Appl Microbiol Biotechnol (1995) 42:756–762

ORIGINAL PAPER

S. Roussos · M. de los Angeles Aquiáhuatl
M. del Refugio Trejo-Hernández
I. Gaime Perraud · E. Favela · M. Ramakrishna
M. Raimbault · G. Viniegra-González

Biotechnological management of coffee pulp – isolation, screening, characterization, selection of caffeine-degrading fungi and natural microflora present in coffee pulp and husk

Received: 18 April 1994/Accepted: 11 May 1994

Abstract A total of 272 strains of filamentous fungi were isolated from soil, leaves of coffee plants and coffee cherries collected in coffee-growing areas of Mexico on three semi-synthetic culture media containing coffee extract, coffee extract with sucrose and coffee pulp extract. The isolated strains were purified by conventional techniques and identified by microscopic examination. Strains were selected on the basis of their caffeine-degrading ability in well-defined liquid medium containing caffeine. Most of the isolated microorganisms belong to *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium*, and *Humicola* genera. Five strains belonging to *Aspergillus* species and two strains belonging to *Penicillium* species had the ability to degrade almost 100% of the caffeine in liquid medium. A comparative study on the evaluation of natural microflora present in coffee pulp and coffee husk revealed the presence of a wide variety of microorganisms. The percentage distribution of fungi, bacteria and yeast was almost similar in all the samples, except in coffee husk where the fungal population was slightly higher than in the other two samples. The yeast population was predominant when the coffee pulp was lyophilized immediately after pulping. However, there was a wide diversity in the microbial population with respect to selective media containing functional nutritional groups like cellulose, starch and pectin.

S. Roussos (✉) · I. Gaime Perraud · M. Raimbault
Biotechnology Unit, ORSTROM Centre de Montpellier
911 Av. Agropolis, BP 5045, 34032 Montpellier Cedex 1,
France. FAX: 33-67617583

M. de los Angeles Aquiáhuatl
M. del Refugio Trejo-Hernández · E. Favela
G. Viniegra-González
Department of Biotechnology,
Autonomous Metrpolitan University, Iztapalapa Campus,
AP 55–535, CP 09340, México, D. F., México

M. Ramakrishna
Department of Microbiology and Bioengineering,
CFTRI, Mysore 570013, India

Introduction

Coffee pulp is some of the most abundantly available agroindustrial waste produced during the pulping operation of the coffee cherries to obtain coffee beans in many coffee-producing areas of the tropics (Zuluaga 1981). According to the data available, the world green coffee and coffee pulp production during 1989–1990 reached a maximum of 5.52×10^6 tons (5.61×10^9 kg) of green coffee and 2.76×10^6 tons (2.8×10^9 kg) of coffee pulp (Roussos et al. 1993). Thus, for every 2 tons coffee cherries processed, nearly 1 ton pulp is generated, whereas in the dry process 0.18 ton coffee husk is generated for every ton of fresh coffee cherries (Adams and Dougan 1981). Coffee pulp is essentially rich in carbohydrates, proteins and minerals (especially potassium) and it also contains appreciable amounts of tannins, polyphenols and caffeine (Bressani, 1979). Owing to the presence of anti-nutritional factors such as caffeine, tannins and polyphenols, its use as an animal feed has been restricted to a large extent. For want of practical and economical avenues, coffee pulp has not been commercially exploited. As this product of the coffee industry does not find any commercial application, it is considered to be the major polluting agent of rivers and lakes located near the coffee-processing regions. The presence of proteins, sugars and minerals in coffee pulp and its high humidity favours the rapid growth of microorganisms and, if it is not utilized immediately, it causes environmental pollution. In a country like Columbia, processing of fresh coffee cherries produces annually the same amount of organic pollution as is produced by 24×10^6 people out of its 30 million total population (Zuluaga 1989). For environmental protection and economic gain, attempts have

been made in the past to utilize coffee pulp as an animal feed. However such attempts met with limited success because of the presence of antiphysiological factors such as caffeine, tannins, chlorogenic acid and high levels of potassium (Bressani 1979; Adams and Dougan 1981).

Even though there have been many reports describing the composition, conservation, up-grading and utilization of coffee pulp, there is not a single report on the natural microflora it contains, the agent responsible for causing environmental pollution if not conserved properly (Calle 1951, 1954; Ledger and Tilman 1972; Bressani 1979; Christensen 1981; Orue and Bahar 1985; Martínez-Carrera 1987). Accurate knowledge about the nature and characteristics of coffee pulp natural microflora is of utmost importance for its further utilization in the biotechnological process.

The main objective of this work was to isolate new strains of filamentous fungi capable of degrading caffeine. Strategies employed for the isolation, screening, purification and characterization of such filamentous fungi are reported in this paper. Further, the natural microflora present in coffee pulp and coffee husk are evaluated and compared.

Materials and methods

Isolation and purification of filamentous fungi

Soil, leaves and coffee cherries were collected from coffee-growing areas of Chiapas and Veracruz in Mexico and used for the isolation of filamentous fungi. Samples of coffee pulp/husk were obtained from three different coffee-processing units located in Xalapa (Mexico), Chinchina (Colombia) and Mysore (India).

Strain isolation and purification were carried out in three different media (Table 1). Media A, B and C were inoculated with about 0.2 g each sample, which was incubated at 25°C and 30°C and allowed to grow for 48–72 h. Well developed colonies were picked and subjected to purification. Pure cultures of filamentous fungi were obtained after successive transfers of individual colonies in the respective isolation media and incubation for 48–72 h at their isolation temperature.

Table 1 Composition of different media used for the isolation and the selection of fungal strains capable of degrading caffeine

Components (g/l)	Media			
	A	B	C	D[a]
KH_2PO_4	1.30	1.30	1.30	1.30
Na_2HPO_4	0.12	0.12	0.12	0.12
$MgSO_4 \cdot 7H_2O$	0.30	0.30	0.30	0.30
$CaCl_2 \cdot 2H_2O$	0.30	0.30	0.30	0.30
Sucrose	–	5.00	–	5.00
Caffeine	–	–	–	1.20
Coffee infusion	40.00	40.00	–	–
Coffee pulp extract	–	–	40.0	–
Streptomycin	0.03	0.03	0.03	–
Agar	15.0	15.00	15.00	15.00

[a] Medium D was used for rapid selection of pure strains

Description and identification of the strains

To obtain data on the description and identification of the strains, cultures were grown on potato/dextrose/agar (PDA) medium, observed under a microscope for morphological characteristics and compared by reference to classical keys reported in the literature (Raper and Fennell 1965; Barnet and Hunter 1972; Ainsworth 1973; von Arx 1974). To obtain microscopic characterization of the cultures, superficial seeding was carried out on PDA in petri dishes at three points in the case of slow-growing fungi (*Aspergillus, Penicillium, Fusarium*) or at the centre of the petri dish for fast-growing fungi (*Trichoderma, Rhizopus, Mucor*). Microculture techniques were used for characterization of mycelium and reproductive structures (Riddel 1950).

Selection of caffeine-degrading strains

The selection of filamentous fungi capable of degrading caffeine was carried out in two steps (Aquiahuatl 1992). In the first step, isolated pure cultures were inoculated in medium D and those cultures that grew well in this solid medium were subjected to further screening in liquid medium, in order to determine quantitatively the caffeine-degrading capacity of the selected strains. In the second step the test cultures were inoculated with 0.5 ml spore suspension into 50 ml medium D (without agar) in conical flasks (250 ml capacity) and incubated in a rotary shaker at 150 rpm at their isolation temperature for 72 h. At the end of the incubation period the culture medium was centrifuged and subjected to further analysis.

Measurement of growth

The growth was quantified, by determining the biomass (dry weight) by filtering and drying in an oven at 105°C for 16 h and weighed to constant weight.

Determination of apical growth

This was measured using Ryan tubes (Ryan et al. 1943) on PDA medium according to the procedure of Smith and Berry (1975). The velocity of apical growth was expressed as millimetres per hour (Roussos and Raimbault 1982).

Sporulation index

Index of sporulation was determined as described by Roussos (1985) and expressed as the number of spores per gram of substrate utilized in the culture medium.

Medium for determination of total microflora

All the media used in the present studies were obtained from Difco laboratories, Detroit, Michigan, USA and prepared according to the instructions given in the data sheet. Plate-count agar medium, pH 6.5–6.8, was used for evaluating bacterial populations PDA medium containing 0.25 g/l chroramphenicol, pH 5.6, for determination of fungi, and Sabouraud dextrose agar medium containing 0.25 g/l chroramphenicol, pH 5.6, for yeasts.

Selective culture medium

Four different culture media were used for the evaluation of micro-organisms with specific functional attributes such as microorganisms of the amylolytic, cellulolytic, pectinolytic and lactic acid bacteria groups (Gaime-Perraud et al. 1993).

Sample preparation

Inoculation at different dilutions and identification were carried out as described elsewhere (Gaime-Perraud et al. 1993). For the lactic acid bacteria group, after inoculation in MRS agar (Difco), the petri dishes were incubated at 28°C in an anaerobic atmosphere using an anaero-culture system and identification of bacteria and yeasts was carried out microscopically at different times.

All experiments were carried out in triplicate and their average values are reported. The number of colonies per gram of dry coffee pulp was calculated according to the following equation.

$$\text{No. of colonies/g dry coffee pulp} = \frac{\text{no. of colonies (minimum of 20 colonies/petri dish)} \times 100}{\text{dilution factor} \times (100 - \text{moisture content})}$$

Caffeine determination

For routine analysis, caffeine was estimated spectrophotometrically after magnesium oxide treatment (Ishler et al. 1948). For quantitative determination, caffeine was estimated by HPLC (Waters) using a Bondapak C18 column (30 cm) with methanol/water (1:1), containing 1% acetic acid as the mobile phase (Smyly et al. 1976). Pure caffeine (Sigma) at a concentration of 0.1 mg/ml was used as the internal standard. All experiments were carried out in duplicate under identical conditions.

Results

Isolation and purification

A total of 272 strains of filamentous fungi were isolated and purified from samples obtained from the Chiapas and Veracruz area in Mexico, using media A, B and C at 25°C and 35°C in order to obtain mesophilic and thermotolerant strains. The isolates thus obtained mainly belonged to the genera *Aspergillus*, *Penicillium*, *Geotrichum*, *Humicola*, *Rhizopus* and *Trichoderma*. Strains belonging to the *Aspergillus* genus were predominant among the isolates. The percentage distribution of different species of pure cultures, isolated from the above two sources using media A, B and C at 25°C and 35°C, revealed that at 35°C the population of *Aspergillus* species predominated (nearly 68% of the total population) in samples from both locations, whereas they constituted about 25% of the total population when the isolation temperature was 25°C. The *Humicola* population was higher at 25°C than at 35°C. At 25°C in the Chiapas sample the populations of *Fusarium* and *Humicola* were similar to that of Aspergillus species, whereas in the Veracruz sample the *Humicola* population was about 40% of the total.

The distribution pattern of different species of filamentous fungi in three different media used for isolation at 25°C and 35°C also shows the predominance of *Aspergillus* in all cases. It was observed that almost all the isolated strains were able to degrade caffeine in liquid media as they were isolated in media containing coffee extract or coffee pulp extract, which provided its nitrogen source. However the ability to degrade caffeine varied from strain to strain. This confirms the importance of medium composition for the isolation and selection from natural ecosystems of micro-organisms with a specific role.

Selection of strains

The various parameters considered at this stage for the evaluation of strains were growth (pellet formation), final pH of the culture medium and caffeine degradation. On the basis of the results of the second step of the screening system, the isolates were grouped into three categories according to their ability to degrade caffeine in the following ranges: (a) 0–30%, (b) 31%–80% and (c) 81%–100%. Only those strains that could degrade caffeine by more than 80% in the liquid medium in 72 h were selected for a study of their physiology and biochemical characteristics. Five strains belonging to *Aspergillus* species and two strains belonging to *Penicillium* species had the ability to degrade almost 100% of the caffeine present in the liquid medium (Table 2). The first three strains listed in Table 2 were from samples

Table 2 Rate of caffeine degradation and growth characteristics of selected filamentous fungi. The sporulation index represent the number of conidia produced per gram of initial substrate

Isolates	Species	Caffeine (%)	Degradation rate ($mg\,ml^{-1}\,day^{-1}$)	Apical growth (mm/h)	$10^{-9} \times$ Sporulation index
V12A25	*Aspergillus* sp.	100	0.157	0.24	1.30
V26A25	*Pencillium* sp.	98	0.126	0.15	5.40
V33A25	*Pencillium* sp.	100	0.126	0.14	9.26
C16A25	*Aspergillus niger*	99	0.123	0.23	3.40
C11B25	*Aspergillus fumigatus*	100	0.120	0.16	18.60
C17B25	*Aspergillus niger*	100	0.103	0.22	15.20
C28B25	*Aspergillus niger*	98	0.119	0.19	8.83

obtained from the Veracruz area and the rest were from the Chiapas area. The first four strains mentioned in Table 2 were isolated using medium A whereas the other three strains were isolated using medium B. The isolation temperature of all the strains was 25°C. It is interesting to note that strains isolated at 35°C were unable to degrade more than 30% of the caffeine present in the liquid medium even though there was very good growth.

Physiology and biochemistry of selected strains

A study of the physiology and biochemistry of selected strains will lead to a better understanding of the behaviour of the fungi and will constitute the basis for further studies of a solid-state fermentation system for decaffeination of coffee pulp. With this objective, preliminary studies were carried out on the kinetics of the degradation of caffeine in liquid medium, apical growth and sporulation indices of the strains in PDA medium. The measurement of apical growth indicates the capacity of filamentous fungi for superficial colonization on solid culture medium. This provides information on the kinetics of biomass production and for better understanding of the growth strategy of fungi in their natural habitat. The data on the rate of degradation of caffeine, apical growth and sporulation index of selected strains are presented in Table 2. The strain belonging to the *Aspergillus* species (V12A25) had the highest rate of caffeine degradation and apical growth but a low sporulation index as compared to the other isolates.

Origin of coffee pulp and coffee husk

Coffee pulp and coffee husk are the solid wastes produced during the processing of coffee cherries by the wet and dry process respectively (Rolz et al. 1988). The technique of wet coffee processing used in Xalapa is different from the one that is used in Chinchina. In the Xalapa coffee-growing area, coffee cherries are processed by adding a large amount of water during the pulping operation and the pulp thus obtained is sun-dried for 10 days to a final moisture content of 10% (pulp A). Two samples of coffee pulp were collected from the Chichina coffee processing unit, where a new method is employed in the processing of coffee cherries. Here the cherries are directly pulped without the addition of water and sun-dried for 2 days to a moisture content of 10% (pulp B). The second sample that was collected from this unit was obtained by lyophilizing the pulp immediately after the pulping operation (pulp C). It had a moisture content of 5%. The coffee husk obtained from Mysore was from the dry process where the coffee cherries are sun-dried and subsequently the outer layers of husk covering the green coffee are removed by a hulling machine as and when needed. This sample had a moisture content of around 14%.

Natural microflora in coffee pulp

The distribution of total microflora present in coffee pulp A, B, C and coffee husk per gram of dry material is presented in Fig. 1 on a logarithmic scale. The purpose of carrying out evaluation studies with coffee pulp C (lyophilized pulp), which is not a commercial sample, is to compare the effect of exposure of coffee pulp samples A and B to the atmosphere for different periods of time during the drying process on the distribution pattern of microflora. Coffee pulp A had the highest total microbial population (1.2×10^8 colonies/g dry pulp) as compared to the other three samples. It was nearly 160 and 17 times higher than that found in coffee pulp B and coffee husk respectively. This is mainly attributed to the processing conditions that are employed during the pulping operation and also to the time involved in drying of the coffee pulp, which in this case was 10 days. In the case of pulp samples A and B and coffee husk the bacterial population was 98.8%, 95.4% and 94.3% respectively of the total microflora. The yeast population was negligible. The distribution of filamentous fungi was almost similar in the above three samples except in coffee husk, were the fungal population was slightly higher (5.2%) than in the other two samples. Pulp C had the highest yeast population (61% of the total microflora) and the bacterial population was only 37%. This was mainly due to the presence of yeast on the coffee cherries and to the fact that pulp C was lyophilised immediately after the pulping operation. Thus it is obvious that the bacterial population becomes predominant when the pulp is exposed to the atmosphere during the drying process.

Functional groups of the natural microflora of coffee pulp

The data on the microbial population with respect to selective media containing specific functional groups (substrates) like cellulose, starch, pectin and coffee pulp, and also on *Lactobacillus* medium, are presented in Table 3. Here again pulp A had the highest population of microflora that grew on cellulose, starch, pectin and coffee pulp as compared to the other three samples. Pulp B had the lowest microbial population on these selective media. It is interesting to note that the numbers of microorganisms that can grow on all these four media were almost equal in the case of pulp C. Even though pulp C was obtained by lyophilizing immediately after the pulping operation, the total microflora with respect to different selective substrates in pulp C is very much higher than that present in pulp B. This may be due to the presence of a very high yeast population

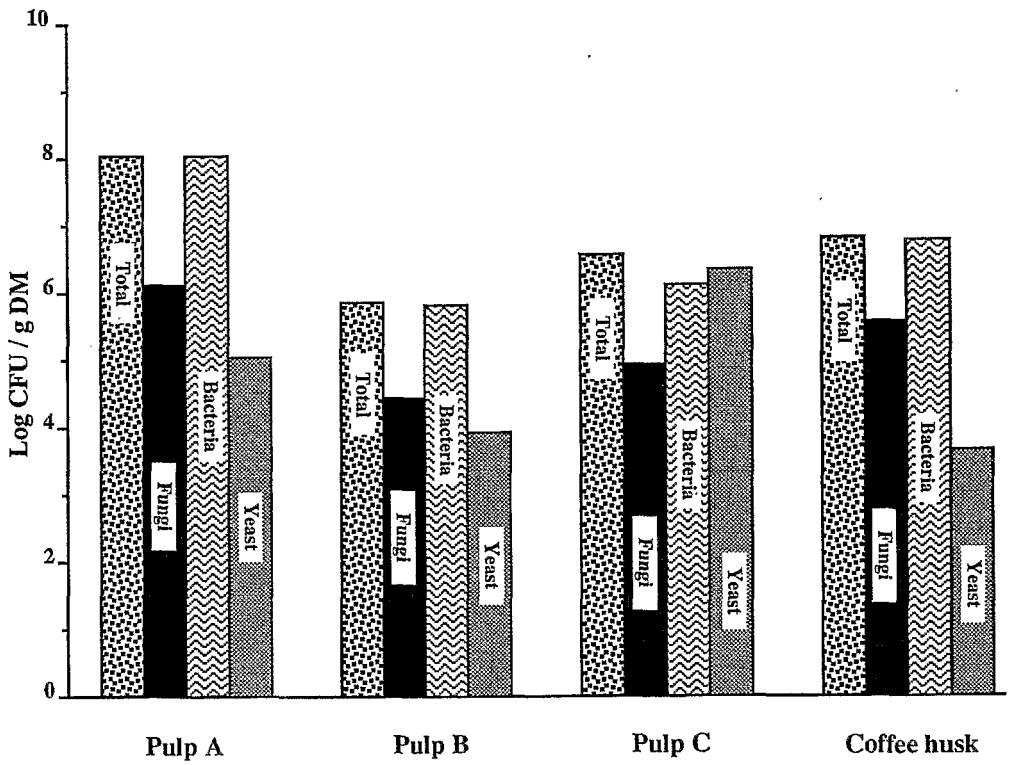

Fig. 1 Distribution of microflora present in coffee pulp and coffee husk

in coffee cherries. During the drying process the yeast population diminishes with a simultaneous increase in bacterial population as, demonstrated in the case of pulp A, where the drying time for the pulp is 10 days. In the case of coffee husk (Table 3) the microbial population that can grow on cellulose medium is much higher than those that can grow on starch, pectin and coffee pulp medium. In spite of coffee pulp medium being a non-specific medium rich in carbohydrates, proteins and minerals, only 10.2%, 8.8% and 5.3% of the total microflora present in pulp A, pulp B and coffee husk respectively can grow in this medium (Table 3). This may be due to the presence of compounds in coffee pulp extract that may inhibit the growth of certain groups of micro-organisms in this medium. The data on the lactic group, representing the anaerobic population present in coffee pulp/husk, reveals that, in the case of pulp B, nearly 30% of the total microflora present in the pulp can grow anaerobicaly in *lactobacillus* MRS medium whereas with coffee husk this population is totally absent.

Discussion

The patterns of distribution of different genera of filamentous fungi isolated from two different coffee-growing areas are almost similar. For selection of strains with high caffeine-degrading ability, medium D with defined composition was used. Thus other easily assimilable nitrogen sources cannot influence the strains at this step even if these strains are isolated on a complex medium. Of the 272 strains isolated only 7 had the ability to degrade caffeine totally in liquid medium (medium D). Out of the 7 strains selected for the best caffeine-degrading capacity 4 strains were isolated using medium A and 3 using medium B (Table 2). None of the strains isolated using medium C were able

Table 3 Coffee pulp and coffee husk microflora population on different selective media. Numbers in parenthesis indicate the percentage with respect to total microflora

Source	10^{-5} × number of microorganisms per gram of dry sample				
	Cellulose medium	Starch medium	Pectin medium	Coffee pulp medium	Lactobacilli MRS medium
Pulp A	464.0 (41.4)	558.0 (49.6)	9.3 (0.8)	115.0 (10.2)	84.4 (7.5)
Pulp B	1.1 (16.4)	0.4 (5.9)	0.3 (3.7)	0.6 (8.8)	2.1 (30.6)
Pulp C	26.2 (72.9)	28.0 (79.7)	19.5 (54.1)	21.3 (59.2)	0.9 (2.4)
Coffee husk	42.7 (65.5)	8.5 (13.1)	3.2 (5.0)	3.4 (5.3)	0.0

to degrade caffeine at appreciable rates even though the isolation medium contained coffee pulp extract as the carbon and nitrogen source. This may be due to the fact that the coffee pulp extract that was used in medium C may contain an easily assimilable nitrogen source other than caffeine, like soluble protein, to meet the nitrogen demand of the organism (Elias 1979). Further, it is likely that if a complex medium C is used at the isolation step, some strains without or with low caffeine-degrading ability may grow and depress the growth of strains having high caffeine-degrading ability.

Strains of *Aspergillus* species have been used by adopting a solid-state fermentation technique for protein enrichment of coffee pulp, for its use in animal feed stock (Guzman 1983; Peñaloza et al. 1985). However caffeine degradation by these strains was not observed. The medium used by these workers contained exogenous mineral nitrogen, thus fungi may have preferred to utilize this nitrogen for growth rather than the nitrogen from caffeine molecules and hence caffeine degradation was not observed. The chemical composition of the medium and the incubation temperature play an important role in the selection of proper filamentous fungi. In the present studies all the selected strains that had the ability to degrade caffeine totally were isolated from culture medium containing extract of coffee without any added mineral nitrogen. This confirms the importance of various components present in the medium when microorganisms with a specific role are selected from the abundant microflora of great metabolic diversity using natural substrates. Considering the economic importance of coffee, the large volumes of coffee pulp produced during the processing of coffee cherries and the problems associated with its management and utilization, an alternative for efficient management of coffee pulp is the biotechnological approach for the elimination of caffeine from the pulp. As a first step in this direction, a strategy has been worked out for the isolation, characterization and selection of filamentous fungi capable of degrading caffeine in liquid medium. Further studies are needed to understand the relation between caffeine degradation and pH variation in the culture medium. Free tannins and polyphenols present in the coffee pulp, which are known to be antinutritional factors for animals, can also be eliminated by using the approach presented here.

Studies on the distribution pattern of microflora in different samples of coffee pulp revealed that pulp A had the highest population of total microflora, which is attributed to the processing conditions of the pulp. It has been demonstrated with fresh sugar-cane bagasse, having an initial moisture content of 44%, that the total microflora present was 2.6×10^6 microorganisms/g dry bagasse (Roussos 1985; Roussos et al. 1991). If this bagasse is stored outside for 15 days the total microflora count increases to 1.89×10^9 microorganisms/g dry bagasse. This population changes rapidly with storage time. Coffee pulp also exhibits a similar trend. In the case of pulp A, where the initial moisture content is comparatively low and the time taken for drying is around 10 days, the microbial count is very high as compared to coffee pulp B, where the initial moisture content is more than 80% and the time taken for drying is only 2 days. Since coffee pulp is rich in carbohydrates and proteins it is essential to dry the pulp immediately or to employ different techniques for its conservation (Daqui 1974; Molina 1979). As drying of coffee pulp is not economically attractive because the drying equipment can be used more profitably for drying the coffee beans rather than the pulp, and also because of space limitation, the coffee pulp is discarded without drying, either in the open air or into rivers and lakes in the case of wet processing of coffee cherries, causing severe environmental pollution (Zuluaga 1989). This situation does not arise with coffee husk where a dry process is employed.

It is advantageous to use a silage method to stabilize coffee pulp/coffee husk and conserve its nutritional characteristics for better utilization of this major agroindustrial waste (Daqui 1974; Murillo 1979; Gaim-Perraud et al. 1993). It is necessary to subject the coffee pulp to silage processing immediately after the separation of the pulp and to create an anaerobic atmosphere to favour lactic fermentation for better conservation.

Acknowledgements We express our gratitude to Dr. Zuluaga, Cenicafé Colombia, for providing the coffee pulp samples B and C. We also would like to express our thanks to Ms. Laure Hannibal, Rosalva Sanchez, Mrs. Jorge Cassagne, Jesus Salas and Wilfrido Rodriguez Perez for excellent assistance during the work. M. Ramakrishna wishes to thank ORSTOM for the award of a Visiting Associateship, and the Biotechnology Unit, ORSTOM Centre, Montpellier, for excellent facilities. The project was funded by CEE (STD1), UAM-Iztapalapa and ORSTOM (Departement MAA, UR 3B2).

References

Adams MR, Dougan J (1981) Biological management of coffee processing. Trop Sci 123:178–196

Ainsworth GC (1973) Introduction and keys to the higher taxa. In: The fungi. An advanced treatise vol 4a. A taxonomic review keys. Academic Press, New York

Aquiahuatl MA (1992) Detoxificación de la pulpa de café: Morfologia, Fisiologia y Bioquimica de hongos filamentosos que degradan la cafeina. Tesis Maestria en Ciencias, Universidad National Autonoma de Mexico (UNAM), Mexico

Arx von JA The genera of fungi sporulating in pure culture. Cramer J, Vaduz 2nd Ed.

Barnet HL, Hunter BB (1972) Illustrated genera of imperfect fungi, 3rd edn. Burgels, Minneapolis

Bressani R (1979) Antiphysiological factors in coffee pulp. Coffee pulp: composition, technology and utilization. Int Dev Res Cent Publ 108e:83–88

Calle VH (1951) Ensayo sobre cultivo de levaduras alimentarias en pulpa de café. Bol Inf CENICAFE 2:33–36

Calle VH (1954) Producción de gas combustible por fermentación metánica de la pulpa de café. Bol Inf CENICAFE 6:198–205

Christensen MS (1981) Preliminary tests on the suitability of coffee pulp in the dies of common carp (*Cyprinus carpio* L.) and catfish (*Clarias mossambicus* Peters). Aquaculture 25:235–242

Daqui LE (1974) Caracteristicas quimicas y nutricionales de la pulpa de café ensilada con pasto napier y pasta de mais. Tesis, Universidad de San Carlos de Guatemala, Facultad de Medi cina Veterinaria. INCAP/CESMA, Guatemala

Elias LG (1979) Chemical composition of coffee-berry by-products Coffee pulp: composition, technology and utilization. Int Dev Res Cent Publ 108e:11–16

Gaime-Perraud I, Roussos S, Martinez-Carrera D (1993) Natural microorganisms of the fresh coffee pulp. Micol Neotrop Apl 6:95–103

Guzman AE (1983) Efectos de nivel y naturaleza de fuentes de nitrogeno sobre el mejoramiento de calidad quimico nutritional de la pulpa de café por fermentation solida usando *Aspergillus niger*. Tesis, Universidad San Carlos de Guatemala, Guatemala

Ishler NH, Finucane TP, Borker E (1948) Rapid spectrophotometric determination of caffeine. Anal Chem 20:1162–1166

Ledger HP, Tilman AD (1972) Utilization of coffee hulls in cattle fattening rations. East Afr Agric For J 37:234–236

Martinez-Carrera D (1987) Design of a mushroom farm for growing *Pleurotus* on coffee pulp. Mushroom J Trop 7:13–23

Molina MR (1979) Drying of coffee pulp. Coffee pulp: composition, technology and utilization. Int Dev Res Cent Publ 108e:63–70

Murillo B (1979) Coffee pulp silage. Coffee pulp: composition, technology and utilization. Int Dev Res Cent Publ 108e:55–61

Orue C, Bahar S (1985) Utilization of solid coffee waste as a substrate for microbial protein production. J Food Sci Technol 22:10–16

Peñaloza W, Molina MR, Gomez R, Bressani R (1985) Solid state fermentation: an alternative to improve the nutritive value of coffee pulp. Appl Environ Microbiol 49:388–393

Raper KB, Fennell DI (1965) The genus *Aspergillus*. Williams and Wilkins, Baltimore

Riddel RW (1950) Permanent stained mycological preparations obtained by slide culture. Mycologia 42:265–270

Rolz C, Leon R. Arriola MC (1988) Biological pretreatment of coffee pulp. Biol Wastes 26:97–114

Roussos S (1985) Croissance de *Trichoderma harzianum* par fermentation en milieu solide: Physiologie, sporulation et production de cellulases. Thèse d'Etat, Université de Provence

Roussos S, Raimbault M (1982) Hydrolyse de la cellulose par les moisissures. I. Screening des souches cellulolytiques. Ann Microbiol 133:455–464

Roussos S, Raimbault M, Viniegra-Gonzalez G, Saucedo-Castañeda G, Lonsane BK (1991) Scale up of cellulases production by *Trichoderma harzianum* on a mixture of sugarcanne bagasse and wheat bran in solid state fermentation system Micol Neotrop Aplic 4:83–98

Roussos S, Gaime-Perraud I, Denis S, Marin B, Marakis S, Viniegra-Gonzalez G, Trejo HM, Aquiahuatl A, Gutierrez RM (1993) Biotechnological advances on coffee byproducts utilization. In: Proceedings of IFCON 93, 7–12 September 1993, Mysore, India

Ryan FJ, Beadle GW, Tatum EL (1943) The tube method of measuring growth rate of *Neurospora*. Am J Bot 30:784–789

Smith JE, Berry DR (1975) The filamentous fungi. I. Industrial mycology. Edward Arnold, London

Smyly DS, Woodward B, Conrad EC (1976) Determination of saccharin, sodium benzoate and caffeine in beverages by reverse phase high pressure liquid chromatography. Journal of AOAC 59:14–19

Zuluaga VJ (1981) Contribution à l'étude de la composition chimique de la pulpe de café *Coffea arabica*. Thèse Doct Sciences. Université Neuchatel, Switzerland

Zuluaga VJ (1989) Utilización integral de los subproductos del café. In: Roussos S, Licona FR, Gutierrez RM (eds), Proceedings of I Seminario Internacional sobre Biotecnologia en la Agroindustria Cafetalera (I SIBAC), Xalapa, Mexico. pp 63–76