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Degradation and product analysis of caffeine and related dimethylxanthines by filamentous fungi

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Twenty strains of filamentous fungi were tested for their ability to grow on a liquid medium containing caffeine (1,3,7-trimethylxanthine) as a sole nitrogen source. Seven strains which were able to grow on caffeine belong to the Aspergillus *and* Penicillium *genuses. They all presented the same degradation pathways but varied in the efficiency by which degradation took place. Caffeine was first demethylated in position 7; this lead to the formation of theophylline (1,3-dimethylxanthine). This compound was next demethylated in position 1 to give 3-methylxanthine. All these strains were also able to grow on theophylline, theobromine (3,7-dimethylxanthine), and paraxanthine (1,7-dimethylxanthine) as a sole nitrogen source. All degradation intermediates detected were methylxanthines.* *© 1998 Elsevier Science Inc.*

Keywords: Caffeine; theophylline; theobromine; paraxanthine; degradation; filamentous fungi

Introduction

Coffee pulp is one of the major agro-industrial wastes produced during the pulping operations of the coffee cherry to obtain coffee bean in many production areas of the tropics.[1] For the 1989–1990 season, 2.8 million tons of coffee pulp were produced in the world.[2] Almost all the pulp produced each year is discarded as a waste product and is considered as the most abundant pollutant material of lakes and rivers located near coffee processing sites. Coffee pulp is rich in carbohydrates and proteins[3,4] but the presence of antinutritional factors such as caffeine (1,3,7-trimethylxanthine, *Table 1*), polyphenols, and tannins restricted its use as animal food. Attempts to reduce caffeine levels in coffee pulp by solid-state fermentation using a *Penicillium* strain have been successful,[2,5,6] but no studies about the degradation products have been performed.

There are few publications about caffeine degradation in filamentous fungi; nevertheless, it has been shown in a strain of *Aspergillus niger*[7] and in a *Penicillium roquefortii*[8] that the first product to appear from degradation of caffeine in liquid cultures is theophylline (1,3-dimethylxanthine). This compound can have greater toxic and pharmacological effects than caffeine.[9] This could also be a problem in trials to obtain a decaffeinated coffee through use of filamentous fungi.

The purpose of this study was to investigate whether the caffeine degradation pathway was the same for filamentous fungi in general. We assessed the ability of several filamentous fungi to degrade caffeine as well as the corresponding dimethylxanthines. We tested 20 different strains for their ability to use caffeine as the sole source of nitrogen in liquid media. The kinetics of caffeine and related dimethylxanthine degradation were established for all caffeine-degrading strains. In each experiment, degradation products were characterized.

Materials and methods

Microorganisms

All fungal strains used are from the ORSTOM-UAM collection.[10]

Culture media

All microorganisms were maintained on coffee infusion medium (CIS) prepared as follows. sucrose, 2.0 g; KH_2PO_4,

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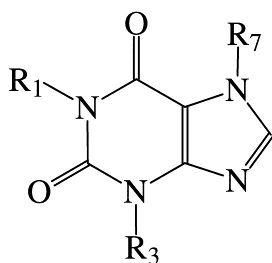

Table 1 Caffeine and derived compounds

Purines	R1	R3	R7
1,3,7-Trimethylxanthine (caffeine)	CH_3	CH_3	CH_3
1,3-Dimethylxanthine (theophylline)	CH_3	CH_3	H
1,7-Dimethylxanthine (paraxanthine)	CH_3	H	CH_3
3,7-Dimethylxanthine (theobromine)	H	CH_3	CH_3
1-Methylxanthine	CH_3	H	H
3-Methylxanthine	H	CH_3	H
7-Methylxanthine	H	H	CH_3
Xanthine	H	H	H

1.3 g; $Na_2HPO_4 \cdot 2H_2O$, 0.12 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; and $CaCl_2 \cdot 2H_2O$, 0.3 g were dissolved in an infusion of commercial ground coffee (Grand'Mère ''familial'', café Grand'Mère S.A., wattinities, France) in distilled water. The pH was adjusted to 5.6 with 1 M KOH and the volume was brought to 1 l. The medium was sterilized at 121°C for 20 min after supplementation with 20 g of agar.

Liquid cultures were performed in a caffeine-sucrose medium (CS) prepared as follows. The chosen methylxanthine, 1 g; sucrose, 28.4 g; KH_2PO_4, 1.3 g; $Na_2HPO_4 \cdot 2H_2O$, 0.12 g; $MgSO_4 \cdot 7H_2O$, 0.3 g and trace elements were dissolved in water. The pH was adjusted to 4 by addition of 1 M H_2SO_4 and the final volume was brought to 1 l. The medium was sterilized at 121°C for 20 min.

Cultures

Spores of 6-day-old cultures on CIS were harvested with 30 ml of distilled water containing 0.2% Tween 80 (w/v) under agitation provided by magnetic stir bar (100 rpm). Erlenmeyer flasks containing 50 ml of liquid media were inoculated with the spore suspension at 10^6 sp ml^{-1}. Flasks were incubated at 27°C with an agitation of 160 rpm. In the case of caffeine degradation experiments, after 48 h of culture, 0.2 g of anhydrous caffeine previously heated to 100°C was added to each flask. This moment was chosen as the initial time for HPLC analysis.

Samples analyzed by HPLC were obtained as follows. A suspension culture (300 μl) was homogeneously removed every 12 h from each incubated flask. Biomass was then discarded from the culture medium by centrifugation followed by filtration (0.45 μm). Filtered samples were stored at −20°C until analysis.

HPLC Analysis of methylxanthines

Chromatographic 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^{-1} by a gradient elution system. The eluents were 1.75 mM KH_2PO_4, acetonitrile, and tetrahydrofuran (98:1:1; v/v/v) for pump A. Acetonitrile and tetrahydrofuran were used (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 appropriate comparison of their retention times to those of the pure products and quantified by comparison to standard curves established for each methylxanthine.

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

Results

Out of 20 strains tested for their ability to grow in a medium containing caffeine as the sole nitrogen source (CS medium), only seven strains were able to grow (*Table 2*). Their ability to degrade caffeine was checked by HPLC analysis of the culture supernatant. In each case, there was caffeine degradation only if fungal growth was observed. The seven strains were not able to grow when caffeine was used as a sole source of carbon and nitrogen.

In later experiments, only the seven strains able to grow on the CS-medium were used. The strains were quantitatively tested for their ability to degrade caffeine, theophylline, theobromine, and paraxanthine as a sole nitrogen source.

Caffeine degradation

We used a high concentration of caffeine in caffeine degradation experiments (4 g l^{-1} after 48 h of culture at 1 g l^{-1}) to be able to see as many intermediates as possible. *Aspergillus tamarii* (V12A25) and all *Penicillium commune* strains showed a good ability to degrade caffeine whereas *A. niger* (C28B25) and *Aspergillus fumigatus* (C25A35) were less effective (*Table 2, Figure 1*). The *A. niger* degradation profile (C28B25) is the same as that of *A. fumigatus* (C25A35) (*Figure 2B*). The *P. commune* degradation profile (V14A35) (*Figure 2A*) is representative of all other strains except *Aspergillus oryzae.* In this case, biomass production was higher than with other strains, sucrose disappeared from the culture medium after 48 h of culture, and no further degradation was noted at this incubation time (*Figure 1).*

Table 2 Caffeine degradation by the selected strains

Code number	G. species	% Degradation at 48 h	*V* mean ($mg/l^{-1}h^{-1}$)
V12A25	*A. tamarii*	67.2	53.6
V33A25	*P. commune*	56.8	35.0
C25A35	*A. fumigatus*	17.6	14.7
V29A25	*P. commune*	67.7	48.0
V14A35	*P. commune*	46.0	39.2
C28B25	*A. niger*	16.1	30.7
C7A25	*P. commune*	61.6	52.1

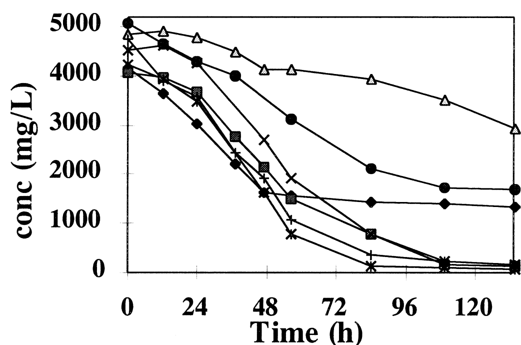

Figure 1 Caffeine degradation by the seven selected strains. C28B25 (●); V12A25 (◆); C25A35 (△); V14A35 (×); V29A25 (*); C7A35 (+); and V33A25 (■)

Figure 2A shows clearly that theophylline appeared first followed by 3-methylxanthine. These two compounds represented up to 60% of initial caffeine present during caffeine degradation by *P. commune* (V14A35). Theobromine and paraxanthine were detected in the culture media at concentrations of about 40 mg l^{-1}. Trace amounts of 1-methylxanthine were also detected. In all HPLC assays, no trimethyluric acid or dimethyluric acids was detected.

Dimethylxanthine degradation

The lower concentration used in dimethylxanthine degradation experiments (1 g l^{-1} for all cultures) was due to the lower solubility of dimethylxanthines. The amount of each degradation product detected in these experiments was usually less than 20 mg l^{-1}. With the initial quantity of each dimethylxanthine being low, the intermediary products are degraded as they appear.

All strains except *A. tamarii* seemed to be more efficient in degrading theobromine than theophylline. In all cases, paraxanthine was the least easily degradable dimethylxanthine (*Figure 3C*). For all strains, degradation of theobromine and theophylline led to the appearance of 3-methylxanthine. Degradation of paraxanthine led to the formation of 1- and 7-methylxanthine. These products were only present in trace amounts (data not shown).

Discussion

Twenty different strains of filamentous fungi were tested for their ability to use caffeine as well as different dimethylxanthines (*Table 1*) as a sole nitrogen source. Only *Aspergillus* and *Penicillium* genuses were able to grow on caffeine as a sole nitrogen source. All *Penicillium* strains tested were *P. commune* and were able to grow. For *Aspergillus,* only four strains out of nine were able to grow. It appears that *Penicillium* and *Aspergillus* are the more frequent caffeine-degrading genuses. It therefore seems logical that the majority of the studies done on caffeine degradation by filamentous fungi are related to *Aspergillus* and *Penicillium* genuses. Kurtzman and Schwimmer[11] isolated a strain of *Stemphylium* able to degrade caffeine which suggests that this characteristic is not restricted to *Aspergillus* and *Penicillium.* In addition, not all *Aspergilli* are able to degrade caffeine. Similar studies have been undertaken by Buchanan *et al.*[12] This capacity is therefore a characteristic of a strain but not of a genus. The *Fusarium* strains tested did not seem to be very efficient in caffeine degradation. None of the fungal strains tested were able to grow when caffeine was used as the sole source of both carbon and nitrogen. It has been reported that on the contrary, specific bacteria can grow under the former conditions.[13,14] This particularly suggests enzymatic differences between such microorganisms.

The seven selected strains do not have the same caffeine-degrading efficiency; nevertheless, they all yield the same degradation products which are theophylline and 3-methylxanthine. This could be due to two successive demethylations. First, a 7-demethylation, giving theophylline from caffeine, followed by a 1-demethylation leads to 3-methylxanthine from theophylline. This hypothesis is supported by data presented in *Figure 2* where theophylline appears first after caffeine degradation. 3-Methylxanthine appears only when theophylline is present in the culture medium. Support for this hypothesis also comes from Schwimmer *et al.*[8] who identified theophylline as the first product in the caffeine degradation pathway by a *Penicillium* strain. *Figure 3* shows that the sum of theophylline and 3-methylxanthine represents more than 50% of the initial caffeine concentration after 110 h of culture. This suggests that other degradation pathways are not quantitatively important. The small

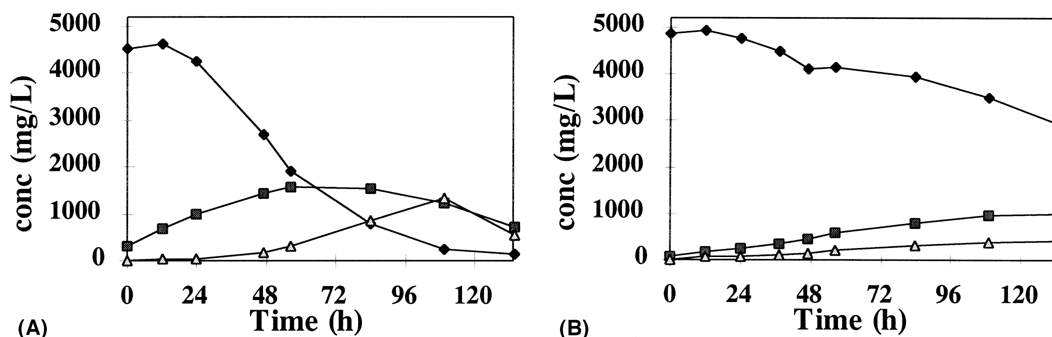

Figure 2 Caffeine degradation and appearance of products. V14A35 (A); C25A35 (B); caffeine (◆); theophylline (■); and 3-methylxanthine (△)

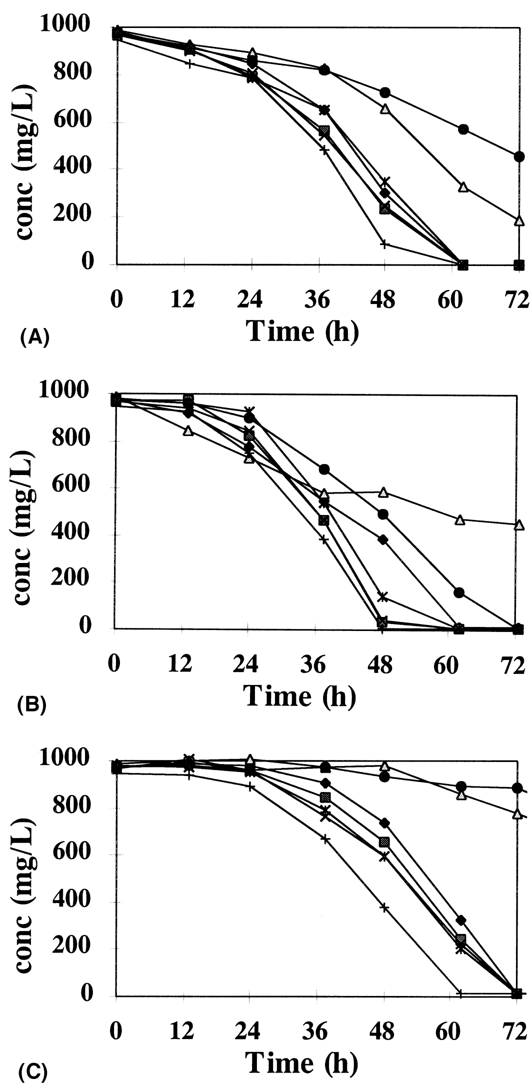

Figure 3 Dimethylxanthines degradation by the seven selected strains. Initial substrates were: theophylline (A); theobromine (B); and paraxanthine (C). Strains tested were: C28B25 (●); V12A25 (◆); C25A35 (△); V14A35 (×); V29A25 (*); C7A25 (+); and V33A25 (■)

amounts of paraxanthine and theobromine also present in the culture medium can be due to an unspecific reaction of the same enzyme which catalyzes the transformation of caffeine into theophylline. It could also be due to specific enzymes which could be weakly expressed or that present a weak affinity for the substrate. In bacteria, the major intermediaries in caffeine degradation are theobromine, 7-methylxanthine, and xanthine[13–16] which is another difference between bacterial and fungal systems.

Although produced at a slow rate, theobromine and paraxanthine can be subsequently degraded by all strains. Theobromine is even more effectively degraded than theophylline. Theobromine and theophylline degradation leads to 3-methylxanthine formation (7- and 1-demethylation, respectively). Paraxanthine degradation leads to formation of 1- and 7-methylxanthine (7- and 1-demethylation). Formation of trimethyluric acid and dimethyluric acids were never detected regardless of the substrates used.

From all these experiments, we can conclude that the first steps of caffeine degradation in filamentous fungi consist of demethylation reactions. Demethylations in position 1 and 7 can occur each time a methyl group is present in this position but the 7-demethylation seems to be preferentially used. Ina[7] identified xanthine as a degradation product of caffeine by a strain of *A. niger*. It would be interesting to further identify the products resulting from caffeine degradation of our seven strains.

It is difficult to say whether caffeine degradation in filamentous fungi is due to one or more enzymes and whether these enzymes are the same as those present in bacteria. In bacteria, caffeine degradation is lead by at least two enzymes and one of these enzymes has been purified.[17] Answers to these questions could come from attempts presently made to purify the enzyme(s) implicated in the first steps of caffeine degradation.

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