Selection of filamentous fungi for decaffeination of coffee pulp in solid state fermentation prior to formation of conidiospores

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SUMMARY

Decaffeination of coffee pulp, to eliminate its antiphysiological effects on animals, was studied by aerobic fungal solid state fermentation, prior to the stage of initiation of conidiospore formation. Comparative data on performance of two strains of *Penicillium* and six strains of *Aspergillus* spp., selected for their high ability to degrade, indicated the potential of *Penicillium* sp V33A25 for caffeine degradation in aerobic solid state fermentation, before the initiation of sporulation by the culture. Kinetic studies pointed out that the evolution of CO_2 is the reliable criterion for the determination of the phase of fermentation, caffeine degradation, increase in medium pH and initiation of sporulation, without taking sample and subjecting it to analyses or disturbing the fermentation. These advantages are not available, if rise in pH the medium is selected as a criterion. Amongst 7 different factors, the fermentation temperature, level of $CaCl_2$ in the medium and autoclaving or non-autoclaving of the medium exhibited strong effects on the initial time of sporulation, extent of CO_2 evolution, pH of the medium and caffeine degradation. The data allow to envisage the use of mixed culture of lactic acid bacteria and filamentous fungi for decaffeination and ensilage of the coffee pulp, or in two stage fermentation, involving any of the simpler order.

Keywords: Solid state fermentation, coffee pulp, aerobic process, filamentous fungi, *Penicillium, Aspergillus*, caffein degradation, spore formation, criterion for fermentation stage, CO_2 evolution, effects of parameters, medium autoclaving, fermentation temperature, calcium chloride.

RESUME

Selection de champignons filamenteux pour la dégradation de la caféine de la pulpe de café en milieu solide et avant la sporulation des souches.

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La décaféination de la pulpe de café, pour éliminer son effet antiphysiologique en alimentation animale, a été étudié en fermentation solide en utilisant des champignons filamenteux. L'objectif est d'obtenir la dégradation de la caféine avant la phase de sporulation de manière à pouvoir tester en association avec des bactéries lactiques au cours d'un procédé de conservation du substrat décaféiné. Des études préalables réalisées en coopération avec l'ORSTOM et la UAM d'Iztapalapa de Mexico ont permis de sélectionner 2 *Penicillium* et 6 *Aspergillus* pour leur haute capacité à dégrader la caféine. Après un criblage en Fermentation en Milieu Solide de ces souches, nous avons sélectionné *Penicillium* sp. V33A25. Ce microorganisme dégrade la caféine de la pulpe de café à 94% après 30 heures de fermentation aérobie, avant la phase de sporulation. La croissance du champignon et la dégradation de la caféine sont parfaitement corrélés à la respirométrie, technique qui nous permet de suivre en continu le développement des microorganismes en FMS. D'autre part, un plan d'expérience nous a permis de constater que la stérilisation du substrat n'a pas d'influence sur la dégradation de la caféine.

Mots clés: Fermentation en Milieu solide, pulpe de café, procédé aérobie, champignons filamenteux, *Penicillium*, *Aspergillus*, dégradation de la caféine, formation de conidiospores, critères d'avancement de la fermentation, effets de paramètres, stérilisation des milieux, température de fermentation, évolution du CO_2, chlorure de calcium

INTRODUCTION

It is of economic and industrial importance to note that only 5.8% of the solids of the coffee berry result in the ultimate coffee drink and the remaining 94.2% forms water and various byproducts (Zuluaga, 1989). Among the latter, the coffee pulp is the maximum and represents 40% of the coffee berry in wet form (Tauk, 1986), corresponding to 29% of dry matter (Bressani *et al*, 1972). This large quantity of the

coffee pulp poses problems of disposal to coffee berry producers, due to putrefaction and causes environmental pollution if not disposed after appropriate treatment (Zuluaga, 1989). Due to its high organic matter content, coffee pulp can be utilized for beneficial purposes and intensive research on this topic has been carried out at ORSTOM (Roussos *et al*, 1995) and also in collaboration with Universidad Autonoma Metropolitana (UAM-I), Mexico (Viniegra-Gonzalez *et al*, 1991).

Direct use of coffee pulp in animal feeding poses problems, due to its chemical composition (Viniegra-Gonzalez *et al*, 1991). For example, the coffee pulp of *Coffea arabica* contains approximatelly 1% caffeine and has antiphysiological effects on the animals (Braham *et al*, 1973; Cabezas *et al*, 1974, 1976; Vargas *et al*, 1982). It is, therefore, necessary to decaffeinate the coffee pulp, before its use as animal feed. Moreover, the coffee pulp gets putrified, because of its high content of water and, hence, needs preservation by appropriate economic technique. At ORSTOM, Montpellier, the techniques of ensilage and fungal degradation of caffein by solid state fermentation (SSF) have been selected for preservation and decaffeination of the coffee pulp, respectively, because of their economic character. If these two techniques are applied in succession, it is of vital importance that the decaffeination by fungi is achieved before the formation of conidiospores. In the case of conidiospore formation, it will be essential to sterilize the decaffeinated coffee pulp, before ensiling. However, mycelial cells of fungi can be eliminated during ensiling and hence sterilization step can be avoided to achieve economy (Perraud-Gaime, 1995).

A total of 350 fungi have been isolated from coffee domains (coffee plants, soils of coffee plantation, coffee byproducts, fermenting coffee berries, etc.) during the research at ORSTOM and UAM (Aquiahuatl *et al*, 1988; Viniegra-Gonzalez *et al*, 1991; Roussos *et al*, 1995). From this collection, a total of 8 filamentous fungi, representing two strains of *Penicillium* and 6 strains of *Aspergillus*, were selected for use in the present studies, based on their higher capacity to degrade caffeine to the extent of 90 to 100% in liquid culture (Roussos *et al*, 1989). One of the *Penicillium* strains selected (V33A25) showed negative effect on caffeine degradation, upon the addition of inorganic nitrogen to the medium in SSF process (Roussos *et al*, 1994).

The objective of this study was to select one or more of the filamentous fungi to grow in SSF and to degrade caffeine to the extent of 80%, before the initiation of conidia formation. Work was also carried out to develop a simple criterion, to correlate growth of the fungi, degradation of caffeine and sporulation time, so that it can be used to stop fermentation at the most appropriate stage.

MATERIALS AND METHODS

MICROORGANISMS

The fungal cultures (2 strains of *Penicillium* and 6 strains of *Aspergillus* species) are maintained on sucrose-coffee-medium plates at 4°C by subculturing every 3 months (Perraud-Gaime, 1995). The methodology for inoculum preparation was, as described elsewhere (Roussos *et al*, 1995).

SUBSTRATE PREPARATION AND FERMENTATION

The coffee pulp used in the present study, was obtained from a industry in Xalapa, Mexico, which processes the berries of *Coffee arabica* by a wet fermentation method. The wet coffee pulp was sun-dried by the industry to contain less than 10% moisture and packed in plastic sacs. The dry coffee pulp was coarsely ground and sieved to obtain a particle size of 0.8 to 2 mm (Perraud-Gaime and Roussos, 1995).

The coffee pulp was moistened to contain 68% final moisture, with a nitrogen source free of mineral salt solution, which contained (g/L) KH_2PO_4 1.3, Na_2HPO_4 0.12, $MgSO_4.7H_2O$ 0.3, $CaCl_2.2H_2O$ 0.3 and distilled water 1000. The moist pulp was sterilized at 121°C for 20 min, inoculated with a spore suspension of a culture to obtain $2x10^7$ spore/g dry matter, 80 g moist inoculated medium was charged in to a column and compacted slightly (Raimbault and Alazard, 1980). The column was installed on the humidifier and placed in a constant temperature water bath. The details are presented in Fig. 1.

The fermenter allows continuous monitoring of the parameters, with display on a computer, throughout the fermentation period and every column functions independently. It is possible to pull out a column and the content of the entire column constituted each sample. Exhaust gas was collected from each column and separately analysed for CO_2 and O_2 in gaseous form in the analyzers. These analyzers are equipped for detectors, thermal conductors and a computer to analyze the gases continously, during the course of fermentation (Dufour, 1990; Saucedo-Castañeda, 1991; Soccol, 1992; Trejo-Hernandez, 1992). Thus, the development of the culture in each column can be visually seen on the computor, in terms of O_2 consumption and CO_2 evolution (Fig. 1).

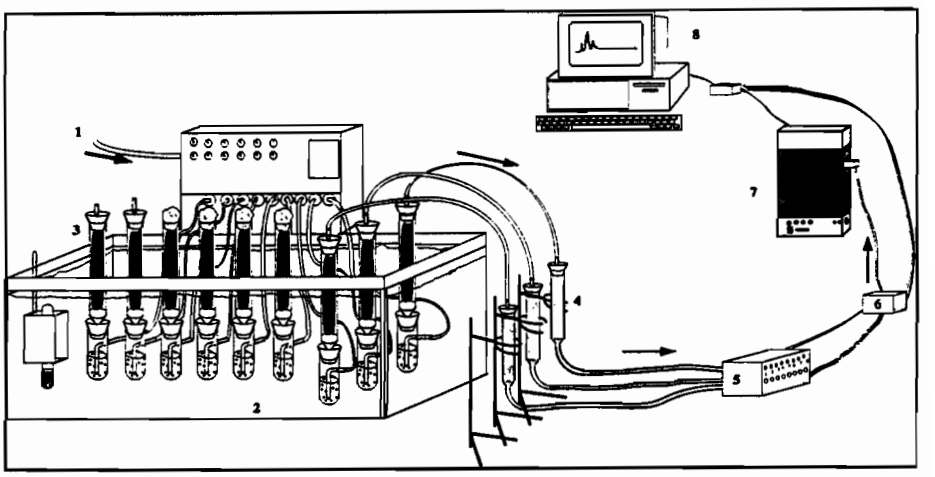

Fig. 1. Protocol for preparation of column for solid state fermentation, with details of set-up and analytical as well as monitoring devices.

1: Air inlet 2: Water bath 3: Column fermenters 4: Silica gel columns 5: Sampler 6: Automatic gas injector 7: Gas chromatograph 8: Computer

SAMPLE PROCESSING AND ANALYSES

Schematic diagram of sample processing and analyses is shown in Fig. 2. Caffeine was estimated by gas chromatography, using the method of Vitzthum *et al* (1974). The details of the methods for estimation of other parameters are similar to those reported elsewere (Perraud-Gaime and Roussos, 1995; Roussos *et al*, 1995).

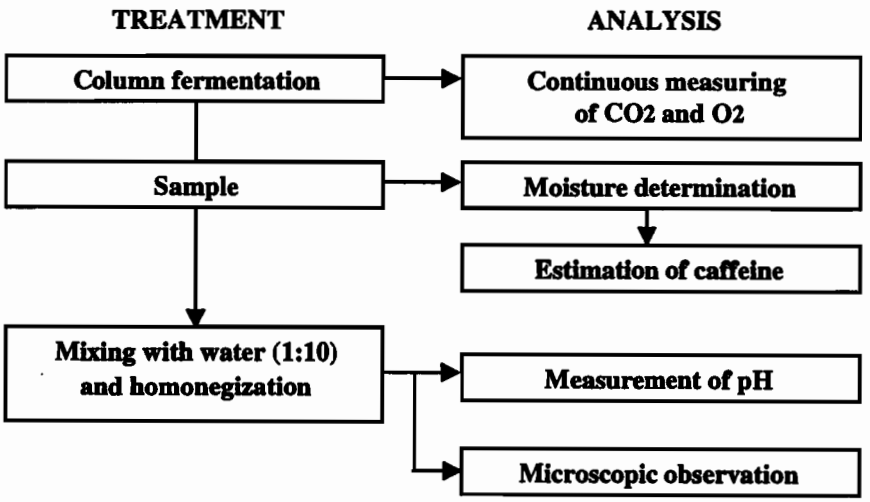

Fig. 2. Schematic diagram of the sample treatment and analyses.

RESULTS AND DISCUSSION

SELECTION OF THE CULTURE

The main objective of the study was to select one or more filamentous fungi, with the ability to degrade caffeine, before initiation of sporulation. This is of importance, as the selected fungal strain is intended to be used for caffeine degradation, before ensiling of the coffee pulp for its preservation. The resulting decaffeinated and ensiled coffee pulp, thus, can find use in animal feeding, without antiphysiological effects of caffeine. The data on growth and metabolism of the 8 fungal cultures studied are presented in Table 1. These fungi have been earlier studied in liquid culture and showed high caffeine degradation capability (Roussos *et al*, 1995).

The comparative analysis of the data allows to eliminate *Aspergillus* sp. strains C16A25 and C23B25, especially because the residual level of caffeine in the medium was higher, even when the strains started to sporulate, as compared to other strains.

The data also allow to eliminate *Aspergillus* sp. C11B25, whose lag phase is very long (20 h) and the level of degradation of caffeine at 30 h is very low (Table 1). *Aspergillus* sp V12A25 sporulates very early (28 h) and is not of interest for the present requirement.

Among the remaining 4 strains, *Penicillium* sp V33A25 and *Aspergillus* sp. C28B25 are the most effective, based on the level of caffein degradation (about 94%) at 30 h (Table 1). These two strains sporulated at 30 and 32 h, respectively, and the lag phase was also shorter (11.0-11.5 h). The data on CO_2 evolution and respiratory coefficient also show a good growth and metabolism of these two fungi on coffee pulp (Table 1).

Table 1. Comparative data on growth and metabolism of the filamentous fungal cultures in column fermenters under solid state fermentation (Perraud-Gaime, 1995).

Strains	Lag phase h	CO_2 production ml/g MSI	Respirometry coefficient h^{-1}	Caffeine degradation at 30 h, %	Time of initiation of sporulation
Penicillium sp. V26A25	12,5	115	0,34	91	32 h
Penicillium sp. V33A25	11,5	95	0,34	94	30 h
Aspergillus sp. C16A25	13,0	100	0,30	80	32 h
Aspergillus sp. V12A25	10,5	130	0,29	82	28 h
Aspergillus sp. C17B25	17,0	65	0,34	87	32 h
Aspergillus sp. C11B25	20,0	65	0,26	12	42 h
Aspergillus sp. C28B25	11,0	100	0,30	94	32 h
Aspergillus sp. C23B25	11,0	85	0,34	79	30 h

Among these two filamentous fungi of good potential, *Penicillium* sp. V33A25, was choosen in the present studies, as this strain is also used in ORSTOM in other studies (Roussos *et al*, 1989; Denis, 1992) and it also fulfills the requirements of this study to the highest extent. For example, the degradation of caffeine in aerobic solid state fermentation is 94% at 30 h, and the sporulation starts only at this stage.

FORMULATION OF CRITERION

The second objective of the study was to formulate a reliable criterion to correlate maximum degradation of caffeine and time of sporulation as an appropriate fermentation parameter, for its use in simpler monitoring of the fermentation of the

culture in columns in solid state fermentation. The results of the experiments conducted for this purpose are presented in Fig. 3, in terms of growth and metabolism of *Penicillium* sp V33A25 on coffee pulp in column fermenters.

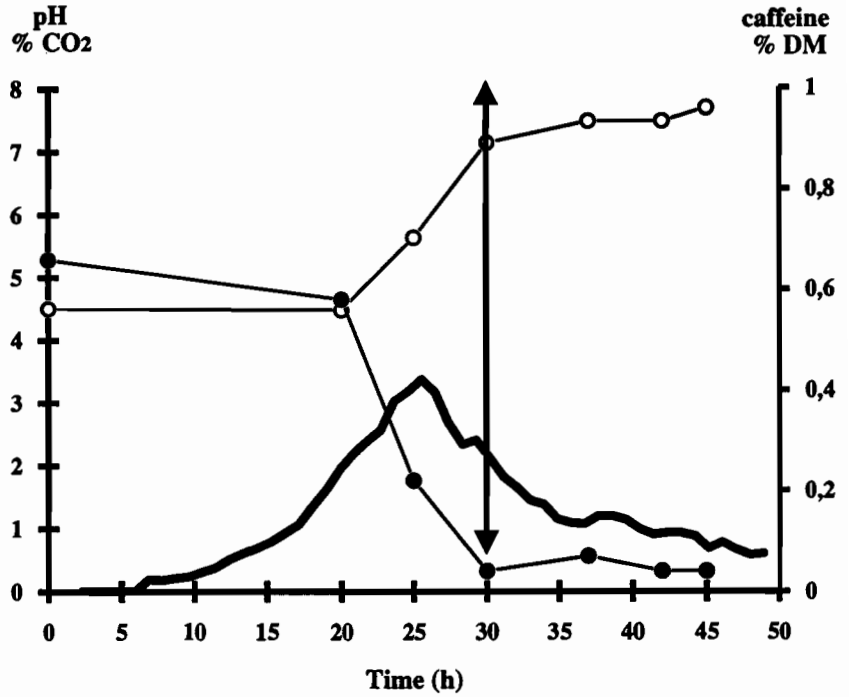

Fig. 3. Profile of pH (m) and caffeine (l) as against CO_2 (—) evolution during the course of the growth and metabolism of *Penicillium* sp. V33A25 on coffee pulp at 25°C. Arrow indicates the phase of initiation of sporulation (Perraud-Gaime, 1995).

Data revealed that the pH of the medium started increasing from 20 h fermentation to reach the level of 6.8 at 30 h, the time at which sporulation was also initiated. The begining of sporulation in the medium at 30 h is represented in Fig. 3 by a verticle arrow. Data on the kinetics of caffeine degradation also perfectly correlate with this stage, at which the pH of the medium starts to increase. Thus, the stage at which pH starts to stabilize could be taken as a criterion to indicate maximum caffeine degradation and initiation of the sporulation stage. However, its utility is

limited, as it is necessary to take the sample and process it to measure the pH before taking the decision to stop the fermentation.

The fermentation system allows to continuously visualize the evolution of CO_2 on the computor, without any necessity to remove a sample, process and analyse. In fact, this does not disturb the fermentation process. Hence, its selection as the reliable criterion is better, as it also allows to distinguish the phases of growth and metabolism during the course of fermentation. For example, the CO_2 evolution curve clearly shows the lag, germination and growth phases, while the last one can also be seen visually by observing the fermenting solids in the column. During the growth phase, the substrate is completely surrounded by the mycelial cells. The spore formation is initiated, after about 5 h from the stage of maximum CO_2 production. Thus, if the fermentation is stopped after 3 h of maximum CO_2 production, it is the fermentation stage at which the level of caffeine in coffee pulp is less than 0.2% on a dry weight basis and there are no spores in the medium.

Hence, data of the respirometry analysis was selected as a criterion to indicate the most important step of the development of fungi and to correlate it with maximum degradation of caffeine, prior to initiation of the conidiospore formation.

EFFECT OF FACTORS ON FERMENTATION EFFICIENCY

As for the last objective of the study, a number of experiments have been carried out to evaluate the effects of seven different parameters on the level of caffeine degradation, CO_2 evolution, final pH of the medium and initiation of sporulation. These seven factors tested were a) with or without the use of mineral solution, b) fermentation under natural light or in darkness, c) aeration at 40 or 70 ml/min, d) fermentation at 25 or 35°C, e) with or without autoclaving of the substrate, f) inclusion of $CaCl_2$ in the mineral solution at 0.1 or 0.3 g/l and h) initial pH of the mineral salt solution at 4.4 or 5.6. All the other factors were constant, i.e., 68% initial moisture content of the medium and inoculum level of $2x10^7$ spores/g dry matter.

Graphic representation of the relative effects of these seven different factors is given in Figure 4. The results show that the incubation temperature is the most important factor. The fermentation needs to be conducted at 25°C for the best degradation of caffeine and good development of the fungi. These observations are in agreement, with those reported by Roussos *et al* (1989). The rate of aeration, initial pH of the medium, presence or absence of mineral salt solution and fermentation under natural light or in darkness have non-significant influence on all the four parameters studied.

It was felt that it is better to increase the concentration of $CaCl_2$ in the medium to favour the mycelial growth and delay sporulation.

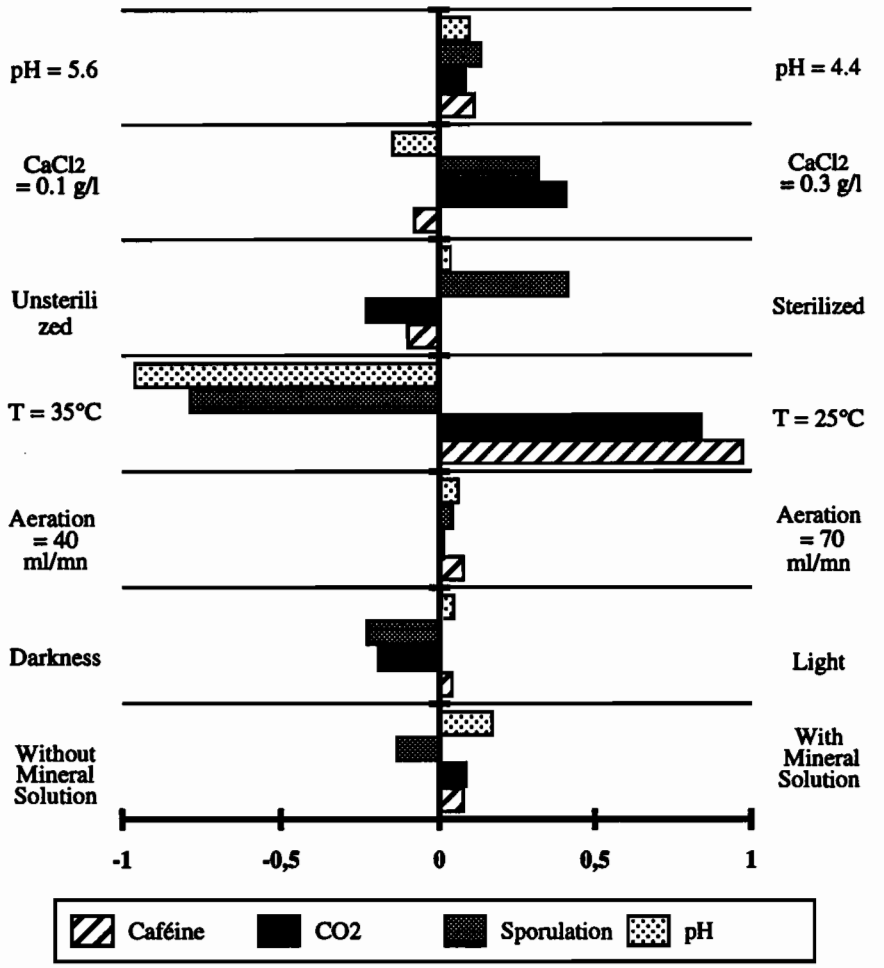

Fig. 4. Graphic analysis of the effects of various factors on the degradation of caffeine, evolution of CO_2, initiation of sporulation and final pH of the moist solid medium (Perraud-Gaime, 1995).

Another economically important result of these experiments is the demostration that the medium sterilization or its use without any sterilization has no influence on the degradation of caffeine (Fig. 4). This observation allows to envisage the use of this process of degradation of caffeine in the coffee pulp by filamentous fungi in SSF,

after its ensilage by lactic acid bacteria, without sterilization. It might be even possible to firstly subject the coffee pulp to degradation of caffeine and then preserve by ensilage.

CONCLUSION

It can be concluded that it is possible to decaffeinate the coffee pulp in 30 h under aerobic conditions by using selected fungal culture, i.e., *Penicillium* sp. V33A25 in solid state fermentation, before initiation of the sporulation by the strain. It is also not necessary to sterilize the substrate. It is, therefore, possible to envisage the inoculation of the coffee pulp with mixed culture of lactic acid bacteria, for the ensilage preservation of coffee pulp, along with the selected filamentous fungi, for degradation of the caffeine. It can lead to decaffeinated and stabilized coffee pulp, which is suitable for animal feeding.

It is also possible that the stages of the fermentation can be observed visually on the computer, through respirometric parameters, without removing the sample and subjecting it to analyses and also without disturbing the culture medium. This factor of CO_2 evolution permits to reliably estimate different phases of the development of *Penicillium* sp. V33A25, in terms of degradation of caffeine and time of the sporulation of the filamentous fungi.

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