EVALUATION OF *Penicillium sp.* V33A25 CAFFEINASE ACTIVITY IN RELATION TO ITS CONSERVATION METHOD

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ABSTRACT

Caffeine degradation by Penicillium sp. strain V33A25 (PV33A25) was evaluated using a liquid culture medium prepared from a coffee infusion with an initial caffeine concentration of 0.5 g/L. After caffeine activity of the strain PV33A25 was induced, strain conservation was carried out four methods: 1)conserved in a coffee-agar medium, 2) coffee pulp-agar medium, and for the next two methods, the spores were harvested from 3) potato dextrose agar (PDA) medium, and 4) coffee-agar. The spores were lyophilized in the last two cases. The strain was adapted three times by subculturing on coffee agar medium before been used for the evaluation of caffeine degradation ability. It was observed that using methods 3 and 4 the fungi begin to degrade caffeine after 60 and 36 hours of culture, respectively. Nevertheless, with method 1, the fungi starts to degrade caffeine 12 hours after the inoculation time, reaching 92 % of caffeine degradation in a 72 hour period; with method 2, 39 % of degradation was observed within 72 hours but it was necessary to make 6 precultures to reach a similar degradation rate with method 2. The results of this study, suggest that the enzymatic activity responsible for caffeine degradation is inducible and once caffeine degradation ability is expressed it is important to conserve the strain in a medium with caffeine.

RESUMEN

Se evaluó la degradación de cafeína por Penicillium sp. strain V33A25 (PV33A25) empleando un medio de cultivo líquido preparado a partir una infusión de café con una concentración inicial de cafeína de 0.5 g/L. Una vez inducida la actividad cafeinasa de la cepa PV33A25, se procedió a la conservación de dicha cepa usando cuatro métodos: 1) la cepa fue conservada en medio café agar; 2) en medio pulpa de café agar y en los otros dos métodos las esporas se cosecharon a partir de medio, 3) agar papa dextrosa y 4) café agar, y las esporas fueron liofilizadas en los estos últimos dos casos. La cepa fue resembrada tres veces en medio café agar, antes de ser cultivada en el medio café líquido para evaluar su capacidad de degradación de cafeína . Se observó que con el método de conservación 3 y 4 el hongo empieza a degradar la cafeína después de las 60 y 36 horas de cultivo, respectivamente. Sin embargo, con el método de conservación 1, el hongo comienza a degradar la cafeína a las 12 horas, llegando a un 92 % de degradación de cafeína en 72 horas; con el método 2, hubo una degradación del 39 % a las 72 horas, pero es necesario hacer 6 resiembras para alcanzar una degradación similar a la obtenida por el método 2. Los resultados de este estudio, sugieren que la actividad enzimática responsable de la degradación de la cafeína es inducible y una vez expresada dicha actividad es importante conservar la cepa en un medio que contenga cafeína.

INTRODUCTION

Coffee pulp is one of the major subproducts available produced during the pulping operation of coffee cherries. One fraction of this material is used for compost production for coffee plant nurseries and the other part is spilled in rivers or piled up near them. For the 1997-98 period 14.92 million tons of fresh coffee pulp were produced in the world (Barreiro, 1999) and for every 2 ton coffee cherry processed, nearly 1 ton pulp is generated. Coffee pulp is essentially rich in proteins, minerals and fiber, nevertheless its utilization in animal feed is reduced notably due to the presence of toxic compounds difficult to degrade, such as caffeine, phenolics and tannins (Roussos, 1989). These compounds cause adverse effects on the animals that consume coffee pulp.

Silage of coffee pulp for conservation and its food value improvement, is an alternative for latter utilization of the coffee pulp. Silage is an anaerobic and fast process, which involves lactic

acid bacteria. It has been widely used for forage preservation in regions of moderate climate. Silage enables prevention of forage putrefaction with minimum degradation of the organic material. The process is faster and improves forage nutritional quality (Perraud-Gaime, 1995).
Peñaloza in 1985, Gómez in 1985, and Aquiáhualt in 1992, carried out solid state fermentation studies with coffee pulp with an *Aspergillus niger* strain, in order to evaluate the improvement of the nutritional value. They reported proteic enrichment of the substrate but with no significant caffeine elimination. In this sense, utilization of coffee pulp for feed is constrained by antiphysiological (caffeine) and antinutritional (polyphenols) compounds.
The aim of this work was to study the effect of the expression of *Penicillium sp.* strain V33A25 caffeine-degrading enzymes after various conservation methods.

MATERIALS AND METHODS

Microorganism

Penicillium sp. strain V33A25 used in this study, came from the IRD-UAM collection (Roussos, 1995) and was selected because of its ability to use caffeine as nitrogen source (Roussos, 1995; and Denis 1996).

Culture media

The culture media composition used in this work are shown in Table 1. For CSA and CS, commercial coffee used was Grand'Mère "familial". The infusion was filtered by Whatman paper No. 41, salts were added, pH was adjusted to 5.5 using KOH, 0.1 M, agar was added and the end. The volume was adjusted to 1 L. In the case of CPA, fresh coffee pulp was milled in a blender for 5 minutes, then was heated until boiling, after addition of 750 mL of distilled water salts solution and agar were then incorporated. The culture medium was sterilized at 121°C for 15 minutes.

Table 1. Culture media used for strain conservation and spore harvest (Aquiahuatl, 1992; Denis, 1996).

Component	Medium (g/L)		
	CPA	CSA	CS
Saccharose	---	2.0	2.0
Milled coffee	---	40.0	40.0
Fresh coffee pulp	427.7	---	---
KH_2PO_4	1.73	1.3	1.3
$Na_2HPO_4 \cdot 2H_2O$	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	---

CPA: Coffee pulp agar medium; **CSA:** Coffee agar medium;
CS: Coffee saccharose medium

Culture conditions and strain conservation

Four methods were used for the evaluation of strain conservation associated to caffeine degradation: 1) strain was conserved on CSA, and 2) on CPA, for the next two methods, the spores were harvested from 3) PDA and 4) CSA and in both cases spores were lyophilized. The selected strain was precultured twice on CSA at 30°C for 6 days and caffeine degradation were evaluated on CS medium according to Denis methodology (Denis, 1996).

Caffeine analysis

Caffeine was extracted from liquid samples with 1 mL of a chloroform/isopropanol mixture (85:15; v/v) mixing in a vortex during 30 seconds and recovering the organic phase until three successive extractions. Solvent was evaporated in a heater and the extract was resuspended in 1.5 mL of deionized water. Caffeine was analyzed by High Performance Liquid Chromatography (HPLC), using a modification to the technique reported by Denis, 1996; Sphersorb ODS-2 column, acetonitrile/tetrahidrofurane/deionized water (5:1:94; v/v/v), 1.5 $mL \cdot min^{-1}$ flow rate, room temperature, diodes array detector at λ=273 nm were used.

RESULTS AND DISCUSSION

In Figure 1, caffeine concentrations related to the conservation methods proposed (see methodology), are shown. When the strain was conserved with the method 1 or 2, that is precultured previously 3 and 6 times on CSA, respectively, the degradation presented a 12 hour delay phase and degradation rates were near 0.01 $g \cdot L^{-1} \cdot h^{-1}$, in both cases, reaching 92 %

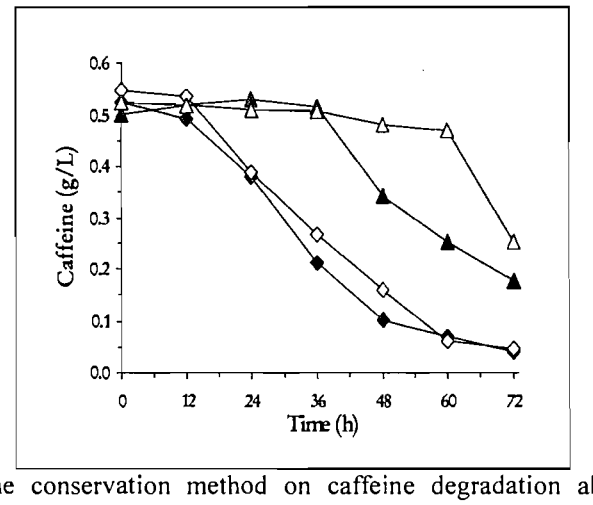

Figure 1. Influence of the conservation method on caffeine degradation ability. Method (◆) CSA, previously cultured three times on CSA; (◇) CPA, previously cultured six times on CSA; (▲) CSA_L, spores harvested from CSA and hlyophilized; (Δ) PDA_L, spores harvested from DA and lyophilized

degradation after 72 hours. In the case of conservation method in which lyophilized spores came from CSA the degradation began later (36 hours), which explains probably the relative spore damage suffered during the lyophilization
rocess. In the case in which the spores are harvested from PDA medium it is observed that caffeine degradation begins after 60 hours. As way of comparison we can mention that Hakil (1998) obtained caffeine degradation greater than 90 % after 80 hour of incubation using CSA method.

In CSA liquid medium (Table 1) caffeine degradation ability was induced due to the presence of caffeine as unique nitrogen source. These studies suggest that enzymatic activity responsible for caffeine degradation is inducible, due to the fact that degradation was increased considerably after repeated cultivation. Therefore, once caffeine degradation ability is expressed is important to conserve the strain on CSA medium, as to have a shorter reactivation period. There are no studies about the influence of the conservation method on the caffeine degradation ability.

Penicillium sp. V33A25 strain was able to degrade 92 % of total caffeine present at the beginning of the culture, after 72 hours of incubation. Therefore this strain will now used for the forthcoming degradation studies of caffeine present in ensiled coffee pulp.

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