BIOTRANSFORMATION OF COFFEE PULP USING FILAMENTOUS FUNGI

By

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ABSTRACT

Efforts devoted to the efficient use of agro-industrial residues have increased, especially if the use contributes to solve pollution problems. Coffee pulp/husk is the main byproduct generated during coffee wet processing. To reduce the pollution from this source it is necessary to establish new treatments to bio-transform these wastes, which contain various toxic substances (e.g. caffeine and lignin). The presence of these substances limits their recycling potential and makes their management an environmental challenge. This study focuses on the characterization of fungal strains which might be able to detoxify coffee processing wastes. Among the activities we screened for are decaffeination and delignification. We have identified *Alternaria alternata* as a potential candidate to degrade caffeine. This isolate was obtained from a ligniculous fungi collection provided by the Forest Products Laboratory in Madison, Wisconsin. *A. alternata* was evaluated in terms of caffeine degradation as carbon and nitrogen source. Under these conditions we observed a decrease near to 45.33% in the caffeine content. After addition of sucrose the caffeine degradation was 60.92%, in the same period (69 days). Analysis in coffee pulp using *Alternaria alternata* showed the ability to caffeine degradation during fermentation time (20 days) obtaining a 44.5% of caffeine degradation.

Keywords: caffeine, lignin, coffee pulp, filamentous fungi

RESUMEN

Investigaciones dedicadas al uso eficiente de residuos agroindustriales han ido aumentando, especialmente aquellas que contribuyen a solucionar problemas de contaminación. La pulpa de café es el principal subproducto generado durante el procesamiento húmedo de café. Para reducir la contaminación de esta fuente es necesario establecer nuevos tratamientos para bio-transformar por medio biológico estos desperdicios, los cuales contienen sustancias toxicas (ej. cafeína y lignina). La presencia de estas sustancias limita su potencial de de ser reciclados, convirtiendo el manejo de estos desperdicios en un gran reto ambiental. Este estudio se enfocó en caracterizar cepas de hongos capaces de detoxificar los desperdicios del procesamiento de café. Entre las actividades de interés se seleccionaron para la decafeinización y delignificación. Se identificó a *Alternaria alternata* como un candidato potencial para la degradación de cafeína. Este aislado fue obtenido de una colección de hongos ligniculosos provistos por The Forest Products Laboratory en Madison, Wisconsin. *A. alternata* fue evaluada en términos de degradación de cafeína como única fuente de carbono y nitrógeno. Bajo estas condiciones se observó una degradación cercana a 45.33% en el contenido de cafeína. Luego de añadir sacarosa la degradación de cafeína fue de un 60.92% en el mismo periodo de incubación (69 días). El análisis en la pulpa de café empleando *Alternaria alternata* mostró habilidad para degradar cafeína durante el tiempo de fermentación (20 días) obteniendo un 44.5% de degradación de cafeína.

Palabras claves: cafeína, pulpa de café, hongos filamentosos.

To God, to my family especially to my parents: Julita (in heaven) and Onofre, and to my love Andres "Osito", for their support in good and bad times whenever they happen,

Love is forever.

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1 INTRODUCTION

Agro-industrial residues have been used as efficient substrates in several bioprocesses such as the production of organic acids, the production of enzymes (Medeiros et al. 2006) and as feedstock for biorefineries (Sandun et al. 2006). The re-utilization of this residual matter into bioprocesses contributes to solve pollution problems, and improves resource management. Furthermore, the reutilization of wastes promotes the concept of integrated biorefineries, where the "wastes" could be used, for example, to generate energy for the plant, allowing a total sustainability of the process.

The accumulation of coffee pulp/husk is a prevalent problem in regions where coffee processing takes place. The large worldwide demand on coffee consumption increase the magnitude of problem exponentially with time (Berecha et al. 2011) wet processing techniques, (that produce coffee pulp/husk) are used for approximately 40 percent of global production generate wastewater with a biological oxygen demand (BOD) up to 150 g/l. (Soccol and Vandenberghe 2003). BOD is defined as the amount of oxygen required to biologically break down organic wastes diluted in water. The dry method results in 40% coffee husk/pulp as the main residue. Coffee growers spread the residue in the field where it is allowed to decompose (Salmones et al. 2005).

The expulsion of high quantities of wastewater in the initial bean processing leads to reduced oxygen levels in water, which can threaten many forms of marine life. Moreover, coffee processing industries are posing environmental hazards due to large-scale disposal of coffee pulp, husk and effluents from these units. This practice poses a greater threat to water and land quality around the coffee processing plants. Presence of toxic compounds like phenols in these byproducts restricts their direct use in agriculture. In addition, the indiscriminate use of fresh coffee pulp also affects crops through acid formation and local heat generation in the process of its fermentation. In order to decrease the adverse effects of coffee-processing wastes, there is a need to develop a healthier and productive way of re-utilizing these wastes (Preethu et al. 2007).

There have been reports in which the feasibility of recycling coffee husk and pulp is explored. Coffee pulp is the main byproduct from the wet processing of coffee beans is a good source of carbohydrates,

protein, fat and fibers, which may be a useful feed supplement for cattle or another livestock and is a good substrate for bioconversion processes (Mazzafera, 2002). Orozco et al. (2008) reported that efforts to recycle coffee pulp and husk include activities such as composting, feeding it to animals, production of organic fertilizers, aroma production, single-cell proteins which refers to the dried cells of microorganisms such as algae, bacteria, and fungi grown in large-scale culture systems for use as protein source in human food or animal feed (Litchfield, 1983), and biogas (Jayachandra et al. 2011).

Brand et al. (2002) explained that coffee husk could be beneficial as animal feed if some anti-physiological factors, such as tannins, polyphenols, and caffeine (highest toxic), among others are partially or entirely removed by biological detoxification. Likewise, Ashihara and Crozier (1999) mentioned ways to remove caffeine from coffee beans. However, differences in relative levels of each anti physiological factor are highly dependent on coffee varieties and thus this fact should be considered during the process. Filamentous fungi have been proposed as detoxifying biological agents on agro industrial wastes (Brand et al. 2000). Several fungal strains have been studied for cellulase or pectinase production from agricultural wastes, but there are no reports of simultaneous production of cellulase, mannase and pectinase by a single strain (Fujii and Takeshi 2007). Furthermore, there are fewer studies that evaluate the production of enzymes using the coffee pulp. For these reasons the authors suggest the use of coffee pulp for biotechnological application. Locally, there are no studies evaluating potential strains to detoxify coffee pulp and their potential for other biodegradation capabilities such as lignocellulose degradation. The study proposed here focuses on the characterization of caffeine biodegradation using fungi and the development of a simple, cost effective and environmentally friendly bioprocess to implement biodetoxification on the reutilization of coffee processing waste.

1.1 Motivation

The adoption of environmentally sustainable production practices holds the promise to improve economic returns to producers over the short and long term. Therefore, certified socially-and environmentally responsible coffees typically receive a considerable higher price over conventional coffees. In Puerto Rico, coffee is one of the most important crops. Twenty-two municipalities compose the coffee production region, located mainly along the western central part of the island. In 2009 the production of coffee estimated by Food and Agriculture Organization (FAO) was 7,523 tons per year and the worldwide coffee production was 8,261,487 tons per year (FAOSTAT)[1]. Only 6% of the coffee cherries constitute the portion used as coffee powder, the remaining 94% was discarded as waste products (husk, pulp-mucilage, etc.) (Brand et al. 2002). As a result, coffee pulp is one of the major agro-industrial wastes produced during the pulping of the coffee cherry to obtain the coffee bean.

In some cases coffee waste management is neglected because the producers are mainly dedicated to their final product. Few of the coffee producers allocate sufficient resources to invest in appropriate waste management, which due to large volumes of wastes generated need more workers, equipment, and increases operating costs. In a similar manner, the costs of sophisticated technologies involved in the treatment of waste are increasingly high. In spite of all the latter, a multi-disciplinary approach including bioprocess development, biotechnology, and microbial technology promises to minimize the costs of waste management by using microorganisms that carry out eco-friendly processes amenable for simple implementation.

We hypothesize that, screening ligniculous fungi, which exist in close proximity to lignin, for their ability to utilize caffeine will result in the development of an eco-friendly, economically feasible and efficient coffee pulp detoxification system. The resulting detoxified material may be a good nutritional supplement for animal feed, suitable compost, and a source of biomass for biofuel.

[1] http://faostat.fao.org

1.2 Literature Review

Some of the studies cited here represent an effort to integrate the principal areas of study including microbiological population, biomass characterization and the integration of both to develop novel technologies to treat this kind of pollution.

1.2.1 Mycology

Fungi are eukaryotic organisms with important ecological roles as saprotrophs, mutualistic symbionts, parasites, or hyperparasites (Webster and Weber 2007). Filamentous fungi consists of rigid tubular hyphae containing varied amounts of cytoplasm that can be moved through the hypha, depending on the environmental conditions, including the levels and ratios of nutrient resources, as well as the physical distribution of nutrients (Klein and Paschke 2004). Hyphae are generally quite uniform in different taxonomic groups of fungi. One of the few features of distinction that they do offer is the presence or absence of cross-walls or septa (Webster and Weber 2007).

Fungi are in majority mesophilic, organisms growing at temperatures within the range of 10-35 °C, with optimum temperatures between 15 and 30°C. Some species (e.g. *Aspergillus fumigatus*, *Talaromyces avellaneus*) are thermo tolerant and will grow at higher temperatures, although they are still capable of growth at room temperature. All filamentous fungi are variable in their pH requirements, growing well over the range pH 3 to 7 (Hawksworth and Kirsop 1988).

Filamentous fungi are capable of colonizing a wide range of living and dead tissues, including plants, wood and paper products, agricultural plant residues, and live or dead animal tissues (Brussaard et al. 1997). In composting, fungi colonize a mixture of heterogeneous substrates such as municipal solid waste and cattle manure with straw (Bonito et al. 2010). Many fungi can grow on solid substrates and secrete extracellular enzymes that break down various polymers to molecules that are then reabsorbed by the fungal colony (Gadd 2001). These microorganisms take an important role in carbon and nitrogen cycles. To mention, nitrogen cycling (Figure 1) involves four microbiological processes: nitrogen fixation, mineralization (decay), nitrification and denitrification. The input of large amounts of nitrogen fertilizers to agricultural fields influences these processes, especially nitrification and denitrification, and results in

increased production of N_2O. Fungi participate in denitrification, which is defined as the dissimilatory reduction NO_3 or NO_2 to N_2O and N_2 (Hayatsu et al. 2008).

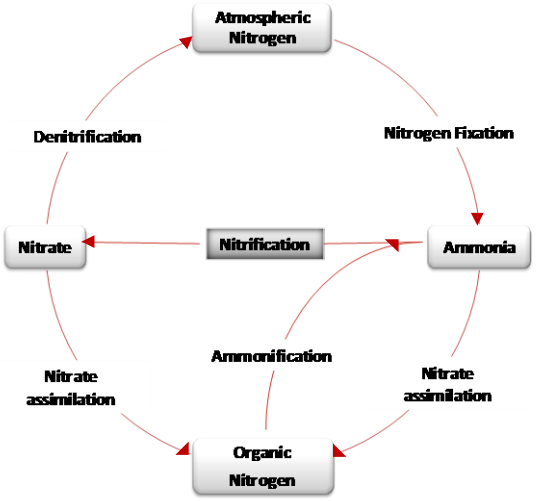


Figure 1 Nitrogen cycle

In the carbon cycle (Figure 2), fungi has the ability to break down dead organic matter into simple compounds (e.g. degrading lignin to CO_2). During the process of decomposing organic matter, fungi returns carbon dioxide to the atmosphere (Sanchez, 2009).

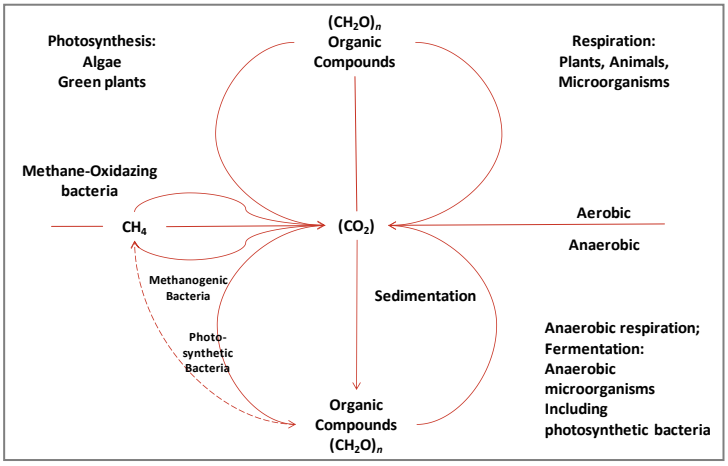


Figure 2 Carbon cycle

Many of bio-industrial fungi belong to sub-phylum Ascomycota, the largest group of fungi. The principal characteristic of the group is the sexually produced spores, called ascospores, within a sac (the ascus). Ascomycetes, grow in soil, are common on the above-ground parts of plants, and are also found in

freshwater and in the sea. Some of them are saprotrophs, others necrotrophic or biotrophic parasites of plants and animals, including humans (Webster and Weber 2007).

1.2.2 Bio-industrial fungi

Filamentous fungi exhibit characteristics that make them good models for industrial applications. Among some of these are their capacity for fermentation, the production of large quantities of extracellular enzymes (e.g. several grams per liter in strains of *Aspergillus*), the feasibility of cultivation, and the low-cost of production in large bioreactors. Fungi most frequently used for the production of polymer-degrading enzymes include *Trichoderma reesei* and several of strains of *Aspergillus* and *Penicillium* (Lara-Marquez et al. 2011).

Fungi have been extensively used as cell factories for different biotechnological products such as enzymes, pharmaceuticals, as well as primary and secondary metabolites. In terms of industrial production, could represent billions of U.S. dollars in annual revenues (Dijksterhuis 2007).

The most important bio-industries are:

1. Food industry: For instance, include alcoholic fermentations by yeasts as the basis of the wine and brewing industries. Some ascomycetes are important in food production as in bread-making by yeast, cheese ripening by *Penicillium roqueforti* and *P. camemberti* and the fermentation of soybeans and wheat by *Aspergillus*, yeasts and bacteria to produce soy sauce (Webster and Weber 2007).
2. Pharmaceutical industry: Include production of antibacterial antibiotics such as penicillin from *Penicillium chrysogenum* and cephalosporin from *Acremonium* spp.
3. Agro industry: Include production of organic acids (e.g. citric acid from *Aspergillus niger*) or another byproducts from agro-industries residues. In particular, use of biotechnological methods represents a major challenge, especially when trying to integrate biology and engineering as tools for cleaner production and environmentally friendly processes. The method of biopulping evidence the successful integration of these two disciplines. Biopulping a method that uses fungi known to be able to degrade wood as well as the lignin constituent of wood. The fungus such as *Ceriporiopsis subvermispora* is non-sporulating and is a selective lignin degrader. It colonizes

either on living or dead wood and decomposes all wood polymers including lignin and extractives, making it to an extreme contender to be used in biopulping. The process of biopulping reduces the utilization of chemicals in the pulping industry and help in decreasing the environmental hazard caused by normal pulping. (Singh et al. 2010)

A wide list of fungi which has been related with any lignin or lignocellulose activity (enzyme production, delignification and others) is showed in Table 1.

Table 1 Fungi involved in delignification, lignin degradation, lignin modifying enzymes, lignocellulosic fungi, plant pathogens and saprophytes.

Microorganism	Bioprocess	Enzyme production	References
Agrocybe aegerita	Lignocellulose degradation	Manganese-oxidizing peroxidases	Liers et al. (2011)
Alternaria alternata	Carbendazim degradation	Beta-Galactosidase Methyltransferase Pectinolic enzymes Laccase Manganese-peroxidase	Bilkay, IS (2009) Hiltunen and Soderhall (1992) Martinez et al. (1988) Atalla et al. (2010) Rezacova et al. (2006) Silva et al. (1999)
Aspergillus sp	Lignin degradation	Manganese peroxidases, Laccase	Yang et al. (2011)
Auricularia polytricha	Lignocellulosic degradation	NR	Ma et al. (2011)
Bjerkandera adusta	Lignocellulolytic biodegradation	Laccase, Manganese peroxidase, and Lignin peroxidase	Chen, et al. (2011)
Ceriporiopsis subvermispora	Lignocellulose degradation Lignocellulolytic degradation Production of ligninolytic enzymes, Fenton reaction, Delignification	Manganese-peroxidase, Cellobiose Dehydrogenase, Mannanase, Xylanases Laccase, Cellulase, Manganese peroxidase	Tanaka et al. (2009) Yaghoubi et al. (2008) Mancilla et al. (2010) Cunha et al. (2010) Harreither et al. (2009) Magalhaes and Milagres (2009) Babic and Pavko (2007) Chmelova,et al. (2011) Wan and Li (2011) Ohashi et al. (2007) Wan and Li (2010)
Cerrena unicolor	Production of lignin modifying enzymes	Manganese peroxidases, Laccase	Winquist, et al.. (2008)
Chaetomium globosum	Lignocellulose degradation	Xylanolytic enzymes	Popescu et al. (2010) Gandhi and Rao(1997)
Coniochaeta ligniaria	Lignocellulose-degrading enzymes production	Cellulase, xylanase, manganese peroxidase and lignin peroxidase	Lopez, et al. (2007)
Coriolopsis byrsina	Ligninases production	Lacasse[1]	Gomes et al. (2009)
Curvularia inaequalis	Wood decay	Chloroperoxidase	Ortiz-Bermudez et al. (2007)
Daldinia concentrica	Wood decay	NR	Nsolomo et al. (2000)
Dichomitus squalens	Lignocellulolytic biodegradation	Laccase, Manganese peroxidase, and Lignin-peroxidase	Chen et al. (2011)

Table 1 (continued)

Microorganism	Bioprocess	Enzyme production	References
Ganoderma australe	Wood decay	Laccase	Elissetche et al. (2007)
Ganoderma lucidum	Delignification	Laccase, Manganese peroxidase, and Lignin peroxidase	Ke et al. (2011) Misra et al. (2007)
Ganoderma ssp	Delignification	NR	Segura et al. (2008)
Gloephyllum trabeum	Delignification Ethanol production Bamboo decay	NR	Monrroy et al. (2010) Shrestha et al. (2010) Cho et al. (2008)
Hypoxylon fragiforme	Lignocellulolytic biodegradation	Laccase, Manganese peroxidase, and Lignin-peroxidase	Chen et al. (2011)
Lecytophora hoffmannii	Lignocellulose degradation	NR	Bugos et al. (1988)
Lentinus edodes	Bioethanol production, Bamboo decay	NR	Hiyama et al. (2011) Kim et al. (2008)
Lentinus ssp	Delignification, Ligninases production	Manganese peroxidases and lignin peroxidase	Segura et al. (2008) Gomes et al. (2009)
Lentinus strigellus	Ligninases production	NR	Gomes et al. (2009)
Microsphaeropsis	NR	Chitinolitic and cellulolytic enzymes	Benyagoub et al. (1998)
Paecilomyces inflatus	Lignocellulose degradation	NR	Kluczek-Turpeinen et al. (2007)
Panus tigrinus CBS 577.79	Enzymes production	Laccase	Quarantino et al. (2007)
Penicillium oxalicum	Enzymes production	Xylanase and Laccase	Dwivedi et al. (2011)
Phanearochaete chrysosporium	Delignification, lignocellulose degradation, Ethanol production Cellulose degradation, Saccharification Lignolytic enzymes production, Wastewater treatment, Lignin-degrading enzymes	Lacassa, Lignin peroxidase, Manganese Peroxidase	Huang et al. (2010) Vanden et al. (2009) Shrestha et al. (2010) Sato et al. (2010) Mena-Espino et al.(2011) Kanmani et al. (2009) Li et al. (2011) Gassara et al. (2011) Sridhar et al. (2011) Gupte et al. (2007) Wu and Yu (2007) Dutt et al. (2007) Kwon et al. (2008) Singh and Chen (2008) Hu et al. (2009)
Phellinus rimosus	Ligninases production	NR	Gomes et al. (2009)
Phialocephala dimorphospora	Decayed wood	NR	Jones and Worrall (1995)
Phlebia brevispora	Delignification	NR	Arora and Sharma (2011)
Phlebia floridensis	Lignocellulosic enzymes production	Manganese peroxidase, CMCase, and Xylanase	Sharma and Arora (2010)
Phlebia radiate	Lignocellulose degradation, Lignocellulolytic biodegradation, Wood decay	Manganese-oxidizing peroxidases Lignin peroxidase Manganese-peroxidase, Laccase	Liers et al. (2011) Chen et al. (2011) Cho et al. (2009) Kang et al. (2010)
Phlebia tremellosa	Lignin degrading enzymes production	Laccase and Manganese peroxidase	Kum et al. (2011)
Physisporinus vitreus	Wood decay	NR	Schwarze and Schuber (2011)
Physisporinus vitreus	Delignification	NR	Lehringer et al. (2011)
Pleurotus eryngii	Lignocellulolytic biodegradation	Laccase, Manganese peroxidase, and Lignin-peroxidase	Chen et al. (2011)

Table 1 (continued)

Microorganism	Bioprocess	Enzyme production	References
Pleurotus ostreatus	Delignification, Fluorene, Lignocellulolytic biodegradation, Lignolytic enzymes production, Lignocellulose degradation	Lacasse,Cellulose Manganese peroxidase, Lignin-peroxidase, Xylanase, endo-1,4 glucanase	Shrivastava et al. (2011) Akgogan and Pazarlioglu (2011) Apohan and Yesilada (2011) Osma et al. (2011) Diaz et al. (2011) Tinoco et al. (2011) Mena-Espino et al. (2011) Dwivedi et al. (2011) Chen et al. (2011) Shrivastava et al.. (2011) Sridhar et al. (2011) Gupte et al. (2007) Zhang et al. (2007) Alexandrino, et al.. (2007)
Pleurotus sajorcaju	Lignolytic enzymes production	NR	Sridhar et al. (2011)
Polyporus brumalis	Lignin degrading enzymes production	Laccase, Manganese peroxidase	Kum et al. (2011)
Poria subvermispora	Ethanol production	NR	Salvachua, et al. (2011)
Postia placenta	Wood decay	Polygalacturonase, Beta-D-glucosidase	Duncan and Schilling(2010) Clausen and Green (1996) Green et al. (1995) Micales (1991)
Pycnoporus cinnabarinus	Delignification	NR	Gupta et al. (2011)
Pycnosporus sanguineus	Ligninases production	Manganese peroxidases, lignin peroxidase	Sharma et al. (2011) Gomes et al. (2009)
Strobilurus ohshimae	Lignin degradation	Laccase	Homma et al. (2007)
Stropharia rugosoannulata	Lignocellulose degradation	Manganese-oxidizing, Peroxidases	Liers et al. (2011)
Trametes versicolor	Lignolytic enzymes production Production of lignin modifying enzymes, Delignification	Manganese peroxidases Laccase, Lignin peroxidase	Staszczak et al. (2011) Shrivastava et al.. (2011) Gupte et al. (2007) van Beek et al. (2007) Jing, et al. (2007) Thiruchelvam and Ramsay (2007) Dominguez et al. (2007) Winquist, et al.. (2008)
Voriallae volvoraceae	Lignolytic enzymes production	NR	Sridhar et al. (2011)
Xylaria polymorpha	Wood decay Lignocellulose degradation	Laccase Manganese-oxidizing peroxidases	Chaparro et al. (2009) Liers et al. (2011)

NR= Not reported

From the previous review we obtained some strains from the Culture Collection at the Center for Forest Mycology Research of the Forest Product Laboratory, Madison –Wisconsin.

1.2.3 Coffee Processing

In the review of Mazzafera (2002) coffee fruits can be processed by two ways, the wet or dry process. The majority of coffee farms use the wet method, in which the fruits are pulped, fermented, washed and sun dried to obtain the coffee seeds (Figure 3).

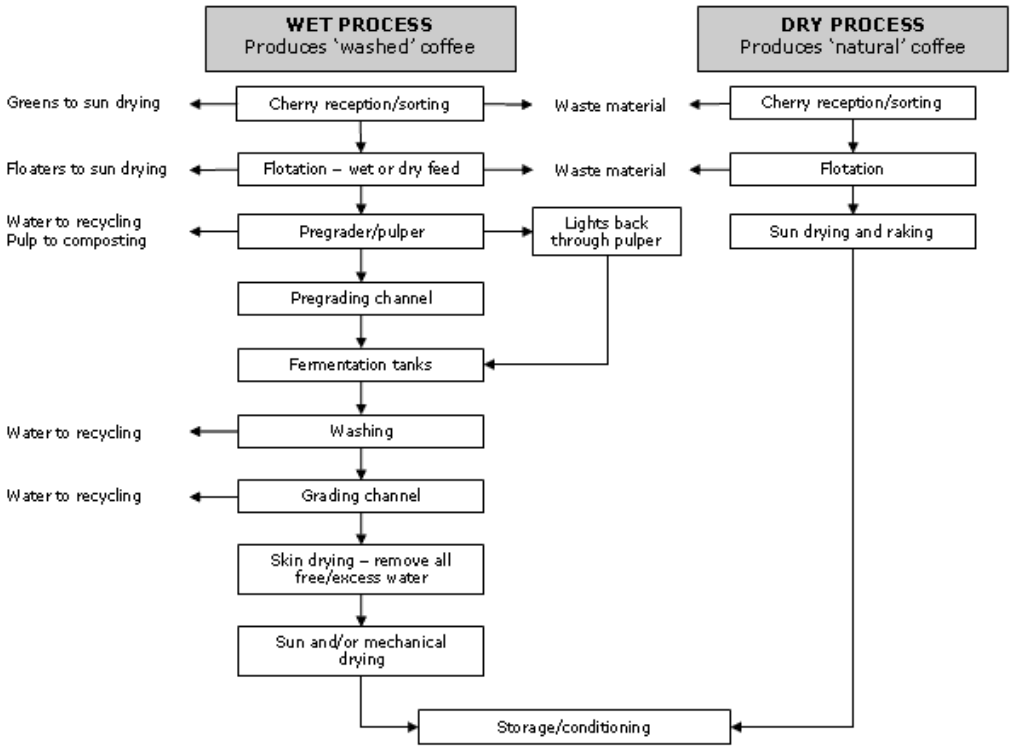


Figure 3 Coffee processing methods.
Source: http://www.coffee-ota.org/grafico_en.htm

The pulping process removes the exocarp (outermost layer or peel), mesocarp (the middle layer or pith), and endocarp (inner layer that surrounds the seed) resulting in coffee pulp (Figure 4), this represents the most abundant waste. Coffee pulp is barely used and it is considered the most abundant pollutant material of lakes and rivers located nearby coffee processing farms (Penaloza et al. 1985).

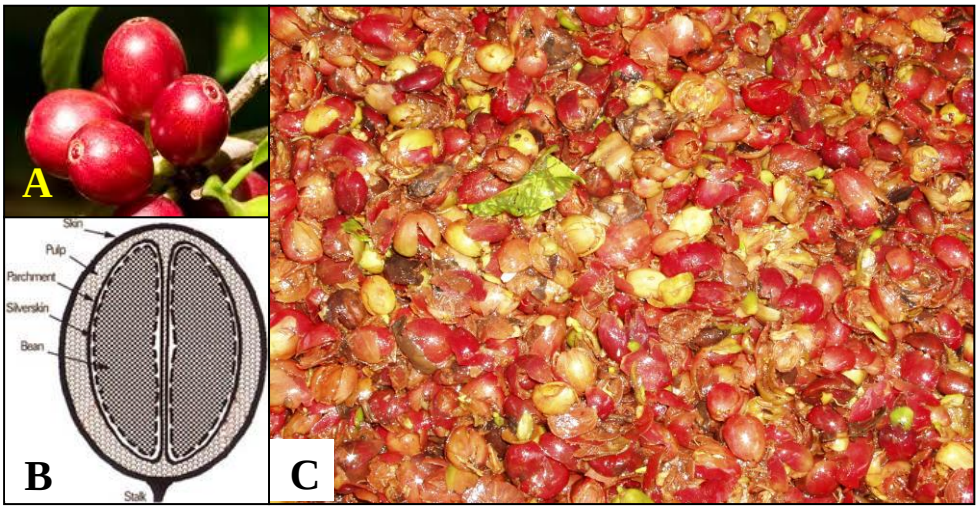


Figure 4 Coffee pulp
A Coffee fruit (http://www.vibrantbeautynetwork.com) **B** Coffee fruit parts (http://farmextensionmanager.com); and **C**. Fresh coffee pulp (Photo by María del Pilar Sierra).

The waste generated in large quantities during wet method (Figure 5) is composed generally of cellulose, galactomannan and pectin and mainly used for producing compost. However, application of untreated coffee residue to soil inhibits the growth of crops (Fujii and Takeshi 2007). Reports of compositional analyses of coffee pulp have been published (see Table 2) concluding that this agro-industrial residues represent a good source of carbohydrates and sugars that can be used as nutritional supplement for animal feed and others.

Figure 5 Coffee pulp wastes after harvesting season
(Photo by Nolberto Figueroa).

Authors have reported that the utilization of coffee pulp is limited by its content of anti-physiological (caffeine) and anti-nutritional (tannins and polyphenols) (Nava et al. 2006).

Table 2 Coffee pulp composition

Components	Percentage (%)	References
Water	77	Braham and Bressani (1979)
Dry matter	23.3	Braham and Bressani (1979)
Ether extract	0.48	Braham and Bressani (1979)
Fiber	3.4, 18	Braham and Bressani (1979), Orozco et al. (2008)
Protein	2.1, 8.25, 9.2, 10	Braham and Bressani (1979), Roussos et al. (1994) Mazzafera (2002), Orozco et al. (2008)
Ash	1.5	Braham and Bressani (1979)
N free extract	15.8	Braham and Bressani (1979)
Tannins	1.80-8.56, 4.5	Braham and Bressani (1979) Mazzafera (2002)
Pectins	6.5, 12.4	Braham and Bressani (1979) Mazzafera (2002)
Reducing sugars	12.4, 23-27	Braham and Bressani (1979) Roussos et al. (1994)
Non reducing sugars	2.0	Braham and Bressani (1979)
Caffeine	1.3, 0.33	Braham and Bressani (1979) Mazzafera (2002) Molina et al. (1974)
Chlorogenic acid	2.6	Braham and Bressani (1979)
Caffeic acid	1.6	Braham and Bressani (1979)
Carbohydrates	57.8, 50	Mazzafera (2002), Orozco et al. (2008)
Fat	2, 2.5	Mazzafera (2002), Orozco et al. (2008)
Lignin	38.6	Preethu et al. (2007)
Cellulose	29.4	Preethu et al. (2007)

1.2.4 Degradation of pollutants of agro industrial residues

During the processing of vegetable matter (e.g. sugar cane, coffee, wheat, corn, rice, etc) huge quantities of solid organic residues are generated. These wastes are structure-less and have high water content (80%). Composting is the most common management approach used, but it represents an environmental issue (Weiland 1993).

In the case of coffee residues, an economical way of dealing with the excessive amount of pulp and solving the environmental problem would be to use it for animal feed. However, chemical components

such as lignin, phenolics and caffeine hamper it utilization by animals. Caffeine and phenolics are known to exert detrimental effects to both the rumen microflora and the host animal (Gadd 2001).

Caffeine (1,3,7-trimethylxanthine) is a natural substance present in leaves, seeds or fruits of over 63 plant species, and belong to a group of compounds known as methylxanthines (Mumin et al. 2006). Caffeine is a powerful stimulant and can be toxic to animals due to its high nitrogen concentration and its diuretic effect. In some cases if this compound is present in animal nutrition the consequence could be a low feed intake (Brand et al. 2002).

There are many methods of caffeine removal, including water decaffeination, solvent extraction and supercritical carbon dioxide extraction (Figure 6).

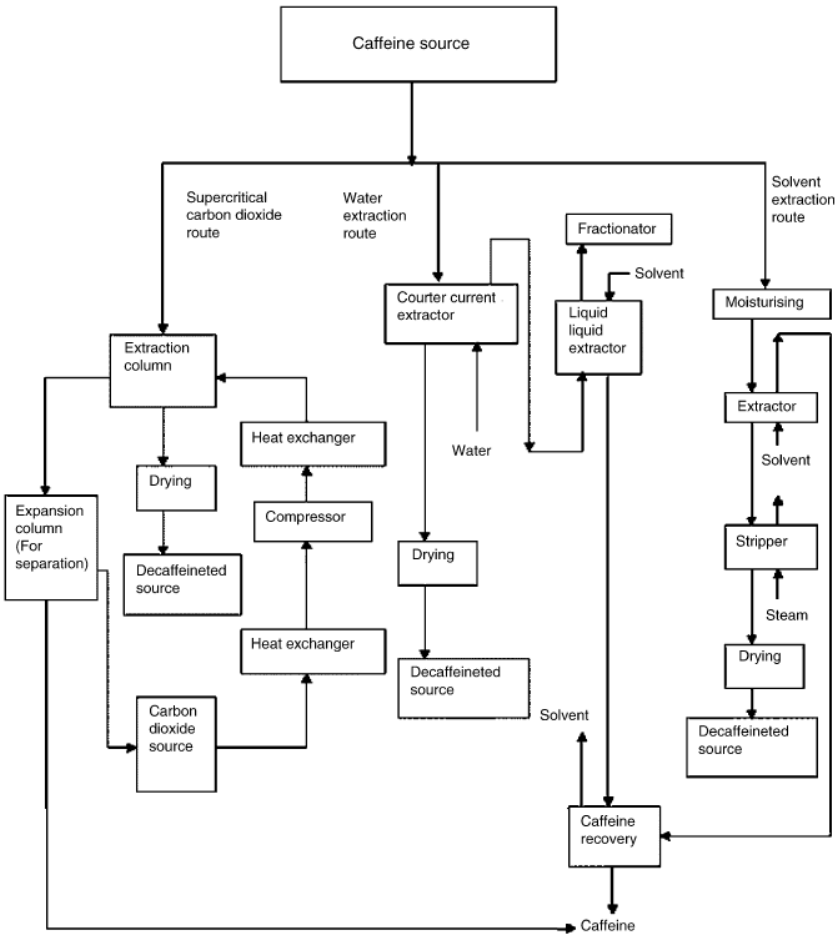


Figure 6 Diagram of various chemical methods for caffeine removal
(Gokulakrishnan et al. 2005)

Also, conventional chemical methods of decaffeination usually involve the use of agents such as methylene chloride, ethyl acetate, charcoal or carbon and triglycerides (Sarath Babu et al. 2005).

Chemical methods for caffeine removal are more effective than non-chemical methods. However, these methods have disadvantages such as the removal of other beneficial compounds (e.g. vitamins, proteins). Additional disadvantages include higher technological demands, expensive process components, (e.g. solvents), and adverse environmental impact (Gokulakrishnan et al. 2005).

1.2.5 Caffeine detoxification by biological methods

In recent years there has been an increased tendency toward the biotransformation of agro-industrial wastes. Many bioprocesses are focused in obtaining commercial products such as ethanol, mushrooms, enzymes, organic acids, amino acids, etc. These processes provide new alternatives to solve pollution problems due to the irresponsible management from processing farms (Brand et al. 2000)

Different pathways to caffeine degradation had been proposed. Some of them conclude that these depend on microorganisms, for instance, in the degradation by demethylation using the theophylline pathway (Figure 7).

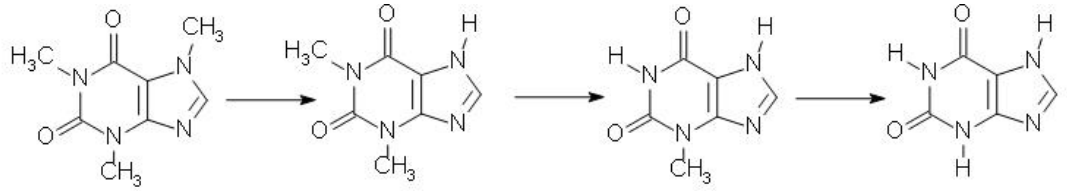
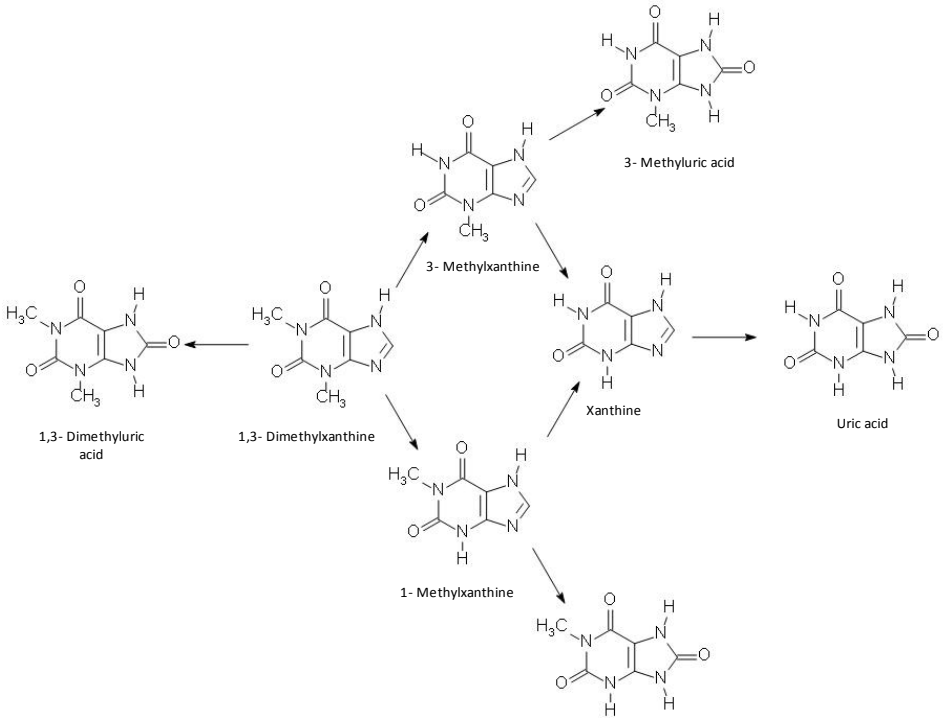


Figure 7 Caffeine degradation pathway by *Rhizopus delemar*
(Reproduced from Tagliari et al. 2003)

However, in the case of bacteria, alternative pathways through theophylline and theobromine have been described. Yu et al. (2009) evaluted two pathways for metabolism of caffeine using *Pseudomonas putida* isolated from soil samples. The study showed that soil bacteria consumed 2.5 g l^{-1} in 8 days using caffeine as sole carbon and nitrogen source. When caffeine was added to the cell suspension theobromine, 7 methylxanthine, xanthine and uric acid were detected, suggesting that caffeine is degraded by the N-

demethylation pathway. Theophylline (1,3-dimethylxanthine) was metabolized to three metabolites 1, 3-dimethyluric acid, 1-methyluric acid and 3-methyluric acid (Figure 8).

A



B

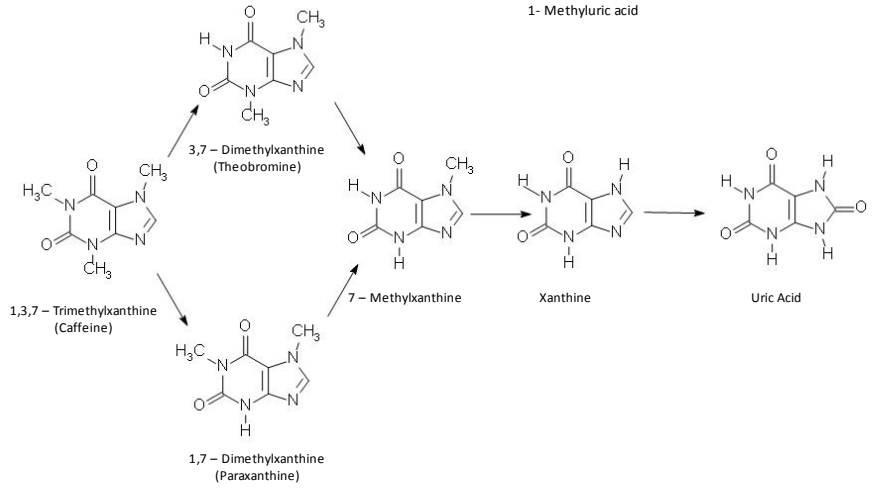


Figure 8 Demethylation pathways by bacteria
A Theophylline pathway; **B**. Theobromine pathway (Reproduced from Yu et al. 2009).

1.2.6 Previous works with coffee pulp and caffeine degradation

There have been ample reports describing the composition, conservation, upgrading and utilization of coffee pulp. Several important studies in bacteria and fungi are mentioned in the table below.

Table 3 Previous reports on caffeine degradation

Reference	Research topic
Penaloza et al. (1985)	Nutritive improvement of coffee pulp under Solid State Fermentation (SSF) using *A. niger*, the fermented product had a higher aminoacid content
Roussos et al. (1994)	Effect of inorganic and organic nitrogen source in decaffeination process using *Penicillium verrucosum*.
Roussos et al. (1995)	Isolation of caffeine degrading fungi belonged to the genera *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, and *Humicola.* Five *Aspergillus* strains could degrade almost 100% of caffeine in liquid media.
Mazafera et al. (1996)	Caffeine degradation by *Serratia marcences*, using 1200 ppm caffeine is toxic and inhibit bacteria growth.
Hakil et al. (1998)	Caffeine degradation by *Aspergillus tamarii*, *Penicillium commune*, *Aspergillus fumigatus*, and *Aspergillus niger* using caffeine as sole source of nitrogen. The highest degradation percentages were 67.2 and 67.7 for *A. tamarii* and *P. commune* respectively
Denis et al. (1998)	New analytical method of methylxanthines quantification by HPLC using *Aspergillus sp* strain to identify the caffeine metabolism suggesting the theophylline pathway in 140 h.
Yamoka-Yano et al. (1999)	Evaluation of strains of *Pseudomonas*, which showed a higher ability to grow on caffeine (20 g/L- 50 g/L). and characterization of xanthine oxidase, responsible of methylxanthines and xanthine oxidation during caffeine degradation.
Brand et al. (2000)	Detoxification of coffee husk in solid state fermentation using *Rhizopus*, *Phanerochaete*, and *Aspergillus* which showed 92% of caffeine degradation
Odafe et al. (2001)	Caffeine demethylases enzymes using *Pseudomonas putida* which is able to use caffeine as carbon and nitrogen source to convert caffeine to uric acid
Brand et al. (2002)	A solid state fermentation carried out in a packed bed column fermenter using coffee pulp as substrate, achieved 90 % of caffeine degradation using *Aspergillus* strain .
Ulloa Rojas et al. (2003)	Evaluation of nutritive value of coffee pulp after a degradation period of 21-28 days for monogastric animals using *Aspergillus* and *Bacillus* sp.
Tagliari et al. (2003)	Evaluation of caffeine degradation using *Rhizopus delemar* in a packed bed reactor with coffee pulp as substrate. The degration pathway suggested was theophylline pathway
Sarath Babu et al. (2005)	Caffeine degradation using *Pseudomonas alcaligenes*, which was able of completely degrading caffeine (1g/L) within 4-6 hours in a pH range of 7.0-8.0

Table 3 (continued)

Reference	Research topic
Nava et al. (2006)	Effect of substrate thermal treatment, and incubation temperature using *Penicillium commune* on coffee pulp by solid state fermentation
Fujii and Takeshi (2007)	Microbial degradation of agricultural residues using strains capable to degrade cellulose and pectin to produce enzymes cellulases and pectinase.
Gummandi et al. (2007)	Effect of glucose on caffeine degradation using *Pseudomonas sp*. Caffeine degradation was inhibited with glucose. The inhibition effect was not seen using sucrose and lactose
Dash et al. (2008)	Assay for demethylases activity, which unraveled the inducible nature of methylxanthine degrading activity in *Pseudomonas sp*
Gummandi and Santhosh (2010)	Effect of various caffeine concentrations on caffeine demethylases production by *Pseudomonas sp* using a bioreactor.the enzyme reported was N-demethylase obtaining a 100% of degradation

1.3 Objectives

1.3.1 Primary Objective

Agro-industrial waste management need to be addressed with concern, in particular coffee pulp which is considered to be one of the most abundant agricultural wastes in Puerto Rico, as well as one of the hardest to handle. To date, most of the by-product remains unused, causing contamination problems. Biological pretreatment could, however, improve the value of this waste product. The presence of proteins, sugars and minerals in coffee pulp and its high humidity favor the rapid growth of microorganisms (Gadd 2001). This project intends to evaluate microorganisms able to degrade caffeine present in coffee pulp residues. The use of coffee pulp is restricted by the high content of fiber and by the presence of antinutritional factors such as phenols, tannins and caffeine, which interfere with food intake and nutrient absorption. To improve its nutritive value for animal feed is necessary to apply biological treatments, to increase the protein content and reduce the fiber or cellulose in the final product (Ulloa Rojas et al. 2003).

1.3.2 Specific Objectives

1.3.2.1 Identify candidate strains

Since one of the main challenges for the reutilization of coffee- processing waste is the presence of lignin, a collection of thirteen filamentous fungi with the ability to degrade/inhabit lignocellulose was obtained from the Mycology Collection at the Forest Products Laboratory in Madison, Wisconsin. These candidates were grown on solid medium with increasing concentrations of caffeine, ranging from 0.5, 1.0, and 2% caffeine. The cultures were scored based on confluent growth with respect to control plates.

1.3.2.2 Assessment of caffeine detoxification by candidate strains in a purified system.

Candidate strains showing confluent growth on plates were ground in a Waring blender and diluted in a completely defined minimum liquid medium, which contain 0.1% of caffeine. This mixture was aliquoted into 250-mL Erlenmeyer flasks, covered with aluminum foil, and was incubated under stationary and shaking conditions at appropriate temperatures. Samples incubated were harvested and cell-free extracts will be tested for caffeine concentration using HPLC.

1.3.2.3 Assessment of caffeine detoxification upon growth of candidate strains under solid-state fermentation (SSF) conditions.

A fresh inoculum from a candidate strain was maintained in malt agar and transferred to a sterile coffee pulp extract agar plate and incubated at the appropriate temperature until confluence was obtained. Confluent plates were ground in a Waring blender and diluted in a completely defined minimum liquid medium. A portion of this suspension was used to inoculate coffee pulp. Dried pulp was weighed, aliquot into 250-mL Erlenmeyer flasks, covered with aluminum foil, and sterilized with steam and pressure in an autoclave. After the sterile coffee pulp aliquots were inoculated, they were incubated stationary at appropriate temperatures. After fermentation time samples of coffee pulp (SmSSF) were harvested and the liquid extracted was tested for caffeine concentration using HPLC.

2 MATERIALS AND METHODS

2.1 Identify candidate strains

2.1.1 Screening

2.1.2.1 Microorganisms and media

Strains obtained from the Culture Collection at the Center for Forest Mycology Research of the Forest Product Laboratory, Madison - Wisconsin (Table 4), were inoculated in a rich media YMPG with different concentrations of caffeine (0.2, 0.5, 1.0, and 2.0 %). Candidate strains were selected because of their ability to grow in the presence of caffeine, and the strains were incubated at 25, 30 and 37 °C during four weeks. *Aspergillus tamarii* (NRRL 559) strain was obtained from the Mycology Laboratory at UPR-Mayagüez and was used as positive control due their ability as caffeine degrader reported in the literature (Gutierrez-Sanchez et al. 2003).

Table 4 Ligniculous fungi from the culture collection (CFMR) of the Forest Product Laboratory (FPL)

ID Collection	Strain
Mad-617-R	*Gloeophyllum trabeum*
FP-101798 sp	*Pleurotus ostreatus*
BKM- F- 1767	*Phanerochaete chrysosporium*
VAG-1005-04	*Alternaria alternata*
ATCC-6205	*Chaetomium globosum*
FS-33	*Microsphaeropsis sp.*
VY-8-1	*Phialocephala dimorphospora*
Wilcox-113	*Lecythophora hoffmannii*
FP-90031-Sp	*Ceriporiopsis subvermispora*
MUCL- 28295	*Xylaria polymorpha*
FP- 140074	*Daldinia concentrica*
Mad-698-R	*Postia placenta*
Y115	*Chaetomium globosum*

2.2 *Assessment of caffeine detoxification by candidate strain in a purified system*

The selected strain was maintained on Potato Dextrose Agar (PDA) and induced on Coffee Pulp Extract (CPE) agar until complete growth. CPE medium was prepared as follows (g /l): an infusion was prepared with Coffee Pulp Fresh (40); supplemented as described by (Gutierrez-Sanchez et al. 2003) sucrose (2.0), KH_2PO_4 (1.3), Na_2HPO_4 (0.19), $MgSO_4$ 7.H_2O (0.15) and $CaCl_2$ (0.2) were dissolved in the coffee infusion. The pH was adjusted to 5.6 and the volume was brought to 1 l. Agar (20 g) was added and the medium was sterilized at 121ºC for 30 min. The candidate strain was inoculated and incubated at 25 C during five to seven days.

2.2.1 Liquid minimal media

2.2.1.1 Liquid media preparation

A master mix was prepared as follows. A plate with the candidate strain was mixed with a minimal media containing (g /l): 20 ml of Salt Solution consisting of (g/l): KH_2PO_4 (76), MgSO4. 7 H_2O (26), KCl (26); and 10 ml of a trace element solution consisting of (g/l): $FeSO_4$ (0.8), $ZnSO_4$ (8), $MnSO_4$ (0.8), $Na_2MoO_4.2H_2O$ (0.8), $CuSO_4.5H_2O$ (0.4). The media was supplemented with caffeine (1 g l^{-1}), and the pH was adjusted to 4 with HCl. The liquid media was sterilized at 121ºC for 30 min.

In a similar manner a liquid minimal media was prepared as describe above supplemented with caffeine (1 g l^{-1}) and sucrose (25 g l^{-1}).

2.2.1.2 Stationary culture assay

Erlenmeyer flasks containing 10 ml of master mix were incubated at 25 °C until the formation of a mycelial mat was observed. Samples were taken periodically to perform analytical assays and determine the percentage of caffeine degradation in triplicate for an incubation period of ten weeks. This assay was conducted using both media mention in section 2.2.1.1.

2.2.1.3 Shaking culture assay

Erlenmeyer flasks containing 50 ml of master mix were incubated at 25 °C in an orbital shaker at 110 RPM. Samples were taken periodically to perform analytical assays to determine the percentage of

caffeine degradation in triplicate. The incubation time was six weeks. This assay was conducted with minimal media supplemented with caffeine and sucrose.

2.2.2 Caffeine determination in purified system

2.2.2.1 Sample Preparation

Flasks containing the minimal media inoculated and incubated were vacuum filtered using a Whatman #44 filter paper. The flasks were washed with water (10-12 ml) (Figure 9). The filtered solutions were placed into tubes and storage at -20°C until analysis. The filters were placed in an oven at 98-100°C during 24 h. Dry samples were stored in a desiccator until samples were weighed.

Figure 9 Filtration minimal media
Alternaria alternata stationary method at 25°C (Day 69); Photo by María del Pilar Sierra-Gómez

2.2.2.2 Solid Phase Extraction

To concentrate the sample and to avoid chromatographic interference from mycelia or medium components, the sample was adsorbed onto SPE cartridges packed with a C18-functionalized resin (Grace Davison Discovery Science, Deerfield, IL)(Figure10). Manufacturer instructions were followed for use and conditioning of the cartridges. Caffeine was eluted with methanol (100%) HPLC grade (Carlin-Sinclair et al. 2009); samples were diluted (1/10), (1/100), (1/1000) to avoid the saturation into the HPLC column.

Figure 10 Solid phase extraction (Cartridge C18)
Photo by María del Pilar Sierra-Gómez

2.2.2.3 High Performance Liquid Chromatography

A High Performance Liquid Chromatograph composed of: Binary HPLC pump, (model Waters 1525), auto sampler (Waters model 717 plus), Ultraviolet detector (Waters, Model 2487), Column Symmetry Shield ^{TM} RP_{18} 5 µm (4.6 x 250mm) at 25°C, and software Breeze 2 was used. An isocratic method using methanol: water (30:70) as mobile phase was used at a flow rate of 1.0 ml/min. Detection was at 254 nm (Tagliari et al. 2003) Caffeine 99.7% was purchased from Alfa Aesar (Ward Hill, MA), Methanol HPLC Grade, Theophylline, 3-methylxanthine, and xanthine were purchased from Sigma-Aldrich (St. Louis, Mo) and theobromine from Acros Organics (New Jersey, USA), sample solutions were injected in triplicate and the retention times and concentrations of caffeine in each sample were compared to those of standards in a calibration curve including dilution factors.

2.3 *Assessment of caffeine detoxification upon growth of candidate strains under Submerged Solid State Fermentation (SmSSF) conditions.*

2.3.1 Coffee pulp

Coffee pulp (*Coffea arabica*) was collected from the coffee processing plant Hacienda Café San Pedro located in Jayuya-Puerto Rico at early season (November, 2010). This material was used for identification of filamentous fungi and as substrate for solid-state fermentation experiments.

2.3.1.1 Biological Characterization: Isolation and identification of filamentous fungi from coffee pulp

2.3.1.1.1 Isolation

Ten grams of coffee pulp added to ninety milliliter of distilled water after mixing at 170 rpm for 10 min were used in perform serial dilutions to isolate the microorganisms. The media used to allow spore production were water agar [WA, (20 g l^{-1}) agar] and Coffee Pulp Extract (CPE). The culture medium CPE was prepared as follows: forty grams of coffee pulp was ground and boiled in one liter double distilled water during ten minutes. This solution was filtered (Filter paper Whatman #1) and adjusted to one liter and mixed with KH_2PO_4 1.3, Na_2HPO_4 0.12, $MgSO_4$ 0.3, $CaCl_2$ 0.3 and agar 15 (Gaime-Perraud et al. 1993). Cultures were incubated and monitored in three temperatures (25, 30 and 37 °C) during 24-48 h necessary to harvest the spores. Then, the spores were inoculated in Potato-Dextrose-Agar (PDA, Difco-Fisher Scientific) at 25, 30 and 37 °C during 5-7 days or until colonies from one spore were observed.

2.2.1.2 Identification of filamentous fungi

The slide culture technique to identify fungi was performed using the methodology described by Riddell (1950) as follows. One PDA plate was divided in squares (1x1 cm) then; each square was placed over the slide, which was inside of glass Petri dish. The isolated strains were inoculated in the PDA squares and were covered with a cover glass. The wet mountings were incubated to 25, 30 and 37 °C for 7-14 days. After, incubation time, flat glass slides were prepared placing one droplet of lactophenol cottonblue on each. Then, the cover glass slip from the wet mount was placed over the droplet, and

observed under the microscope (Olympus CH). Fungi were identified at the genus level using descriptions on Barnett and Hunter (1986) for imperfect fungi.

2.3.1.2 Physical-chemical Characterization

2.3.1.2.1 Moisture content

Five grams of sample were placed in the pan (weighed previously) and weighed accurately. Then, the sample was placed in an oven at 98 - 100°C for 24 h. Dry samples were stored in a desiccator until samples were weighed. Percentage moisture (wt/wt) was calculated as described below.

$$\% \text{ moisture} = \frac{(\text{wt of wet sample+ pan}) - (\text{wt of dried sample+ pan}) \times 100}{(\text{wt of wet sample + pan}) - (\text{wt of pan})} \qquad \textbf{Equation 1}$$

2.3.1.2.2 Nitrogen content

I. Digestion

Approximately 0.1 g coffee pulp dry was weighted. Then, coffee pulp was placed into digestion tube, adding a catalyst and appropriate volume (e.g., 7 ml) of concentrated sulfuric acid to each tube with coffee pulp. The rack of digestion tubes was placed on a digestion block which was place in the fume hood. Samples were digested completely. Samples were taken off the digestion block and were cooled. Carefully, samples were diluted with an appropriate volume of distilled-deionized water, and well mixed (See figure 11).

Figure 11 Nitrogen content (sample digestion)

II. Distillation

An appropriate volume of boric acid solution was added into the receiving flask, which was placed on the distillation system. Sample tubes were placed in the distillation equipment making sure it was seated securely and proceeds with the distillation until completed. In this distillation process, a set volume of NaOH solution was delivered to the tube and a steam generator was distilled the sample for a set period of time. Upon completing distillation of one sample, the procedure was repeated with a new sample tube and receiving flask (Figure 12).

Figure 12 Nitrogen content (sample distillation)

III. Titration

Using a standardized HCl solution each sample and blank was titrated to the first faint gray color. Use HCl volume was recorded and used for N determination.

$$\% \text{ Nitrogen} = \frac{\text{Normality of HCl* [(ml acid for sample)- (ml acid for blank)]}}{\text{g of sample}} \qquad \textbf{Equation 2}$$

$$\% \text{ Protein} = \% \text{ Nitrogen} * \text{Protein factor (6.25)} \qquad \textbf{Equation 3}$$

2.3.1.2.3 pH

The procedure used to determinate the pH was according to the Official method AOAC 981.12 (AOAC, 1990)

2.3.2 Submerged Solid State Fermentation (SmSSF)

Based on the results from the screening tests, the candidate strain was mixed with a solution enriched with salts and trace components. Coffee pulp was used as carbon and nitrogen sources.

2.3.2.1 Inoculum preparation

The candidate strain was maintained on PDA and inoculated on Coffee Pulp Extract agar described above. The candidate strain was incubated at 25 °C during five to seven day.

2.3.2.2 Substrate (Coffee pulp) preparation

Coffee pulp was dried in an oven at 100 °C during 24 hours, milled and sieved to obtain fractions between 0.8 - 2.0 mm particle size (Figure 13).

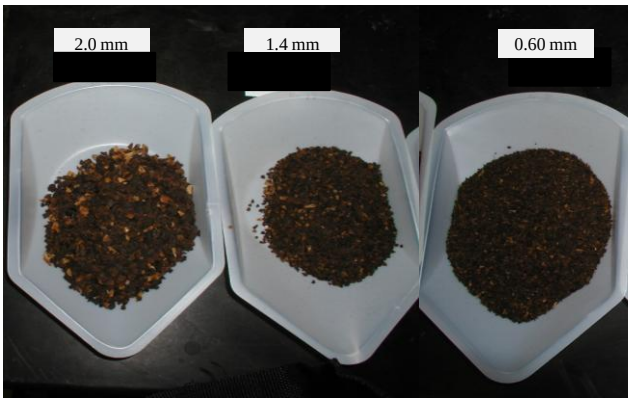


Figure 13 Particle size
Photo by Erick N Zorrilla

2.3.2.3 Sample preparation

The candidate strain-CPE agar was blended with a solution containing 10 ml of salt solution, and sucrose (2 g l^{-1}) was added into 2.0 g of coffee pulp in 250 ml Erlenmeyer flasks. This experiment was conducted with six replicates. Flasks were incubated at 25°C, for a period of 20 days. Samples from 0, 3, 6, 10, 13, and 20 days, for each sampling day flasks were placed in a water bath at 80-90°C for 30 min, then, each flask was washed with hot water (10-12ml) and filtered using Whatman#2 filter paper (weighted accurately) to calculate the dry weight (Figure 14) The filtered solution was placed into tubes and storage at -20°C until further analysis.

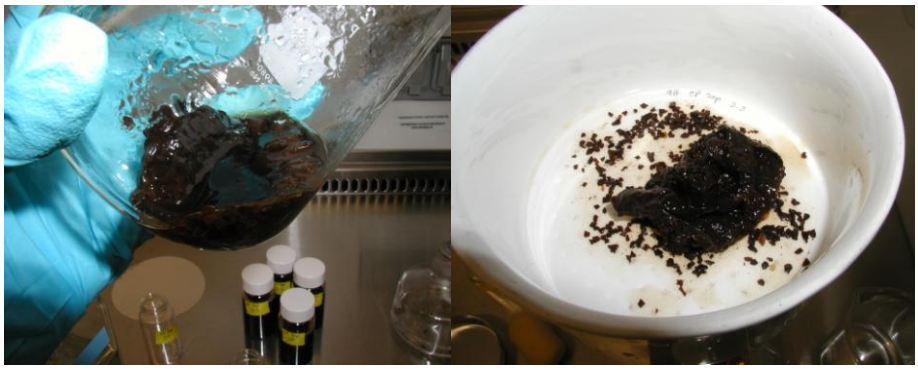


Figure 14 SmSSF with *Alternaria alternata* (sample preparation)
Left: Flask after bath water and hot water addition (Day 3). Right: coffee pulp filtered (Day 3). Photos by María del Pilar Sierra-Gómez

2.3.2.4 Dry weight

Coffee pulp inoculated was placed in the filter (weighed accurately previously). Then, the sample was placed in an oven at 98-100°C for 24 h. Dried samples were stored in a desiccator until samples were weighted.

2.3.2.5 Ergosterol determination

Ergosterol determination was adapted from S. Gors et al. (2007) as described below.

2.3.2.5.1 Ergosterol extraction

0.5grams of each dried fermented replicate of coffee pulp was refluxed in 50 ml of methanol (100%) for 2 h. 25 ml of methanolic KOH solution 0.2 M was added and refluxed for 30 min. Subsequently, the sample was transferred to centrifuge tubes and two extractions were conducted with n-pentane (1.5 ml each) under rigorous shaking using a vortex. One ml of distillated deionized water was added to the extract (Figure15).

Figure 15 Ergosterol extraction
Photos by Erick N. Zorrilla

Afterwards, the extract was centrifuged for 2 min at 6200 rpm. Non polar phase was transferred into micro tubes and evaporated in an air stream (Figure 16). Extracts were storage at -20°C for HPLC analysis.

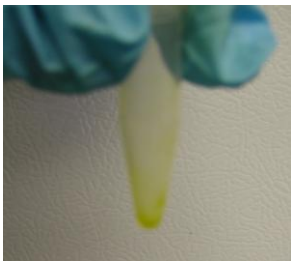


Figure 16 Ergosterol extracted and dried
Photo by Erick N. Zorrilla

2.3.2.5.2 Ergosterol analysis by HPLC

Dried extracts were dissolved in (0.5-1 ml) methanol 100% by sonification for 5 min. Tubes were centrifuged at 7200 rpm for 2 min. Supernatant was placed into the vials to proceed with the quantification.

HPLC- Waters and column described in the section 2.3.2.3 were used for ergosterol determination, under isocratic conditions, methanol 100% was used as mobile phase at a flow rate of 0.8 ml min^{-1}, at 25°C, and 282nm by injection of 20 µl sample. Calibration was conducted with an external standard of ergosterol purchased from Sigma-Aldrich (St. Louis, Mo).

2.3.2.6 Caffeine determination in coffee pulp from SmSSF

Caffeine determination in coffee pulp from SmSSF, was conducted as describe in sections 2.2.2.2 and 2.2.2.3. A few modifications were done to the original procedure, including a filtration before passing the

sample through the SPE cartridge to avoid clogging the cartridge by any solid in the extract from SmSSF of coffee pulp.

2.4 Statistics

The data is presented as average values ± standard error of the mean. Analysis of variance was performed using statistical software MiniTab14 to compare the degradation activity by the candidate strain during the incubation time.

3 RESULTS

3.1 Identify Candidate strains

3.1.1 Screening

Table 5 shows some fungal strains from CFMR-FPL collection, which were grown under 25°C, 30°C, and 37°C using a Yeast Malt Peptone Glucose Agar (YMPG) with the addition of different caffeine concentrations; the concentration range was chosen based on the caffeine content in coffee pulp reported previously, between 0.5 to 1.5%.

Table 5 Fungal screening from FPL collection under different temperatures and caffeine concentrations

Strains	25°C				30°C				37°C			
	Caffeine (%)				Caffeine (%)				Caffeine (%)			
	0.0	0.5	1.0	2.0	0.0	0.5	1.0	2.0	0.0	0.5	1.0	2.0
Alternaria alternata	+	+	+	±	+	+	±	-	±	-	-	-
Ceriporiopsis subvermispora	+	-	-	-	+	-	-	-	+	-	-	-
Chaetomium globosum	+	+	±	-	+	-	-	-	±	-	-	-
Daldinia concentrica	+	±	-	-	+	-	-	-	+	-	-	-
Gloeophyllum trabeum	+	-	-	-	+	-	-	-	+	-	-	-
Lecythophora hoffmannii	+	+	-	-	+	-	-	-	+	-	-	-
Microsphaeropsis sp	+	-	-	-	+	-	-	-	±	-	-	-
Phanerochaete chrysosporium	+	±	±	-	+	±	-	-	-	-	-	-
Phialocephala dimorphospora	+	±	-	-	+	-	-	-	+	-	-	-
Postia placenta	+	-	-	-	+	-	-	-	+	-	-	-
Xylaria polymorpha	+	+	±	-	+	±	-	-	+	-	-	-

(++) Abundant growth; (+) Moderate growth; (±) Poor growth; (--) No growth

The effect of temperature and caffeine concentration for *Alternaria alternata* is shown in Figure 17. The growth was determined based on colony diameter and by comparison with control. The highest growth occurred at 25°C. At 30°C the growth observed was fair and at 37°C no growth was observed in the presence of caffeine indicating that the ideal temperature for the filamentous fungi tested was between (25- 30 °C). In terms of caffeine concentration, the inhibitory concentration was 2.0 % and the concentration where the fungi showed better growth was 0.5%.

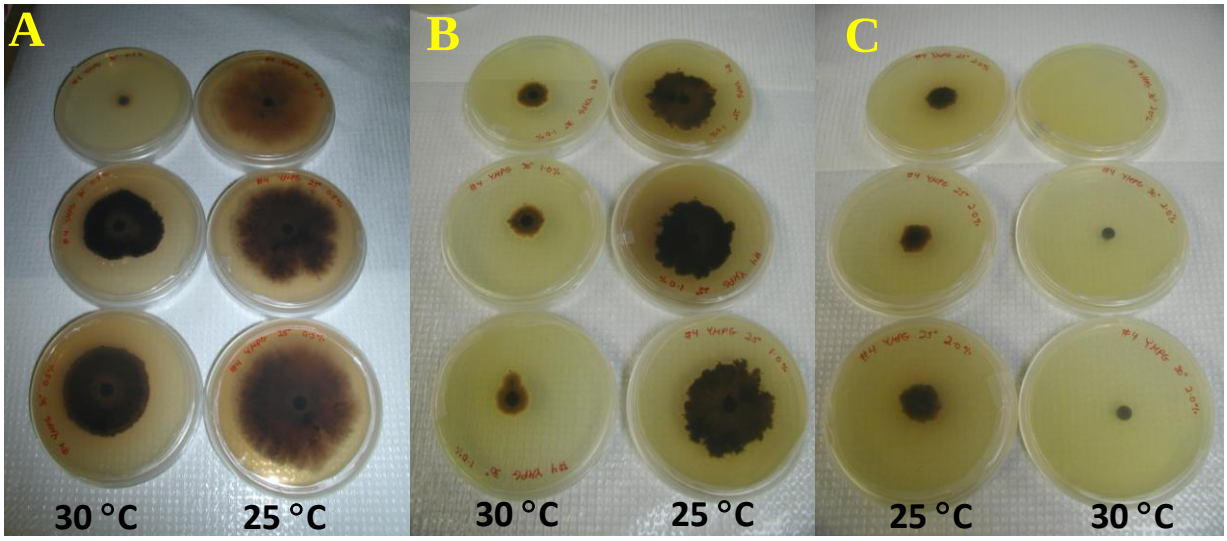


Figure 17 *Alternaria alternata* under different temperatures and caffeine concentrations
A (0.5% Caffeine); **B** (1.0% Caffeine); **C** (2.0% Caffeine)

After evaluation of our screening results, after incubation time of four weeks, we can highlight four strains *Alternaria alternata*, *Phanerochaete chrysosporium*, *Chaetomium globosum* and *Xylaria polymorpha* which exhibited significant growth in caffeine concentrations similar to those found in coffee pulp (0.30- 1.6%).

Alternaria alternata grew on YMPG-caffeine 2.0% (±) in small proportion. For *Phanerochaete chrysosporium*, *Chaetomium globosum* and *Xylaria polymorpha* the highest growth was 1.0% (±), but, in a small proportion.

Based on these results *Alternaria alternata* was selected as a candidate strain for decaffeination studies in coffee pulp and was further used to conduct the experiments developed in this study.

3.2 Assessment of Caffeine Detoxification by Candidate Strain in a Purified System

Figure 18 illustrates the growth of *Alternaria alternata* in presence of different concentrations of carbon and nitrogen source using minimal media. Here the carbon source was glucose and nitrogen source was asparagine. Under these conditions mat formation was observed when carbon and nitrogen sources were in ideal proportions (C 2%, N: 1.0%).

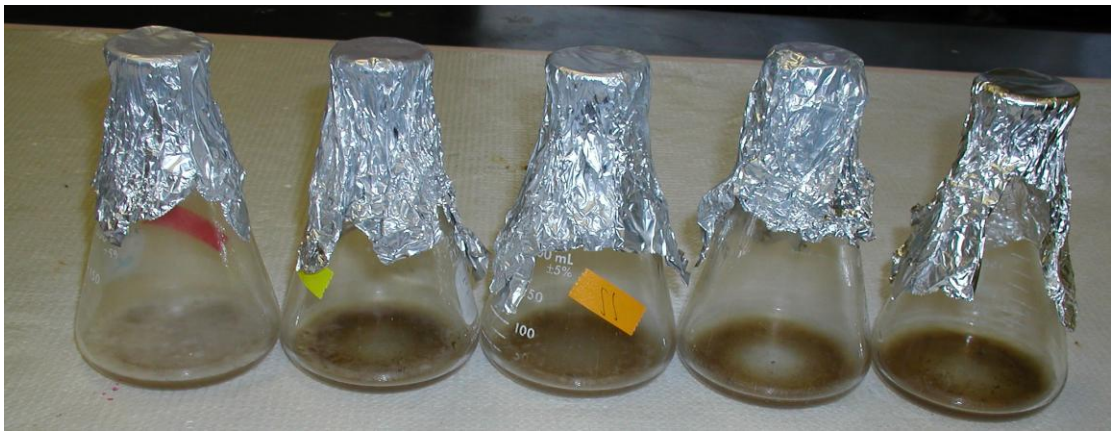


Figure 18 *Alternaria alternata* growing at different concentration of carbon and nitrogen sources. Left to the right: (C: 2%, N: 1%), (C: 1.5%, N: 0.75%), (C: 1.0 %, N: 0.5%), (C: 0.5%, N: 0.25%), and (C: 0%, N: 0%). Photos by María del Pilar Sierra-Gómez

3.2.1 Liquid minimal media (caffeine) stationary culture assay

Alternaria alternate, the candidate strain selected from our initial screening for further study, was grown in liquid media in a purified completely defined system with caffeine 1g l^{-1}. During the incubation period (12 weeks), the candidate strain showed poor growth (weak structure, limited filamentous formation) because of nutrients limitation. Figure 19 shows the minimal media appearance on day 14, was similar throughout the whole incubation period.

Figure 19 *Alternaria alternata* minimal media supplemented with caffeine (day 14)

Media containing caffeine as a sole carbon and nitrogen source seemed to inhibit the growth of the fungus and the caffeine degradation observed lagged on day 69. At the end of the incubation time a caffeine content of 566 ± 41.16 (mg l^{-1}) was determined. This amount represented near 45.33% degradation, as shown in figure 20. Caffeine concentration was calculated using a calibration curve (y=31603x + 9221).

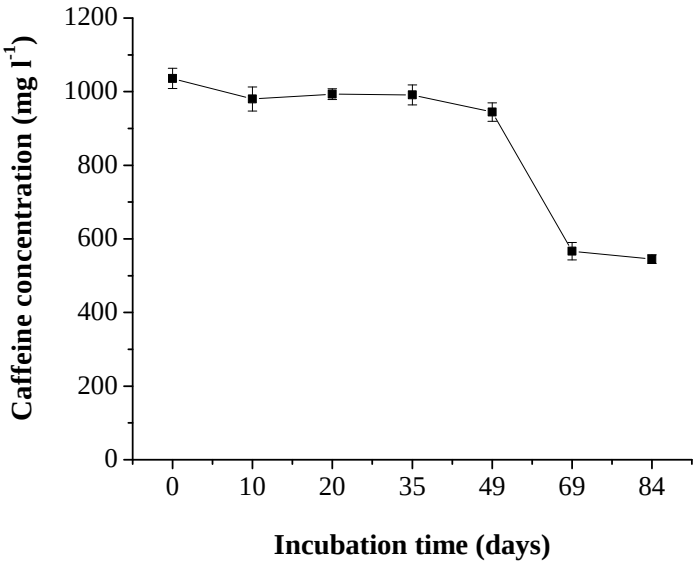


Figure 20 Caffeine concentration using *Alternaria alternata*
In a minimal media (purified system) with caffeine

To assess the detoxification capabilities of *A. alternata*, we included in our study the fungus *Aspergillus tamarii* as a positive control since it has been reported in the literature as a caffeine detoxifier (Gutierrez-Sanchez et al. 2003). Both were evaluated under the same conditions in terms of maintenance media, temperature, and volume of inoculum. Figure 21 shows the caffeine concentration for the candidate strain *Alternaria alternata* and *Aspergillus tamarii*.

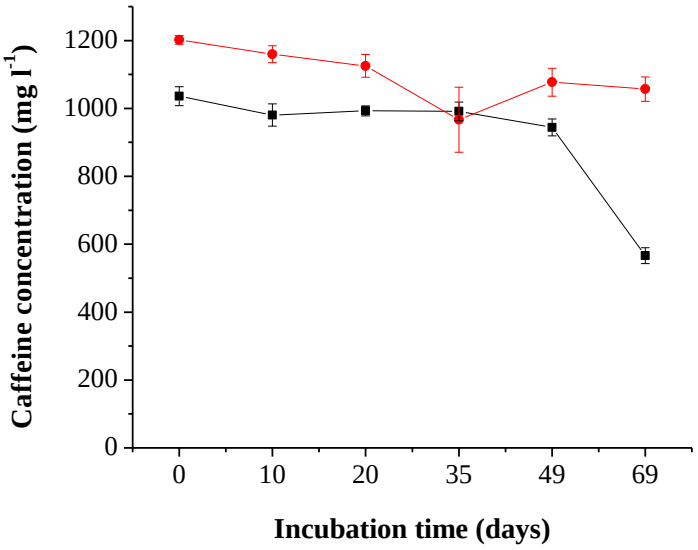


Figure 21 Caffeine concentration using minimal media (caffeine) at 25°C stationary *Alternaria alternata* (-■-) and *Aspergillus tamarii* (-●-)

In the initial caffeine concentration was observed a difference which was related to caffeine residual from the induction media CPE.

Aspergillus tamarii produced a mild caffeine degradation on day 69; a caffeine amount of 1050.28 ± 45.58 (mg l^{-1}) was determined, representing 11.13% degradation, which means that under these specific conditions *Aspergillus tamarii* is not an effective degrader.

3.2.2 Liquid minimal media (caffeine-sucrose) stationary culture assay

Figure 22 presented the two strains involved in this research growing in a purified system with caffeine and sucrose.

Figure 22 Appearance of liquid minimal media (caffeine-sucrose) after incubation
Left: *Alternaria alternata*, right: *Aspergillus tamarii* both growing at 25 °C. Photos by María del Pilar Sierra-Gómez

Figure 23 shows caffeine degradation supplemented by sucrose as carbon source. A decrease in caffeine concentration (increase of caffeine degradation) was observed to when caffeine was used as a sole carbon and nitrogen source. For instance, *A. alternata* showed caffeine content of 437 ± 29.20 mg l^{-1} on day 69, which represents 60.92 % caffeine degradation. For *A. tamarii* on day 69, caffeine content was 1056.83 ± 62.23 mg l^{-1} representing 12.05% of degradation.

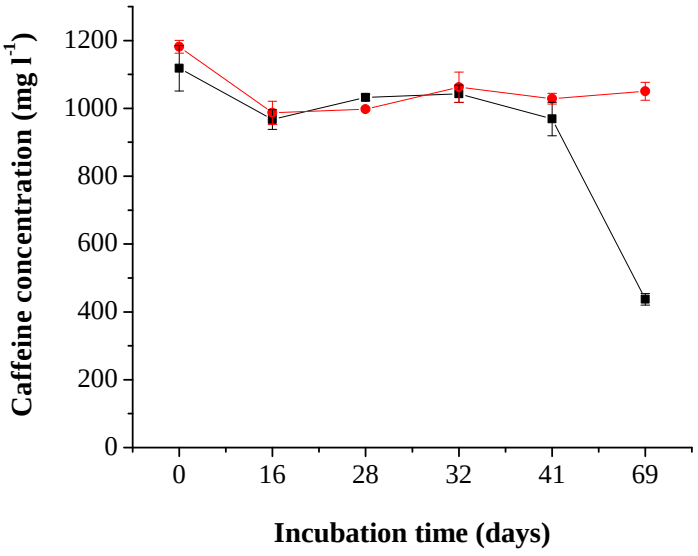


Figure 23 Caffeine degradation using liquid minimal media (caffeine-sucrose)
Caffeine (1 g l^{-1}) and sucrose (25 g l^{-1}) under stationary method inoculated at 25°C. *Alternaria alternata* (-■-) and *Aspergillus tamarii* (-●-)

3.2.3 Liquid minimal media (caffeine-sucrose) shaking culture assay

In an attempt to improve the behavior of the positive control a new set of experiments were set up under different growth conditions. Fungi were grown under agitation in larger volumes. The purpose of this experiment was to assess optimal conditions for caffeine detoxification based on optimal growth conditions of our positive control, *A. tamarii*. Figure 24 illustrates caffeine degradation of both fungi under agitation and at larger culture volumes. Approximately 46.95% of the initial caffeine was degraded. Nevertheless a highest variability was observed, since the growth under these conditions was highly heterogenous, and large mycelia clumps were usually observed (See Figure 25).

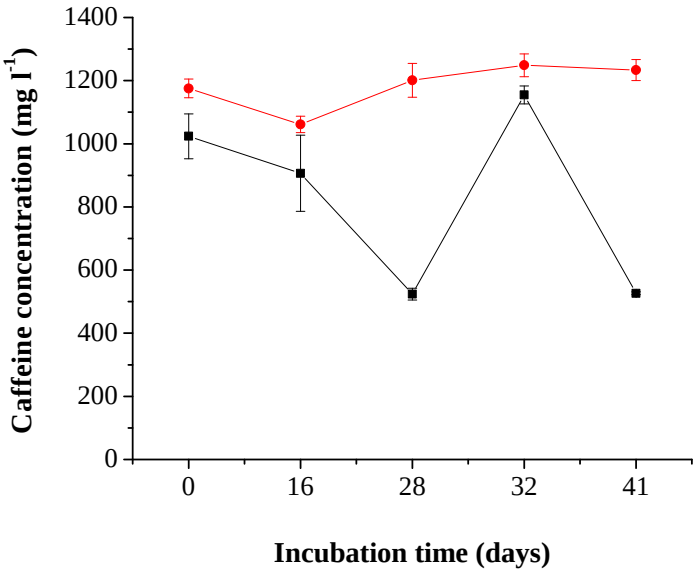


Figure 24 Caffeine concentration in minimal media incubated at 25°C and 110 RPM *Alternaria alternata* (-■-) and *Aspergillus tamarii* (-●-)

Figure 25 Clumps formed by *Alternaria alternata* in minimal media under agitation. Photo by María del Pilar Sierra-Gómez

3.3 Assessment of Caffeine detoxification Upon Growth of Candidate Strains under Submerged Solid State Fermentation (SmSSF)

3.3.1 Fresh Coffee Pulp

3.3.1.1 Biological Characterization: Isolation and identification of filamentous fungi from coffee pulp

From fresh coffee pulp a total of 22 isolates were obtained (Table 6). These isolates were described as yeast like or filamentous.

Table 6 Fungal isolates from coffee pulp at different temperatures.

Isolate	**Temperature (°C)**			**Sporulation**	**Observation**
#	**25**	**30**	**37**	**Media**	(Appearance)
1	x			WA	Yeast like
2	x			WA	Yeast like
3	x			WA	Powder-cotton
4	x			WA	Filamentous
5	x			WA	Yeast like
6	x			WA	Yeast like
7	x			CPE	Yeast like
8	x			WA	Yeast like
9	x			WA	Yeast like
10	x			WA	Filamentous
11	x			WA	Filamentous
12	x			WA	Yeast like
16		x		CPE	Yeast like
17		x		CPE	Filamentous
18		x		CPE	Yeast like
19		x		CPE	Yeast like
20			x	WA	Yeast like
21			x	WA	Yeast like
22			x	WA	Yeast like
23			x	WA	Yeast like
24			x	CPE	Yeast like
25			x	CPE	Yeast like

The isolates above were extracted from fresh coffee pulp, using water agar (WA) and CPE as sporulation media. These isolates were selected from germinated spores on coffee pulp extract (CPE) media.

Table 7 Total of isolates from fresh coffee pulp by temperature and media

Isolation	Temperature (°C)		
Media	25	30	37
WA	11	-	4
CPE	1	4	2
Total	22		

WA = Water agar, CPE= Coffee Pulp Extract

Isolates from coffee pulp extract (CPE) (Table 7) were inoculated in slide culture using moist chamber. Figure 26 shows the microscopic structures after incubation.

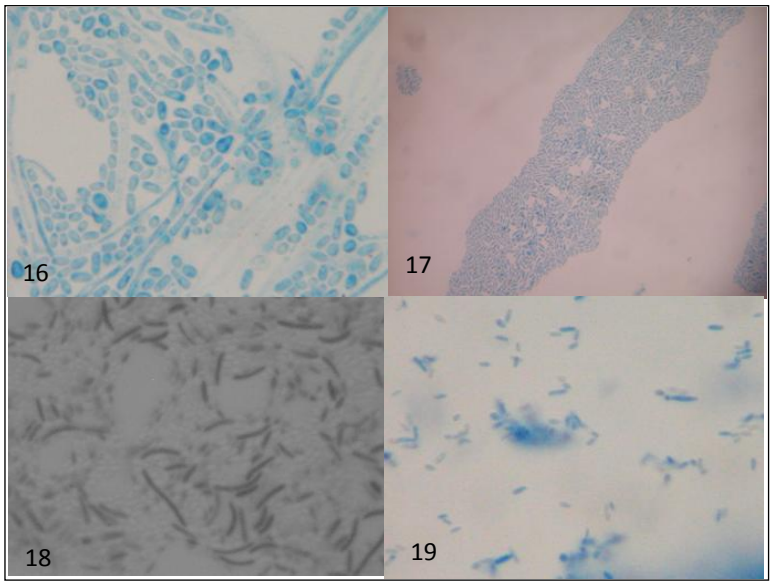


Figure 26 Fungal isolates from fresh coffee pulp in CPE at 30°C
Photos by María del Pilar Sierra-Gómez

During this study we identified two isolates (17 and 18) based on the morphology as *Fusarium* sp from CPE media.

In addition, the same procedure was performed using coffee composted pulp. Five fungi were isolated identified as *Aspergillus fumigatus*. Four fungi were identified as (*Geotrichum* Link, *Oidioendron* Robak (2), and *Chrysosporium* Corda) two *Oidium* Sacc., *Basipetospora* Cole and Kendrick, and one possible blastospora (*Blastomyces* Cost and Roll) (See Appendix A).

3.3.1.2 Physical-chemical Characterization

Fresh coffee pulp was evaluated in terms of moisture, protein, pH and caffeine composition (Table 8), parameters important in solid state fermentation and future studies related to bioreactor design.

Table 8 Coffee pulp fresh composition

Component	***Content***
Moisture (%)	81.24 ± 0.87
Protein (%)	5.01 ± 0.81
pH	4.73 ± 0.03
Caffeine (mg l^{-1})	536.44 ± 29.65

3.3.2 Submerged Solid State Fermentation (SmSSF)

Dried and ground (850μm -1.4 mm) coffee pulp was used as substrate, to inoculate the candidate strain (*Alternaria alternata*). The control strain (*Aspergillus tamarii*) was also grown under these conditions. Figure 27 shows the organisms used as potential caffeine degraders in the context of coffee pulp. Growth occurred uniformly on the material.

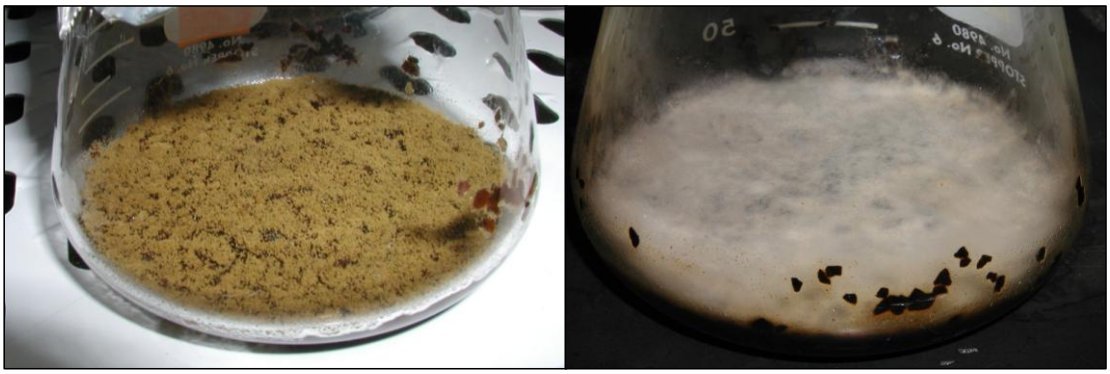


Figure 27 Solid State Submerged Fermentation
Left: *Aspergillus tamarii* Right: *Alternaria alternata* growing in coffee pulp at 25 °C stationary incubator.
Photos by María del Pilar Sierra-Gómez.

Total fermentation time was three weeks. *Alternaria alternata* was sampled twice during two weeks and once for the other week, to measure caffeine, dry weight, and ergosterol content (as a sign of positive growth).

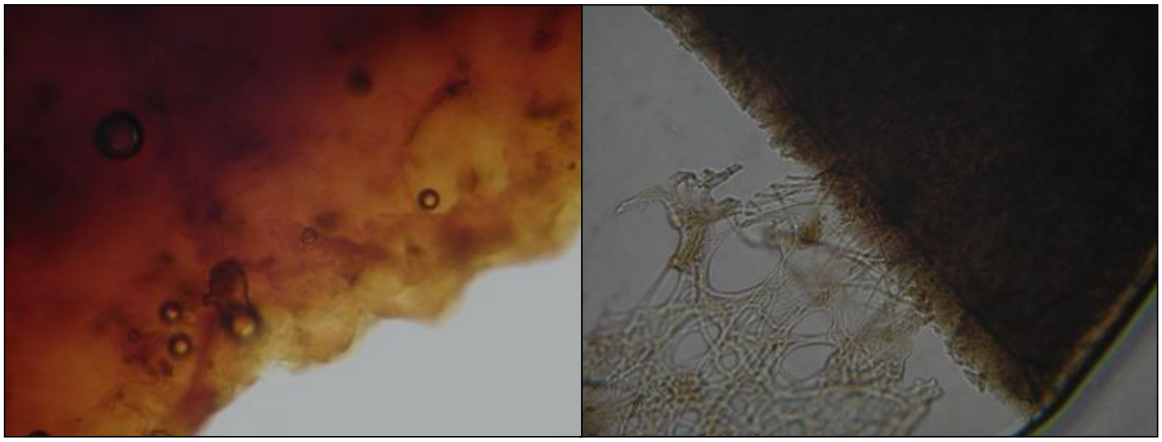


Figure 28 Microscopic slides of coffee pulp.
Left: coffee pulp without growing (day 0) 40X and Right: Coffee pulp with *Alternaria alternata* (day 20) 40 X.
Photos by María del Pilar Sierra-Gómez

Figure 28, shows light microscopy images in which non-inoculated and inoculated coffee pulp is observed. During day 0 (left) no fungal filaments or hyphae present. The "flake" of coffee pulp remained clear on the surface and on the edge. For day 20 (right) the slide shows the presence of hyphae on the edge, and on the surface of the flake of coffee pulp.

A relative decrease in the caffeine content was observed during the fermentation time, and the caffeine decreased on day 20. However, *Aspergillus tamarii* under these fermentation conditions exhibited less caffeine degradation (25.6%) than that of *A. alternata* (See Appendix B). *Alternaria alternata* showed a better performance, with caffeine content of 637.83 ± 125.67 mg l^{-1}, representing 44.5% of caffeine degradation (Figure 29A).

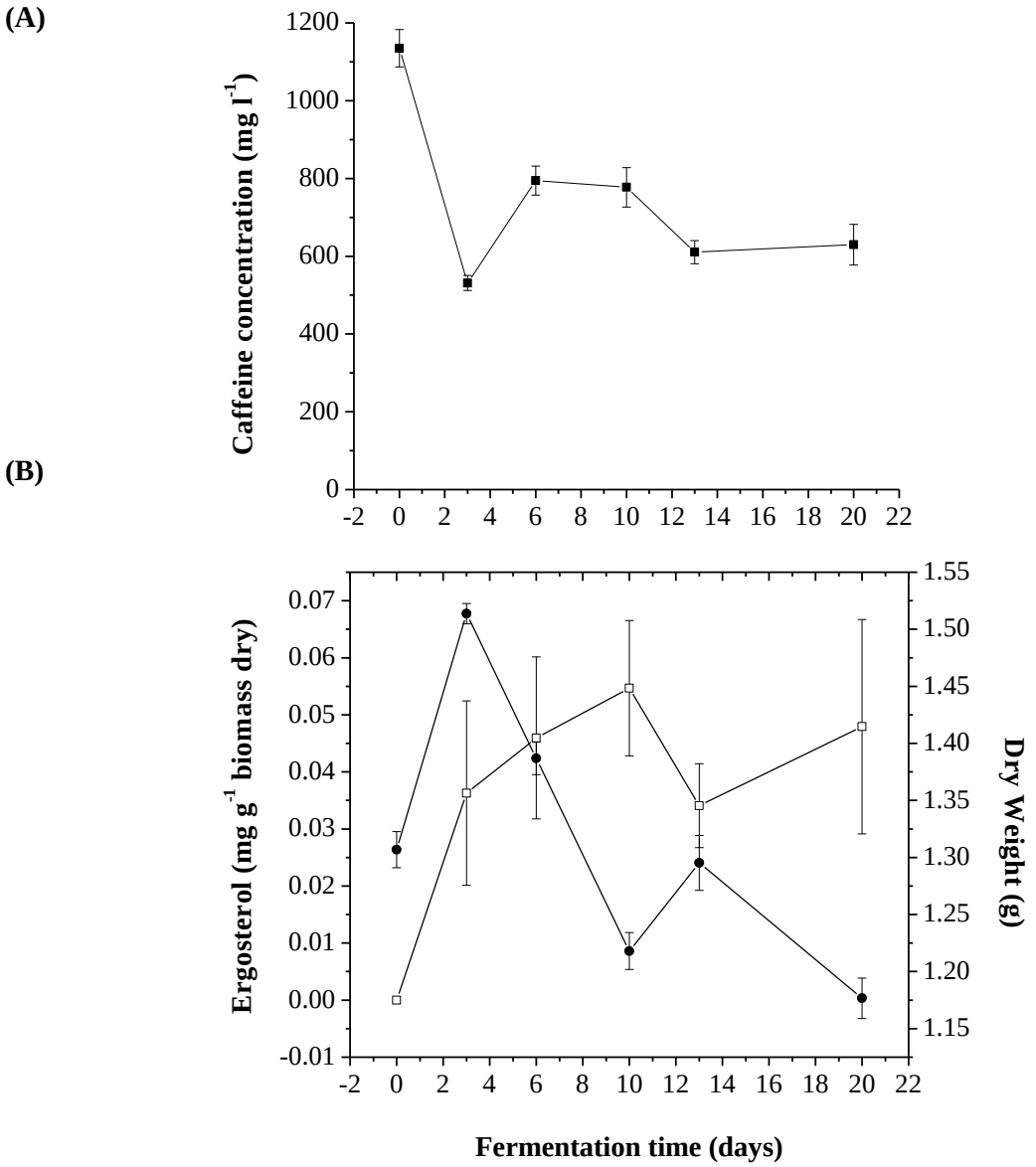


Figure 29 Caffeine degradation, Ergosterol content and weight dry by *Alternaria alternata* SmSSF **(A)** Caffeine degradation. **(B)** Ergosterol and weight dry as an indicator of growth (-●-) Dry weight, and (-□-) Ergosterol content.

Figure 29B illustrates the variation of dry weight compared with ergosterol content during the fermentation time. Dry weight decreased with fermentation time. In the case of ergosterol content, six replicates were measured for each day except for day 13 which used four replicates. A typical microbial growth curve was observed with increasing fermentation time, where active growth was indicated by a positive slope (log phase), which eventually reached a stationary phase. Ergosterol concentration was calculated using a calibration curve (y=2.00e7x +54656).

3.4 Statistics

Assessment of Caffeine Detoxification by Candidate Strain in a Purified System

ANOVA was conducted to determine if there were differences between fungi and incubation time. Given the results there are evidence of difference between *Alternaria alternata* and *Aspergillus tamarii* (p=0.000), also there are differences between incubation time (p=0.000) in minimal media containing caffeine as sole carbon and nitrogen source (See appendix C).

At the same time ANOVA was conducted to determine any differences between *A. tamari* and *A. alternata* and incubation time in minimal media containing sucrose and caffeine as carbon and nitrogen source. As result there are evidence of difference between fungi (p=0.000) and between incubation time (p=0.000) (See Appendix D).

4 DISCUSSION

4.1 Identification of Candidate Strains

4.1.1 Screening

Preethu, et al. (2007) reported 38.6% of lignin in coffee pulp, Salmones et al. (2005) recorded lignin content for *Coffea arabica* between (25-26% dw), Mazzafera (2002) reported the contents for carbohydrates (57.8% dw) and caffeine between (0.33-1.3% dw) (Molina et al. 1974) (Mazzafera, 2002). Based on these contents, coffee pulp is an alternative substrate to use in new trends of efficient use of agro-industrial residues. Effective re-utilization of this substrate will reduce its environmental impact (presence of agro-toxic such as caffeine), and will decrease the dependece of fossil fuels (coffee pulp as potential source of lignin).

A literature review was conducted using as criteria lignin and caffeine degradation, lignocellulosic activity, lignin-degrading enzymes, production of enzymes (such as peroxidase, laccase), fenton reactions, saprophytes, plant pathogens, and caffeine degradation enzymes in fungi. This review was done considering the toxic (caffeine) and antinutritional (lignin and derivatives) factors present in coffee pulp. From this review and in collaboration with the University of Wisconsin-Madison (Forest Products Laboratory) we decided to test thirteen strains which had reported the activities mentioned before. The collection used for this study represents ligniculous fungi which have been isolated from the natural environments. From this collection the ability to produce enzymes like laccases, peroxidases, cellulases, and others is known (Table 1). These enzymes are recognized in processes of delignification and studies of lignin degradation. From our results we can narrow our list to four candidates with caffeine degrader potential: *Alternaria alternata*, *Phanerochaete chrysosporium*, *Chaetomium globosum* and *Xylaria polymorpha*. *Alternaria alternata* grew on 2.0% of caffeine to a small extent. *Phanerochate chrysosporium*, *Chaetomium globosum* and *Xylaria polymorpha* exhibited growth until 0.5% also to a small extent.

Phanerochaete chrysosporium is the ligniculous fungi mostly used in studies of lignin degradation (Table 1), also, it has been reported in studies of caffeine degradation, obtaining a caffeine detoxification of 70.8% (Brand et al. 2000). *Chaetomiun globosum* has been involved in lignocellulose degradation (Popescu et al. 2010) and has been studied for xylanolitic enzymes production (Gandhi and Rao, 1997). *Xylaria polymorpha* has been related on laccase production and wood decay studies (Chaparro et al. 2009). To our knowledge, there are no records of the caffeine degradation potential of *Alternaria alternata, Chaetomium globosum* and *Xylaria polymorpha*.

Alternaria alternata is known to grow well on PDA at 25 ºC in darkness and mycelial mats covered all the media in Petri dishes in two weeks. The color of fungal colonies was usually dark brown to dark olive green brown, but often lighter and almost white colonies sometimes appear under the same conditions with the same medium. Its conidial size, shape, and segmentation varies considerably, depending on factors such as fungus strain, age of spores, substrate, pH, temperature, humidity, and light (Slavov et al. 2004). Laccase activity from this fungi has been reported by Atalla et al. (2010).

Alternaria alternata was selected to continue this study following the main objective of this study which focused on finding a fungal isolate that can coexist on a substrate with lignin and caffeine and can potentially oxidize or modify the material to make it more usable. Given the fact that *Alternaria alternata* was the candidate that showed the highest caffeine tolerance in our screening we decided to continue the characterization of its potential as a caffeine detoxifier of coffee pulp. In future studies we intend to pursue detoxification experiments with the other candidates (*Chaetomium globosum* and *Xylaria polymorpha*).

While we understand that the logical approach to find a candidate for the degradation of a material is by looking directly into the microbial population present in the material, in the case coffee pulp, we did not have adequate tools to conduct these experiments at the beginning of the present study. We decided to design an indirect approach to go around our limitations, which is the object of this research. However, we reported as part of our results work done to screen the fungal community present in the coffee. Our laboratory has begun work on establishing protocols for identification using molecular tools to continue a comprehensive study of the population of microorganisms in coffee pulp.

4.2 Assesment of Caffeine Detoxification by Candidate Strain in a Purified System

The purpose of this experiment was to determine directly whether there was any change in the concentration of pure caffeine during the growth of our candidate fungus in a purified system. Minimal media was set up with a small quantity of liquid, to provide support for mat formation.

In terms of culture conditions, the depth of fluid media is an important factor in chemical degradation by fungi. When fungi grew in deep (50 ml) liquid suspensions a higher variability was observed. In shallow (10 ml) suspensions less growth variability but a higher degradation time was observed. Logan et al. (1994) mention that there is not a reason to explain this phenomenon, but they make the assumption that oxygen transfer is an important factor. Additionally, based on Leisola et al. (1983), the mat formation limited oxygen transfer into the fluid of non-agitated cultures and inhibit the compound degradation. Therefore, we chose to use a shallow culture with no agitation to begin our studies like wood degradation and enzymatic studies (Sato et al. 2009) (Rothschild et al. 1999) Also, we noted that evaporation was an issue, especially for the later time points. For this reason the experiments were incubated until day 69.

Alternaria alternata decreased the caffeine content in minimal medium on day 69 (566 ± 41.16 mg l^{-1}), near to 45.33% of caffeine degradation, after that the caffeine content was stabilized at day 84 (544.73 ± 20.60 mg l^{-1}) approximately 47.42% of caffeine degradation.

Many authors had reported *Aspergillus tamarii* as good caffeine degrader, (Denis et al. 1998) and (Hakil et al. 1998) *Aspergillus tamarii* was integrated in this study to evaluate the caffeine degradation in comparison with *Alternaria alternata. Aspergillus tamarii* was selected based on studies which show the ability to caffeine degradation of 67.2% of caffeine degradation was degradation in a period of 48 h (Hakil et al. 1998). For Gutierrez-Sanchez et al.(2003) in a period of 6 days and after adding a pulse of caffeine the degradation was approximately 50%. Given these results *Aspergillus tamarii* was taken as control microorganism (caffeine-degrading fungi) under stationary shallow culture. *Aspergillus tamarii* did not exhibit mat formation, reflected in the poor degradation of caffeine near to 12.05%. Likely, the media and conditions in our experiment were not ideal for its development. The literature suggests the

addition of sucrose to improve the caffeine degradation, further, simple sugars such as glucose, fructose, sucrose, and sorbitol as sole carbon sources support high fungal growth; and sporulation (Gummadi et al. 2007). Thus, we conducted another experiment using caffeine as nitrogen source (Gutierrez-Sanchez et al. 2003), (Roussos et al. 1994), and sucrose (and caffeine) as a carbon source. For *Alternaria alternata* the kinetics of the degradation did not change. However, we observed an enhancement in caffeine degradation (60.92% as opposed to 45.23%). In the case of *A. tamarii*, it reached a maximum degradation of 11.13% was reached.

Based on these results with *A. tamarii* a new experiment was designed under agitation for both fungi, to assess whether the degradation time could be optimized. The results showed a high variability between samples and days, mainly due to the formation of aggregates, which limit the mass transfer and thus limit caffeine degradation. The lack of homogeneity of the medium in agitation, the high variability in caffeine degradation, and low feasibility of implementation in coffee farms (cost in energy from agitation) led us to discard the agitation method and continue our studies in solid state fermentations using a stationary format.

4.3 Assesment of Caffeine Detoxification upon Growth of Candidate Strain under Submerged Solid State Fermentation (SmSSF) Conditions

4.3.1 Coffee Pulp

In Puerto Rico, the predominant coffee specie is *Coffea arabica* which was introduce in 1736 from Martinica through Dominican Republic. (Monroig-Inglés n.d.). The cultivation temperature in Jayuya PR during the year is 73 °F (22.8 °C) which is the ideal temperature for the growth of mesophillic fungal strains. Roussos et al. (1995) isolated mesophillic and thermotolerant (25 and 37°C) strains from a mixture of soil, leaves and coffee cherries, thus some of the obtained strains belonging to *Aspergillus, Penicillium, Geotrichum, Humicola, Rhizopus* and *Trichoderma*. Silva et al. (2008) isolated and identified *Aspergillus*, *Penicillium*, *Cladosporium, Fusarium, Pestalotia* and *Paecelomyces* in coffee beans, where predominant fungi species during day zero were *Cladosporium* and some species of *Fusarium*. Also mentioned, that difference in moisture content and chemical composition of coffee affect the colonization by these microorganisms. Isolates of *Penicillium* were found during the whole fermentation process of fermentation and isolates of *Aspergillus* were detected on the 8th fermentation day. Microbial succession

was established with the predominance of bacteria at the initial fermentation time with the presence of filamentous fungi and yeast during the fermentation process.

In this research with coffee pulp from Jayuya, PR a temperature of 28 °C was recorded during the sampling. Most of the isolated strains grew at 25-30°C. In fresh coffee pulp found mainly yeast-like microorganisms which were not identified to genus using the proposed methodology. In this substrate (in fresh coffee pulp during day 0) *Fusarium* and *Aspergillus fumigatus* were also found in composted coffee pulp. Some isolates did not show all the stuctures in wet mounts and many of them showed only spores. Based on Watanabe (2000) spores may be one of the most important morphological characteristics for identification. However, some fungi may be identified on the basis of other morphological characteristics, for instance, the presence of hyphal septum which varies by species. Nevertheless, in some cases the fungi does not have spores making the identification process very difficult. Although, *Aspergillus fumigatus* has been reported as caffeine degrader, this fungi is recognized also as human pathogen and the use of this fungi require special conditions such as biohazard laboratory level 2.

Currently, molecular studies are a better tool to characterize the microbial population of these environmental isolates. Molecular studies are needed to identify the organisms from coffee pulp, especially in those where spores were abundant but hyphaes are not well defined in the slide (due to a possible artifact), which can result in a wrong identification.

4.3.2 Submerged Solid State Fermentation (SmSSF)

Experiments using submerged solid-state fermentation (SmSSF) was conducted under stationary conditions based on the results with the defined minimal medium with *Alternaria alternata*. Coffee pulp was dried, milled, and sterilized before being utilized in the fermentation. In preliminary experiments the most efficient particle size of the coffee pulp was determined. The particle size chosen based on the preliminary data was between 0.850 mm and 1.4 mm. Many authors suggest a particle size range between 0.8 -2.0 mm (Brand et al. 2000, Orozco et al. 2008). Large particle size increases the porosity of the cell surface, providing higher respiration/aeration efficiency. Small particles result in substrate accumulation, and poor growth (Murthy et al. 2009).

Alternaria alternata was grown in a coffee pulp extract agar (CPE-A) containing sucrose and other salts as decribed in the methodology section. This growth prior to inoculation in coffee pulp was carried out to allow an adaptation or induction period (Gokulakrishnan et al. 2005). In this research the inoculum was ground up mycelium growing on CPE. After incubation, samples were taken to measure the caffeine content. A reduction in the degradation time of caffeine was obtained. This result may be attributed to the nutritional composition of the media (greater availability of nutrients, or other potential precursors of caffeine degradation) (Braham and Bressani, 1979).

During the first three days an accelarated caffeine degradation occurs, which could be related to the high concentration of soluble caffeine and nutrients derived from the pulp after it had been processed (dried, milled and sterilized). Then, with the addition of water, sucrose and salts the media promotes a rapid induction of growth. This is evidenced by the maximum (day 3) in the ergosterol curve in Figure 29B. Ergosterol is a steroid present in fungi (Volker et al. 2000), which has been used as biomarker for quantification for biomass (Gors et al. 2007). Figure 29B also shows that, ergosterol increases with fermentation time and remained stable (day10) as the caffeine is degraded. This behavior suggests that the fungus uses soluble nutrients (including caffeine soluble in the media) rapidly causing a degradation of 53.19%. This result also corresponds to the weight loss observed in the sample. (Fig. 29B). In dry weight, which is a mix of pulp and fungi, there is a notably higher weight on day 3 presumably due to fungal growth. During the growth process occurs a substrate weight loss which is attributed to the fungus respiration which produces CO_2 and water (Boyle and Kropp, 1992). However, the behavior observed in dry weight can also be explained by the lack of any nutrient that can stimulate growth such as a nitrogen source as mentioned by Bjurman, (1994).

After the dissolved nutrients are depleted from the medium (day 6) we believe that the fungus begins to attack or consume nutrients such as carbon and nitrogen sources sequestered in the pulp (day 10). At this point more caffeine is released into the medium to be consumed again by the fungus. This is reflected by the increase in caffeine concentration at day 6. After day 6 the reduction of caffeine continues obtaining a degradation of 44.5% based on the initial concentration of caffeine extracted from the fresh pulp in day 20 (Figure 29A).

5 CONCLUSIONS AND RECOMMENDATIONS

Based on this study, the implementation of a stationary system for the treatment of coffee pulp is recommended. This recommendation is based on the fact that this system achieved a caffeine decrease of 43% over a period of 20 days using *Alternaria alternata* as caffeine degrader. The potential of the proposed system but more studies are done to optimize the percentage of degradation of caffeine and to evaluate the lignin degradation, or the removal of other anti-nutritional factors under these conditions.

Several parameters to optimize caffeine degradation in an SSF include pH, moisture content, temperature, and inoculum effect. With regards to this last parameter the development of methods to stimulate and measure the sporulation process of *Alternaria* and to establish different percentage of inoculum in term of weight volume ratio (w/v) are recommended (Sarath Babu et al. 2005).

As part of this work a study of the fungal population in coffee pulp was initiated resulting in the identification of fungi that occur naturally in fresh coffee pulp, most of which were yeast. Additional studies are in progress to assess the identity of microorganisms from coffee pulp using molecular biology tools. Furthermore, other candidate strains such as *Chaetomium globosum* and *Xylaria polymorpha* will be evaluated in coffee pulp using the suggested methodology to compare with *Alternaria alternata* in terms of caffeine and lignin degradation

To elucidate the metabolism and enzymes that are produced during caffeine degradation its necessary to carry out other studies that integrate the use of other more sensitive equipment such as Gas Chromatography- Mass Spectrometry (GC-MS) that can be used to determine the identity of eluents in the HPLC profiles that result as the reduction of caffeine takes place.

Finally, based on the work presented here a model for coffee pulp detoxification was constructed. The model is based on biopulping. The biopulping procedure involves decontaminating wood chips with steam to eliminate competitive naturally occurring bacteria and fungi. Then the chips are sprayed with an inoculum of a selected fungus, and then inoculated chips are incubated for 2 weeks. Under warm, moist

conditions lignin degrading fungi colonize the chips' surfaces and penetrate their interior with a network of hyphae (Singh et al. 2010). With this model in mind we proposed a design to be implemented by coffee farmers for the management of coffee-processing waste (Figure 30). The model works as follows: (1) represents the coffee pulp inlet, which pass through a grinding mill, (2) reduces the particule size, and will be attached to a screw conveyor (4) the coffee pulp will be decontaminated with steam (3) and sprayed with the inoculum (6) which will be a suitable candidate (e.g. *Alternaria alternata)* lyophyllized and reconstituted in an inoculum tank (5) to finally transport the material to the incubation tanks (7).

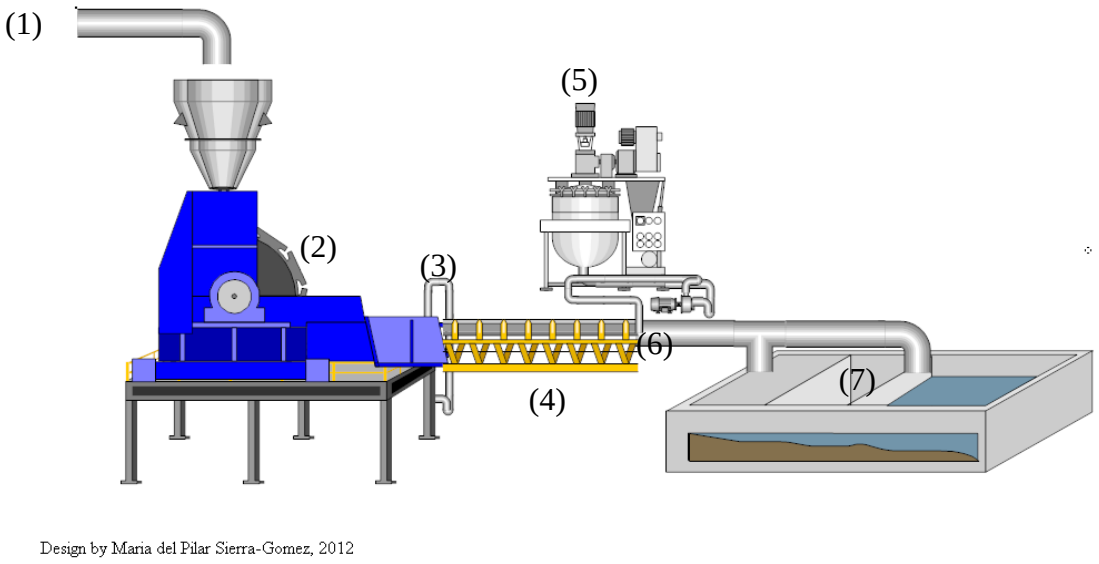


Figure 30 Detoxification process
(1) Coffee pulp flow, (2) Gringind mill, (3) Steam inlet, (4) Screw conveyor, (5) Inoculum preparation tank, (6) Inoculum inlet (spray), (7) Fermentation tank (Made with SmartDraw)

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APPENDIXES

APPENDIX A

Identification of filamentous fungi presented in composted coffee pulp

The methodology used to identify filamentous fungi from composted coffee pulp (collected on January 2010) was described in section 2.3.1.1.; preliminarily twelve isolates were identified, which were classified as follows: five fungi were identified as *Aspergillus* (Isolates C01, C02, C10, C11, C12), and seven (C03, C05, C06, C08, C04, C09, C07) were classified as others.

1. ***Aspergillus*** Link.

Aspergillus genus has the following characteristics: conidiophores upright, simple, terminating in a globose or clavate swelling, bearing phialides at the apex or radiating from the apex or the entire surface; conidia (phialospores) 1-celled, globose, often variously colored in mass, in dry basipetal chains (Barnett and Hunter, 1986). During the identification process we found five isolates belonging to this genus which were inoculated and incubated under selective media: CY20S, CYA, and Malt Extract Agar (MEA).

1.1. Isolate C01

Micro morphology - Figure 31 shows the specific characters described as follows: stipes are uncolored and yellowish near to the apices, longer than 250 μm, expanding gradually into pyriform, vesicles 20 μm in diameter; uniseriate; phialides 9 μm, conidia globose 4-5 μm in diameter.

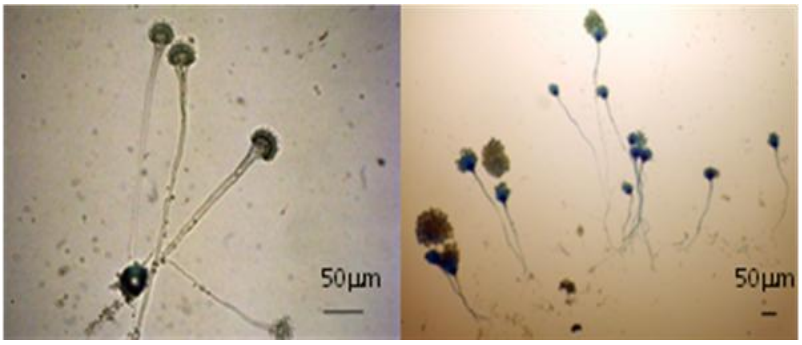

Figure 31 Isolate C01 microscopic characters.
MEA 40x (left) wet mounting 10x (right)

Macro morphology – The fungus growth was evaluated based on colony color obtaining as a result dark green colonies, with a white mycelium (CY20S and CYA), also yellow pale pigmentation on the reverse was observed. CYA at 37 °C a grayish colony and yellow reverse was observed. MEA at 25 °C shows light green colonies with a green pigmentation on reverse. The colony diameter was 26.5, 50.3, 47.5 and 48.2 mm for CY20S, CYA 25°C, MEA, and CYA 37°C respectively (See Figure 32).

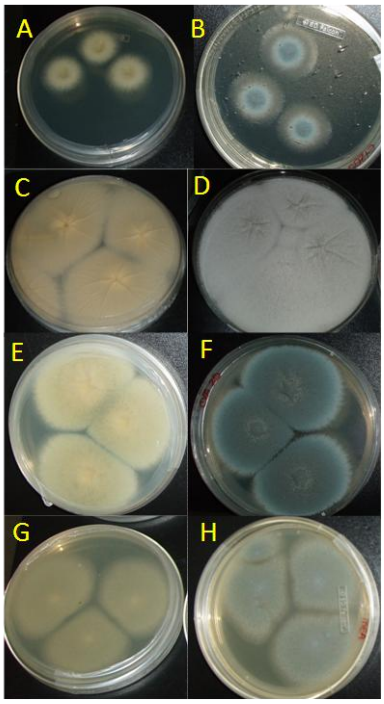

Figure 32 Isolate C01 colonies adverse and reverse.
(**A-B**) CY20S at 25°C, (**C-D**) CYA at 37 °C, (**E-F**) CYA at 25 °C and (**G-H**) MEA 25°C after 7 days inoculated.

1.2. Isolate C02

Micro morphology - Figure 33 shows the specific characters described as follows: stipes are uncolored, expanding gradually into pyriform, vesicles 48-34 μm in diameter; uniseriate; phialides 10-11 μm, conidia globose 3-4 μm in diameter.

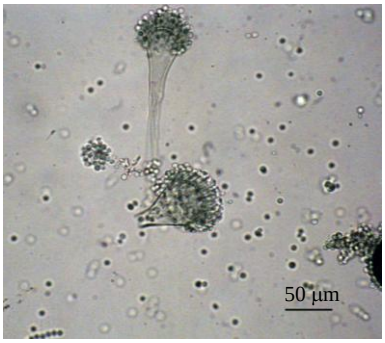

Figure 33 Isolate C02, inoculated on CYA 25°C (40x)

Macro morphology - The fungal isolate C02 was inoculated on the same media used for isolate C01. Dark green colonies were observed on CY20S, CYA and MEA at 25 °C some colonies showed a white mycelium and a protuberance. Colonies observed on CY20S and CYA yellow pale pigmentation was showed on the reverse. CYA at 37 °C a grayish colony and yellow reverse was observed. MEA at 25 °C light green colonies, yellow reverse and green pigmentation center was showed. The colony diameter was 29.3, 47.3, 49.3 and 32.5 mm for CY20S, CYA 25°C, MEA, and CYA 37°C respectively. (Figure 34)

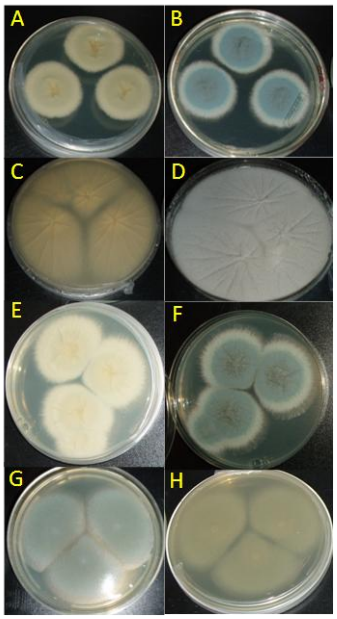

Figure 34 Isolate C02 colonies adverse and reverse.
(A-B) CY20S at 25°C, (C-D) CYA at 37 °C, (E-F) CYA at 25 °C and (G-H) MEA 25 °C after 7 days inoculated.

1.3. Isolate C10

Micro morphology - Figure 35 shows the specific characters described as follows: stipes are uncolored and greenish near to the apices, long 385 μm x 9 , expanding gradually into pyriform, vesicles 20 μm in diameter; uniseriate; phialides 8-9 μm, conidia globose 3-4 μm in diameter.

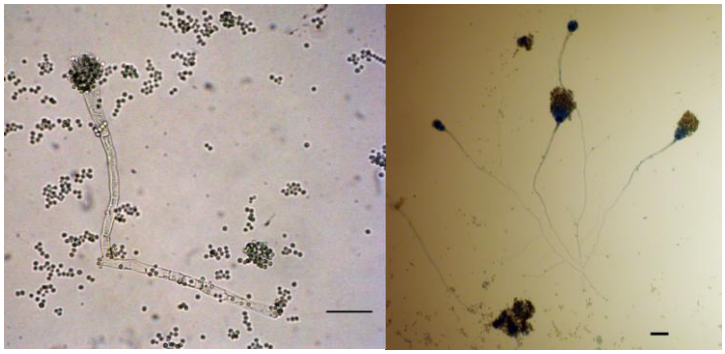

Figure 35 Isolate C10 microscopic characters: CYA 25° C 40x (left) wet mounting 10x (right)

Macro morphology – The fungus isolate C10 showed dark green colonies on CY20S, CYA and MEA at 25 °C some colonies showed white mycelium (CY20S and CYA), also yellow pale pigmentation with olive green center on the reverse was observed. CYA at 37 °C a grayish colony and yellow reverse was observed. MEA at 25 °C light green colonies, and yellow reverse was showed. The colony diameter was 28, 47.2, 50.8 and 59.7 mm for CY20S, CYA 25°C, MEA, and CYA 37°C respectively (See Figure 36).

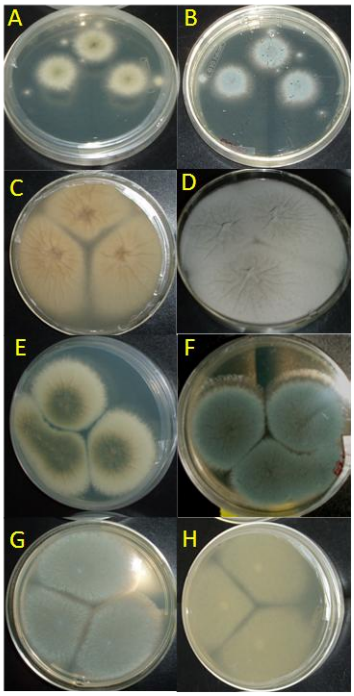

Figure 36 Isolate C10 colonies adverse and reverse.
(A-B) CY20S at 25°C, (C-D) CYA at 37 °C, (E-F) CYA at 25 °C and (G-H) MEA 25 °C after 7 days inoculated.

1.4. Isolate C11

Micro morphology - Figure 37 shows the specific characters describe as follows: stipes are uncolored and greenish near to the apices, longer than 250 μm, expanding gradually into pyriform, vesicles 28 μm in diameter; uniseriate; phialides 10 μm, conidia globose 3-4 μm in diameter

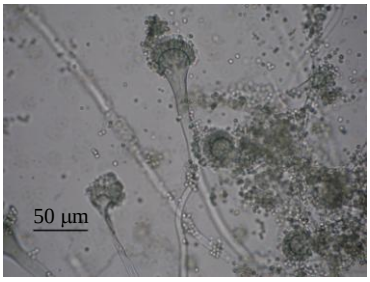

Figure 37 Isolate C11 microscopic characters: MEA 25° C 60x

Macro morphology – Isolate C11, shows dark green colonies on CY20S, CYA and MEA at 25 °C, some colonies showed white mycelium (CY20S and CYA), also yellow pale pigmentation with olive green center on the reverse was observed. CYA at 37 °C a grayish colony and yellow reverse was observed.

MEA at 25 °C light green colonies and on yellow reverse were showed. The colony diameter was 42.3, 50, 60 and 64.3 mm for CY20S, CYA 25°C, MEA, and CYA 37°C respectively (See Figure 38).

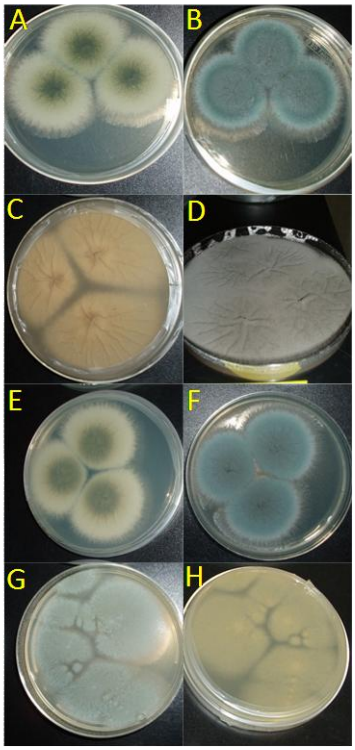

Figure 38 Isolate C11 colonies adverse and reverse.
(A-B) CY20S at 25°C, (C-D) CYA at 37 °C, (E-F) CYA at 25 °C and (G-H) MEA 25 °C after 7 days inoculated.

1.5. Isolate C12

Micro morphology - Figure 39 shows the specific characters describe as follows: stipes are uncolored and greenish near to the apices, longer than 280 µm, expanding gradually into pyriform, vesicles 24 µm in diameter; uniseriate; phialides 8 µm, conidia globose 4-5 µm in diameter.

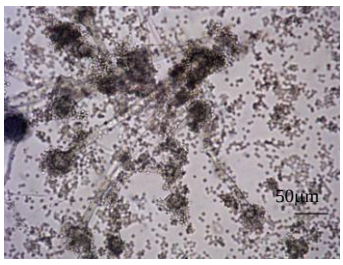

Figure 39 Isolate C12 microscopic characters: MEA 25° C 40x

Macro morphology – Isolate C12 showed dark green colonies on CY20S, CYA and MEA at 25 °C, CY20S the color was more intense, some colonies showed white mycelium and a protuberance. For CY20S and CYA yellow pale pigmentation on the reverse was observed. CY20S olive green pigmentation in the center was observed, on CYA green- brown pigmentation was observed. CYA at 37 °C a grayish colony, yellow reverse was observed. MEA at 25 °C shows light green colonies, on the reverse, yellow with green pigmentation on the center were observed. The colony diameter was 43.5, 45.2, 50.2 and 57.8 mm for CY20S, CYA 25°C, MEA, and CYA 37°C respectively (See Figure 40).

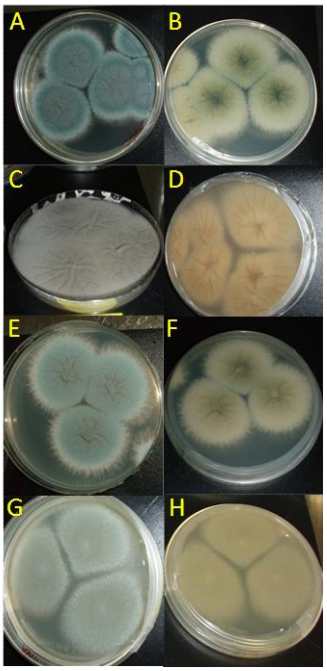

Figure 40 Isolate C11 colonies adverse and reverse.
(A-B) CY20S at 25°C, (C-D) CYA at 37 °C, (E-F) CYA at 25 °C and (G-H) MEA 25 °C after 7 days inoculated.

After inoculation and incubation in selective media for *Aspergillus* we concluded that the isolates described above had the characteristics of *Aspergillus fumigatus*, this conclusion was based on the *Aspergillus* key.

2. Other fungi isolated from composted coffee pulp

The isolates presented below were inoculated on Potato Dextrose Agar (PDA) under slide culture conditions.

2.1. Isolate C03

This isolate showed hyaline hyphae, conidia (arthrospores) formed by segmentation of branches (See figure 41).

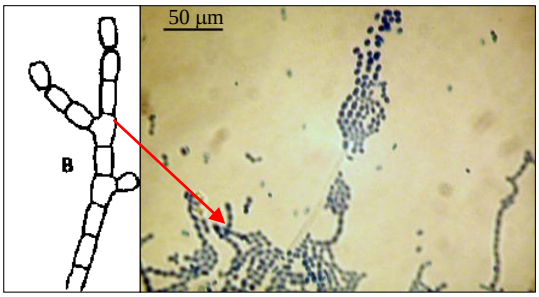

Figure 41 Isolate C03 wet mounting 40 x at 25°C (right) structure from Barnett and Hunter (1986) (left)

Based on the characteristics described above we concluded that the fungus isolate C03 belong to O*idioendron* Robak

2.2. Isolate C05

Isolate C05 shows characteristics described as follows: white mycelium, septate, conidiophores absent, conidia (arthrospores) hyaline, 1-celled, short cylindrical with truncate ends, formed by segmentation of hyphae (see figure 42); are mostly saprophytic, common in soil. Some basidiomycetes form conidia in this manner (Barnett and Hunter, 1986).

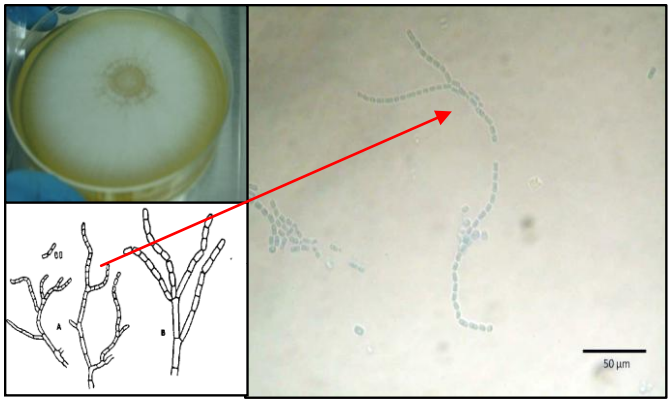

Figure 42 Isolate C05 wet mounting 40 x on PDA (right), culture on PDA at 25°C (upper left) and structure from Barnett and Hunter (1986)(Bottom left)

Based on the characteristics described above we concluded that the fungus isolate C05 belong to *Geotrichum* Link.

2.3. Isolate C06

Isolate C06 shows characteristics described as follows: conidiophores poorly differentiated, much like vegetative hyphae, mostly erect and branching irregularly, hyaline; conidia (aleuriospores or arthrospores) hyaline, 1-celled, globose to pyriform, terminal or intercalary, single or in short chains, usually with a broad basal scar; saprophytic (see figure 43).

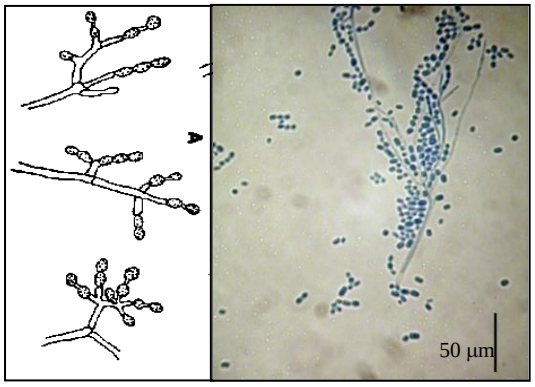

Figure 43 Isolate C06 wet mounting 40 x on PDA
(Right) and structure from Barnett and Hunter (1986) (left)

Based on the characteristics described above we concluded that the fungus isolate C06 belong *Chrysosporium* Corda.

2.4. Isolate C08

Isolate C08 show the characteristics described as follows: hyaline to brown mycelium; conidiophores sparsely branched only on upper portion, rebranched irregularly, branches segmenting into rod-shaped or rounded conidia, remaining in chains; conidia (arthrospores) 1-celled, hyaline or subhyaline (see figure 44); is a saprophytic microorganism.

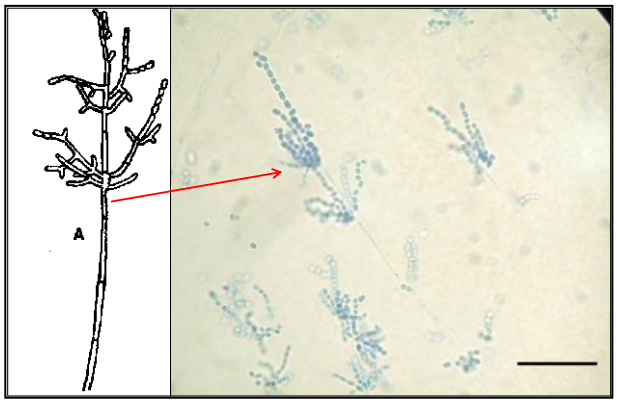

Figure 44 Isolate C08 wet mounting 40 x on PDA at 25 °C
(Right) structure from Barnett and Hunter (1986) (left)

Based on the characteristics described above we concluded that the fungus isolate C08 belong to *Oidiodendron* Robak.

2.5. Isolate C04

Isolate C04 shows the characteristics described as follows: mycelium external on host, white; conidiophores upright, simple; upper portion increases in length as conidia are formed; conidia (meristem arthrospores) cylindrical, 1-celled, hyaline, produced in basipetal chains (see figure 45); this organism is parasitic on higher plants, producing powdery mildews.

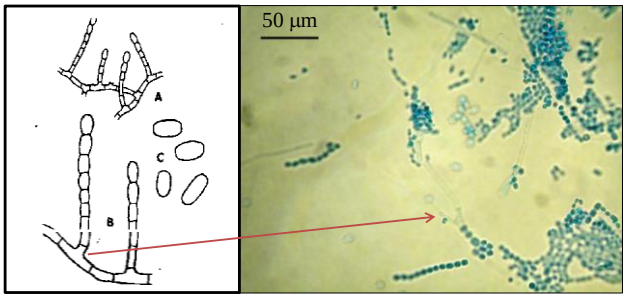

Figure 45 Isolate C04 wet mounting 40 x at 25°C
(Right) structure from Barnett and Hunter (1986) (left)

Based on the characteristics described above we concluded that the fungus isolate C04 belong to *Oidium* Sacc.

2.6. Isolate C09

Isolate C09 shows characteristics described as follows: conidiophores simple, resembling vegetative hyphae elongating slightly at apex as conidia are formed; conidia (arthrospores) globose, with truncate base, hyaline to pale brown, l-celled in simple basipetal chains; saprophytic; *B. rubra* is conidial state of *Monascus rubra* (see figure 46).

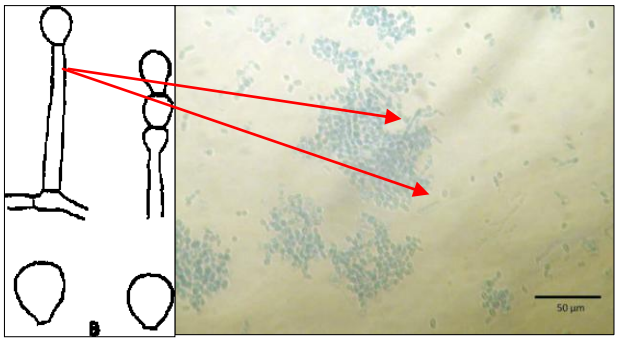

Figure 46 Isolate C08 wet mounting 40 x on PDA at 30 °C
(Right) structure from Barnett and Hunter (1986) (left)

Based on the characteristics described above we concluded that the fungus isolate C08 belong to *Basipetospora* Cole and Kendrick.

2.7. Isolate C07

Isolate C09 shows characteristics described as follows: white mycelium (culture), filamentous at room temperature, yeastlike at 37 °C; conidia (aleuriospores) thick-walled, budding cells (blastospores) found in lesions; pathogenic in man, causing blastomycosis (See figure 47).

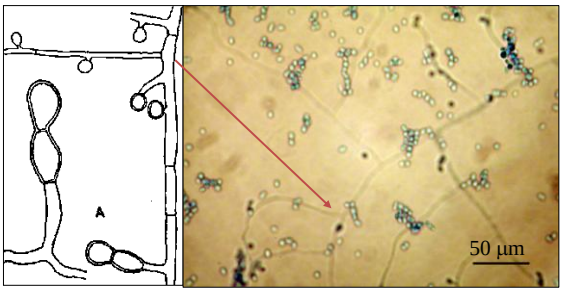

Figure 47 Isolate C07 wet mounting 40 x on PDA at 25 °C
(Right) structure from Barnett and Hunter (1986) (left)

Based on the characteristics described above we concluded that the fungus isolate C07 belong to *Blastomyces* Cost and Roll.

APPENDIX B

Caffeine degradation by *Aspergillus tamari*

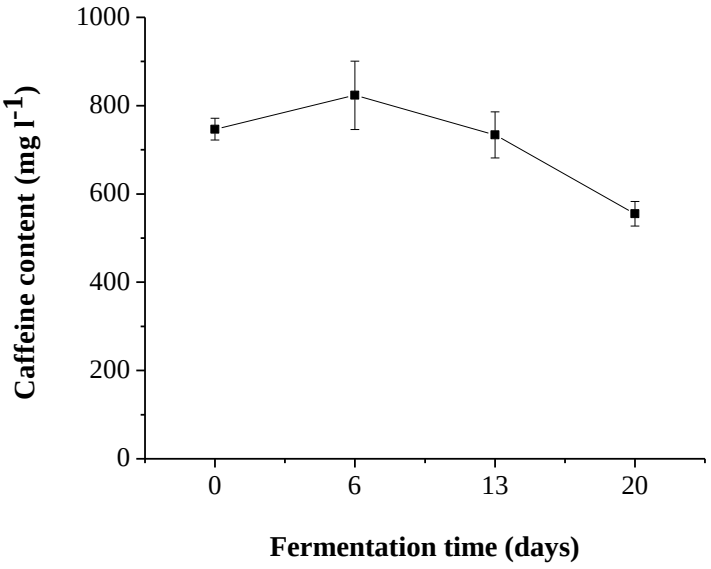

Figure 48 Caffeine content during fermentation of coffee pulp under SSSmF conditions for *Aspergillus tamarii* (6 replicates per time sample).

APPENDIX C

Two-way ANOVA: Caffeine (mg/l) versus Incubation time(days), Fungi minimal media with caffeine

Source	DF	SS	MS	F	P
Incubation time	5	348986	69797	15.46	0.000
Fungi	1	289403	289403	64.11	0.000
Interaction	5	214398	42880	9.50	0.000
Error	24	108333	4514		
Total	35	961120			

S = 67.19 R-Sq = 88.73% R-Sq(adj) = 83.56%

Individual 95% CIs For Mean Based on Pooled StDev

Incubation time	Mean	----------+---------+---------+---------+--
0	1118.84	(---*----)
10	1069.94	(----*----)
20	1059.18	(---*----)
35	978.91	(----*---)
49	1010.80	(---*----)
69	811.61	(----*---)

840 960 1080 1200

Individual 95% CIs For Mean Based on Pooled StDev

Fungi	Mean	----+---------+---------+---------+------
1	918.55	(---*----)
2	1097.87	(----*----)

910 980 1050 1120

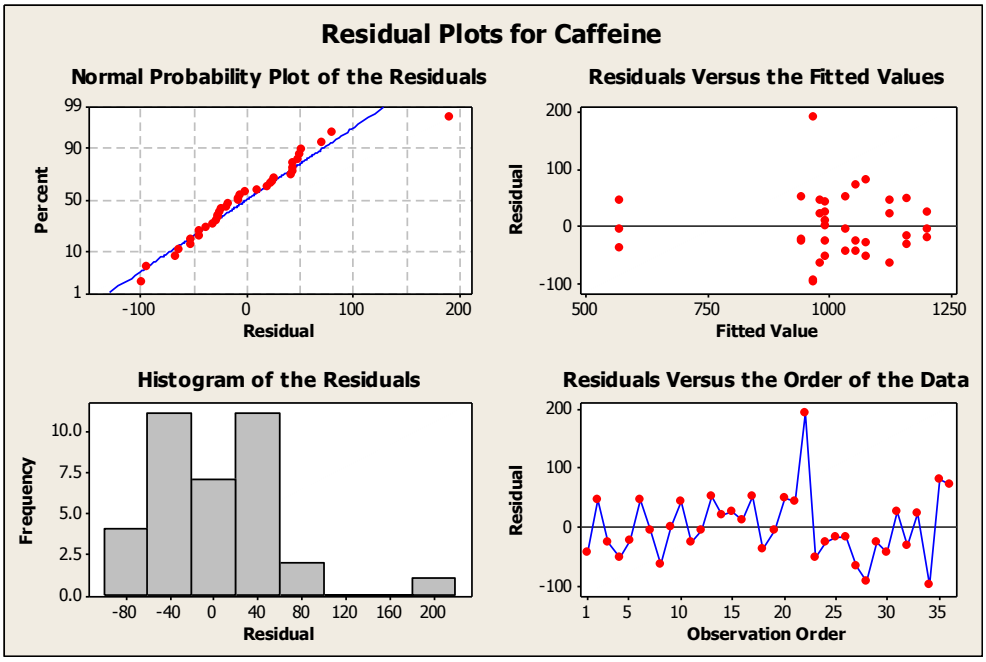

Figure 49 Residual Plots for minimal media (caffeine)

APPENDIX D

Two-way ANOVA: Caffeine (mg/L) versus Incubation time (days), Fungi (Caffeine sucrose)

Source	DF	SS	MS	F	P
Incubation time	5	546886	109377	32.94	0.000
Fungi	1	137303	137303	41.35	0.000
Interaction	5	441160	88232	26.57	0.000
Error	24	79696	3321		
Total	35	1205045			

S = 57.63 R-Sq = 93.39% R-Sq(adj) = 90.36%

Individual 95% CIs For Mean Based on Pooled StDev

Incubation time	Mean	----+---------+---------+---------+-----
0	1150.18	(---*--)
16	977.03	(--*--)
28	1014.64	(---*--)
32	1052.88	(--*--)
41	998.54	(---*--)
69	743.65	(---*--)

----+---------+---------+---------+-----
750 900 1050 1200

Individual 95% CIs For Mean Based on Pooled StDev

Fungi	Mean	+---------+---------+---------+---------
1	927.73	(-----*----)
2	1051.24	(----*-----)

+---------+---------+---------+---------
900 950 1000 1050

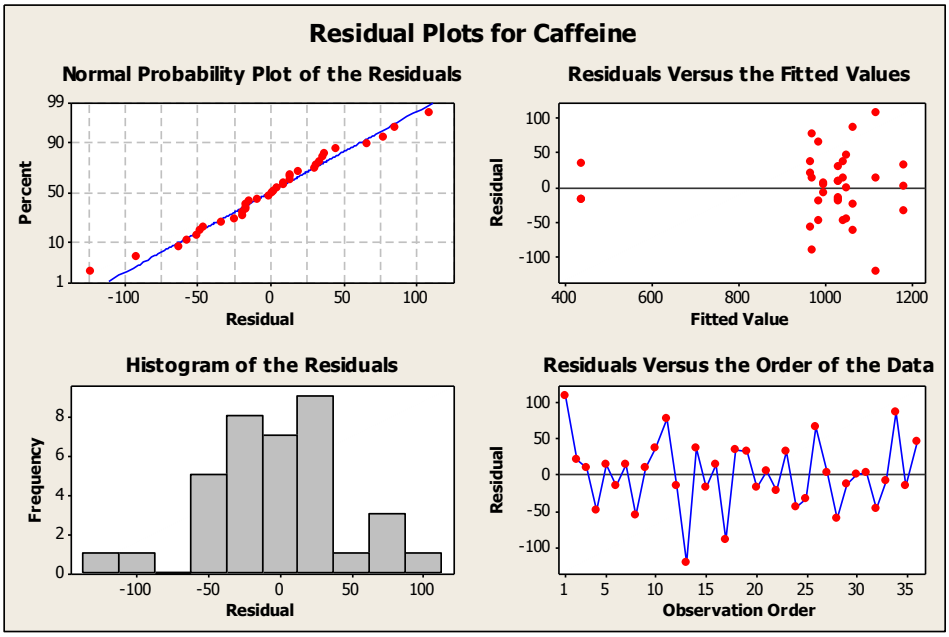

Figure 50 Residual Plots for minimal media (caffeine-sucrose)