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Caffeine degradation in solid state fermentation by *Aspergillus tamarii*: effects of additional nitrogen sources

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Abstract

The ability of *Aspergillus tamarii* V12A25 to use caffeine as sole nitrogen source was investigated in solid state fermentation (SSF) using two different supports, polyurethane foam (PUF) and sugarcane bagasse. Caffeine can be used in SSF as the sole nitrogen source, the carbon source being saccharose. If a simpler nitrogen source (ammonium sulphate, urea) is added to the medium containing caffeine, this source will be used first allowing fungal growth. If saccharose is still present when the simple nitrogen source has been degraded, caffeine will be used as the nitrogen source, together with saccharose. Caffeine at a concentration of 8 g/l has no effect on fungal growth when a simple nitrogen source is not limiting. The main difference between the two solid supports was the time required to complete the fermentation. With sugarcane bagasse as the support, substrates (caffeine, ammonium sulphate, urea) were degraded almost twice as fast as with PUF as the support. Theophylline and 3-methylxanthine, the major products from caffeine degradation, disappeared completely from the culture medium shortly after caffeine degradation stopped. The major advantage of using PUF support over sugar cane bagasse is that the former allows determination of fungal biomass which is not possible with the latter.

Keywords: Caffeine; Degradation; *Aspergillus*; Polyurethane; Sugarcane bagasse; Solid state fermentation

1. Introduction

For over 20 years, attempts have been made to detoxify by-products of the coffee industry, such as coffee pulp, in order to use it as a source of animal feed [1,2]. One aspect of this detoxification is the removal of caffeine, which has anti-physiological effects on animals. Caffeine (Fig. 1) can be degraded by various microorganisms including bacteria [3], yeast [4] and fungi [5]. Decaffeination of coffee pulp was successfully carried out [6,7] using a solid state fermentation (SSF) process with fungal strains. Coffee pulp was both support and substrate for the fermentation as reported by Roussos et al. [6] and Perraud-Gaime [7]. These demonstrated that total degradation of caffeine in coffee pulp was possible. Moreover, Roussos et al. [6] have reported on the effect of addition of simple nitrogen sources (urea, ammonium) on caffeine degradation. At a concentration of 3.75 g/l, ammonium sulphate completely inhibited caffeine degradation in coffee pulp. However, in all fermentation studies involving caffeine degradation with coffee pulp, no particular attention was given to its degradation products.

Theophylline (1,3-dimethylxanthine) is the major degradation product of caffeine by various filamentous fungi grown in liquid medium [5] resulting from a demethylation of caffeine in position 7 (Fig. 1). Theophylline may still be present at the end of fermentation when caffeine has been totally removed, which poses the problem of theophylline toxicity. Lethal doses (LD_{50}) of caffeine and theophylline have been reported to be 200 and 206 mg/kg, respectively, in rat [8]. The presence of theophylline in the fermented coffee pulp would be as undesirable as the presence of caffeine.

Coffee pulp is a complex substrate and its composition varies depending on its origin [9,10]. It contains nitrogen that could inhibit the degradation of caffeine.

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To solve this problem, it was decided to monitor caffeine degradation in SSF by a fungal strain using specific supports impregnated with defined media in order to study the effect of nitrogen additions interfering with caffeine degradation. Such fermentations enabled the definition of the exact concentrations of all elements present in the growth medium in order to study and understand the physiology of decaffeination. Two different supports that have been previously used in SSF experiments were chosen. The first, sugarcane bagasse, has been used for years as a support for SSF using fungal strains in order to produce secondary metabolites [11], enzymes [12] or biomass [13]. The second support, polyurethane foam, has recently been used by a few groups [14–16] to produce enzymes (nuclease, glucose oxidase) or metabolites (lactic acid, citric acid). This support seems to have some advantages when compared to the previous support. The main advantage is the ability to measure biomass easily. Polyurethane foam is also an ideal support to grow *Aspergillus tamarii* in order to better understand, on a laboratory scale, the physiology of decaffeination. These two different supports impregnated with different liquid media were used to study caffeine degradation by *A. tamarii* V12A25. Effects due to the addition of a simple nitrogen source were evaluated. Degradation kinetics and biomass production were monitored. Special attention was given to caffeine and theophylline degradation.

2. Materials and methods

2.1. Microorganism

The *A. tamarii* strain V12A25 used in this study was obtained from the IRD-UAM collection [17] and was selected because of its ability to use caffeine as a sole source of nitrogen [7,18].

2.2. Culture media

The fungal strain was maintained on coffee infusion medium (CIM) prepared as follows: An infusion of commercial ground coffee (Grand'Mère 'familial', café Grand'Mère S.A., Wattinities, France) was prepared by adding the ground coffee to distilled water (40 g/l). The solution was then brought to boiling point under agitation then cooled and filtered through a commercial coffee filter. Caffeine, 0.5 g; sucrose, 2.0 g; KH_2PO_4, 1.3 g; $Na_2HPO_4.2H_2O$, 0.12 g; $MgSO_4.7H_2O$, 0.3 g; and $CaCl_2.2H_2O$, 0.3 g were then dissolved in the filtered infusion. The pH was adjusted to 5.6 with 1M KOH and the volume was brought to 1 l. The medium was sterilized at 121°C for 20 min after supplementation with 20 g agar.

Solid fermentations were performed with five different media. The basic medium contained (g/l) saccharose, 66; KH_2PO_4, 9.28; $MgSO_4.7H_2O$, 0.57; and $FeSO_4.7H_2O$, 0.75. Different nitrogen sources were added to this medium as shown in Table 1.

2.3. Cultures

Spores of 6 day-old cultures on CIM were harvested with 30 ml distilled water containing 0.2% Tween (w/v) under agitation provided by a magnetic stir bar (100 rpm). Liquid medium to be added to each SSF support was inoculated with the spore suspension at 6.6×10^6 spores/ml.

2.4. Polyurethane foam (PUF) cultures

Inoculated medium (7 ml) was added to flasks containing 0.4 g PUF (17 kg/m^3; PUF cubes: $5 \times 5 \times 5$ mm). The flasks were shaken to allow medium adsorption and incubated at 30°C. At selected times, the PUF was compressed in a 50 ml syringe to recover extracellular fluids. After filtration at 0.22 μm the extracts were stored at −20°C. The PUF was washed three times by water re-adsorption and dried at 90°C to a constant weight. The calculated biomass is the difference between initial and final PUF weight.

2.5. Sugarcane bagasse cultures

Inoculated medium was added to sterile sugarcane bagasse to reach a water content of 68%. After homogenization, inoculated bagasse was introduced in sterile flasks (11.2 g/flask). These flasks were incubated at 30°C along with the PUF containing flasks. At selected times the samples were harvested, 10 ml water was

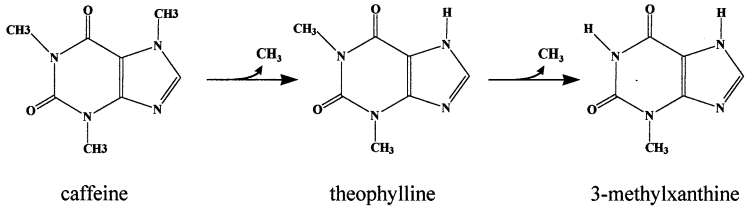

Fig. 1. First steps in the degradation pathway of caffeine by *A. tamarii*.

Table 1
Nitrogen source concentration of different fermentation media and growth characteristics in SSF

Medium	1	2	3	4	5
Caffeine (g/l)	8	8	0	0	8
Ammonium sulphate (g/l)	0	11.9	11.9	0.4	0.4
Urea (g/l)	0	1.4	1.4	0	0
N from caffeine (%)	100	42.5	0	0	96.5
μ Biomass (per h)	0.35	0.25	0.21	0.15	0.35
X_{max} (mg DCW/g PUF)	564	300	325	74	540

added to the sugarcane bagasse to allow recovery of extracellular fluids. After homogenization, the bagasse was compressed in a 50 ml syringe for medium recovery. After filtration (0.22 μm) the extracts were stored at −20°C for further analysis. In the present case, syringe compression did not allow complete medium recovery, but saccharose was directly measurable after recovery. A standard curve for caffeine extraction from sugarcane bagasse was set up.

2.6. Kinetic models of fermentations

Evolution of biomass, $X = X(t)$ was followed by the Velharst–Pearl or logistic equation:

$$dX/dt = \mu(1 - X/X_{max})X \quad (1)$$

where μ is the specific growth rate and X_{max} is the maximal value of X. Integration of Eq. (1) yields the following explicit relation:

$$X = X_{max}/(1 + (X_{max} - X_0)/X_0)\ e\ (-\mu t))$$

where X_0 is the initial biomass ($t = 0$). In order to estimate the parameters X_0, X_{max} and μ, a non linear regression routine was used (Solver algorithm in Excel 6.0 data sheet).

For the five media, correlation coefficients between calculated and experimental curves were at least equal to 0.985. All biomass units are in mg of dry cell weight/g PUF (mg DCW/g PUF).

2.7. HPLC analysis of methylxanthines

2.7.1. Chromatographic analysis conditions

HPLC analyses were performed on a Beckman GOLD system with a 116 pump, a 507 autosampler with a 100 μl injection loop, and a 168 detector. Compounds were separated on a C18 (5 μm) Ultrasphere column (1.6 mm × 25 cm) at room temperature with a flow rate of 2 ml/min, by a gradient elution system. The eluent was 1.75 mM KH_2PO_4, acetonitrile and tetrahydrofurane (98:1:1; v/v/v) for pump A and acetonitrile–tetrahydrofurane (99:1; v/v) for pump B. Solvent A was used first for 7 min before starting a linear gradient reaching 20% of solvent B in 5 min. Methylxanthines were identified by comparison of retention times with those of the pure products and quantified by comparison to standard curves established for each methylxanthine.

2.7.2. Sample preparation

Samples were diluted in solvent A in order to obtain a concentration in the range of the standard curves of each methylxanthine.

2.8. Sugar determination

Sugar content was determined by a colorimetric method using an anthrone based assay [19].

2.9. Ammonium sulphate determination

Determination of ammonium sulphate content was based on 'Berthelot Determination' (SIGMA diagnostics, procedure no. 640).

3. Results

A fungal strain of *A. tamarii* V12A25 was grown on different solid supports (sugarcane bagasse and polyurethane foam) in order to study the parameters controlling caffeine degradation. High and low levels of simple nitrogen sources were used (Table 1) with or without caffeine to determine their effects on fungal growth and caffeine degradation. In all experiments, the carbon source was saccharose as *A. tamarii* is unable to grow on caffeine as the sole carbon and nitrogen source.

3.1. Caffeine as sole nitrogen source

When caffeine was used as the sole nitrogen source (medium 1, Table 1), both saccharose and caffeine were completely degraded in 54 h when the fungus was grown on PUF and 27 h when it was grown on bagasse (Fig. 2). This corresponded to the time of maximum biomass production observed on PUF where the biomass could be directly measured. This maximum corresponded to 564 mg DCW/g PUF. The specific growth rate (μ) was 0.35/h. Biomass from the spores

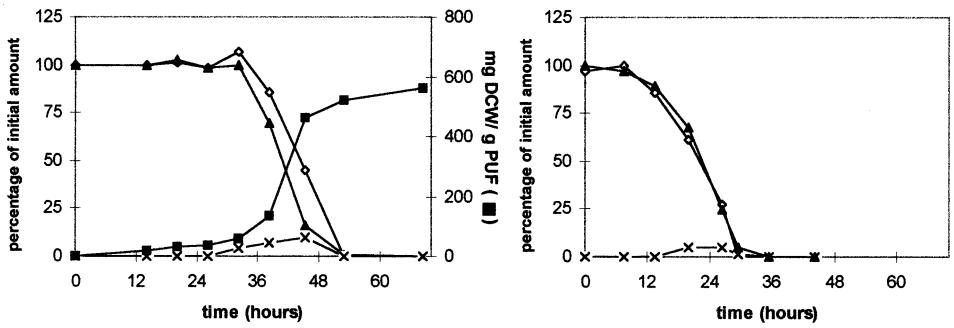

Fig. 2. Growth of *A. tamarii* on solid supports using medium 1. Nitrogen source: caffeine 8 g/l. (A) PUF; (B) sugarcane bagasse: ◇, saccharose; ▲, caffeine; ■, biomass; ×, theophylline; and ×, 3-methylxanthine. Theophylline and 3-methylxanthine are represented as a percentage of the initial caffeine concentration. The first two products of caffeine degradation are present over time in very similar amounts.

was not completely taken into account when calculating the total biomass as the spores were washed off prior to biomass determination. Biomass was not directly measurable in SSF with bagasse as the support. During caffeine degradation, theophylline (1,3-dimethylxanthine) and 3-methylxanthine were the main products formed but there were also traces of paraxanthine and theobromine. At their highest, theophylline and 3-methylxanthine each represented almost 10% of the initial caffeine concentration. Intermediary products of caffeine degradation disappeared completely from the culture medium a few hours after the caffeine disappeared.

3.2. Caffeine with different levels of urea–ammonium as nitrogen source

The profiles of caffeine degradation depended on the presence of simple nitrogen sources. In fermentations on medium 2 using PUF as the support (Fig. 3), 57.5% of the nitrogen was provided by urea and ammonium. The specific growth rate (μ) was 0.25/h. Caffeine was not degraded whereas saccharose was degraded within 42 h, the time at which biomass reached a maximum of 300 mg DCW/g (Fig. 3). No sporulation was observed. The experiment was carried out with sugarcane bagasse as the support and caffeine was not degraded (data not shown). As caffeine was not used by the fungus in this medium, fermentation was carried out with the same concentrations of simple nitrogen sources but without caffeine (Table 1, medium 3). The calculated μ and maximum biomass for this fermentation were 0.21/h and 325 mg DCW/g PUF, respectively, indicating that no differences appeared between fermentations with medium 2 and 3. If urea and ammonium were present in the culture medium and allowed complete saccharose degradation, the presence of caffeine at a concentration of 8 g/l had no effect on biomass growth. If a low concentration of ammonium sulphate was used and no caffeine was present (medium 4), the nitrogen source was rapidly depleted by the fungus, but only about 10% of the saccharose was degraded and biomass reached only 74 mg DCW/g with a μ of 0.15/h (Fig. 4). Nitrogen was therefore the limiting factor. When caffeine was added to this medium (medium 5), fermentations followed the same time course as in medium 1 in which no ammonium sulphate was present. The biomass then reached a maximum of 540 mg DCW/g PUF with a μ of 0.35/h (Fig. 5). The presence of ammonium sulphate at this concentration (0.4 g/l) did not change the time required for complete degradation of caffeine and saccharose.

4. Discussion

When simple nitrogen sources were present in quantities high enough to allow complete saccharose degradation (medium 2), caffeine was not used by the fungus. However, if the simple nitrogen source was not in sufficient quantity to allow maximal assimilation of the carbon source (medium 5, Table 1), caffeine was degraded after the primary nitrogen source had disappeared (Fig. 5). Results in the present study confirmed those obtained by Denis [18] who showed, in liquid cultures, that caffeine degradation by fungi was inhibited by the presence of ammonium sulphate. In the

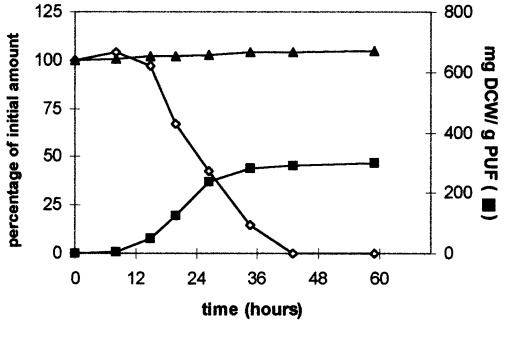

Fig. 3. Growth of *A. tamarii* on PUF using medium 2. Nitrogen source: caffeine 8 g/l, ammonium sulphate 11.9 g/l, urea 1.4 g/l. ◇, Saccharose; ▲, caffeine; and ■, biomass.

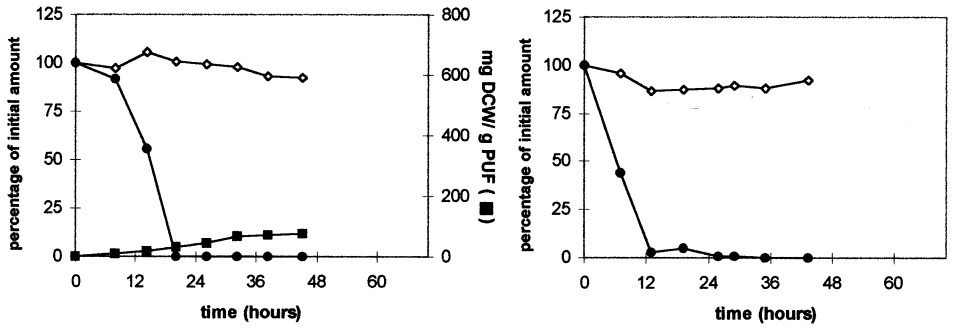

Fig. 4. Growth of *A. tamarii* on solid support using medium 4. Nitrogen source: ammonium sulphate 0.4 g/l. (A) PUF; (B) sugarcane bagasse: ◇, saccharose; ●, ammonium sulphate; and ■, biomass.

present study, we observed that caffeine degradation was completely inhibited in the presence of high concentration of urea and ammonium sulphate (Fig. 3). There was no apparent inhibition of caffeine degradation when ammonium sulphate was present at a concentration allowing its rapid degradation (Fig. 5).

Aguilar [20], Peñaloza et al. [1] and Roussos et al. [6] observed an inhibition of caffeine degradation by different fungal strains in the presence of inorganic nitrogen sources in SSF using coffee pulp as the substrate. This inhibition seems to be a function of the ratio of the available carbon source to the nitrogen source present and is not an irreversible inhibition, caffeine being degraded when the inorganic nitrogen source disappeared. From both the results of Roussos et al. [6] and the present study, it would seem logical that no exogenous nitrogen source should be added in order to degrade the caffeine present in coffee pulp.

Roussos et al. [6] and Perraud-Gaime [7] demonstrated that complete caffeine degradation in coffee pulp was possible, but none of the degradation products were characterized. In liquid fermentation the main products formed by *A. tamarii* were theophylline and 3-methylxanthine [5]. Theophylline was still present in liquid cultures more than 12 h after the caffeine completely disappeared. Theophylline has been reported to be even more toxic than caffeine. For example, theophylline has a greater potential to cause central nervous system seizures and is significantly more active than caffeine in cardiac stimulation or coronary dilatation [21]. This is why it seemed important to verify the theophylline levels during caffeine degradation in SSF. In the present study on solid supports, theophylline was rapidly degraded and totally removed from the culture medium a few hours after caffeine degradation stopped (Figs. 2 and 5). This is particularly important if, as in the studies cited, the main goal in coffee pulp detoxification is the removal of caffeine and its degradation products, mainly the ones with anti-physiological effects, in order to use it as animal feed.

Solid fermentations were also much faster than liquid fermentations with regards to caffeine degradation. In liquid cultures, caffeine at 5 g/l was not totally degraded after more than 100 h [18] whereas, at a concentration of 8 g/l, caffeine was totally degraded in 54 and 27 h in SSF using PUF and sugarcane bagasse, respectively. Increasing concentrations of caffeine present in liquid fermentations were correlated with a decrease in the ability to degrade caffeine. At 10 g/l no caffeine degradation was observed [18]. No such inhibitions seemed to take place in solid fermentation at these concentrations.

Fermentation kinetics using two different supports were compared. The major difference between fermentation using PUF or sugarcane bagasse as the support was that the kinetics were almost twice as fast for the former compared with the latter support. In a preliminary experiment, medium 1 (Table 1) was used to set up fermentation with bagasse as the support in columns with forced aeration as described by Raimbault et al. [22] and Saucedo-Castañeda [23]. Results of these fermentations showed that caffeine and saccharose were degraded within the same timeframe in columns and in Erlenmeyer flasks (data not shown) leading to the conclusion that aeration was not a limiting factor for fermentation on sugarcane bagasse in flasks, whereas it could be with polyurethane.

The major advantage of fermentations with PUF as support is that the biomass could be directly measured [24]. The biomass was not directly measurable in SSF with bagasse as the support. Additionally, recovery of culture media was much easier when *A. tamarii* was grown on PUF because of the proprieties of the support in which only water is adsorbed, whereas media is absorbed on sugarcane bagasse. Moreover, caffeine extraction from sugarcane bagasse was difficult and a standard curve had to be established for caffeine recovery from this support. This may be due to hydrophobic interactions between caffeine and bagasse. This problem was not encountered when caffeine was extracted from PUF and could be a great advantage in physiological studies where fermentation products have to be analyzed or if extracellular proteins have to be recovered.

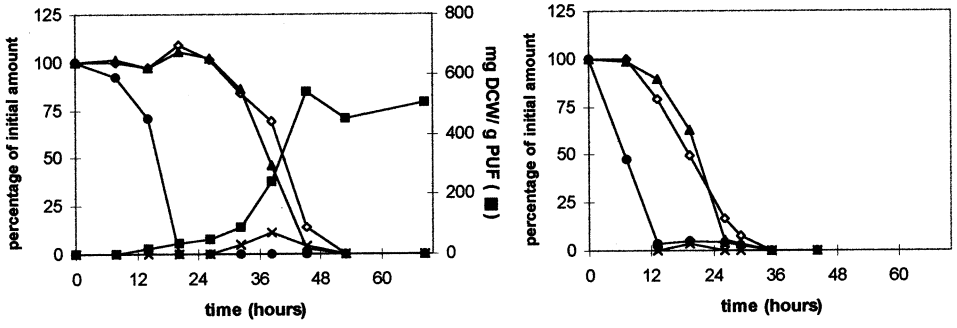

Fig. 5. Growth of *A. tamarii* on solid supports using medium 5. Nitrogen source: caffeine 8 g/l. (A) PUF; (B) sugarcane bagasse: ◇, saccharose; ▲, caffeine; ■, biomass; ●, ammonium sulphate; ×, theophylline; and ×, 3-methylxanthine. Theophylline and 3-methylxanthine are represent as a percentage of the initial caffeine concentration. The first two products of caffeine degradation are present over time in very similar amounts.

PUF is an inert carrier [14] of known composition, whereas sugarcane bagasse contains products that could interact with microorganisms (residual sugars, cellulose, hemicellulose, lignin). Furthermore, PUF can be used repeatedly [24] whereas fermentation with sugarcane bagasse generates unusable solid waste. In PUF fermentations, another advantage of using polyurethane is that it can easily be ground in liquid nitrogen for extraction of intracellular enzymes such as those that degrade caffeine [18]. Interactions between such enzymes and the support should be less than with sugarcane bagasse, a support that is hard to grind. Extraction of caffeinase enzymes from SSF using sugarcane bagasse as the support have been attempted but no enzyme activity was recovered. For this purpose, fermentations using PUF as the support should be a good alternative. Studies are presently underway to characterize caffeinase enzymes from fungi grown on PUF.

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References

[1] Peñaloza W, Molina M, Gomez R, Bressani R. Solid state fermentation, an alternative to improve the nutritive value in coffee pulp. Appl Environ Microbiol 1985;49:388–93.
[2] Bressani R. Coffee pulp: composition, technology, and utilisation. Publication 108e, Ottawa: International Development Research Centre, 1979:83–88.
[3] Asano Y, Komeda T, Amada H. Microbial production of theobromine from caffeine. Biosci Biotech Biochem 1993;57(8):1286–9.
[4] Sauer M, Kappeli O, Fietcher A. Comparison of the cytochrome P-450 containing monooxygenase originating from two different yeasts. Develop Biochem 1982;23:452–7.
[5] Hakil M, Denis S, Viniegra-Gonzalez G, Augur C. Degradation and product analysis of caffeine and related dimethylxanthines by filamentous fungi. Enzyme Microb Technol 1998;22:355–9.
[6] Roussos S, Hannibal L, Aquiahuatl MA, Hernandez MRT, Marakis S. Caffeine degradation by *Penicillium verrucosum* in solid state fermentation of coffee pulp—critical effect of additional inorganic and organic nitrogen sources. J Food Sci Technol 1994;31(4):316–9.
[7] Perraud-Gaime I. Cultures mixtes en milieu solide de bactéries lactiques et de champignons filamenteux pour la conservation et la décaféination de la pulpe de café. PhD Thesis, Université de Montpellier II, Montpellier, France, 1995:209.
[8] Starvic B. Methylxanthines: Toxicity to humans. 3: theobromine, paraxanthine and the combined effects of methylxanthines. Food Chem Toxicol 1988;26(8):725–33.
[9] Aguirre BF, La utilización industrial del grano de café y de sus subproductos, vol. 1. Guatemala: Invest Tecnol ICAITI, 1966:1–43.
[10] Zuluaga J, Bonilla C, Quijano RM. Contribución al estudio y utilización de la pulpa de café. In: 7th Col Int Quem Cafe, Hambourg: ASIC, 1975:233–242.
[11] Sarhy-Bagnon V, Lozano P, Pioch D, Roussos S. Coconut-like aroma production by *Trichoderma harzianum* in solid state fermentation. In: Roussos S, Lonsane BK, Raimbault M, Viniegra-Gonzalez G, editors. Advances in Solid State Fermentation. Montpellier, France: Kluwer, 1997:379–91.
[12] Roussos S, Raimbault M, Viniegra-Gonzalez G, Saucedo-Castaneda G, Lonsane BK. Scale-up of cellulases production by *Trichoderma harzianum* on a mixture of sugarcane bagasse and wheat bran in solid state fermentation system. Micol Neotrop Appl 1991;4:83–98.
[13] Gonzalez-Blanco P, Saucedo-Castaneda Gand, Viniegra-Gonzalez G. Protein enrichment of sugar cane by -products using solid-state cultures of *Aspergillus terreus*. J Ferment Bioeng 1990;70:351–4.
[14] Zhu Y, Smits JP, Knol W, Bol J. A novel solid-state fermentation system using polyurethane foam as inert carrier. Biotechnol Lett 1994;16:643–8.
[15] Haapala R, Parkkinen E, Suominen Pand, Linko S. Production of extracellular enzymes by immobilized *Trichoderma reesei* in shake flask cultures. Appl Microbiol Biotechnol 1995;43:815–21.
[16] Dong X-Y, Sun SBY. Production of L(+)-lactic acid with *Rhizopus oryzae* immobilized in polyurethane foam cubes. Biotechnol Lett 1996;18:225–8.

[17] Aquiahuatl MA, Raimbault M, Roussos S, Trejo-Hernandez MR. Coffee pulp detoxification by solid state fermentation: isolation, identification and physiological studies. In: Raimbault M, editor. Proceedings of the Seminar Solid State Fermentation in Bioconversion of Agroindustrial Raw Materials. Montpellier, France: ORSTOM, 1988:13–26.

[18] Denis S. Dégradation de la caféine par *Aspergillus* sp. et *Penicillium* sp. Etude physiologique et biochimique. PhD Thesis, Université de Montpellier II, Montpellier, France, 1996.

[19] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–6.

[20] Aguilar M. Estudios sobre la utilización de carbohidratos, por *Aspergillus niger* en sistemas de fermentación sólida, usando pulpa de café como sustrato y diferentes niveles de nitrógeno. PhD Thesis, Universidad de San Carlos de Guatemala, Guatemala, 1983.

[21] Starvic B. Methylxanyhines: toxicity to humans. 2: caffeine. Food Chem Toxicol 1988;26:645–62.

[22] Raimbault M, Roussos S, Oriol E, Barrios J, Gutierrez-Rojas M, Viniegra-Gonzalez G. Procédé de culture de micro-organismes sur supports solides imprégnés, en vue de la production et de l'extraction d'enzymes et de métabolites. Patent No. 89.06558, 1989.

[23] Saucedo-Castañeda G. Contrôle du métabolisme de *Schwanniomyces castelli* cultivé sur support solide. PhD Thesis, Université de Montpellier II, Montpellier, France, 1991.

[24] Zhu Y, Knol W, Smits JP, Bol J. Medium optimization for nuclease P1 production by *Penicillium citerinum* in solid-state fermentation using polyurethane foam as inert carrier. Enzyme Microb Technol 1996;18:108–12.