Chapter 42

EFFECT OF CONSERVATION METHOD ON CAFFEINE UPTAKE BY *PENICILLIUM COMMUNE* V33A25

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Running title: Caffeine degradation

1. Introduction

Coffee pulp is one of the major by-products obtained during the pulping operation of coffee cherries. A fraction of this material is used for compost production for coffee plant nurseries and other part is spilt in rivers or piled up near them. For every two tons of coffee cherry processed, nearly one ton of pulp is generated. During the 1997-98 period, 10.4 million of sacks (60 kg each) of fresh coffee pulp were produced in Mexico (Barreiro, 1999). Coffee pulp is rich in proteins, minerals and fibre that can be used for animal feeding, but utilization of coffee pulp for feed is constrained by anti-physiological (caffeine) and anti-nutritional (polyphenols) compounds (Roussos *et al.*, 1989). These compounds cause adverse effects on the animals that consume coffee pulp. Caffeine (1,3,7-trimethylxanthine, Figure 1) concentration varies according to the coffee variety (0.9-2.4% dry wt. basis). Its physiological role is probably to defend coffee plants from predators and to inhibit growth of other plants. In humans, caffeine is demethylated into three primary metabolites: theophylline, theobromine, and paraxanthine. At 100 $mg.kg^{-1}$ teophylline is toxic to rats. Theobromine has been related with headache, insomnia, restlessness, excitement, mild delirium, muscle tremor, tachycardia and extrasystoles in man and caffeine has been reported to have many other activities including mutagenic, teratogenic and carcinogenic capacities.

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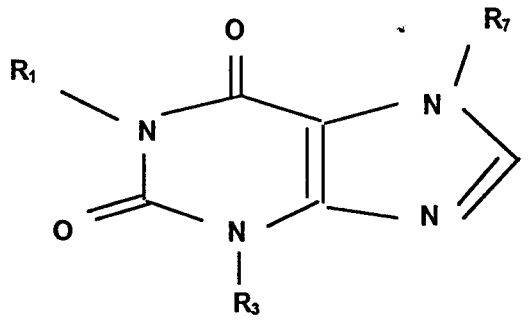

Figure 1. Caffeine structure and related compounds. (*Gutiérrez-Sánchez Gerardo et al.*).

Compounds	R1	R3	R7
Caffeine	CH3	CH3	CH3
Theophylline	CH3	CH3	H
Paraxanthine	CH3	H	CH3
Theobromine	H	CH3	CH3
1-metil-xanthine	CH3	H	H
3-metil-xanthine	H	CH3	H
7-metil-xanthine	H	H	CH3
Xanthine	H	H	H

Coffee pulp could be silage for conservation and nutrimental improvement. Silage is an anaerobic fast process, which involves lactic acid bacteria. It has been widely used for forage preservation in regions of moderate climate and enables prevention of forage putrefaction with minimum degradation of the organic material and a reduction in the polyphenols compounds but not in the caffeine content (Perraud-Gaime, 1995). In order to improve the nutritional value, Peñaloza *et al.* (1985), Gómez *et al.* (1985) and Aquiáhualt (1992) carried out solid state fermentation (SSF) studies with coffee pulp using a strain of *Aspergillus niger*. They reported protein enrichment of the substrate but with no significant caffeine elimination.

The removal of caffeine from coffee pulp appears attractive because it could then be used as animal feed. Caffeine elimination could be carried out by two ways, chemically and microbiologically. The first way is not recommended due to the use of heavy salts, solvents and high cost. Microbial caffeine degradation by filamentous fungi such as Penicillium and Aspergillus, which were able to use caffeine as sole nitrogen source has been reported by Hakil *et al.* (1998).

Of the thousands of enzymes a cell is capable of producing, a certain number are always presented in substantial concentration, regardless of what medium in which the organism is growing. Enzyme induction is defined as a relative increase in the rate of synthesis of a specific enzyme, resulting from exposure to a chemical substance, inducer. Inducible enzymes are necessary when the organism finds itself limited. Induction insures that energy and amino acids are not wasted in making unnecessary enzymes but that, when needed, these enzymes can be formed rapidly (Wang *et al.*, 1979). On the other hand, the ability to preserve successfully a wide range of micro-organisms and cell cultures has been a major achievement in microbiology over the last century that many have taken for industrial development (Smith, 1991). The choice of preservation method depends on many factors. For example, industrial collections place much emphasis on techniques that maintain genetic stability, especially for strains that have special features required for industrial processes such as the production of antibiotics and enzymes.
The aim of this work was to study the effect of the conservation method on caffeine uptake by *Penicillium commune* strain V33A25.

2. Experimental

2.1. MICRO-ORGANISM

Penicillium commune strain V33A25 (IRD-UAMI Collection) was selected because of its ability to use caffeine as nitrogen source (Roussos *et al.* 1995, Denis 1996).

2.2. CULTURE MEDIA

The culture media composition used in this work is shown in Table 1.

2.3. CULTURE CONDITIONS AND STRAIN CONSERVATION

Four methods were used for the evaluation of strain conservation associated to caffeine degradation, which included strain conservation on a) CSA, b) CPA, and lyophilizing the spores harvested from c) PDA, and d) CSA. *P. commune* was sub-cultured three times on CSA medium (except for method b using CPA, where strain was sub-cultured six times on CSA medium) at 30°C for six days and caffeine degradation were evaluated on CS broth according to the methodology reported by Denis (1996).

Table 1. Culture media used for strain conservation, spore harvest and caffeine evaluation (medium $g.l^{-1}$)

Component	CPA	CSA	PDA	CS
Saccharose	—	2.0	—	2.0
Milled coffee	—	40.0	—	40.0
Fresh coffee pulp	427.7	—	—	—
$Na_2HPO_4 \cdot 2H_2O$	0.16	0.12	—	0.12
$MgSO_4 \cdot 7H_2O$	0.4	0.3	—	0.3
$CaCl_2 \cdot 2H_2O$	0.4	0.3	—	0.3
Agar	26.7	20.0	—	—
Distilled water (ml)	1000	1000	1000	1000
Potato Dextrose Agar	—	—	39	—

CPA: Coffee pulp-agar medium. **CSA:** Coffee saccharose-agar medium (Aquiáhuatl, 1992; Denis, 1996). **PDA:** Potato Dextrose Agar. **CS:** Coffee saccharose broth.
For coffee-saccharose agar (CSA) and coffee-saccharose broth (CS), commercial coffee (Grand Mère "familial", France) was used. The infusion was filtered using Whatman paper No 41, salts were added and pH was adjusted to 5.5 using KOH (0.1 M). The volume was adjusted to 1-l. In the case of coffee pulp-agar (CPA), fresh coffee pulp was milled in a blender for 5 minutes and then heated until boiling. To this 750 ml distilled water (incorporating salts and agar) were added and pH was adjusted to 5.5 using KOH (0.1 M). The culture media were sterilized at 121°C for 15 minutes.

2.4. CAFFEINE ANALYSIS

Caffeine was extracted from liquid samples (0.5 ml) with 1 ml of a chloroform isopropanol mixture (85:15, v/v), mixing during 30 seconds and recovering the organic phase. The extraction was repeated three times. Solvent was evaporated in a heater and the extract was re-suspended in 1.5-ml de-ionized water. Caffeine was analyzed by High Performance Liquid Chromatography (HPLC), using a modification of the technique reported by Denis (1996). A Sphersorb ODS-2 column, acetonitrile tetrahidrofurane deionized water (5:1:94, v/v) as mobile phase at 1.5 ml min^{-1}, room temperature and diodes array detector at λ=273 nm were used during analysis.

3. Results and discussion

P. commune V33A25 was subjected to four different conservation methods prior to growth in a liquid medium in presence of caffeine. The results are presented in Fig. 2. When spores were conserved on PDA, lag phase of nearly sixty hours before degradation

was noted. However, when spores were conserved on CSA, the lag phase was shorter and degradation was observed after 36 hours of cultures. In the case of method c, degradation began later (36 hours). It was evident that the methods c and d were not good alternatives to conserve the capacity of degradation of the caffeine in short periods. These results could be explained probably by the relative damage suffered by the spores during the lyophilization process. When spores were harvested from PDA medium, it was observed that caffeine degradation began after 60 hours, however, the lyophilization process allowed the conservation of strains for long periods of time, as lyophilization allowed the dehydration of fungi to level that halted metabolism (Smith, 1991).

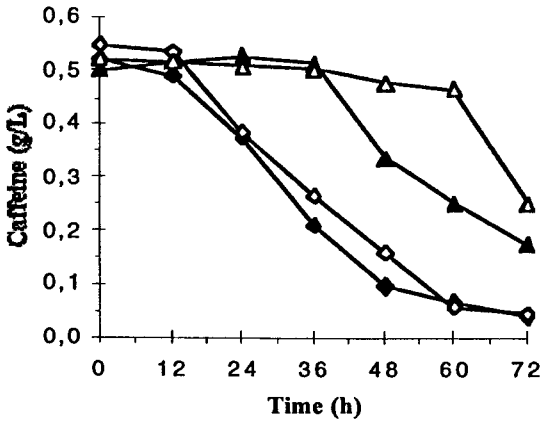

Figure 2. Influence of the conservation method on caffeine degradation ability. Strain conserved on CSA (◆); on CPA (◇); spores harvested from CSA (▲) and from PDA. Cultures were activated three times on CSA medium, except for CPA were inoculum was sub-cultured six times on CSA medium. *Gutiérrez-Sánchez Gerardo et al.*

When the strain was conserved with the methods a or b, degradation presented a 12-hour lag phase and degradation rates were closed to 0.01 $g.l^{-1}$ h^{-1}, reaching 92 % degradation after 72 hours in both cases. Method b, however, required more time of adaptation than for the method a. These two methods could be used for short periods of conservation. Hakil *et al.* (1998) reported 90% caffeine degradation in 80 h using an *A. tamarii* strain conserved with the method a. Filamentous fungi (especially *Penicillium* genus) have been reported to possess the ability to use caffeine as sole nitrogen source, yielding teophylline and 3-methylxantine (Hakil *et al.*, 1998). Caffeine degradation pathway begins with two successive demethylation. In the first step teophylline is produced by a 7-demethylation from caffeine, followed by a 1-demethylation leads to 3-methylxantine from teophylline (Fig. 3) (Hakil *et al.*, 1999).

This way it could be established that teophylline is the first step in caffeine degradation (Schwimmer *et al.*, 1971). Results obtained suggested that enzymatic activity responsible for caffeine degradation was inducible due to the fact that degradation was increased considerably after repeated cultivation on CSA medium. Therefore, once

enzymatic mechanism for caffeine degradation is expressed, it is important to conserve the strain on CSA medium in order to have a short reactivation period. This is the first report about the influence of the conservation method on the caffeine degradation ability.

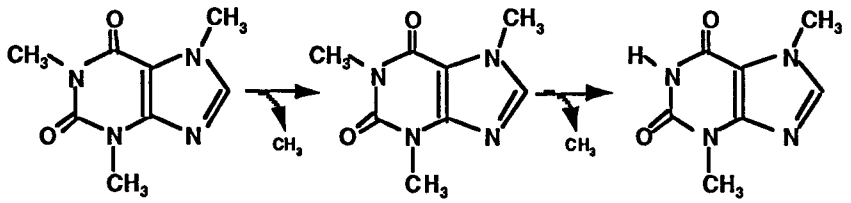

Figure 3. Steps in caffeine degradation by fungi. (Gutiérrez-Sánchez Gerardo *et al.*).

4. Summary

Caffeine degradation by *P. commune* V33A25 was evaluated using a liquid culture medium prepared from a coffee infusion with an initial caffeine concentration of 0.5 $g.l^{-1}$. Once caffeinase activity was induced, four different conservation strategies were tested. Two involved the conservation of the mycelium grown either on a coffee saccharose-agar medium (method a) or on a coffee pulp-agar medium (method b). The other two methods involved spore lyophilization, after having grown the strain either on potato dextrose agar (method c) or on coffee saccharose-agar medium (method d). In each case, before evaluating the ability of the strain to degrade caffeine, *P. commune* was sub-cultured on coffee-agar medium. Spores were then harvested and *P. commune* was grown in liquid medium. Caffeine degradation over time was measured. It was observed that using methods c and d, the mould began to degrade caffeine after 60 and 36 hours of culture, respectively. With method 1, the fungus started to degrade caffeine 12 hours after the inoculation, reaching 92% of caffeine degradation in 72 hours; with method b, 39% degradation was observed in 72 hours. The results suggested that enzymatic activity responsible for caffeine degradation was inducible and once caffeine degradation ability was expressed it would be important to conserve the strain in a medium with caffeine.

5. Acknowledgements

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