CAFFEINE DEGRADATION IN FRESH AND ENSILED COFFEE PULP BY SOLID STATE FERMENTATION: INFLUENCE OF SUBSTRATE PRETREATMENT AND INOCULUM LEVEL

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SUMMARY

Caffeine degradation in fresh (FP) and ensiled (EP) Coffee Pulp (CP) using Solid State Fermentation (SSF) was studied. Thermal treatment and different inoculum levels were also tested in a SSF process. With SSF a 95 % in EP and 55 % in FP of caffeine degradation was reached using a Penicillium comune (V33A25) strain after ten days of fermentation without thermal treatment. Presence of enzymatic activities like tannase (tannin-acyl hydrolase) and exopectinase shown the capacity of strain to utilize tannins and pectins constituents of CP. Initial massive inoculation of 10^{-} spores/ g moist substrate only 56 % of caffeine was degraded in four days of SSF. Nevertheless the diminishing in natural microflora of CP due to thermal treatment produced a significative increase in degradation up to 94 % for EP and 100 % for FP in four days of fermentation. Preservation trough silage and the combination of proper thermal treatment and the use of selected strains can reduce contents of antinutritional and antiphysiological compounds in CP establishing its application potential in animal feed.

RESUMEN

Se estudió la degradación de cafeína en Pulpa de Café (PC) fresca (PF) y ensilada (PE) utilizando la Fermentación en Medio Sólido (FMS). Se probó el tratamiento térmico y diferentes niveles de inóculo en FMS. Por este método de fermentación fue posible degradar 95 % de la cafeína en PE y 55 % usando PF con la cepa de Penicillium comune (V33A25) sin un tratamiento térmico previo y después de 10 días de incubación. Además la presencia de actividades enzimáticas tanasa (tanin-acil hidrolasa) y exopectinasa mostró que el hongo es capaz de utilizar además pectinas y taninos presentes en la PC. La inoculación masiva inicial permitió degradar 56% de la cafeína, en 4 días de FMS, con un nivel de inoculo de 10^{-} esporas/g de sustrato húmedo. Por otra parte el tratamiento térmico disminuyó significativamente la microflora nativa lo que evitó contaminaciones en la FMS y permitió aumentar significativamente la degradación a 100 % en PF y 94 % en PE en 4 días de fermentación. La conservación por ensilaje y la combinación de un tratamiento térmico adecuado y el uso de microorganismos seleccionados permite reducir el contenido de compuestos antifisiológicos y antinutricionales en la PC estableciendo su potencial de aplicación en la alimentación animal.

INTRODUCTION

Coffee Pulp (CP) is an agroindustrial waste obtained from coffee fruits benefaction process and represent 40 % of the whole fruit mass (Zuluaga, 1989). CP represents a serious pollution problem in tropical areas taking to account the large volumes of grain produced each season (Aquiahualt *et al.*, 1988; Rosussos *et al.*, 1995). Conservation and utilization of CP has been difficult due to, among other factors, its scatter and seasonal production, high moist content (80-85 %), presence of nutrient factors (carbohydrates, proteins and minerals) which facilitates its destruction for a wide variety of microorganisms. Alternatives proposed for the utilization of CP include its use as animal food, organic compost, substrate for enzyme production, biogas, mushrooms, vermiculture (Gaime-Perraud *et al.*, 1993). Nevertheless its utilization as animal food is limited by the presence of antiphysiological and antinutritional compounds as caffeine (1,3,7-trimethylxantine), tannins and polyphenols (Roussos *et al.*, 1995). Porres *et al.* in 1993 found reduction in caffeine contents up to 63 % in 223 days of silage for conservation of 3 t of CP supplemented with cane molasses. More recent studies for conservation of CP followed by SSF for reduction of caffeine content shown that in anaerobic fermentation with lactic acid bacteria inocula during silage of dried CP did not reduce caffeine content but permit conservation of the ensiled CP (Gaime-Perraud, 1995). SSF studies for caffeine reduction developed include isolation and screening of fungi strains able to degrade caffeine in dried fresh CP and complete degradation of caffeine in 45 h of fermentation with highly degrading strains

(Aquiahualt *et al.*, 1988; Rosussos *et al.*, 1995). Experiments with EP shown significative caffeine reductions in longer fermentation times (48 to 120 h) due to a retard in germination of spores from inoculum caused by the presence of organic acids produced during silage (Gaime-Perraud, 1995).
One strategy proposed for valorization of CP is conservation of large volumes of CP produced during crop season trough silage then use SSF for detoxification of EP and improve its potential of use in animal feed. In present work caffeine degradation in fresh and ensiled CP was analyzed. Thermal treatment and inoculum level were tested as strategies for improve caffeine degradation and standardization of SSF conditions for scale-up the process.

METHODOLOGY

Strain source

A *Penicillium comune* strain (V33A25) isolated from the CP and belonging to the IRD-UAM collection was used. It was considered because of its high efficiency of caffeine degradation reported previously (Aquiahuatl *et al.*, 1988; Roussos *et al.*, 1995; Gaime-Perraud, 1995; Hakil *et al.*, 1998).

Solid fermentation substrates preparation

The fresh coffee pulp (PF) samples were obtained from a coffee processing plant in Jalapa, Veracruz state, Mexico during the 1997-1998 coffee season (September 97 - March 98). Samples were frozen at -20°C until their utilization. A batch of 400 kg of PF was inoculated with a lactic acid bacterium strain, isolated from the ensiled pulp by Gaime-Perraud in 1995 and named as B01. Inoculation level was 2.6-5.2x10^7 cellg^{-1} wet basis corresponding to 5 % (v:w). Material was ensiled, in 20 kg plastic containers for its conservation and stored until utilization. Lixiviates from 9 containers were taken for enzymatic determination of dextrose (D+glucose) and L-lactic acid by using a YSI instrument. A 10 cm thick samples of 1 kg of FP and EP were treated in an autoclave at atmospheric pressure (95°C) for 1 hour using a direct steam stream. Samples were taken to analyze UFC and moisture content, after treatment samples were dried at ambient temperature during 24 hour. Samples from the same batch without thermal treatment were used as controls for SSF.

Inoculum development and SSF conditions

Strain was 4 times subculture on CSM (Aquiahuatl *et al.*, 1988): commercial coffee used was Grand'Mére "familial"^{MR}, infusion of 40 gl^{-1} was filtered by Whatman paper No. 41, then salts and agar were incorporated in following concentrations (gl^{-1}): sucrose 2.0; KH_2PO_4 1.3; $Na_2HPO_4 \cdot 2H_2O$ 0.12; $MgSO_4 \cdot 7H_2O$ 0.3; $CaCl_2 \cdot 2H_2O$ 0.3; agar 20.0. pH was adjusted to 5.6 using KOH, 0.1 M and the volume was adjusted to 1 liter. The culture medium was sterilized at 121°C for 15 minutes. Cultures were grown at 30°C for 7 days using Erlenmeyer flask or Rusk bottles inoculated with 10^6 spores cm^{-3} The spores were harvest with Tween 80 solution (1 %) and magnetic stirring for 20 minutes. They were centrifuged to reach concentrations of 10^4,10^5,10^6,10^7 and 10^8 spores g^{-1} wet solid. SSF was achieved according to the methodology described by Raimbault and Alazard in 1980, using cylindrical glass laboratory reactor incubated at 30°C and with humid saturated air flow of 0.5 vkgm (dm^3 of air g^{-1} of medium min^{-1}). Samples in duplicate were taken for analytical determinations.

Humidity determination, pH and samples treatment.

Humidity of solid samples was determined by gravimetry. Wet solid samples were milled and resuspended in water in 1:10 (w/w) ratio and pH was determined with a Conductronic pH 20 potentiometer previously calibrated. The supernatant were recovered by centrifugation at 5000 rpm for 20 minutes and stored at -20°C until their analysis.

Volatile fatty acids (VFA) determination

The volatile fatty acids (VFA) concentration, glucose and fructose were determined by HPLC using a Rezek Organic Acid column and H_2SO_4 30 mM as mobile phase at 0.6 cm^3min^{-1} flow rate. Analyses were carried out at 50°C using a refraction index detector. A flame ionization detector (FID) gas chromatograph was also used for VFA analysis.

Caffeine and metabolic intermediaries analysis

Dried and milled (mesh 35) solid samples were extracted with a chloroform/isopropanol mixture (50:50, v/v) doing three successive extractions. Solvent was evaporated by heating and the extract was resuspended in equivalent Milli-Q water volume. Caffeine and intermediary molecules was analyzed by HPLC, using a modification of the technique reported by Denis, 1996: Spherisorb ODS-2 column, acetonitrile/tetrahydrofurane/deionized water (5:1:94 v/v/v), 1.5 cm^3 min^{-1} flow rate, room temperature, diodes array detector at λ=273 nm were used.

CO_2 determination by gas chromatography
Gow-Mac 580 chromatograph was used, equipped with a Alltech (CTR1) concentric column and a thermal conductivity detector (TCD) as previously reported (Saucedo-Castañeda *et al.*, 1994).

Enzymatic activities
Tannase (tannin-acyl hidrolase) activity was achieved using the technique reported by Beverini and Metche, 1990. It is based on the galic acid liberation which is determined by HPLC at λ=280 nm. Results were expressed as mM cm^{-3} min^{-1} of galic acid released at the reaction conditions. Exopectinase activity was determined modifying the technique described by Schwan and Rose, 1994. Based in the galacturonic acid liberation from the pectin hydrolysis. Results were expressed as mM cm^{-3} min^{-1} of galacturonic acid released at reaction conditions. Proteolytic activity was determined using Azocoll as substrate according as described previously by Bidochka and Khachatourians, 1987. A proteolytic unity was defined as the enzyme require to increase 0.001 optical density units at λ =580 nm, at reaction conditions.

Microorganisms counting
Cultivation was done following the methodology described by Perraud-Gaime in 1995, using Petri dishes. Standard nutritional agar (Bioxon) was used for total microorganisms and PDA (Bioxon) added with antibiotic for plating fungi and yeast. Plate count was done after 24 hours of incubation at 30°C. Results were expressed as UFC g^{-1} dry matter.

RESULTS AND DISCUSSIONS

Characterization of inoculated coffee pulp silage using a homolactic bacteria
The aim of this trial was to set up the methodology for in silage inoculation in coffee benefaction plants. A total quantity of 400 kg of coffee pulp, wet basis, was ensiled using a homolactic LAB strain B01. Requiring nearly 20 l of lactic acid bacteria culture. Liquid medium was prepared using a diluted MRS broth (25%) for all the nutrients exception made of glucose concentration. Results of glucose uptake and lactic acid formation are shown in Figure 1. Glucose was consumed almost completely in first four days of silage. Concentration of L-lactic acid in lixiviates increased up to 7.4 g l^{-1} in 11 days of silage, then no further accumulation was observed. A high microbial activity was observed through the liberation of a large quantity of CO_2 which was not measured. This fact caused difficulties for control of anaerobiosis in the silages.

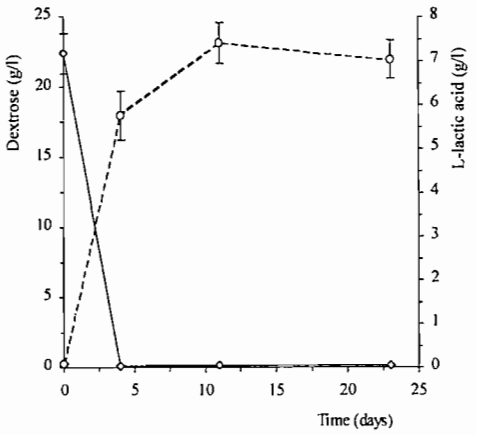

Figure 1. Glucose uptake (-◊-) and lactic acid formation (••O••) during coffee pulp silage inoculated with LAB strain B01.

Caffeine degradation in FP and EP
Experiences of SSF were done using FP and EP as potential available substrates coming from areas of pulping processing plants of coffee cherries. Fermentations were run until the apparition of green-blue color characteristic of *Penicillium* genus spores. Inoculum size was fixed at initial concentration of 10^7 spores g^{-1} moist solids.
Figure 2 shows kinetics of caffeine degradation in SSF using both substrates. Degradation was higher by using EP rather than FP, where degradation of caffeine was 96 and 66 % after 10 days of fermentation. In this case, the duration of the culture required for obtain a significative reduction of caffeine content in EP was greater than in previous works with sun dried CP. Aquiahualt *et al.* in 1988 found that 40 h of culture are required for complete degradation of caffeine in FP, using a *Penicillium roquefortii* strain isolated from CP. In more recent studies the duration of cultivation required for complete caffeine depletion ranged between 48 and 120 hours depending on the source of lactic acid bacteria used for CP silage (Gaime-Perraud, 995).Bacterial contaminants appearing during the culture were the most probable cause for the long term of the SSF process. The origin of the contamination was the natural microflora of FP and EP, on the other hand, this natural microflora was seriously diminished when using CP sun dried was , used avoiding problems of contamination (Aquiahualt *et al.*, 1988; Gaime-Perraud, 1995).

Initial moisture contents of FP and EP were 80.5 and 84 % respectively and were slightly raising during the process up to a final value of 86 and 990%, respectively (data not shown). These excess of moisture produced liquid running out from solid bed during fermentation and given an undesirable conditions for developing of the culture in solid media.

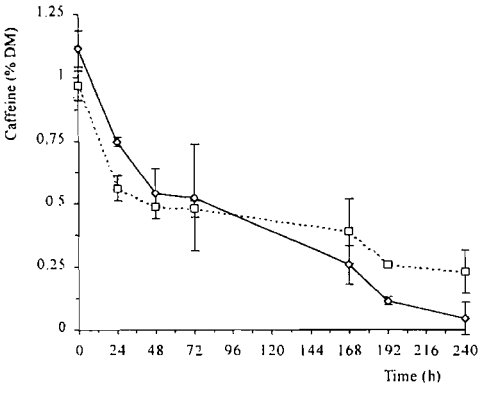

Figure 2. Kinetics of caffeine degradation in ensiled (-◊-) and fresh (•• ••) coffee pulp during solid state fermentation with *P. comune*.

Initial pH in FP and EP were 4.8 and 3.7, respectively (Fig 3a). Decreased of pH in the first hours of culture in FP can be explained by the acetic acid production (data not shown). The pH of culture in EP increased rapidly as a result of VFA consumption produced during previous silage of CP (Fig 3b). Organic acids detected were: lactic, acetic and propionic at initial ratios of 1:1.7:0.25, in any case butyric acid was found. Organic acids were consumed during the first 72 h of fermentation. Increased in pH from 168 h of fermentation was probably the result of the released of urea due to complete caffeine degradation.

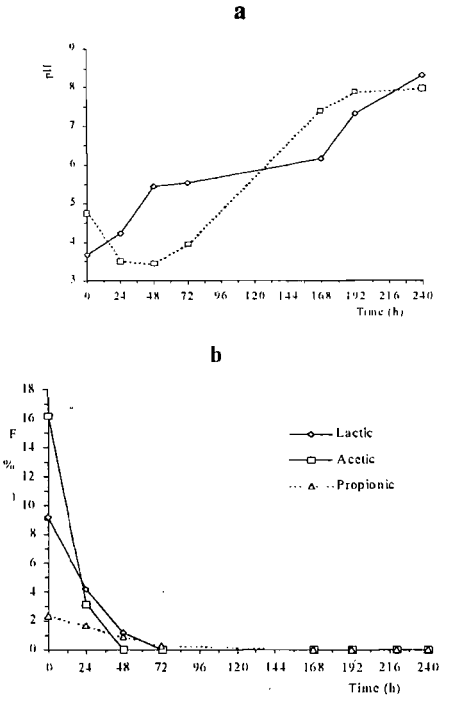

Figure 3. Kinetics of pH evolution in coffee pulp ensiled (-◊-) and fresh (••••) (a) and volatile fatty acids composition in PE (b) during solid state fermentation with *P. comune*.

Metabolic activity of *P. comune* during SSF was monitored through CO_2 evolution in fermentation using both substrates PE y PF (Figure 4). Maximum velocity of carbon dioxide production in EP (11.52 mg g^{-1}IDM h^{-1}) was obtained at 22 h of culture since in FP was the half (5.66 mg g^{-1}IDM h^{-1}) and was reached later (44 h). Decreased of metabolic activity in FP could be associated with the presence of a higher charge of contaminants, as indicated above. This situation changed in EP because the production of organic acids during silage probably reduced the microbial population (Gaime-Perraud, 1995). In the case of EP a lag phase of 12 h was observed as a result of inhibition of spore germination probably produced by organic acids content of EP.

Figure 5a and b show preliminary results obtained in enzymatic activity determinations of tannase and exopectinase during the course of fermentations of EP and FP with the *P. comune* strain used. Expression of these enzymatic activities revealed that this mold is able to utilize other nutrients presents in CP rather than caffeine, like tannins and pectic substances.

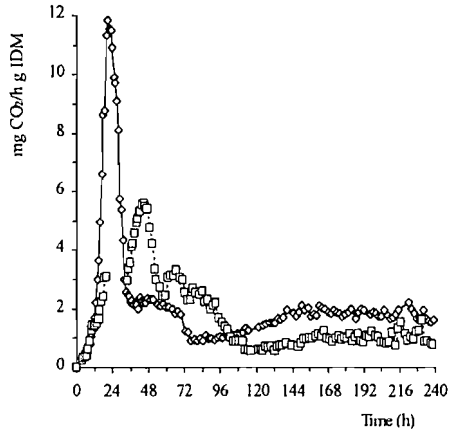

Figure 4. Kinetic of CO_2 evolution rate in coffee pulp ensiled (-◊-) and fresh (••••) during solid state fermentation with *P. comune*.

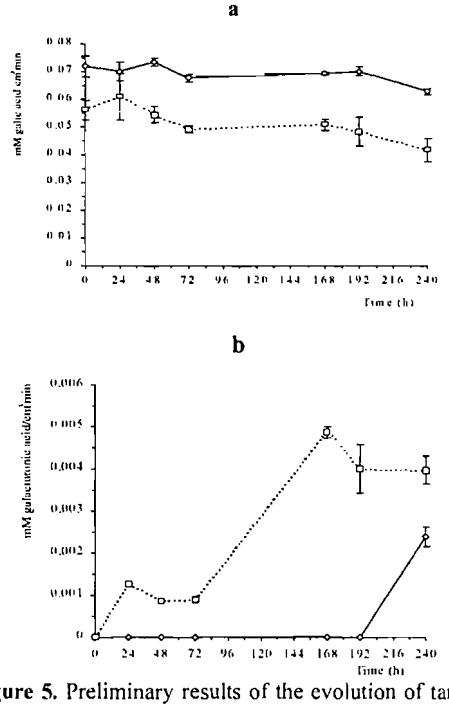

Figure 5. Preliminary results of the evolution of tannase (a) and exopectinase (b) enzymatic activities in ensiled (-◊-) and fresh (•• ••) coffee pulp during solid state fermentation with *P. comune*.

At the moment fermentations experiences are performed for modification of enzymatic analysis according to the characteristics of enzymatic extracts obtained from SSF with CP. More studies are required in order to set up the methodology of production of enzymes using natural substrates as raw and ensiled coffee pulp.

Results presented in this section show a long process for caffeine degradation using EP and FP as substrates of SSF, probably due to bacterial contaminations appeared in both substrates. Although these contaminants are present in less extent in EP its effects are also negatives. This information led to realize studies of thermal treatment of the CP before its utilization as substrate in SSF.

Effect of thermal treatment of substrate: FP y EP

As can be see in Figure 6 initial content of total microorganisms is very high (10^{10} CFU g^{-1} DM), it is twice higher in FP that in EP. During thermal treatment of the EP and the FP a significative reduction of the microbial content was observed after 20 min of exposition to direct steam stream in both substrates. It allowed to reduce natural microflora of CP in a short time treatment and standardize initial conditions for SSF. For later studies, a thermal treatment was used as previously to the fermentation process in further experiences.

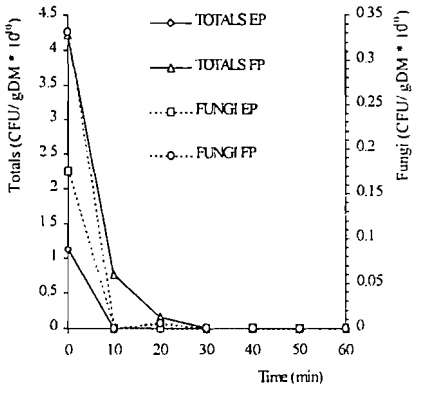

Figure 6. Reduction of microflore present in ensiled (EP) and fresh (FP) coffee pulp during thermal treatment with steam stream at 95 °C.

On the other hand pasteurization process did not caused an increase of moisture content of substrates, and kept values around 80 % during the process (data not shown). However substrates still had high water content at the end of thermal treatment and it can be responsible of water and nutrients losses during SSF, as have been stated in previous paragraphs. To avoid this problem pasteurized substrates were dried at room temperature for 24 h with a reduction of moisture up to 75 % avoiding risks of running off in SSF. Conclusion of this results is the need of pasteurization with steam stream at 95 °C during 20 min and further drying at room temperature for 24 h.

Effect of inoculum level in caffeine degradation in FP and EP with and without thermal treatment.

Experiments with EP and FP with or without thermal treatment were performed in order to asses the effect of heat treatment on caffeine degradation, using different inoculation levels in SSF. Figure 7 shows results obtained after 4 days of fermentation. A significative increase in caffeine degradation of 30% (α=0.05) for EP was observed when thermal treatment and partial drying were used (Fig 7a). These results suggested the need of reduction of the initial

charge of contaminants in order to allow the growth of the mold used ant therefore caffeine degradation can occurred. In EP without thermal treatment caffeine reduction was around 65 %. Increase of inoculum concentration had a positive effect in caffeine degradation starting from a concentration of 10^5 spores g^{-1} of moist substrate. Increment of inoculum level up to 10^8 spores g^{-1} of moist substrate did not produce a significative increment in caffeine degradation. Besides only 40 % in caffeine degradation was reached when EP without thermal treatment was non inoculated, caused by the action of natural microflora. This finding confirm the proposal of inoculation of EP with thermal treatment using the *P. comune* strain which has the potential for degrading caffeine.

Thermal treatment caused a total caffeine degradation when FP was inoculated at every levels but in FP without thermal treatment the results were quite random and lower than in FP with thermal treatment (α=0.05) due to the differences in natural microflora behavior (Fig 7b). Only with a very high inoculum level of 10^8 spores g^{-1} of moist substrate in FP without thermal treatment could reach the same caffeine reduction as in EP without thermal treatment. The use of thermal treatment and inoculation of CP, FP or EP, is well stated as this point showing the necessity of standardize initial conditions of SSF for caffeine degradation in CP in large volumes.

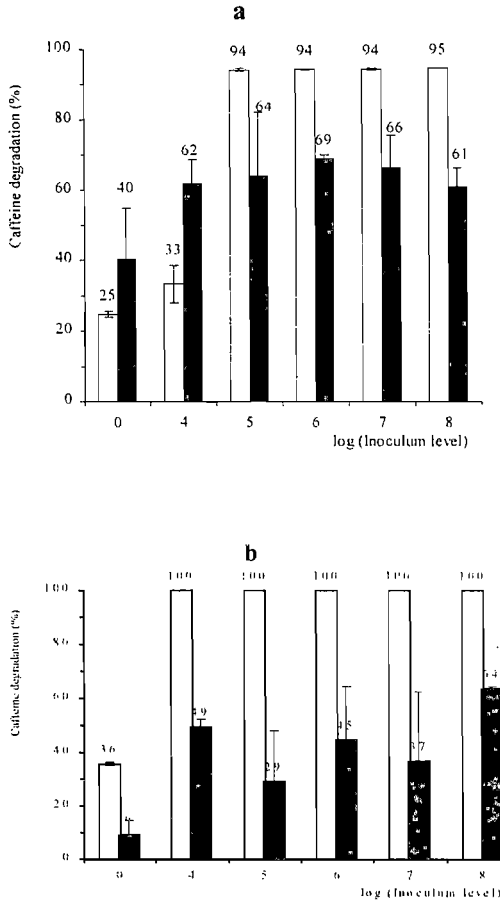

Figure 7. Caffeine degradation by *P. comune* in 4 days of solid state fermentation of ensiled (a) and fresh (b) coffee pulp with (empty bars) and without (filled bars) thermal treatment using direct steam stream at 95 °C during 20 min.

Figure 8 shows kinetic of CO_2 evolution rates during SSF of EP with (Fig 8a) and without (Fig 8b) thermal treatment. Inoculation of 10^4 spores g^{-1} moist substrate caused an increment in maximum CO_2 evolution rate when EP was previously pasteurized but further increments up to 10^8 spores g^{-1} moist substrate did not improve maximum rates (Fig 8a), similar results to that already presented were obtained for caffeine degradation in FP. A direct relationship between maximum CO_2 evolution rate and caffeine degradation could be establish at this point, taking to account that caffeine is use as nitrogen source by the fungus (Denis, 1996; Hakil, 1998; Roussos *et al.*, 1995).

In EP without thermal treatment a random behavior of maximum CO_2 evolution rate with increment of inoculum level was obtained probably because the difficulties encountered for standardize initial microbial content of solids (Fig 8b). In case of inoculum levels of 10^5 spores g^{-1} moist solid for EP without thermal treatment were found higher maximum CO_2 evolution rates (near

15 mg h^{-1} g^{-1} IDM) than in EP with thermal treatment probably because the contribution to respiration of natural microflora of EP without hermal treatment.

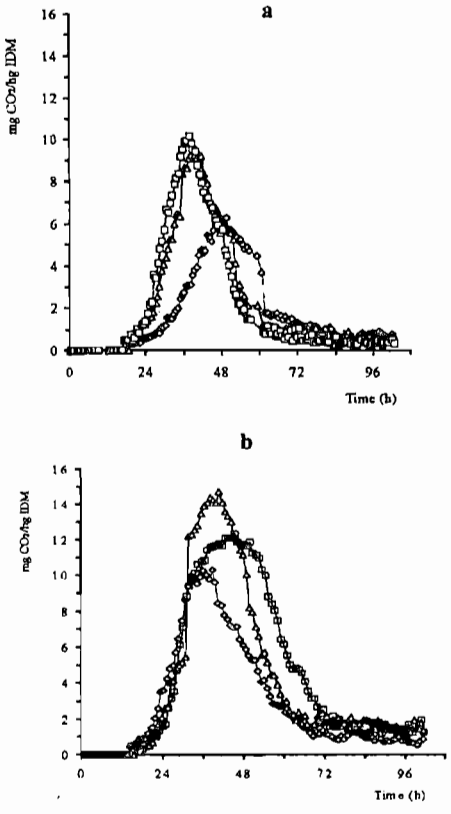

Figure 8. Kinetic of CO_2 evolution rates in 4 days of solid state fermentation of ensiled coffee pulp with (a) and without (b) thermal treatment with different inoculum levels (no inoculum -◊-, 10^5 -Δ-, 10^7 - -) of *P. comune*.hermal treatment and partial drying improved caffeine degradation.

Inoculation levels since 10^5 spores g^{-1} moist substrate increased significatively efficiency of caffeine degradation in EP but inoculum level was set at 10^6-10^7 spores g^{-1} moist substrate in further experiments. Because lesser levels facilitate development of contaminants in SSF (Soccol, 1992) and higher levels possibly cause inhibition phenomena in spores germination (Barrios *et al.*, 1989).

Results obtained show the viability of utilize SSF to reduce caffeine content of CP without alteration in substrate characteristics and with the possibility to use first silage, as a simple method of conservation, and then controlled conditions of SSF for detoxifying or production of enzymatic extracts able to be applied during silage for partial or total degradation of caffeine. A similar experimental plan could be employed for reduction of poliphenols content in coffee pulp. On the other hand these results help to face scale up of SSF for coffee pulp detoxification

CONCLUSIONS

A number of methodologies have been set up in order to carried out microbiological and biochemical analysis in fermented and raw substrates. In the same sense, the methodology of inoculation of coffee pulp using lactic acid bacteria had been tested, resulting in an useful experience for future studies.

Strain of *Penicillium* V33A25, identified as *Penicillium comune* was selected for future studies. It was confirmed that this strain can utilized caffeine by solid culture using natural substrates.

The high natural microflora present in raw coffee pulp or ensilaged coffee pulp requires a heat treatment in order to allow the development of selected strain. A thermal treatment is recommended previously to the fermentation process for further studies.

The effect of inoculation level on caffeine degradation was tested in raw coffee pulp or ensiled coffee pulp, results indicated that an inoculation of level of 10^6-10^7 spores g^{-1} moist substrate is required to degraded more than 94 % of the caffeine contained in ensiled coffee pulp after 4 days of culture.

More enzymatic studies are required in order to optimize the methodology of production of enzymes using natural substrates as raw and ensiled coffee pulp. Experimental evidence presented in this report contributed to set up the condition of solid state culture in order to set the basis of the scale up process for coffee pulp detoxification..

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