Effect of the nitrogen source on caffeine degradation by *Aspergillus tamarii*

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ABSTRACT

G. GUTIÉRREZ-SÁNCHEZ, S. ROUSSOS AND C. AUGUR. 2003.

Aims: To evaluate caffeine degradation and nitrogen requirements during *Aspergillus tamarii* growth in submerged culture.

Methods and Results: *Aspergillus tamarii* spores produced on a coffee infusion agar medium added with sucrose were used. Several caffeine and ammonium sulphate concentrations (0–1 and 0–1·36 g l^{-1}, respectively) were tested simultaneously on fungal biomass production and caffeine degradation. An additional caffeine pulse (4 g l^{-1}) was added for all experiments after 48 h of fermentation. Results revealed that when using 0·90 g l^{-1} of caffeine and 0·14 g l^{-1} of ammonium sulphate, biomass production and caffeine degradation were enhanced. Highest biomass production ($X_{max} = 9·87$ g l^{-1}) with a specific growth rate (μ) of 0·073 h^{-1} and caffeine degradation rate of 0·033 g l^{-1} h^{-1}, was observed under these conditions.

Conclusions: Caffeine degradation as well as biomass production were characterized.

Significance and Impact of the Study: These studies set the stage for future characterization studies of intracellular enzymes involved in caffeine degradation. Moreover, results observed may help in the biotreatment of residues from the coffee agroindustry.

Keywords: biodegradation, caffeine, filamentous fungus, nitrogen source, purine.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a major pharmacologically active ingredient in coffee. This purine is a stimulant of the central nervous system and occurs naturally in leaves, seed or fruit of more than 60 plant species, including cocoa beans, tea leaves, kola nuts, guarana seeds, mate and coffee beans (Steffen 2000).

The presence of caffeine in coffee agroindustry residues is one of the major limiting factors in the use of these by-products as animal feed, because of antiphysiological effects. These effects include stimulation of the central nervous system, muscle contraction (heart and blood vessels), as well

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as diuretic effects (Mazzafera 2002). The presence of caffeine in waste products such as coffee pulp, have hindered its transformation into a higher added value co-product. During the last 3 years, the total quantity of coffee pulp produced in Latin America has reached 1 million metric tons per year (Gutiérrez-Sánchez 2000). In the last two decades, many attempts have been made to give coffee pulp an added value. This agroindustrial residue is rich in carbohydrate but also contains around 10% protein (Elías 1979; Zuluaga 1981). According to Bressani (1979) it should be possible to use coffee pulp as a component of mixed livestock feed replacing cereal grains, frequently imported to Latin America. However, so far, the use of coffee pulp has had limited success, primarily because of its content of specific anti-physiological compounds such as caffeine and tannins (Roussos *et al.* 2000; Mazzafera 2002) interfering with food intake and nutrient absorption (Bressani 1979;

Vargas *et al.* 1982). Therefore, there is a need for new technologies that can detoxify coffee pulp and at the same time, improve the nutritional quality of the final product. Microbial decaffeination could be a good alternative. However, literature on the subject is scarce and limited (Ina 1971; Gomez-Brenes *et al.* 1985; Perraud 1995; Roussos *et al.* 1995; Sideso *et al.* 2001). Although degradation of caffeine within coffee pulp by solid-state fermentation system has been reported (Roussos *et al.* 1994; Perraud 1995; Hakil *et al.* 1999), the addition of a mineral nitrogen source has been shown to affect caffeine degradation (Roussos *et al.* 1994; Hakil *et al.* 1999).

Fungal demethylases are known intracellular enzymes (Denis 1996; Hakil 1999) and biomass production is a bottleneck for the purification of demethylases because of toxicity of the nitrogen source, namely caffeine. However, Denis (1996) proposed the use of ammonium sulphate in submerged culture, enhancing caffeine degradation, with limited biomass production at initial caffeine concentration as low as 1 g l^{-1}.

In order to enhance biomass production and circumvent the toxicity effect of the alkaloid, a two-step fermentation process was tested using different caffeine/ammonium sulphate ratios. After 48 h of culture a pulse of caffeine was added. Degradation kinetics and biomass production were monitored.

MATERIALS AND METHODS

Micro-organism and media

Aspergillus tamarii V12A25 strain was from the IRD–UAM [Institut de Recherche pour le Développement (France)– Universidad Autónoma Metropolitana (Mexico)] collection (Roussos *et al.* 1995) and was selected because of its ability to grow in the presence of caffeine (Hakil *et al.* 1998). The strain was maintained on coffee infusion agar medium (CIAM) at 4°C, as described by Denis (1996). CIAM medium was prepared as follows (g l^{-1}): a coffee infusion was prepared with ground coffee (40); and sucrose (2·0), KH$_2$PO$_4$ (1·3), Na$_2$HPO$_4$·7H$_2$O, (0·19), MgSO$_4$ (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 121°C for 20 min.

Culture conditions

Spores of 6-day-old cultures grown on CIAM were harvested as follows: 30 ml sterile distilled water containing 0·01% Tween 20 (w/v) was added to the CIAM culture flasks and spores were gently suspended with a magnetic stirrer.

First stage. Erlenmeyer flasks containing 50 ml of liquid media (described below) were inoculated with the spore suspension at 10^6 spores ml^{-1} and incubated at 30°C in an orbital shaker at 110 rev min^{-1}. The growth medium was essentially as described by Denis (1996) containing (g l^{-1}): sucrose (25), KH$_2$PO$_4$ (1·3), MgSO$_4$ (0·15); and 10 ml of a trace element solution consisting of (g l^{-1}): FeSO$_4$· 7H$_2$O (3), ZnSO$_4$·7H$_2$O (2·2), MnSO$_4$·H$_2$O (1·54), (NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O (0·37), CaCl$_2$ (0·15), CuSO$_4$ (0·1) and CoCl$_2$·6H$_2$O (0·4). The medium was supplemented with nitrogen sources as shown in Table 1. The pH was adjusted to 4 with H$_2$SO$_4$. The medium was sterilized at 121°C for 15 min.

Second stage. After 48 h of culture, a pulse of caffeine was added by sterile injection of a concentrated caffeine solution (4 g l^{-1}) to each flask. Sampling for each of the two stages was carried out every 24 h. Each experiment was carried out in duplicate and the average values are reported as mean values.

Biomass evaluation

Fungal biomass was measured gravimetrically by filtering the cell mass through a Whatmann no. 41 filter paper. The mycelium was washed three times with 0·9% NaCl solution (w/v) and samples were dried to constancy at 60°C for at least 24 h. Biomass concentration is reported as grams of dry weight per litre of culture medium (g l^{-1}).

Substrate evaluation

Sucrose concentration was estimated by the phenol–sulphuric acid assay described by Dubois *et al.* (1956).

Ammonium sulphate concentration was analysed by a Sigma Kit, based on 'Berthelot Determination' (Kit no. 640; Sigma Chemicals Co., St Louis, MO, USA).

Caffeine analysis was performed on a HPLC Waters Millennium (Milford, MA, USA) system with a 626 pump, a 717 plus auto sampler with 100 µl injection loop, a 600s controller and a 996 PDA detector. Methylxanthines were separated on a prodigy column (C18, 5 µm, 240 × 4.6 mm)

Table 1 Initial nitrogen source concentrations for *Aspergillus tamarii* growth prior to caffeine pulse

	Culture media				
Nitrogen source	1	2	3	4	5
Caffeine (g l^{-1})	1	0·90	0·50	0·03	0
(NH$_4$)$_2$SO$_4$ (g l^{-1})	0	0·14	0·69	1·32	1·36
% of N from caffeine	100	90	50	10	0

from Phenomenex (Torrace, CA, USA) at room temperature with a flow rate of 1.5 ml min^{-1} by a gradient elution system. The mobile phase used was the one described by Denis *et al.* (1998). Samples were diluted in deionized water in order to obtain a concentration within the range of the standard curve.

Growth parameters estimation

Evolution of biomass, estimated as X (g l^{-1}) was followed by the Velhurst-Pearl or logistic equation logistic equation:

$$\frac{dX}{dt} = \mu\left(1 - \frac{X}{X_{max}}\right)X \qquad (1)$$

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

$$X = \frac{X_{max}}{\{1 + [(X_{max} - X_0)/X_0]e^{(-\mu t)}\}} \qquad (2)$$

where X_0 is the biomass ($t = 0$). A nonlinear regression routine was used based upon the Solver algorithm (Excel 2000 data sheet; Microsoft, Seattle, WA, USA) to estimate the parameters X_{max} and μ. The coefficient of the nonlinear fitting function was determined by an iterative minimizing the chi-squared merit function.

Caffeine degradation rate values were obtained by dividing caffeine consumed by time (t).

RESULTS

Aspergillus tamarii strain was previously isolated from coffee-growing areas of Mexico (Roussos *et al.* 1995) and showed the ability to grow in the presence of caffeine. Growth of *A. tamarii* on a synthetic medium with caffeine as sole nitrogen source resulted in maximal biomass production of 6.2 g l^{-1} after 120 h of fermentation (Gutiérrez-Sánchez, G., unpublished results). Moreover, a 60-h adaptation period or lag phase was observed. However, when presented with a mixture of ammonium sulphate and caffeine, *A. tamarii* preferentially utilized ammonium sulphate (Hakil *et al.* 1999). Consequently, a two-stage fermentation process was developed to evaluate the effect of the nitrogen source ratio on caffeine degradation and biomass production (Table 1).

Table 2, shows the effect of the caffeine/ammonium sulphate ratio on nitrogen and sucrose consumption, and biomass production by *A. tamarii* after 48 h of fermentation (end of first-stage fermentation). Strain growth was limited when caffeine was used as sole nitrogen source. Caffeine consumed was around 12%. In contrast, when ammonium sulphate was used as sole nitrogen source, highest biomass production values were obtained. In relation to the values

Table 2 Effect of the caffeine/ammonium sulphate ratio on nitrogen and sucrose consumption by *Aspergillus tamarii* and biomass production

| Culture medium | Percentage of nitrogen and carbon source degraded* | | | Biomass (g l^{-1})* |
	Caffeine	Ammonium sulphate	Sucrose	
1	12.4 ± 0.7	np	18.7 ± 1.1	0.2 ± 0.01
2	48.4 ± 2.9	100 ± 6.0	26.8 ± 1.5	2.1 ± 0.1
3	16.6 ± 1.0	87.8 ± 5.3	1.8 ± 0.1	2.0 ± 0.1
4	81.3 ± 4.9	34.4 ± 2.1	1.75 ± 0.1	1.2 ± 0.1
5	np	85.6 ± 5.1	10.43 ± 0.6	3.6 ± 0.2

np, not present.
*After 48 h of fermentation.

obtained with the mixtures (caffeine and ammonium sulphate), better caffeine degradation results were obtained (48%), associated to high biomass values, when a low concentration of ammonium sulphate (0.14 g l^{-1}) was mixed with 0.9 g l^{-1} of caffeine (culture medium 2). Under these conditions, the lag phase was reduced to 24 h and the capacity to degrade caffeine was increased. It is important to note, that under these conditions, *A. tamarii* rapidly consumed all available ammonium sulphate. In addition, higher caffeine degradation values were obtained with the mixture of nitrogen source, than when caffeine was used as sole nitrogen source.

The second stage of the process was initiated when a caffeine pulse (4 g l^{-1}) was given after 48 h of culture to enhance biomass production. Figure 1 shows biomass production of *A. tamarii* over time. Experimental data points are represented as symbols. When caffeine was added to the reactors containing ammonium sulphate as sole nitrogen source, *A. tamarii* growth reached the stationary phase in a shorter time than in the other conditions. Biomass production values were lower when initial ammonium sulphate concentration was above 0.14 g l^{-1}. However, highest biomass production values (9.87 g l^{-1}) were obtained when culture medium 2 was used (see Table 1). Fungal growth curves were fitted by eqn (1) with a correlation coefficient of $R^2 > 0.99$, showing that the logistic model was adequate to describe this fermentation process using different caffeine/nitrogen ratios.
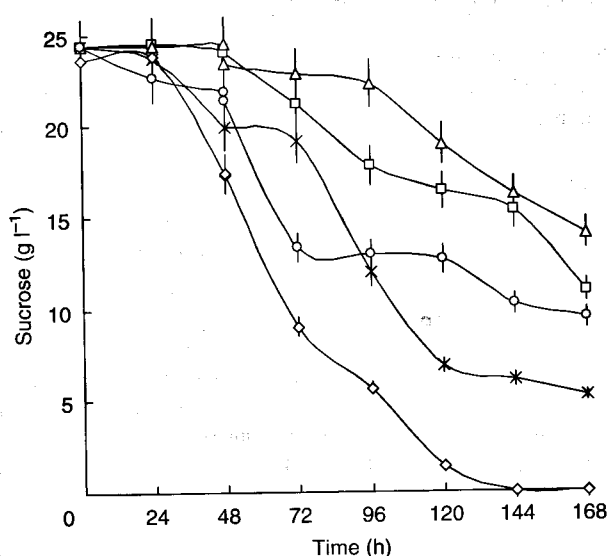
It is important to highlight that ammonium sulphate concentrations above 0.14 g l^{-1} at the first stage were followed by a sudden drop of pH in the medium (data not shown), resulting from ammonium being transported into the fungus as ammonia, leaving the hydrogen ion behind. The medium is therefore acidified. As a result, minimal nitrogen assimilation and poor growth were observed. In addition to the pH shock, *A. tamarii* had to adapt to the new
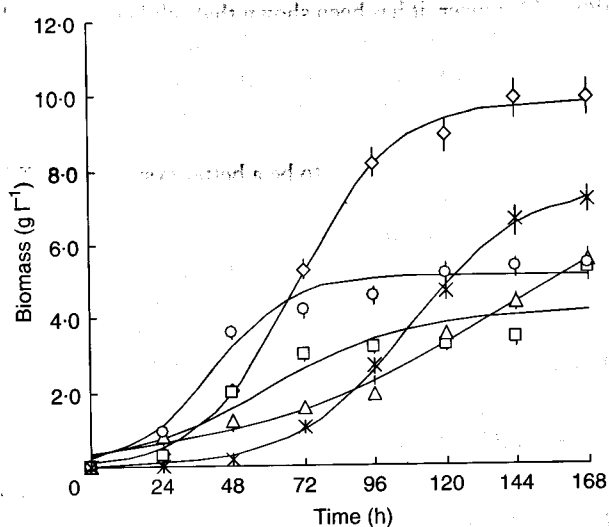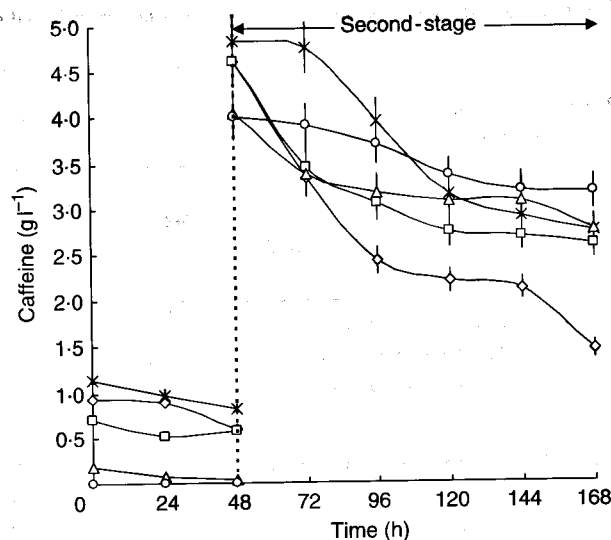
Fig. 1 Growth curves of *Aspergillus tamarii*. Symbols correspond to (∗) 100, (◇) 90, (□) 50, (△) 10 and (○) 0% of initial nitrogen contributed by caffeine. Solid lines correspond to the fit of experimental data by a numerical solution to the logistic equation

Fig. 3 Profiles of caffeine consumption by *Aspergillus tamarii*. (∗) 100, (◇) 90, (□) 50, (△) 10 and (○) 0% of initial nitrogen contributed by caffeine

nitrogen source, resulting in growth arrest and inhibition of caffeine degradation.

It is to be noted that *A. tamarii* uses caffeine solely as a nitrogen source (not as a carbon source). If the carbon source is consumed, caffeine degradation ceases (Hakil *et al.* 1999). Therefore, it is important to measure the consumption of sucrose by the fungus. Figure 2 shows sucrose consumption by *A. tamarii* as a function of fermentation

Fig. 2 Sucrose consumption by *Aspergillus tamarii*. (∗) 100, (◇) 90, (□) 50, (△) 10 and (○) 0% of initial nitrogen contributed by caffeine

time. In medium containing 90% of nitrogen from caffeine (see Table 1) sucrose was totally consumed after 144 h of fermentation. Under these conditions, highest caffeine degradation rate (0.033 g l^{-1} h^{-1}) and maximal biomass production (0.97 g l^{-1}) were also observed. If initial ammonium sulphate concentrations were above 0.14 g l^{-1} (media 3–5), easily assimilated nitrogen source was degraded within the first 96 h (data not shown). Furthermore, sucrose and caffeine consumption were much slower than with media 1 and 2 (Figs 2 and 3).

An overview of caffeine degradation spanning both fermentation stages is represented in Fig. 3. During the first stage (first 48 h of culture) caffeine is supplied (with or without ammonium sulphate) in low amounts (<1 g l^{-1}) in order to minimize toxicity due to caffeine. After 48 h, the second stage of the fermentation is initiated with a caffeine pulse of 4 g l^{-1}. Optimal caffeine degradation was observed when the fungus was grown with a low initial concentration of ammonium sulphate (medium 2).

Table 3 shows kinetic data obtained through fermentation process. *Aspergillus tamarii* grew faster on culture medium 2 ($\mu = 0.073$ h^{-1}) with highest X_{max} value (9.87 g l^{-1}). These results were associated to the highest caffeine degradation rate (0.033 g l^{-1} h^{-1}).

DISCUSSION

This study describes a simple two-step method, set up for efficient degradation of caffeine and enhancement of biomass production. Previous studies on this topic (Denis 1996;

Table 3 Growth kinetic parameters of *Aspergillus tamarii* cultured on liquid culture using different nitrogen sources ratio

Parameters during the second stage	Culture media				
	1	2	3	4	5
X_{max} (g l^{-1})	7·15	9·87	5·23	5·48	5·40
μ (h^{-1})	0·051	0·073	0·042	0·022	0·077
Caffeine degradation rate (g l^{-1} h^{-1})	0·021	0·033	0·021	0·013	0·008

Each value is the average of two experiments. Maximal biomass production (X_{max}) and specific growth rate (μ) are given. The logistic equation was used to estimate μ.

Hakil 1999) had shown that fungal biomass production was a critical step for caffeine degradation due to its toxicity. Results of the present study indicate that caffeine degradation and biomass production by *A. tamarii* were affected by addition of a simple nitrogen source, ammonium sulphate. If a mixture of caffeine/ammonium sulphate is added (90/10% of nitrogen) at the beginning of the fermentation, fungal growth is positively affected, reducing substantially the adaptation or lag phase. Moreover, when a simple nitrogen source was present, caffeine was not used by *A. tamarii*. In contrast, if the simple nitrogen source was not enough to allow total carbon source assimilation, caffeine was degraded after the simple nitrogen source had been metabolized (Table 2 and Fig. 3). It is to be noted that when only ammonium sulphate was present (culture medium 5), biomass production was highest. When only caffeine was present as nitrogen source (culture medium 1), biomass production was lowest. However, when 10% of nitrogen source was ammonium sulphate and 90% was supplied by caffeine (culture medium 2), biomass production was increased 10-fold, compared with culture medium 1. Over the first 48 h of culture, ammonium sulphate provides a simple nitrogen source, accounting for rapid biomass production, consequently enabling efficient caffeine degradation.

Studies carried out in solid-state fermentation using coffee pulp as support and substrate, showed that the addition of ammonium sulphate (3·75 g l^{-1}) inhibited caffeine degradation by *Penicillium verrucosum* V33A25 (Roussos *et al.* 1994). A similar effect was observed by Hakil *et al.* (1999) who showed, in solid-state fermentation using polyurethane foam, that caffeine was not used by *A. tamarii* if there was a simple nitrogen source in the medium and neither the caffeine degradation rate, nor the specific growth was affected. Results obtained by Denis (1996) showed that no inhibition of caffeine was measured when an easily assimilable nitrogen source was added in low concentrations.

However, complete caffeine degradation in solid media (coffee pulp) has been observed (Roussos *et al.* 1994; Perraud

1995). Moreover, it has been shown that solid cultures enable faster degradation of caffeine than liquid cultures, degrading 8 g l^{-1} of caffeine in 27 h using polyurethane foam (Hakil *et al.* 1999). Denis (1996) observed that there was no caffeine degradation using 10 g l^{-1}.

Solid-state culture seems to be a better system for caffeine degradation and could be a great system for physiological studies. The main disadvantage lies in the separation of fungal biomass from the solid support; such a step is necessary to isolate intracellular enzymes. Extraction of caffeinases resulting from solid state culture has been attempted without success (Hakil *et al.* 1999).

CONCLUSIONS

The present study shows the sensitivity and versatility of *A. tamarii* towards nitrogen sources. If one wants to use *A. tamarii* to produce or purify one of the enzymes involved in caffeine metabolism, it is important to know how the micro-organism responds to changes in nitrogen sources. By using a two-stage process, in submerged culture, caffeine degradation could be enhanced increasing fungal capacity to degrade caffeine. This new protocol presents data that may be used to increase fungal biomass and therefore increase caffeine-degrading potential as a result of increased enzyme levels. Studies are presently being carried out in order to purify enzymes from *A. tamarii* involved in caffeine degradation.

The present knowledge concerning the production of fungal biomass, able to degrade caffeine can be applied to the degradation of caffeine present within coffee pulp.

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