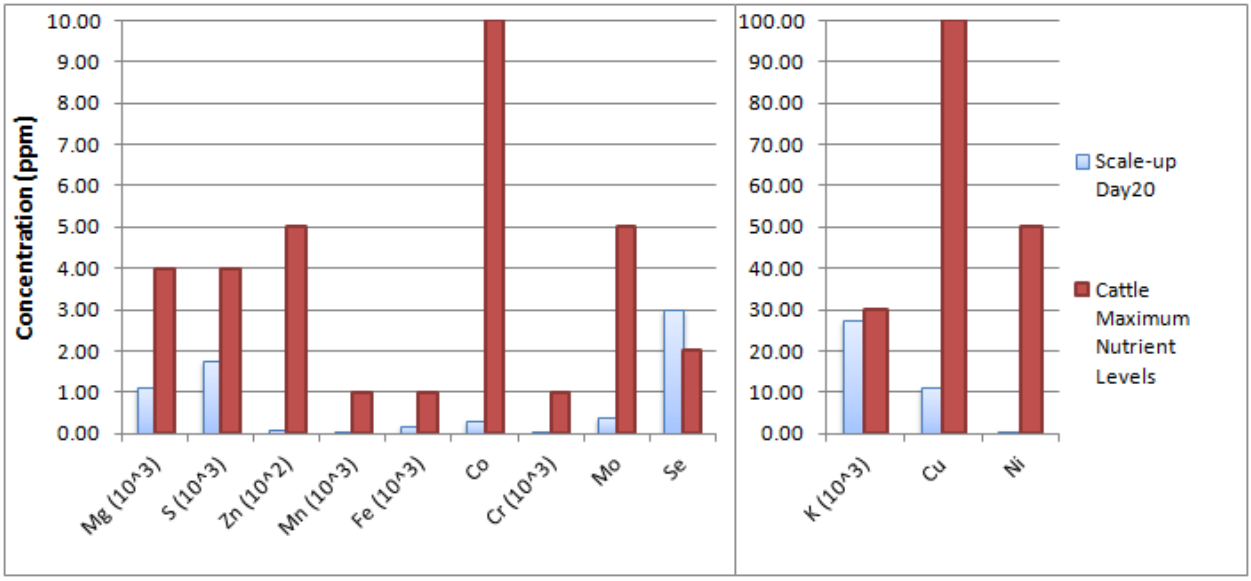


**Figure 23.** Cattle maximum nutrient levels versus scaled-up solid-state fermentation at day 20. Analysis conducted by University of Wisconsin Soil and Plant Analysis Laboratory.

In terms of the total nitrogen present on the coffee pulp, it is important to note, as presented in Table 2, that it content was slightly increased after the fermentation. This is important because of the need protein that animals have. It is necessary for it maintenance and production. As it is known, all proteins are formed by different combinations of aminoacids, which have in their structure at least one nitrogen atom. Furthermore, the microorganisms in the rumen also require nitrogen for growth (Salazar, 2006). Nitrogen obtained values also meet the nutritional needs of cattle used for milk production, which according to The Merck Veterinary Manual is about 1.6% on a dry basis. In the case of the total carbon, from the data shown on Table 2, it is not evident a reproducible behavior between the fresh and the fermented coffee pulp, however a slight decrease can be observe from day 0 to day 20 of fermentation.

**Table 2.** Total carbon and total nitrogen for the unfermented and fermented coffee pulp.

| Sample | Total Carbon (%) | Total Nitrogen (%) |
|---|---|---|
| **Fresh dried pulp** | 36.72 | 1.65 |
| **SSF day 0** | 36.84 | 1.80 |
| **SSF day 20** | 36.41 | 2.00 |
| **Scale-up day 0** | 38.10 | 1.72 |
| **Scale-up day 20** | 37.26 | 1.86 |

### *3.3.2.2. Lignin Content*

In Figure 24, lignin and cellulose content are compared for laboratory-scale and for scale-up experiments. Through this study and the structural analysis of the pulp we can mention that it is clear that the fungus is growing. However, growth apparently has no adverse effect on the concentration of cellulose, which is important to continue considering the material as animal feed. Even both the concentration of the lignin and cellulose remain constant. However, it is possible that this fungus is consuming lignin and cellulose at the same rate. We recommend a future study to determine if the fungus expresses some mechanism of degradation of lignin. There are several metrics to determine whether the lignocelluloses degrading mechanism is active under the particular fermentation conditions. The measurement of the activity of lignocelluloses degrading enzymes such as laccase may be a good indicator in the case of *Alternaria alternata* (Walker, 2010). However, according to the candidate fungus this assessment may vary.

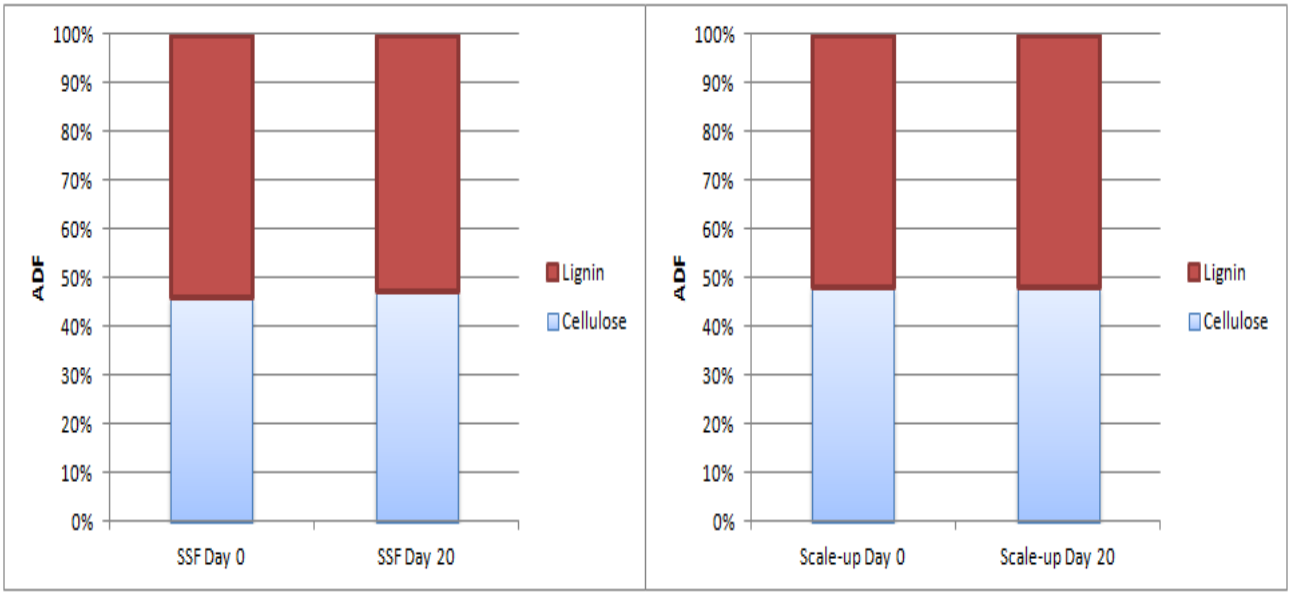


**Figure 24.** Lignin and cellulose profile for solid-state fermentation experiment.

From this analysis, we can also compare the values reported on the literature (Table 1) for lignin on coffee pulp. Our analysis reports a lignin content of about 20-30% of dry matter (DM) while other researchers like Preethu et al (2007) have reported a 38.6%.

# 4. Conclusions and Recommendations

Several scenarios were evaluated to optimize factors such as humidity and coffee pulp size with the filamentous fungi *Alternaria alternata* as caffeine degrader microorganism. The degradation of caffeine in coffee pulp was achieved in each of the studied scenarios. The option that turns out to be the best was the one in which the pulp was not ground and with an 80% relative humidity. For this option the obtained degradation was of an average of 53.5%. This finding can be considered a great contribution. The conditions that were found to be most desirable require less pre-processing of the pulp to be fermented while fermentation requires a smaller amount of liquid to be carried out, which is a great advantage.

Even though the studied scenario represents a great contribution, it could even considered other options for optimizing the degradation of caffeine. These options are based on experiments that are proofs of concept in which all the parameters cannot be evaluated due to restrictions, either of time, availability of materials and resources, among others. Based on this, new questions may arise about what else can be done. In the case of the laboratory-scale experiment it is recommended future experimentation for determining how parameters like inoculum concentration, inoculum type, fermentation temperature, ambient humidity, pH and others can affect fermentation and caffeine degradation.

It is noteworthy that even though the decaffeination of coffee pulp was achieved it does not mean the final product is safer because the fungus and its potential production of mycotoxins. According to http://www.micotoxinas.com.br/altertoxins.htm, among the *Alternaria* species, *Alternaria alternata* is probably the most important mycotoxin-producing species. Some of the metabolites that it can produce are: alternariol monomethyl ether, altertoxins I, altenuene, alternariol and tenuazonic acid.

Although *Alternaria alternata* was used as part of a proof of concept, the option still remains to use another species of filamentous fungi with ability to decrease the indigestible fiber content, i.e. lignin, even though *Alternaria alternata* has been identified in literature as a lignin-degrader microorganism, this ability is not evident under the studied conditions. Assessments of lignocelluloses degrading activities can be established to further explore the potential of this agroindustrial waste. One activity that has been identified in lignocellulosic degrader fungi is Laccase. Laccases are blue copper oxidases that catalyze the one-electron oxidation of phenolics and other electron-rich substrates. Laccases contain multiple copper atoms which are reduced as the substrates are oxidized. After four electrons have been received by a laccase molecule, the laccase reduces molecular oxygen to water, returning to the native state (K. Hammel, 1997).

On the other hand, based on the results of the laboratory scale experiment, a proof of concept for the scaling-up of the fermentation process was carried out. For this purpose, the proposed system consists of trays filled with unground coffee pulp and a humidity of 80% inoculated with filamentous-caffeine degrader fungi *Alternaria alternata*. The preliminary study of the proposed system results in an average of 41.2% caffeine reduction. Future work for the construction of a larger reactor (fermentor) can be based on this proof of concept and adjusted according to coffee farms needs, as the rate of coffee production and its byproducts, knowing that larger quantities must be degraded in order to propose a detoxification model for the coffee-processing sites. Also, future experimentation is proposed based on the reality that the scale-up parameters were increased linearly and other models and parameters can be explored to accomplish maximum optimization of the system.

For the compositional study of coffee pulp, it can be concluded that it use as animal feed may be considered for certain species such as cattle. This recommendation is based on the analysis of

the values obtained from the degraded material against the values reported in the Merck Veterinary Manual (http://www.merckmanuals.com/vet/index.html). However, it will be necessary to supplement the feed to meet other requirements and balance the concentrations of selenium and potassium as the case may. In terms of the total nitrogen, it was slightly increased during fermentation which is important because of the need protein that animals have for it maintenance and production. But in the case of total carbon, a light decrease was observed at the end of the fermentation process.

Further study is suggested based on the, already mention, fact that *Alternaria alternata* may produce mycotoxins; a toxicity study is suggested to determine whether the final product is safer for the proposed reutilization purposes. Also, it is suggested to conduct additional experimentation to determine how cattle can assimilate pulp and in which ratio it can be incorporated into cattle diet. For this latter query, previous studies cited by Mazzafera (2002) suggested a utilization of coffee pulp at a 60% level when supplemented with highly palatable feeds and forage. Ingestion of coffee pulp on the cited study was estimated to be 1.3kg per 100kg of live weight which is equivalent to about 0.017kg of caffeine per each 100kg of live weight.

In addition, it is important to remark, from the point of view of the use of coffee pulp as animal feed, that lignin is an undesired component because it is known that it affects negatively the digestibility of the materials. As mention before, lignin-degradation mechanisms of *Alternaria alternata* are not evident under the studied conditions. For this purposes, it may be necessary to determine by enzymatic activity studies if *Alternaria alternata* is expressing it lignin-degrading ability. If it is not, it is recommended to conduct experiments to determine how lignin degradation can be observed. Also, a screening of fungi with the ability to detoxify coffee pulp in terms of caffeine content and lignin may be carry.

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# APPENDIXES

## **APPENDIX A** Submerged solid-state fermentation raw data

**Table 3.** Caffeine percentage for submerged solid-state fermentation experiment. Coffee pulp size for this experiment was between 0.85 – 1.41mm.

| Sample | Day 0 | Day 3 | Day 6 | Day 9 | Day 12 | Day 20 |
|---|---|---|---|---|---|---|
| **1.1** | 0.498 | 1.173 | 1.407 | 0.603 | 1.236 | 1.046 |
| **1.2** | 0.522 | 1.164 | 1.417 | 0.610 | 1.245 | 1.003 |
| **1.3** | 0.534 | 1.209 | 1.445 | 0.606 | 1.237 | 0.991 |
| **2.1** | 1.180 | 1.050 | 1.094 | 0.600 | 1.117 | 1.020 |
| **2.2** | 1.188 | 1.090 | 1.076 | 0.600 | 1.119 | 1.048 |
| **2.3** | 1.171 | 1.146 | 1.086 | 0.595 | 1.129 | 1.057 |
| **3.1** | 1.137 | 1.012 | 0.781 | 0.878 | 1.160 | 0.999 |
| **3.2** | 1.115 | 0.996 | 0.797 | 0.879 | 1.137 | 1.009 |
| **3.3** | 1.088 | 1.012 | 0.788 | 0.891 | 1.112 | 0.958 |
| **4.1** | 0.991 | 1.119 | 0.599 | 1.097 | 0.957 | 0.900 |
| **4.2** | 0.958 | 1.138 | 0.632 | 1.047 | 0.969 | 0.867 |
| **4.3** | 1.011 | 1.139 | 0.588 | 1.067 | 0.959 | 0.844 |
| **5.1** | 1.147 | 0.789 | 0.902 | 1.523 | 1.025 | 1.089 |
| **5.2** | 1.160 | 0.787 | 0.904 | 1.545 | 1.028 | 1.096 |
| **5.3** | 1.158 | 0.772 | 0.884 | 1.520 | 0.995 | 1.111 |
| **6.1** | 1.037 | 0.799 | 1.207 | 1.313 | 0.537 | 0.992 |
| **6.2** | 1.051 | 0.801 | 1.233 | 1.309 | 0.524 | 0.985 |
| **6.3** | 1.054 | 0.803 | 1.160 | 1.314 | 0.516 | 0.985 |
| **Average Caffeine Percentage (%)** | 1.000 | 0.910 | 0.543 | 0.453 | 0.579 | 0.525 |
| **Standard Deviation (%)** | 0.232 | 0.162 | 0.278 | 0.355 | 0.237 | 0.074 |
| **Average Caffeine ± Standard Deviation** | 1.000 ± 0.232 | 0.910 ± 0.162 | 0.543 ± 0.278 | 0.453 ± 0.355 | 0.579 ± 0.237 | 0.525 ± 0.074 |

## **APPENDIX B** Solid-state fermentation raw data

**Table 4.** Caffeine percentage for solid-state fermentation experiment with a coffee pulp size between 0.85 – 1.41mm.

| Sample | Day 0 | Day 3 | Day 6 | Day 9 | Day 12 | Day 20 |
|---|---|---|---|---|---|---|
| **1.1** | 0.899 | 1.057 | 1.572 | 1.142 | 1.311 | 0.911 |
| **1.2** | 0.883 | 1.048 | 1.536 | 1.141 | 1.313 | 0.919 |
| **1.3** | 0.889 | 1.058 | 1.559 | 1.147 | 1.323 | 0.913 |
| **2.1** | 0.951 | 0.927 | 1.117 | 0.781 | 1.103 | 0.958 |
| **2.2** | 0.940 | 0.916 | 1.126 | 0.845 | 1.083 | 0.957 |
| **2.3** | 0.939 | 0.909 | 1.127 | 0.844 | 1.096 | 0.965 |
| **3.1** | 1.100 | 0.879 | 0.911 | 0.996 | 1.003 | 0.949 |
| **3.2** | 1.106 | 0.882 | 0.912 | 1.018 | 1.013 | 0.951 |
| **3.3** | 1.078 | 0.906 | 0.914 | 0.999 | 0.999 | 0.964 |
| **4.1** | 1.084 | 0.992 | 0.690 | 0.990 | 0.888 | 1.148 |
| **4.2** | 1.069 | 0.994 | 0.674 | 0.993 | 0.888 | 1.144 |
| **4.3** | 1.069 | 0.990 | 0.678 | 0.994 | 0.882 | 1.149 |
| **5.1** | 1.138 | 1.073 | 1.126 | 1.228 | 0.651 | 1.023 |
| **5.2** | 0.958 | 0.998 | 1.123 | 1.235 | 0.651 | 0.865 |
| **5.3** | 1.140 | 0.986 | 1.134 | 1.230 | 0.654 | 0.961 |
| **6.1** | 0.917 | 1.149 | 0.601 | 0.808 | 1.049 | 1.063 |
| **6.2** | 0.925 | 1.124 | 0.596 | 0.805 | 1.047 | 1.091 |
| **6.3** | 0.914 | 1.112 | 0.605 | 0.804 | 1.047 | 1.067 |
| **Average Caffeine Percentage (%)** | 1.000 | 0.628 | 0.507 | 0.586 | 0.709 | 0.685 |
| **Standard Deviation (%)** | 0.094 | 0.085 | 0.328 | 0.159 | 0.208 | 0.089 |
| **Average Caffeine ± Standard Deviation** | 1.000 ± 0.094 | 0.628 ± 0.085 | 0.507 ± 0.328 | 0.586 ± 0.159 | 0.709 ± 0.208 | 0.685 ± 0.089 |

**Table 5.** Caffeine percentage for solid-state fermentation experiment where coffee pulp was unground.

| Sample | Day 0 | Day 6 | Day 12 | Day 20 |
|---|---|---|---|---|
| **1.1** | 1.045 | 1.057 | 1.217 | 1.027 |
| **1.2** | 1.041 | 1.038 | 1.218 | 1.021 |
| **1.3** | 1.041 | 1.043 | 1.234 | 1.022 |
| **2.1** | 0.970 | 1.135 | 0.918 | 0.882 |
| **2.2** | 0.953 | 1.151 | 0.920 | 0.883 |
| **2.3** | 0.951 | 1.152 | 0.913 | 0.877 |
| **3.1** | 1.067 | 1.146 | 1.080 | 0.946 |
| **3.2** | 1.065 | 1.151 | 1.098 | 0.938 |
| **3.3** | 1.089 | 1.148 | 1.085 | 0.950 |
| **4.1** | 1.123 | 0.770 | 1.086 | 1.085 |
| **4.2** | 1.119 | 0.891 | 1.084 | 1.077 |
| **4.3** | 1.112 | 0.893 | 1.076 | 1.072 |
| **5.1** | 0.939 | 1.040 | 0.750 | 0.993 |
| **5.2** | 1.035 | 1.065 | 0.889 | 0.993 |
| **5.3** | 1.012 | 1.042 | 0.857 | 0.994 |
| **6.1** | 0.815 | 0.753 | 0.852 | 0.944 |
| **6.2** | 0.815 | 0.762 | 0.862 | 1.148 |
| **6.3** | 0.807 | 0.764 | 0.860 | 1.148 |
| **Average Caffeine Percentage (%)** | 1.000 | 0.746 | 0.514 | 0.465 |
| **Standard Deviation (%)** | 0.103 | 0.152 | 0.147 | 0.084 |
| **Average Caffeine ± Standard Deviation** | 1.000 ± 0.103 | 0.746 ± 0.152 | 0.546 ± 0.147 | 0.465 ± 0.084 |

## APPENDIX C Solid-state fermentation scale-up design raw data

**Table 6.** Caffeine percentage for scale-up of solid-state fermentation experiment. Coffee pulp for this experiment was not grind.

| Sample | Day 0 | Day 6 | Day 12 |
|---|---|---|---|
| **1.1** | 0.831 | 1.068 | 1.020 |
| **1.2** | 0.834 | 1.076 | 1.019 |
| **1.3** | 0.835 | 1.071 | 1.020 |
| **2.1** | 1.160 | 0.923 | 0.981 |
| **2.2** | 1.173 | 0.927 | 0.980 |
| **2.3** | 1.166 | 0.935 | 0.979 |
| **Average Caffeine Percentage (%)** | 1.000 | 0.913 | 0.588 |
| **Standard Deviation (%)** | 0.183 | 0.079 | 0.022 |
| **Average Caffeine ± Standard Deviation** | 1.000 ± 0.183 | 0.913 ± 0.079 | 0.588 ± 0.022 |

## APPENDIX D Compositional analysis

**Table 7.** Elemental analysis for total minerals raw data.

| Sample | P (%) | K (%) | Ca (%) | Mg (%) | S (%) | Zn (ppm) | B (ppm) | Mn (ppm) | Fe (ppm) | Cu (ppm) | Al (ppm) | Na (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1.SSF Day0** | 0.14 | 2.59 | 0.48 | 0.11 | 0.15 | 7.11 | 26.24 | 89.14 | 191.1 | 9.06 | 199.3 | 124.7 |
| **2.SSF Day20** | 0.23 | 3.58 | 0.47 | 0.13 | 0.19 | 9.25 | 27.31 | 86.41 | 190.0 | 10.70 | 326.9 | 182.9 |
| **3.Dried coffee pulp** | 0.18 | 3.22 | 0.39 | 0.10 | 0.17 | 8.61 | 26.30 | 48.96 | 123.2 | 10.14 | 70.2 | 109.6 |
| **4.Scale-up Day0** | 0.12 | 1.84 | 0.48 | 0.09 | 0.13 | 6.99 | 26.34 | 57.05 | 176.2 | 9.72 | 117.4 | 102.5 |
| **5.Scale-up Day20** | 0.24 | 2.75 | 0.48 | 0.11 | 0.17 | 7.52 | 24.96 | 63.55 | 189.8 | 11.06 | 213.2 | 149.1 |

**Table 8.** Elemental analysis for heavy metals raw data.

| Sample | Cd (ppm) | Co (ppm) | Cr (ppm) | Mo (ppm) | Ni (ppm) | Pb (ppm) | Li (ppm) | As (ppm) | Se (ppm) | Ba (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|
| **1.SSF Day0** | <0.4 | <0.3 | 12.81 | <0.4 | 0.82 | <2 | 0.34 | <3 | <3 | 30.11 |
| **2.SSF Day20** | <0.4 | <0.3 | 13.24 | <0.4 | 6.74 | <2 | 0.38 | <3 | <3 | 24.87 |
| **3.Dried coffee pulp** | <0.4 | <0.3 | 12.12 | <0.4 | <0.3 | <2 | 0.31 | <3 | <3 | 18.19 |
| **4.Scale-up Day0** | <0.4 | <0.3 | 12.45 | <0.4 | 0.39 | <2 | 0.36 | <3 | <3 | 14.22 |
| **5.Scale-up Day20** | <0.4 | <0.3 | 10.61 | <0.4 | <0.3 | <2 | 0.32 | <3 | <3 | 12.90 |

**Table 9.** Total carbon and nitrogen content.

| Sample | Total N (%) | NH4-N (ppm) | Total C (%) | TOC[a] (%) | IC[a] (%) |
|---|---|---|---|---|---|
| **1.SSF Day0** | 1.80 | 4.00 | 36.84 | | |
| **2.SSF Day20** | 2.00 | 3.70 | 36.41 | | |
| **3.Dried coffee pulp** | 1.65 | 5.95 | 36.72 | | |
| **4.Scale-up Day0** | 1.72 | 3.48 | 38.10 | | |
| **5.Scale-up Day20** | 1.86 | 4.90 | 37.26 | | |

[a] *Total Organic Carbon is about the same value as Total C and thus Inorganic C is negligible*

**Table 10.** Lignin and cellulose content.

| Sample | DM (%) | ADF (% of DM) | Lignin (% of DM) | Cellulose (%) |
|---|---|---|---|---|
| **1-SSF Day 0** | 94.14 | 53.43 | 28.79 | 24.64 |
| **2-SSF Day 20** | 94.13 | 58.32 | 30.70 | 27.62 |
| **3-Dried coffee pulp** | 93.89 | 43.14 | 20.24 | 22.90 |
| **4-Scale-up Day 0** | 94.46 | 55.47 | 28.69 | 26.78 |
| **5-Scale-up Day 20** | 93.98 | 55.44 | 28.73 | 26.71 |