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Production of Pectinase from Coffee Pulp in Solid State Fermentation System : Selection of Wild Fungal Isolate of High Potency by a Simple Three-step Screening Technique

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Screening of 248 cultures, isolated from Mexico's coffee growing areas, was carried out to select potent culture for production of pectinase from coffee pulp by solid state fermentation. A pectolytic strain of *Aspergillus niger* CH 4 was used as reference to evaluate the potential of the wild strains. The screening involved three simple and rapid steps. The first step involved the qualitative evaluation of pectolytic activities of 248 fungal strains on a selective solid agar medium, while the second one consisted of the quantification of the pectolytic activities of selected 13 isolates in a submerged fermentation medium, with pectin as the sole carbon source. The third step involved the assay of four selected fungal isolates for their capacity to produce pectinase from coffee pulp by solid state fermentation. *Aspergillus niger* V 22 B 35, a wild strain, was found to produce 4 times more pectinase than the reference strain.

Keywords : Coffee pulp, Solid state fermentation, Wild fungi, Pectinase, Simple three-step screening technique, *Aspergillus niger.*

Wet processing of coffee cherries, involving solid state fermentation and the action of pectolytic microorganisms, has been the preferred method over the dry method in several countries, as it leads to the final product of better quality. The wet method, however, leads to generation of huge quantity of coffee pulp, which poses severe disposal problems. For example, the coffee plantations in Mexico, Central America and Columbia generate coffee pulp to the tune of approximately 40% of several million tonnes of coffee cherries processed (Martinez - Carrera et al. 1989). The coffee pulp is barely used in these countries for want of practical and economic avenues. Due to presence of 23-27% fermentable sugars on dry weight basis (Zuluaga-Vasco 1989), the coffee pulp forms a major source of the pollution of rivers and lakes, located near the coffee processing sites, as well as the environment.

Due to its rich organic matter content, the coffee pulp can form an excellent substrate for production of value-added microbial metabolites by solid state fermentation (SSF) system. Microbial enzymes constitute one of the industrially important groups of microbial metabolites. In fact, one such microbial enzyme, i.e. pectinase, plays an important role in the processing of coffee cherries. In the wet fermentation method of coffee cherries, the natural pectolytic microflora present on the cherries are allowed to grow and metabolize for facilitating pectinase production. This enzyme, then, hydrolyses the mucilage, that envelops the coffee bean and consists mainly of pectins. The degree of hydrolysis of this pectic envelope has an ultimate economical importance to the coffee processing industry, due to its significant role on the yield and the quality of the end product. The modern practice is to use externally added microbial pectinase for hydrolysing the pectic envelop of the coffee beans. Consequently, a large quantity of microbial pectinase is consumed by the coffee processing industry.

There will be a tremendous gain to the industry, if some by-product from coffee processing is used as substrate for microbial production of pectinase. Such an approach will be highly economical and may lead to cost reduction of the end product. Such possibility may also prove useful in overcoming the present economic difficulties faced by coffee processing industry. The presence of around 6.5% pectin in coffee pulp on dry basis (Pulgarin et al. 1991) and its moist solid nature allows such possibility, as pectinase is an inducible enzyme, which requires pectin as an inducer (Fogarty and Kelly 1983). The use of coffee pulp as a substrate for pectinase production may also lead to economy in the enzyme production, as isolated pectin is a rather costly chemical. In addition, pectinases also

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find extensive applications in fruit processing industries for clarification of fruit juices and wines, in the manufacture of pectin-free starch and in the curing of cocoa and tobacco (Ghildyal et al. 1981; Joshi et al. 1991). It has been estimated that the pectinase market for various kinds of industrial processes is about 165 million pounds per annum (Fogarty and Kelly 1983).

It is worth mentioning that SSF system is preferred for production of pectinases due to a number of beneficial reasons (Ghildyal et al. 1981). Production of pectinase by SSF of wheat bran has been studied extensively in India (Ghildyal et al. 1981). It has been earlier reported that the pectinase productivity by SSF is much higher than that in submerged fermentation (SmF) process (Trejo-Hernandez et al. 1991). These facts justify the selection of SSF technique in the present studies.

Screening of large number of microorganisms is an important step in selecting a highly potent culture for developing efficient fermentation process. It, however, is highly time-and resources- consuming. A simple and rapid screening method wlll be of high utility. Since no such method is available, the present three-step approach has been designed by us for screening of potent pectolytic cultures.

The objectives of the present investigations, therefore, were to test the potential of using coffee pulp for production of pectinase in SSF system and to evaluate the efficiency of three-step approach for rapid screening of wild fungal isolates as well as selection of the potent strain.

Materials and Methods

Microorganisms : A total of 248 fungal cultures were isolated directly from coffee plants and the soil samples from coffee plantation areas in Mexico, as reported elsewhere (Aquiahuatl et al. 1988). A pectolytic strain of *Aspergillus niger* CH 4, which is used as a reference culture, was provided by Dr Carlos Huitron, Department Biotechnologia, Instituto de Investigaciones Biomedicas, UNAM, Mexico, and it was isolated from agave pulp (Saval et al. 1981). All these cultures were maintained on potato-dextrose-agar (PDA) slants at 4°C by subculturing every alternate month in the ORSTOM-UAM collection, ORSTOM, Montpellier, France. For inoculum development, the cultures were grown on PDA slants at 25°C or 35°C for 96 h and the conidia were dispersed in 0.01% Tween 80 solution. All the experiments were carried out in quadruplicate.

First-step screening technique : Agar medium, designated as medium I, was used and it contained (g/l) : pectin citrate 2.0, urea 0.05, ammonium sulphate 0.15, agar 20.0 and distilled water 1000. The pH of the medium was adjusted to 5.5 and it was autoclaved at 121°C for 15 min in 4 Erlenmeyer flasks of 500 ml capacity. The medium was allowed to cool to about 55°C, poured in 16 ml quantity in sterilized petri dishes and allowed to solidify. The medium in plates was streaked with the test cultures and the plates were incubated at 25°C and 35°C for 72 h.

At the end of the incubation, the diam of the hydrolyzed zone of pectate around the colony was measured, as an indicator of the pectinase activity (Antier et al. 1992). This activity was measured through a coefficient called A'. It represents % higher activity with respect to *A. niger* CH 4 (reference culture) and is defined as below :

$$A' = (Hi/Ci - Hc/Cc)/Hc/Cc$$

Where A' = coefficient of pectolysis; Hi = pectolysis zone diam of wild strain; Ci = growth diam of wild strain; Hc = pectolytic zone diam of *A. niger* CH 4; Cc = growth diam of *A. niger* CH 4.

The A' value of the reference strain was taken as zero. As a confirmative test and also to determine the apical growth, the selected isolates (13 Nos) were grown on the agar medium to note their apical growth, in comparison with the reference culture.

Second-step selection technique : A liquid medium, designated as medium II, was used. For its preparation, 50% of pectin citrate solution was pasteurized twice at 70°C for 30 min. In addition, stock solution of urea (5%) was sterilized by membrane filtration. Both the solutions were mixed to contain a final composition, consisting of 20 g pectin citrate and 0.5 g urea in 1l medium. The pH of the mixture was adjusted to 5.8 with 1 N NaOH. The medium was, then, inoculated at a rate of 2 x 10^7 spores/ml medium, using the spore suspension from freshly grown PDA slants. The flasks were incubated at 25°C or 35°C (depending upon the results of the first-step screening technique) for 40 h on rotary shaker (220 rpm). At the end of incubation period, the culture broth was filtered through Whatman No. 1 filter paper to obtain cell-free liquor for assay of the enzyme by viscosity reduction method (Ghildyal et al. 1981). Pectinase activity was determined by using 18 ml of the culture filtrate in a Brookfield R. V. rotational viscometer at 45°C for 10 min. The substrate used was 1 ml of 2% pectin solution prepared in

0.1 M citrate-phosphate buffer (pH 5.5). One enzyme unit (U) was defined as the amount of the enzyme necessary to reduce the initial viscosity by 50%.

Third-step final selection technique : Coffee pulp-based solid state fermentation medium, designated as medium III, was used. It consisted of 34 g dry coffee pulp, 0.8 g urea, 3.3 g ammonium sulphate and 30 ml distilled water. Thoroughly mixed medium was autoclaved at 121°C for 20 min, as described elsewhere (Raimbault and Alazard 1980). After cooling to ambient temperature, it was inoculated with the spore suspension at a rate of 2 x 10^7 spores/g dry coffee pulp. The inoculated medium was packed in 2.0 cm diam and 15 cm long glass column fermenters, which were placed in a temperature controlled water bath (Raimbault and Alazard 1980). Humidified air, at the rate of 60 ml/min was passed through each column. The fermentation was carried out at 25°C or 35°C, based on the requirement of the culture under study. For extraction of the enzyme from moist fermented solids, 30 g portion of the fermented material was mixed with 30 ml of water and pressed in hydraulic press for 2 min at 1000 psi (Roussos et al. 1992). The extract, thus obtained, was filtered and used for enzyme assay, as per the methodology described above.

Statistical analysis : All the experiments were conducted in quadruplicate and the significance of data was tested by the analysis of variance (Snedecor and Cochran 1968).

Results and Discussion

Fungal isolates : Work on isolation of fungal cultures for their ability to grow on coffee pulp was undertaken earlier, with a view to isolate wild fungi capable of degrading organic matter and caffeine, with a a view to reduce the pollution potential of the treated coffee pulp. A novel isolation strategy was designed for this purpose and is described elsewhere (Aquiahuatl et al. 1988). It resulted in isolation of 248 wild fungi and the success of the strategy can be judged by the fact that it yielded a few cultures which were able to completely degrade caffeine in coffee pulp under SSF technique (Aquiahutal et al. 1988). Morphological study of these 248 isolates permitted to group them in ten different filamentous genera viz., *Acremonium, Aspergillus, Drechslera, Fusarium, Geotrichum, Mucor, Penicillium, Rhizopus, Trichoderma* and *Zygomycetes* (Roussos et al. 1989). These isolates have been screened in the present studies to select the potent fungi, capable of producing pectinase in moist coffee pulp medium in SSF system.

Result of first-step screening programme : Data on the growth and enzyme production of the isolates on pectin-containing agar medium are summarized in Table 1. Out of 248 wild fungal isolates screened, 5% did not grow (Ci = o) on this selective medium.This might be due to the absence of pectolytic enzyme system in these isolates, which otherwise, is essential for growth on this medium, because of the use of pectin citrate as sole carbon source. Further, 47% of the total strains screened were able to grow on this selective medium, but they did not produce any visible pectin hydrolysis zone (Hi/Ci =1). Such behaviour is attributed to the ability of these isolates to use pectin citrate as a sole source of carbon to produce pectolytic enzymes, albeit at extremely poor levels. The remaining isolates (48%) grew profusely and produced a clear zone of pectolysis (Hi/Ci > 1). Among these good pectolytic enzyme producing isolates, the coefficient of pectolysis (A') values were < 0, in case of 97 isolates (39% of total isolates screened). The value of these cultures for industrial exploitation was, therefore, ruled out in comparison with the reference culture. A total of 9 isolates showed the coefficient of pectolysis values, which were equal to those of the reference strain (A' = 0) and these cultures, therefore, do not offer any advantage over the reference culture. In contrast, 13 isolates showed the coefficient of pectolysis values, which were > 0, thereby indicating their better pectolytic abilities, as compared to the reference culture.

TABLE 1. SCREENING OF 248 WILD FUNGAL ISOLATES IN FIRST-STEP SCREENING TECHNIQUE INVOLVING CULTURING ON SELECTIVE AGAR MEDIUM IN PETRI DISHES

Attribute	Wild fungal isolates: Number	Wild fungal isolates: Approximate % of total isolates screened
Growth		
Ci = 0	12	5
Hi/Ci = 1	117	47
Hi/Ci > 1	119	48
Coefficient of pectolysis, A'		
A' < 0	97	39
A' = 0	9	4
A' > 0	13	5

It is, thus, evident that the strategy used for first-step screening programme proved highly efficient, as it was able to select a group of better enzyme producers, which amounted to merely 5.2% of the total isolates, for further studies. Rest of the 94.8% isolates were eliminated by the strategy, based on their worth, as compared to the reference

culture. The strategy is also quicker, simple and much less laborious, as compared to the conventional screening methods.

The selected isolates (13 Nos) were found to belong to two fungal genera (Table 2); *Aspergillus* (7 isolates) and *Penicillium* (6 isolates). These genera of filamentous fungi have been also reported to produce pectolytic enzymes (Fogarty and Kelly 1983). When the apical growths of these selected 13 strains were compared to the reference culture, it was found that only two isolates (*A. niger* C 28 B 25 and C 16 A 25) colonised the solid surface more rapidly and better than that by the reference culture (Table 2). These characteristics are of importance in industrial fermentation, as the economy of the process is determined, to a large extent, by the batch time.

TABLE 2. GROUPING OF 13 ISOLATES, SELECTED AFTER FIRST-STEP TECHNIQUE, AND THEIR APICAL GROWTH AS COMPARED WITH REFERENCE CULTURE (*A. niger* CH 4)

Wild fungal ioslates		Apical growth as compared
Strain No.	Genus/Species	to reference culture
V 16 A 25	*Penicillium* sp	Inferior
V 23 A 25	*Penicillium* sp	Inferior
V 34 A 25	*Penicillium* sp	Inferior
C 14 A 25	*Penicillium* sp	Inferior
C 16 A 25	*Aspergillus niger*	Superior
V 12 A 35	*Aspergillus niger*	Equal
V 22 B 35	*Aspergillus niger*	Equal
V 32 B 35	*Aspergillus niger*	Equal
C 15 C 25	*Penicillium* sp	Inferior
C 16 C 25	*Aspergillus niger*	Equal
C 15 B 25	*Penicillium* sp	Inferior
C 17 B 25	*Aspergillus niger*	Equal
C 28 B 25	*Aspergillus niger*	Superior

Results of the second-step selection technique: A total of 13 strains, selected after first-step screening programme, were further studied in liquid medium containing 2% pectin citrate under SmF for specific reasons. For example, the viscosity of the liquid medium will decrease significantly during growth of the selected culture, due to hydrolysis of pectin. Such decrease in viscosity by the selected isolates can be compared with that by the reference culture, with a view to select one or two cultures, which are most potent. The results of such studies indicated that only four isolates (three isolates of *A niger* : C 16 C 25, C 28 B 25, V 22 B 35 and one isolate of *Penicillium* sp. : C 15 B 25) displayed a significant decrease in viscosity of the medium, than that by the reference culture (Table 3). Among these four isolates, the greater reduction in viscosity was given by two strains of *A. niger* i.e. V 22 B35 and C 28 B 25 (Table 3).

TABLE 3. ABILITY OF THE SELECTED 13 WILD FUNGAL ISOLATES TO REDUCE THE VISCOSITY OF LIQUID MEDIUM IN SUBMERGED FERMENTATION DURING SECOND-STEP SELECTION TECHNIQUE

Genus/Species	Comparative pectolytic activity*	Comparative difference in final pH**	Classification***
Aspergillus niger			
V 22 B 35	+ 1 ± 0.17	0.2	1
C 28 B 35	+ 1 ± 0.15	0.1	2
C 16 C 25	0 ± 0.17	0.2	3
V 17 B 25	0 ± 0.14	0.1	4
V 32 B 25	- 0.5 ± 0.17	0	6
V 12 A 35	- 6.0 ± 0.13	- 1.0	10
Penicillium sp			
C 15 B 25	0 ± 0.14	0.1	4
C 15 C 25	- 0.5 ± 0.14	0	5
C 16 A 25	- 1.0 ± 0.15	0	7
V 34 A 25	- 1.0 ± 0.14	0	7
V 23 A 25	- 1.5 ± 0.15	- 0.3	8
V 16 A 25	- 2.0 ± 0.15	- 0.6	9
V 14 A 25	- 54.0 ± 0.11	- 4.0	11

* Values reported are units produced by the strain under studies (-) units produced by the reference strain. ** Values represent the differences in final pH of the strain under studies (-) that by the reference strain. *** The strains are classified based on their performance, as compared to the reference strain. Class 1 strain scored highest difference and thus is most potent.

Though the second-step selection technique allows efficient and reliable selection of two isolates (*A. niger* V 22 B 35 and C 28 B 35) as most potent cultures among the whole of 248 isolates screened, all the four isolates, which gave significant decreases in the viscosity of the medium, as compared to the reference culture, were selected for third step final selection technique. This was done by keeping in view, the recent report that the cultures, which are the best producers of the metabolite in SmF are usually not efficient, when used in SSF and *vice versa* (Shankaranand et al. 1992). It was, thus, possible to eliminate more than 98% of the 248 isolates and zeroed down on selected four culture in a most efficient way at the end of second-step screening, due to the screening strategy employed in the present studies.

Results of third-step final selection technique : The data in Table 4 give the comparative production of pectolytic enzyme at 72 h in coffee pulp medium under SSF technique by the four filamentous fungi, selected after second-step selection technique. It is evident that all the four wild fungal isolates studied were able to produce more pectolytic enzyme than

TABLE 4. ABILITY OF FOUR WILD FUNGAL ISOLATES, SELECTED AFTER SECOND-STEP SELECTION TECHNIQUE, TO PRODUCE PECTOLYTIC ENZYME AT 72 h IN COFFEE PULP MEDIUM UNDER SOLID STATE FERMENTATION, AS COMPARED TO THE REFERENCE CULTURE (*A. niger* CH 4)

Culture	Pectolytic activity in coffee pulp medium under SSF at 72 h
A. niger CH 4 (Reference culture)	12.1 ± 0.3
Penicillium sp. C 15 B 25	14.9 ± 0.4
A. niger C 16 C 25	20.5 ± 0.5
A. niger C 28 B 25	43.3 ± 1.1
A. niger V 22 B 35	47.7 ± 1.2

the reference culture (*A. niger* CH 4). The highest enzyme production was shown by *A. niger* V 22 B 35, followed by the strains C 28 B 25 and C 16 C 25, in decreasing order. The enzyme production by *Penicillium* sp. C 15 B 25 was slightly higher than that by the reference culture. The enzyme production by *A. niger* V 22 B 35 was about 4 times higher than that by the reference culture. It, therefore, has most potential for industrial exploitation.

The trend of data allows to infer that the three-step screening strategy used is most suitable for screening a large number of isolates in a simple, reliable and quick manner. The isolate selected was subsequently mutated to obtain pectinase hyper producing mutants (Antier et al. 1992), which is being profitably used currently in the coffee curing industry (Roussos et al. 1989).

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