COFFEE PULP DETOXIFICATION BY SOLID STATE FERMENTATION : Isolation, Identification and physiological studies.

Aquiahuatl, M.A., Raimbault, M., (*), Roussos, S., (*) & Trejo, M.R.

Departamento de Biotecnologia, Universidad Autonoma Metropolitana - Iztapalapa; A.P.55-535; 09340 Mexico DF (Mexique).

(*) ORSTOM: Laboratoire de Biotechnologie, Centre ORSTOM de Montpellier, BP 5045; 34032 Montpellier Cedex, Paris (France)

ABSTRACT :

Coffee pulp is a greatly abundant agroindustrial waste with a limited use as feed, due to its high contents of toxic compounds such as caffeine, tannines and polyphenols. An alternative to increase its utilization could be a detoxification process through Solid State Fermentation, using filamentous fungi. These microorganisms are able to degrade caffeine when it is used as nitrogen source. Some degradation pathways are known, and urea is one of the ultimate products of this degradation.

Soil, leaves and coffee fruits samples was recolected from Xalapa, Ver. and Soconusco, Chis., Mexico; the isolation was done by inoculating samples on 3 semisynthetic culture media contening coffee extract (A), coffee extract with sucrose (B) and coffee pulp extract (C) with mineral salts and streptomycine (30 ppm). Cultures were incubated at 25°C and 35°C.

The purification was made through succesive replication with the same isolation culture medium. In this form, 280 pure strains were isolated and identified by macroscopic and microscopic observations on standard culture media plates and microculture.

Subsequently, a physiologic study of these strains was made in liquid medium with caffeine and other controled conditions. Strains were selected for their caffeine degradation capacity in this selective liquid medium.

After 70 hours of incubation, strains with nearly 80% of caffeine degradation were found and in some cases correlated with a notable increase in pH. Most of the isolated microorganisms were *Aspergillus, Penicillium, Trichoderma and*

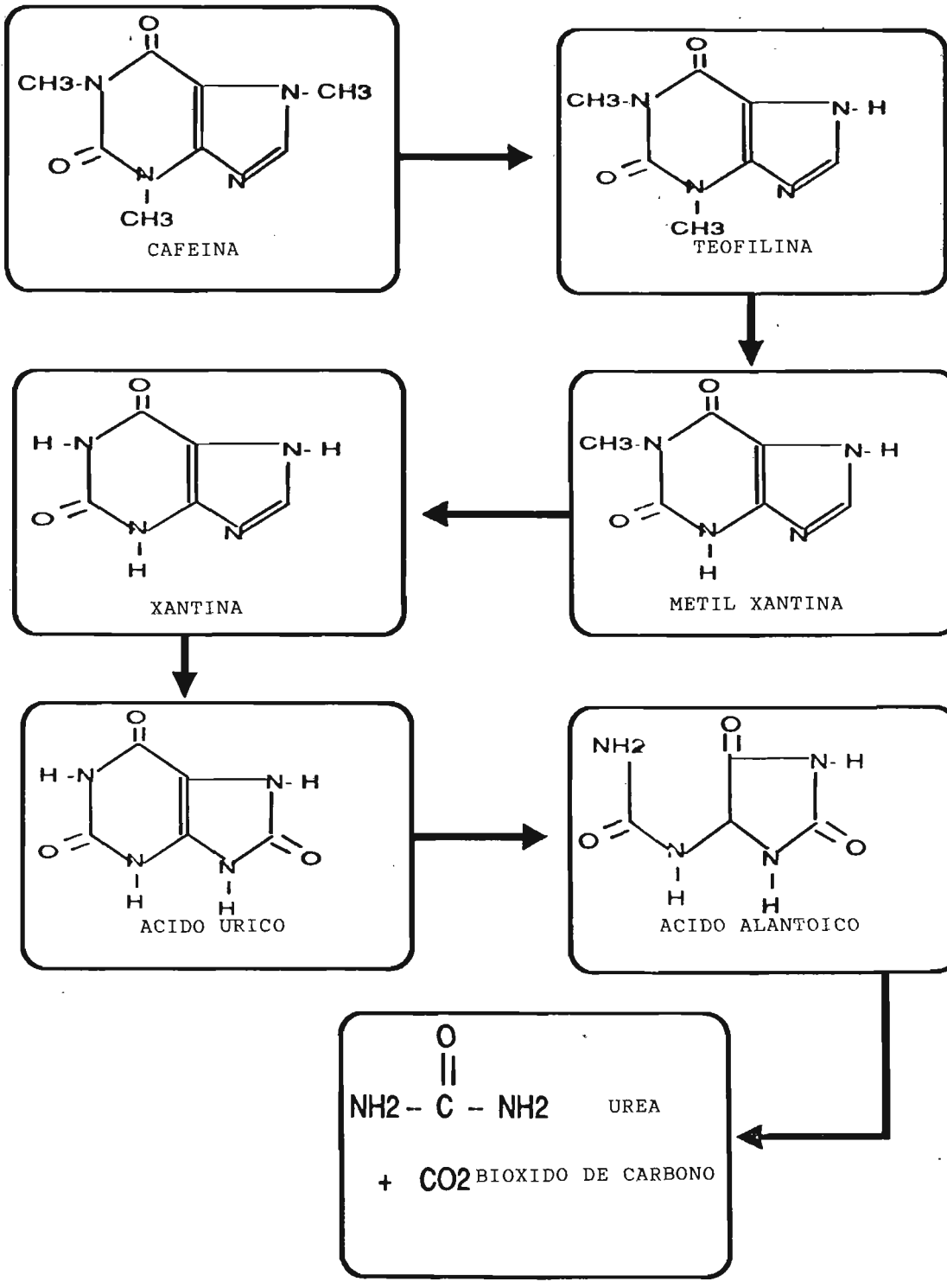

fig. 1 : Biodegradation of caffeine

Fusarium strains, which correspond to Kurtzman y Schwimmer,(1971) who considered biological alternatives to decaffeination with *Penicillium crustosum* strain NRRL 5452 with coffee infusions.

INTRODUCTION :

Coffee pulp represents the most abundant waste produced during the pulping operation of the coffee cherry needed to separate the coffee grain (Bressani, 1979). In Mexico as well as in other coffee producing regions, coffee pulp is barely used and, therefore, it is considered to be the most abundant polluting factor for rivers and lakes located near the coffee processing sites.

The utilization of coffee pulp as an animal feed has been mentioned as an atractive possibility. However, such utilization is limited by antiphysiological factors naturally ocurring in the material such as caffeine, tannins, chlorogenic acid, caffeic acid, and an excess of potassium (Adams and Dougan, 1981; Bressani, 1979).

Many works were performed for elimination of the antiphysiological compounds of coffee pulp using decaffeination (Molina, 1974), silage, as well as treatments of the material with calcium hydroxide or potassium bisulfite, water, and the combination of these compounds with physical treatment: grinding, extrusion, heating and drying (Gomez, 1979). But all methods were either inefficient in reducing the toxicity or too expensives.

Some reports indicate that the biological methods could be successfuly used in detoxification of coffee pulp. Bergmann and al. (1962) reported a *Pseudomonas aeruginosa* strain which oxidize 2-aminopurine as well the 2-methylamino purine and 2-dimethylamino purine derivative at position 8, with a xanthine oxidase system. This bacteria degraded caffeine via theobromine. Schwimmer and Kurtzman (1972) isolated and studied the metabolism of a *Penicillium crustosum* strain, which efficiently utilized caffeine via theophyline as source of nitrogen in clearly defined growth media with coffee infusions.

Recently a cytochrome P-450, a flavoprotein cytochrome mediated oxidase systems, has been detected in enzyme extracts of a caffeine-degrading yeast (Sauer and al. 1982) which implies that caffeine metabolism in yeast might be similar to the human one. As it could be expected from the literature on purine metabolism in both fungi and in higher organisms, xanthine was found to be dehydrogenated to uric acid, which was further metabolized to allantoin, then to allantoic acid, and finally to urea and carbon dioxide. The metabolism of methylated xanthines such as caffeine may involve a direct oxidation to methylated allantoine (Franke, W, 1955) or demethylation, following the catabolic pathway shown in Fig.1.

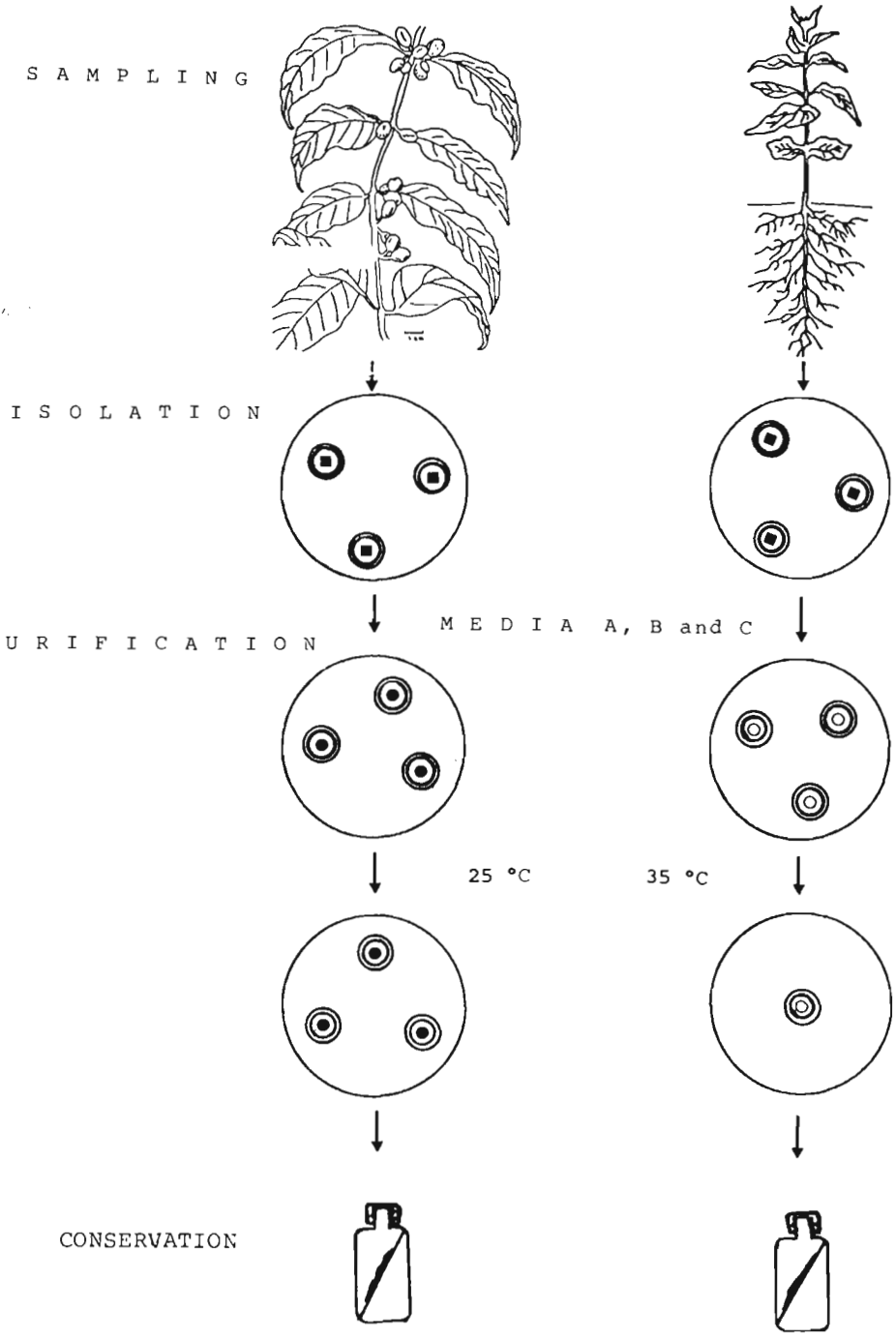

Fig. 2. Isolation, Purification and Conservation of filamentous fungi Diagra

The present work was undertaken to obtain new filamentous fungi strains with ability for caffeine degradation. The isolation, purification and physiological characterization of microorganisms was also necessary. Some 280 strains were screened, 8 of them were chosen for their high capacity for caffeine degradation. The best one was assayed in solid state fermentation using coffee pulp as solid substrate.

MATERIAL AND METHOD :

Isolation and purification of fungi strains.

Leaves, fruits and coffe pulp were sampled from coffee industry in Jalapa, Veracruz and Soconusco, Chiapas in Mexico. Strain isolation and purification was made in a basic medium containing: 1.3 g KH_2PO_4, 0.12 g Na_2HPO_4, 0.3 g $MgSO_4$, 0.3 g $CaCl_2$, in 1000 ml of water (pH 5.6) with coffee extract (medium A), coffee extract and sucrose (medium B), or coffe pulp extract (medium C). Each media were supplemented with 30 mg/l of streptomycin and cultured at 25 and 35°C. The isolation flow sheet is shown in fig. 2.

Identification

The strains identification was made in plate and microculture on Potato-Dextrose Agar Medium, and cultured at same isolation temperature. The conservation strains was made in slants containing the same isolation media and maintained at 4°C

Screening of caffeine degrading strains

The screening of caffeine degrading strains was carried out in 250 ml. shaker flasks containing 50 ml of liquid medium with mineral salts with the same composition as the isolation medium; caffeine (1.2 mg/ml) was the nitrogen source; the incubation was 2-3 days at 25 and 35°C (fig.3).

Solid state fermention

Solid State Fermentation (SSF) was carried out at a laboratory scale, using the unit device described by Raimbault and Alazard (1980), in which a constant temperature can be obtained through forced convection water bath. Regulation of the air flow in each fermenting column could be set by independent valves. Fermentations were run at 25°C and moistened saturated air flow rate was 4 liters/min/device.Each device contained 20 g of the moist coffee pulp (60 mesh). The substrate was mixed with the same mineral salts solution than used in the isolation medium. Inoculation was performed with a suspension of

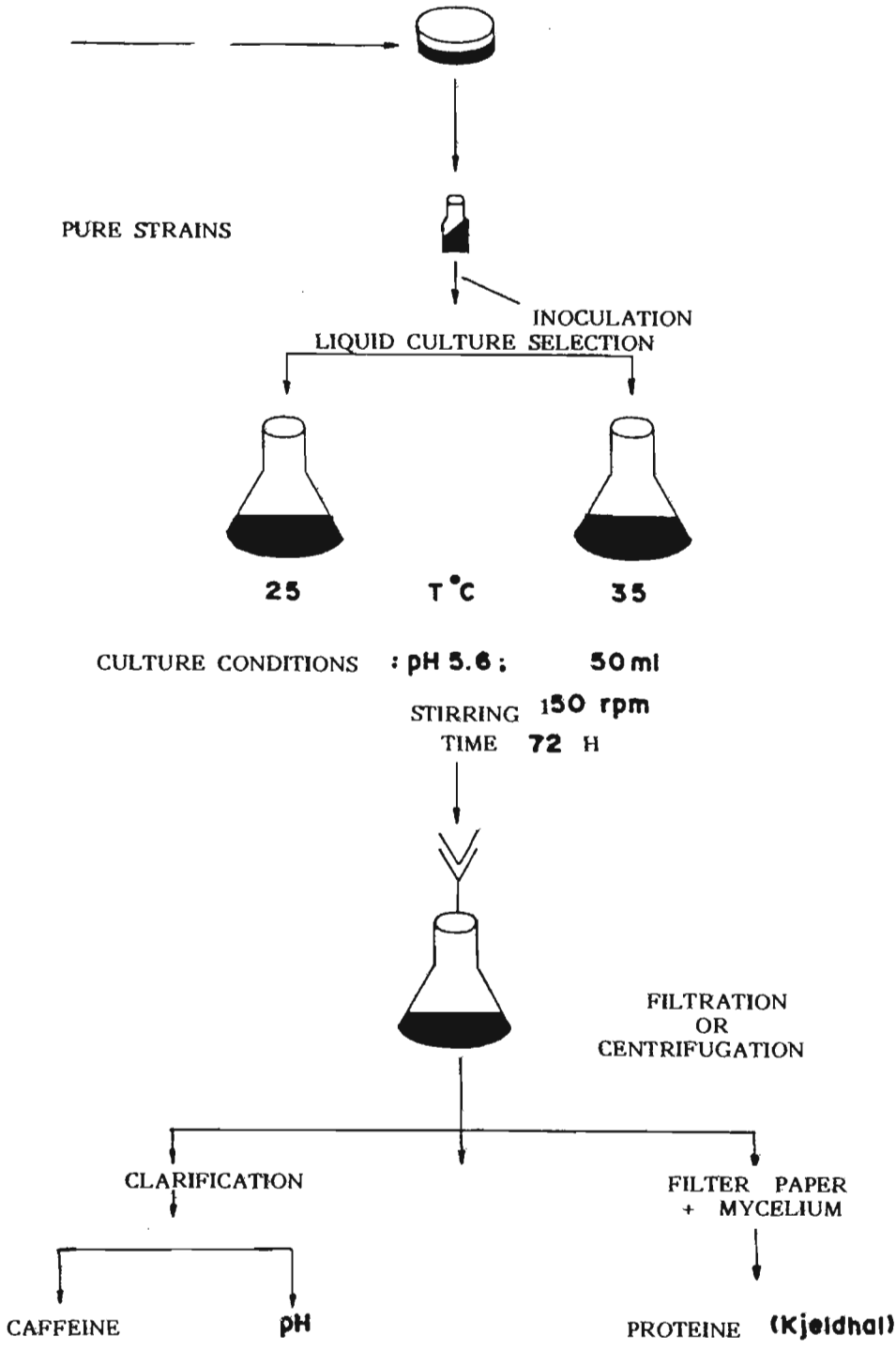

Fig. 3 ISOLATES TREATMENT

conidiospores of the selected strain (2.107 106 spores per g of dried coffee pulp), coffee pulp initial moisture was adjusted to 68-70% and the pH to 4.4 with diluted HCl solution.

Analysis

In selection trials, biomass was measured as crude protein by using standard Kjeldahl method (Bremer, J.M. 1960). Caffeine was measured spectrophotometrically after decoloration with magnesium oxide (Isler, 1948).

For caffeine analysis in solid state fermented products, 5 g of sample were mixed with 75 ml of water and homogenized with Ultra-turrax (IKA, W.Germany) during 2 min at 5000 rpm then the pH was measured, heated to boiling temperature during 10 min and finally filtered, rinsed and adjusted to 100 ml with water (Smyly, Dan S., 1976).

Moisture content was determined in an oven at 100°C during 24 h.

RESULTS AND DISCUSSION :

According to the isolation protocole used, 280 strains of filamentous fungi in pure culture were isolated from Veracruz and Chiapas samples.The table I summarizes the results of isolated and identificated strains: *Aspergillus* and *Penicillium* strains were predominant, but other genera could be identified as *Fusarium*, *Trichoderma*, *Geotrichum* and some Zygomycetes. These results agree with litterature report of Schwimmer and Kurtzman (1972).

The list of filamentous fungi with high capacity to degradate caffeine in liquid medium are reported in tables II and III. This experiment was made with caffeine as sole nitrogen source in mineral salts of basic media containing sucrose as carbon source. The best caffeine degrading strain was Penicillium roquefortii with 95.25% efficiency and a rate of 0.224 mg/ml/day. Other strains such *Aspergillus* and *Penicillium* species also degraded caffeine with similar efficiency and rate. In some cases it should be noted that the growth was associated with an increase in pH.

Figures 4, 5 and 6 show the pH, caffeine depletion and moisture evolution with *Penicillium roquefortii* in solid state fermentation. The coffee pulp required a higher moisture content (70%) than the 50% reported for A. niger grown on starchy substrates by Raimbault (1980). That indicates that the constituents of the pulp bound a larger amount of moisture than the starchy materials and, need more water to allow growth of *Penicillium roquefortii*. Since coffee pulp has been reported to contain 6-8% mucilagenous content, it is possible that these

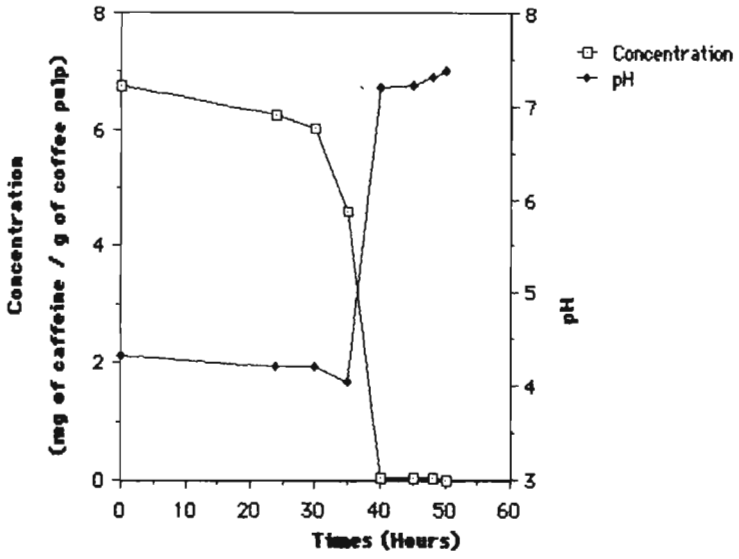

Fig 4 : Kinetics of caffeine degradation and pH evolution during coffee pulp Solid State Fermentation

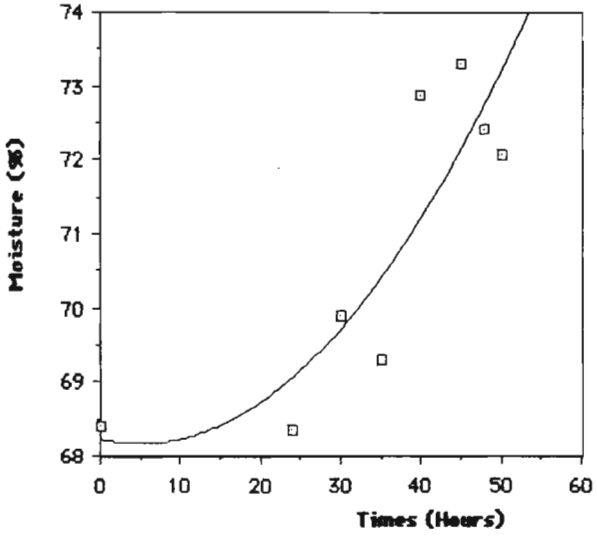

Fig. 5 : Moisture evolution during coffee pulp SSF

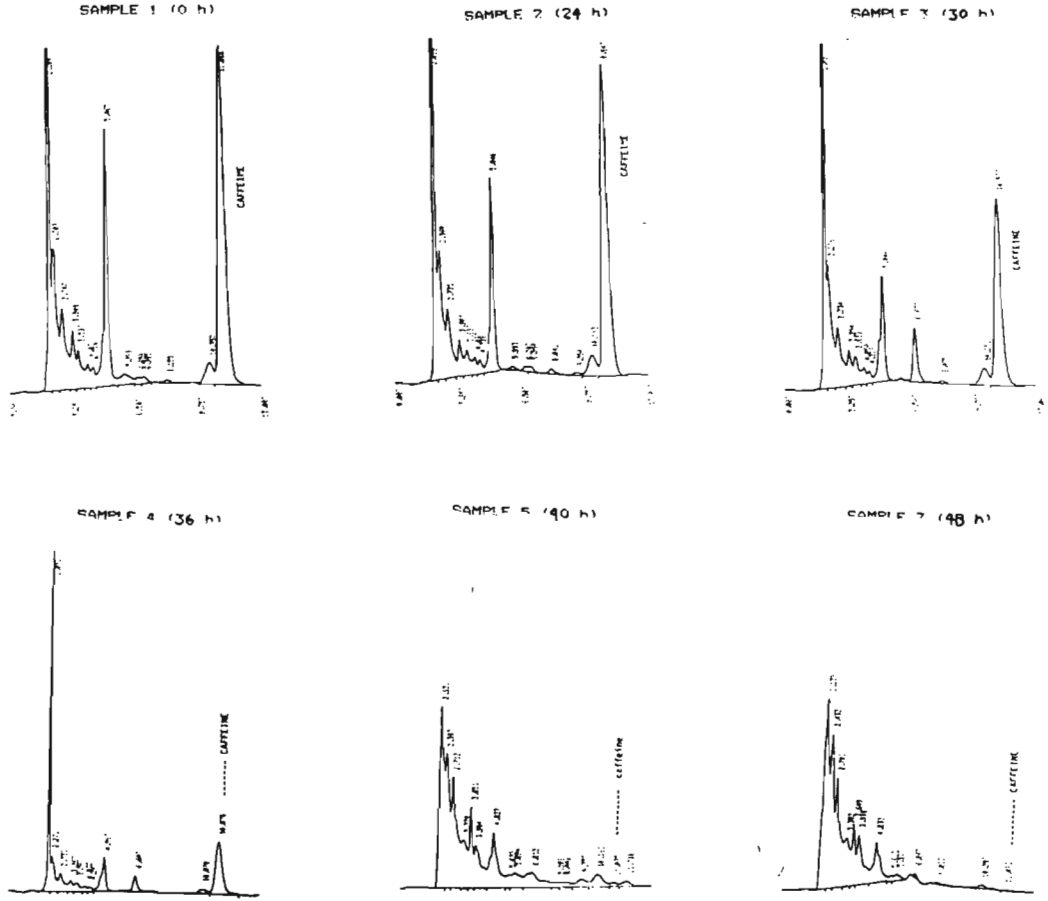

fig. 6 : Determination of caffeine by HPLC Method

TABLE I Isolation and identification of filamentous fungi growed on media A, B y C in 25º C. (% isolated strains).

	VERACRUZ			CHIAPAS		
	A	B	C	A	B	C
Aspergillus	15.2	2.1	2.1	--	14.2	3.2
Penicillium	10.8	2.1	--	4.7	--	--
Fusarium	8.6	--	--	7.9	17.4	11.0
Trichoderma	--	--	--	--	1.6	3.2
Geotrichum	4.3	2.1	13.0	--	1.6	3.2
Zygomycetes	15.2	23.5	--	9.5	3.2	14.2

TABLE II LIST OF FILAMENTOUS FUNGI WITH HIGH CAPACITY TO DEGRADATE CAFFEINE IN LIQUID MEDIUM. STIRRED CULTURES AT 25ºC, pH=5.6,150 RPM AND CAFFEINE AS NITROGEN SOURCE (1.2 g/l).

STRAIN	NAME	% CAFFEINE CONSUMPTION	FINAL pH	DEGRADATION VELOCITY mg/ ml/ days
V12A25	Aspergillus oryzae	77.75	6.7	0.157
V26A25	Penicillium sp.	62.13	7.2	0.126
V33A25	Penicillium roquefortii	95.25	6.5	0.126
C16A25	Penicillium sp.	61.66	2.5	0.123
C28B25	Aspergillus fumigatus	69.60	6.1	0.119
C11B25	Aspergillus sp.	70.66	6.3	0.120
C23B25	Aspergillus niger	63.58	3.4	0.108
C17B25	Aspergillus fumigatus	60.50	2.4	0.103

TABLE III Quantitative selection of strams of filamentous fungi, with hability to deplete caffeine in liquid media, by the use of stirred flasks at 35ºC during 4 days.

STRAIN No.	% OF DEPLETED CAFFEIN	pH FINAL	GROWTH	
V20A35	18.25	6.56	+++	(pellets)
V25A35	17.08	6.63	+++	pellets
V3A35	10.83	6.27	+++	pellets
V2A35	6.66	6.34	++	pellets
V12A35	4.58	3.65	++	pellets
V29B35	18.66	6.28	+++	pellets
V31B35	16.25	6.34	+++	pellets
V20B35	14.33	6.30	++	pellets
V12B35	4.16	3.06	++	pellets
V33B35	2.5	6.3	+	difuso
V29C35	26.5	6.49	+++	pellets
V25C35	25.16	6.31	+++	pellets
V26C35	21.0	6.16	+++	pellets
V13C35	19.83	6.16	+++	pellets
V10C35	14.83	6.3	+++	pellets
V15C35	12.50	6.23	++	pellets
V16C35	11.64	6.35	++	pellets
V23C35	4.41	6.15	+++	pellets

(+) low growth
(++) medium growth
(+++) high growth

Conditions: pH=5.6, 2,00 rpm.

Caffein concentration: 1.2 g/l.

components are mainly responsible for the difference in binding water with the starchy materials (Penaloza,W and all, 1985).

Solid state fermentation process caused an increase of pH after from 35 h correlated with caffeine decrease initiation just in the same way than observed in liquid culture. This could be due to by-products of the degradation such as area, according to the proposed metabolic pathway (Fig. 1). After a fermentation period of 45 hr, the caffeine was completely consumed (Fig.6).

From the evidence presented here, we may conclude that this isolation, purification and selection methodology allowed to find highly degrading strains of caffeine (80-100%).

The present investigation established that caffeine can be removed from coffee infusions as well as synthetic growth media mainly by Aspergillus, Penicillium and Trichoderma strains.

Also results proved that filamentous fungi could be used to degrade caffeine by solid state fermentation processe vith the objective of improving nutritional value of coffee pulp for animal feed.

ACKNOWLEDGMENTS :

This work was carried out as part of the cooperation agreenment between the Consejo Nacional de Ciencia y Tecnologia (CONACYT, México) and the Institut Francais de Coopération Scientifique pour le Développement en Coopération (ORSTOM, France) with a specific research program agreed between the Universidad Autonoma Metropolitana, Mexico and ORSTOM. The authors wish to thank CEE for financial supports (projects n° TSD-A-106 and TSD-A-418).

REFERENCES :

Adams, M.R. and J. Dougan, 1981. Biological management of coffee processing wastes. Trop. Sci. 23: 178-196

Bremmer, J.M. 1960. Determination of nitrogen in soil by the Kjeldahl method. J. Agric. Sci. 55: 11

Bressani R., 1979. " Antiphysiological factors in coffee pulp." p. 83-88. In J.E. Braham and R. Bressani (eds.) Coffee pulp: composition, technology, and utilization. IDRC Publ. 108e. International Development Research Centre,

Bresanni R. 1979. " Potential uses of coffee-berry by-products." p. 17-24. In J.E. Braham and R. Bressani (eds.) Coffee pulp: composition, technology, and utilization. IDRC Publ. 108e. International Development Research Centre, Ottawa.

Gomez R. 1979. " Processing of coffee pulp: Chemical Tratments."p. 71-81. In J.E. Braham and R. Bressani (eds.) Coffee pulp: composition, technology, and utilization. IDRC Publ. 108E. International Development Research Centre, Ottawa.

Isler N.H., T.P. Finucane and E. Borker, 1948. Rapid Spectrophotometric Determination of Caffeine. Anal. Chem., 20(12), 1162-1166

Molina, M., De la Fuente, G., Batten, M.A. & Bressani R. 1974 Decaffeination. A Process to Detoxify Coffee Pulp. J. Agr. Food Chem. 22:1055-1059

Penaloza W., Molina M., Gomez R., Bressani R. 1985. Solid-State fermentation an alternative to improve the nutritive value in coffee pulp. Applied and Environmental Microbiology 49:388-393

Raimbault, M. 1980. Fermentation en milieu solide: Croissance des champignons filamenteux sur substrat amylacé. Paris ,ORSTOM, 291 p. (ORSTOM Travaux et Documents n°127).

Raimbault, M., and D. Alazard. 1980. Culture method to study fungal growth in solid fermentation. Eur. J. Appl. Microbiol. Biotechnol.9 :199-209

Sauer M. Kappeli O, Fietcher A. 1982. Comparison of the cytochrome P-450 containing monooxygenase originating grom two different yeast. Developments in Biochem. 23:452-457

Schwimmer S., Kurtzman Jr. and Heftmann E. 1971. Caffeine metabolism by Penicillium roqueforti. Arch. Biochem. Biophysics., 147, 109-113

Schwimmer S. and Kurtzman R. H. Jr. 1972. Fungal decafeinizacion of roast coffee infusions. J. Food Science.37, 921-923

Smyly, Dan S., Betsy B. Woodward and Edward C. Conrad .1976. Determination of Sacharin, Sodium Benzoate, and Caffeine in Beverages by Reverse Phase High-Pressure Liquid Chromatography. Journal of the AOAC;