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Caffeine Degradation by *Penicillium verrucosum* in Solid State Fermentation of Coffee Pulp : Critical Effect of Additional Inorganic and Organic Nitrogen Sources

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Effect of additional inorganic and organic nitrogen sources, either individualy or in combination, on the degradation of caffeine pulp by *Pencillium verrucosum* in solid state fermentation was studied with a view to decaffeinate this agro-industrial waste for potential utilization as animal feed and overcome the problem of environmental pollution. The results revealed full inhibition of caffeine degradation with all the different strategies adopted for incorporation of additional nitrogen sources in the medium, in spite of good fungal growth. In contrast, full degradation of caffeine occurred in the absence of any added nitrogen source, in spite of the limited growth of the culture. The data on changes in the pH and moisture content of the media eliminated any possible role of these parameters in inhibiting caffein degradation in media with additional nitrogen sources. The results indicate utilization of caffeine by the culture as nitrogen source, which ultimately leads to its complete degradation.

Keywords : Coffee pulp, Caffeine degradation, *Penicillium verrucosum*, Solid state fermentation, Effect of nitrogen supplementation, Nitrogen sources.

Coffee pulp, the waste generated in large quantities during wet method of coffee cherry processing (Aguilar 1983), contains 8.25% protein and 23-27% fermentable sugars on dry weight basis (Zuluaga-Vasco 1989; Bressani 1979). In spite of such high nutrient content, the coffee pulp can not be an animal feed, mainly due to its toxic components such as caffeine, tannins, phenols and other polyphenols (Bressani 1979). Consequently, most of the coffee pulp remains unutilized in many countries of the world and a need exists for its treatment by appropriate biological waste treatment processes to overcome severe environmental pollution problem (Zuluaga-Vasco 1989). Being cost-intensive in nature, the treatment of the waste adversely affects the cost of production of coffee and hence, it is generally avoided by the coffee processors. It is worth noting that huge quantities of coffee pulp, to the tune of about 40% of the several million tonnes of coffee cherries, is generated in the coffee plantations of Mexico, Central America, Columbia and India (Zuluaga-Vasco 1989). Consequently, the coffee pulp forms a major source of the pollution of rivers, lakes and environment in the vicinity of the coffee processing sites (Boccas et al. 1994).

Elimination of the toxic and anti-physiological factors in the coffee pulp can lead to much needed cheaper and abundant alternative source of nutrients for use as animal feed. Such a possibility has been correctly recognized by many workers throughout the world and culminated in several studies for total elimination or at least partial removal of the anti-physiological factors in coffee pulp (Molina et al. 1994; Udaya Shankar et al. 1986). Most of the successful methods, which have emerged from these studies, involve either physical or chemical treatments of the coffee pulp and are characterized either as inefficient in eliminating the toxicity or too expensive in nature. The possibility of microbial detoxification of coffee pulp was also recognized and some efforts have been msde in this direction. For example, the degradation of caffeine via theobromine by *Pseudominas aeruginosa* and *P. sputida* has been reported (Blecher and Lingens 1977: Kurtzman and Schwimmer 1971). The latter has been stated to possess the enzymes such as xanthine dehydrogenase and uricase (Kurtzman and Schwimmer 1971). Sauer et al (1982) have stressed that the degradation of caffeine by yeast is similar to its metabolism in human beings. Blecher and Lingens (1977) have reported urea as one of the end products of caffeine degradation by *P. aeruginosa* in synthetic liquid medium. *Bacillus coagulans*, *Penicillium roquefortii*, *Stemphyllium* sp. (Kurtzman and Schwimmer 1971) and various other filamentous

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fungi have been reported to possess caffein degradation abilities (Aquiahuatl et al. 1988). Even solid coffee wastes are used as a substrate for microbial proteins production (Orue and Bahar 1985). These microorganisms use caffeine as a source of nitrogen in media containing sucrose. Schwimmer and Kurtzman (1972) have reported total degradation of caffeine by *Penicillium curstosum* in roast coffee infusions, containing caffeine at the level of 0.45-0.59 mg/ml.

Solid state fermentation (SSF) technique is known to offer many advantages of commercial importance, as compared to submerged fermentations (Lonsane et al. 1985; Mitchell and Lonsane 1992). Its ability to overcome catabolic repressions, to end product inhibition and ability to tolerate high metal salt concentrations (Ramesh and Lonsane 1992,1993; Shankaranand and Lonsane 1993) are noteworthy. Consequently, SSF may prove useful in detoxification of coffee pulp. It is worthwhile noting that the researchers in Mexico (Guzman and Martinez Carrera 1985) and Guatemala (Rolz et al. 1988) have reported the possibility of edible mushroom production on a commercial scale from coffee pulp by SSF. Its use as substrate for pectinase production by SSF is also reported (Boccas et al. 1993; Antier et al. 1993). A few preliminary reports are also available on detoxification of coffee pulp in SSF process (Aquiahuatl et al. 1988 ; Gaime-Perraud et al. 1993; Penaloza et al. 1985), in addition to ensiling of coffee pulp (Gaime-Perraud et al. 1993). Coffee pulp contains caffeine (1,3,7 trimethylxanthin) in the range of 0.6-1.3% on dry weight basis (Bressani 1979) and caffeine is one of the highly active anti-physiological factors in coffee pulp (Molina et al. 1974). The present work on degradation of caffeine by *P. verrucosum* in SSF system was, therefore, undertaken. During the course of this investigation, a critical effect of additional nitrogen sources on caffeine degradation was observed and these results are reported in the present communication.

Fermentation aspects : Caffeine degrading strain of *Penicillium verrucosum* (V 33 A25), isolated earlier in Mexico (Aquiahuatl et al. 198), was used. Five different liquid media, designated as A,B,C,D and E, were used. All these contained (g/l) : KH_2PO_4 1.30, Na_2HPO_4 0.12, $MgSO_4$ $7H_2O$ 0.30, $CaCl_2$ 0.30 and varying nitrogen sources. The concentration (g/l) and type of nitrogen sources used are given in Table 1. One Kg ground (60 mesh) freeze-dried coffee pulp was mixed with 1 l of medium containing different levels of the nitrogen sources and autoclaved at 121°C for 1 h. After cooling to about 30°C, it was inoculated with 2 l of the spore suspension so as to provide 2×10^7 spores of *P. verrucosum*/g dry coffee pulp and mixed thoroughly for uniform distribution of the inoculum. The initial moisture content of the media in all the cases was 70% (w/w) and the pH (initial) of each medium was adjusted to 4.4. The inoculated moist medium in 20 g quantity was transferred to glass column fermentor of size 2 cm diam and 20 cm height. All the column fermentors were placed in the water bath as per the details described elsewhere (Raimbault and Alazard 1980) for fermentation at 25°C for 72 h. Humidified air was passed through each column fermenter at a rate of 60 ml/min. The temperature control and other methodological details were as per Raimbault and Alazard (1980).

Analytical aspects: For leaching of caffeine and pH determination, 75 ml distilled water was added to 5 g fermented solids and homogenized at 5000 rpm, for 2 min, using Ultra turrax (IKA-WERK, Germany). The pH of the homogenized mixture was determined and then it was boiled for 10 min. The boiled mixture was filtered through Whatman filter paper No 1, the solids left-over on the filter paper were washed with distilled water and the volume of the filtrate was made up to 100 ml (Smyly et al. 1976). Caffeine present in the filtrate was measured spectrophotometrically (Beckman DU 70, USA) as per the methodology of Isler et al (1948), after decolouration with magnesium oxide. Moisture content of the fermented solids was determined by oven-drying at 105°C to constant weight. The growth of the culture was observed visually.

Growth pattern : In all the five media, the germination of the spores of *P. verrucosum* was seen at 11 h after inoculation. The growth of the culture in media A, B and C was good throughout the fermentation time and also at the end of fermentation (Table 1). In contrast, the growth was comparatively poor in media D and E during 48-72 h fermentation, though it was comparable to media A, B and C during the first 48 h fermentation period in case of medium D. The growth in medium E, however, was comparatively poor throughout the fermentation period (Table 1). It is stressed that medium E does not contain any added nitrogen source and the results indicate deficiency in nitrogen concentration of coffee pulp for supporting good growth of *P. verrucosum*. Medium C was enriched with ammonium sulphate, while urea was added to medium D. The comparatively better growth on medium C, therefore, indicates that ammonium

TABLE 1. EFFECT OF ADDITIONAL INORGANIC AND ORGANIC NITROGEN SOURCES ON DEGRADATION OF CAFFEINE BY *PENICILLIUM VERRUCOSUM* AND OTHER PARAMETERS IN SOLID STATE FERMENTATION OF COFFEE PULP

Medium	Added nitrogen source g/l		Degree of cell growth at			Final pH of the medium	Final moisture content,%	Caffeine degradation at 72 h, %
	Ammonium sulphate	Urea	0-48h	48-72h	Overall, 0-72 h			
A	7.50	3.50	+++	+++	+++	7.80	73.4	0
B	3.75	1.75	+++	+++	+++	8.10	78.0	0
C	3.75	0	+++	+++	+++	5.26	76.6	0
D	0	1.75	+++	+	++	8.54	74.7	0
E	0	0	+	+	+	8.21	75.6	100

sulphate is better nitrogen source than urea for supporting the growth of *P. verrucosum*. It is well known that the fungi, in general, prefer ammonium sulphate as nitrogen source (Roussos 1982).

Moisture content of the medium : The fermentation of the moist coffee pulp medium, without any additional nitrogen source, was carried out at less than and higher than 70% initial moisture levels in the preliminary experiments. However, the visual growth of the culture on media below and above 70% initial moisture was poor. Thus, the growth of *P. verrucosum* on coffee pulp medium with 70% initial moisture was best. This is in contrast to the best growth of *Aspergillus niger* in moist starchy substrates at 50% initial moisture content as reported by Raimbault and Alazard (1980). The difference is probably due to the different fungal species involved. It may also be possibly due to the ability of coffee pulp to bind larger amount of water than that by the starchy substrate, because coffee pulp contains 6-8% mucilagenous substances and cellulose (Zuluaga-Vasco 1989).

Data on the final moisture content of the fermented solids at 72 h indicate negligible differences (Table1). This not only shows the efficiency of the methodology used for controlling moisture content of the medium during the course of fermentation, but also eliminate any possible role of the moisture content of the medium on the differences observed in caffeine degradation among the media studied.

pH of the fermented media : The data on changes in the pH of the fermenting solids gave interesting information. The pH, of all the five media studied, remained constant at the initial value of 4.4 during the first 30 h fermentation. It started increasing in the subsequent 18 h fermentation and reached alkaline values in the range of 7.80-8.54 at the end of 72 h, except in medium C (Table1). The increase in medium pH towards alkaline side could be due to production of some alkaline compounds during the growth and metabolism of the culture. Urea has been reported as one of the products of caffeine degradation (Aquiahuatl et al. 1988). It is, therefore, probable that the increase in pH of medium E towards alkaline side is due to production of urea. The increase in pH of medium E from 18 h indicates that the caffein degradation by the culture started after 18 h. The lower pH (5.26) in the case of medium C, as compared to those of the other media studied (Table1), is possibly due to the presence of ammonium sulphate as solely added nitrogen source and the formation of sulphuric acid during its metabolism.

The trends of the final pH of the fermented media (Table 1) eliminated any possible effect of these differences in caffeine degradation in the five media studied. For example, the final pH[s] of media E and B were nearly equal (8.10-8.21), but the degradation of caffeine was complete in medium E, while it was completely absent in medium B (Table 1).

Caffeine degradation : Data on the level of additional inorganic and organic nitrogen sources on the extent of caffeine degradation by *P. verrucosum* in SSF of coffee pulp gave interesting results (Table 1). The degradation of caffeine was completely inhibited by added nitrogen source, both organic and inorganic, and in combination or when used individually. In contrast, the degradation of caffeine was complete (100%) at 72 h fermentation in the absence of any added nitrogen sources. The trends of the results indicate preferential utilization of inorganic and/or organic nitrogen sources by the culture, when the media contained added nitrogen sources along with the caffeine originally present in coffee pulp. Moreover, the uptake of caffeine by the culture seems to be repressed in such media. These results are in agreement with those reported

by Penaloza et al (1985) and Aguilar (1983). These workers have observed the inhibition of the degradation of caffeine by *A. niger* in solid state fermentation, when inorganic nitrogen sources were added to the moist coffee pulp media. The data also reveal the utilization of caffeine by the culture as nitrogen source, probably due to their ability to produce responsible enzymes such as xanthin dehydrogenase and uricase (Kurtzman and Schwimmer 1971). The data establish the requirement of not adding any nitrogen sources to the medium for complete degradation of caffeine in solid state fermentation of coffee pulp. The decaffeinated coffee pulp, thus obtained, may find gainful use as animal feed.

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