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Biological detoxification of coffee husk by filamentous fungi using a solid state fermentation system☆

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Abstract

Studies were carried out on detoxification of coffee husk in solid state fermentation using three different strains of *Rhizopus*, *Phanerochaete*, and *Aspergillus* sp. Fungal strains were selected by their ability to grow on a coffee husk extract-agar medium. Using *R. arrizus* LPB-79, the best results on the degradation of caffeine (87%) and tannins (65%) were obtained with pH 6.0 and moisture 60% in 6 days. When *P. chrysosporium* BK was used, maximum degradation of caffeine and tannins were 70.8 and 45%, respectively, with coffee husk having 65% moisture and pH 5.5 in 14 days. The *Aspergillus* strain, isolated from the coffee husk, showed best biomass formation on coffee husk extract-agar medium. Optimization assays were conducted using factorial design, and surface response experiments with *Aspergillus* sp. The best detoxification rates achieved were 92% for caffeine and 65% for tannins. The results showed good prospects of using these fungal strains, in particular *Aspergillus* sp., for the detoxification of coffee husk.

Keywords: Coffee husk; Solid state fermentation; Filamentous fungi; Detoxification; Caffeine; Tannins

1. Introduction

Brazil is the largest producer of coffee in the world, contributing approximately 25% of the world's production. During 1999, its production reached two million tons. Only 6% of the coffee cherries (fresh weight basis) constitute the portion produced as coffee powder; the remaining 94% is obtained as by-products (water and subproducts such as mucilage, husk, etc.) [1]. Processing of coffee cherries by the "dry-process" results in coffee husk as the main residue in about 40% quantity [2]. Although a small quantity of coffee husk is used as cattle feed, or in compost [3], it does not find any adequate application. Mostly it is discarded by the processing units in the rivers and lakes located near the coffee processing regions, which causes a serious environmental concern.

In recent years, there has been an increasing trend toward efficient use of agro-industrial residues [4–6]. Several processes have been developed that use these as raw materials for the production of bulk chemicals and value-added fine products such as ethanol, single-cell protein (SCP), mushrooms, enzymes, organic acids, amino acid, biologically active secondary metabolites, etc. [4,7–12]. Application of agro-industrial residues in bioprocesses at one hand provides alternative substrates, and on the other side helps to solve pollution problem, which otherwise their disposal may cause. With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new avenues have opened.

With its richness in carbohydrate, protein, fat, and fibers, coffee husk seems to be a useful feed supplement for cattle and other livestock and substrate for bioconversion processes. However, presence of antiphysiological and antinutritional factors such as caffeine, tannins, and polyphenols makes it unsuitable for such applications. Several studies have been carried out to evaluate coffee husk from an

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animal nutrition point of view. Low feed intake, protein digestibility, and nitrogen retention are major factors limiting its use [13,14]. Consequently, most of the husk remains unused or poorly used. If the toxic constituents could be partially removed at least, new avenues would open for its utilization.

Attempts have been made to detoxify the coffee husk by physical, chemical, and microbial methods [15–20]. Some of the physical and chemical methods achieved good results but were expensive. This resulted in a focus on microbial methods. Solid state fermentation (SSF) has been frequently used for the biological detoxification of coffee pulp using fungal strains [17,20–22].

The objective of the present work was to study the biological detoxification of coffee husk in solid state fermentation using strains of *Rhizopus*, *Phanerochaete*, and *Aspergillus* sp.

2. Materials and methods

2.1. Coffee husk

Coffee husk was obtained from Café Damasco, Curitiba. It was dried in an air oven at 55°C for 48 h, milled manually, and sieved to obtain the fractions between 0.8–2.0 mm particle size. It was assayed for pH, moisture, sugars, ash, lipids, fibers, nitrogen, tannins, and caffeine.

2.2. Microorganisms and media

A total of 14 microbial strains belonging to *Rhizopus* sp. (11 strains), *Phanerochaete* sp. (2 strains), and *Aspergillus* sp. (one strain, isolated from the coffee husk) were used in this study. The strains of *Rhizopus* and *Phanerochaete* sp. were maintained on Potato-Dextrose-Agar (PDA) medium. The strain of *Aspergillus* sp. was maintained on coffee husk extract-agar (CHA) medium, which was prepared by cooking 100 g (dry weight.) of coffee husk in 1-l distilled water for 1 hour at 100°C. The resulting solution was then filtered and after adjusting the pH as desired, was supplemented with bacteriological agar (20 g/l). The medium was sterilized at 121°C for 20 min.

2.3. Selection of strains

All the strains were screened for their ability to grow on CHA medium by observing their mycelial spreading rate and biomass production. Twenty milliliters of CHA medium was taken in Petri dishes (7.5 cm diameter) that were inoculated in the center and incubated for 5 days at 28, 32, and 35°C for *Aspergillus*, *Rhizopus*, and *Phanerochaete* strains, respectively. The mycelial growth was measured every 2 h for *Rhizopus* strains and every 12 h for *Phanerochaete* and *Aspergillus* strains. The biomass was measured by the dissolution of the agar and filtration on weighed filter papers. All experiments were made in three sets.

2.4. Inoculum preparation

Spores from the Petri dishes with actively growing 10-day-old cultures of *Rhizopus* and *Phanerochaete* on PDA medium were collected in 50 ml of distilled water with a platinum loop. Spores of *Aspergillus* sp. growing on CHA medium were harvested by homogenization with distilled water (50 ml with five drops of Tween 80) and glass beads. The spores were counted in a Neubauer cell.

2.5. Solid state fermentation (SSF)

Based on the results of screening studies, three strains, namely *Rhizopus arrhizus* LPB-79, *Phanerochaete chrysosporium* BK, and *Aspergillus* sp., were chosen for SSF studies. Coffee husk was used as the sole source of carbon and nitrogen source in SSF. SSF was carried out by taking 20 g substrate in 250 ml Erlenmeyer flasks. All experiments were conducted with two replicates.

2.6. SSF using R. arrhizus

Fermentation was carried out by inoculating the substrate (pH 6.0, adjusted with 3-N NaOH and moisture 70%) using 1.25×10^7 spores/g substrate and incubating at 32°C for 6 days. Experiments were conducted to optimize the initial pH (5.5–6.5) and moisture (60–70%) of the substrate and addition of nutrient solution containing (g/l) KH_2PO_4 2, Na_2HPO_4 0.2, $MnSO_4 \cdot 7H_2O$ 0.5 [modified from Soccol [23]]. A total of 26 runs were done.

2.7. SSF using P. chrysosporium

Fermentation was carried out by inoculating the substrate (pH 5.0, adjusted with 3-N NaOH; moisture 70%) using 1.29×10^7 spores/g substrate and incubating at 35°C for 14 days. Experiments were conducted to optimize the initial pH (4.5–5.5) and moisture (60–70%) of the substrate, and on the addition of nutrient solution. This contained (g/l) KH_2PO_4 1, $CaCl_2 \cdot 2H_2O$ 0.7, $MgSO_4 \cdot 7H_2O$ 3.5, $FeSO_4 \cdot 7H_2O$ 0.35, $ZnSO_4 \cdot 7H_2O$ 0.23, $MnSO_4 \cdot 5H_2O$ 0.17, and $CuSO_4 \cdot 5H_2O$ 0.35 [24]. A total of 26 runs were made.

2.8. SSF using Aspergillus sp.

The best results on growth in CHA medium, and in the initial studies on SSF using coffee husk were obtained with *Aspergillus* sp., so this was chosen for factorial design studies. The inoculum size was 1.75×10^7 spores/g substrate.

Table 1
Experimental design for the optimization of physical parameters on the detoxification of coffee husk by *Aspergillus* sp.

Parameters	Low level	Central point	High level
Factorial design 1			
pH	3.5	4.5	5.5
Temperature, °C	24	26	28
Moisture	60	65	70
Factorial design 2			
pH	3.0	4.0	5.0
Temperature, °C	26	28	30

2.9. Factorial design 1

A 2^{3-0} factorial design based on the surface response with three experimental factors at two levels and a medium point was used to optimize the first step of conditions for pH and moisture of the substrate, and temperature of fermentation to achieve the best detoxification rate (the response variable). Table 1 shows the real and coded experimental variables. A total of 22 runs were made.

2.10. Factorial design 2

To achieve the best parameters for kinetic studies, the second experimental design was a 3^{2-0} that involved the studies on the influence of pH and temperature that were significant by the analysis of the first factorial design experiments at three levels. Table 1 shows the real and coded experimental variables.

2.11. Kinetics of caffeine and tannin degradation of coffee husk by Aspergillus sp.

The parameters used in the kinetics studies were: substrate pH and moisture 4.0 and 65%, respectively, temperature 28°C, and inoculum size 1.75×10^7 spores/g of substrate. Samples were collect every 18 h and analyzed for pH, caffeine, and tannins.

2.12. Analytical methods

Lipids, ash, and caffeine assays were carried out following the methodology described by Instituto Adolpho Lutz [25]. Tannins were determined by Lowenthal method [26] and proteins by Stutzer method [27] (total proteins as N x 6.25 by Kjeldahl method). Total and reducing sugars were analyzed by Somogyi method [28].

3. Results and discussion

3.1. Chemical composition of coffee husk

Results of chemical analysis showed that the experimental sample of coffee husk contained (% w/w): moisture, 11.98; lipids 1.5; fibers 31.86; ash 6.03; total sugars 26.5; protein (total N x 6.25) 6.8; protein (Stutzer) 4.8; caffeine 1.2; and tannins 9.3. The chemical composition of the coffee husk has not been extensively studied in comparison to the coffee pulp, although they seem to have similarities in their compositions. It was interesting to note a difference between the values obtained for protein (total N × 6.25) determined by the Kjeldahl method and for true protein determined by the Stutzer method, possibly due to the nitrogen content present in caffeine and other nitrogenous compounds present in the coffee husk. The high content of tannins was probably because the coffee grains were sun dried, which favored the production of these compounds in the coffee husk. It has been reported that there could be a difference in percent composition of the constituents, depending on the processing mode and efficiency, crop variety, and cultivation conditions such as soil type, etc. [29]. The content of nutrients of the coffee husk compares favorably with other agricultural products such as oats, rice meal, rice bran, and wheat bran, all of which are increasingly being used in the diet of man [30]; however, due to the presence of caffeine and tannins, it cannot compete with these residues.

Table 2
Growth of fungal strains on CHA medium

Strain	Biomass (mg/plate)
P. chrysosporium HD	1.83 ± 0.56
P. chrysosporium BK*	2.21 ± 0.76
Rhizopus sp.	2.80 ± 0.20
R. formosa LPB 22	2.90 ± 0.80
R. arrhizus LPB 82	3.20 ± 0.20
R. oligosporus LPB 67	3.60 ± 0.20
R. arrhizus LPB-25	6.60 ± 0.40
R. oryzae LPB 27	7.90 ± 0.04
R. oryzae LPB 95	8.70 ± 0.57
R. circicans LPB 75	9.20 ± 0.07
R. oryzae LPB 68	10.20 ± 0.02
R. delemar LPB 12	10.80 ± 0.12
R. arrhizus LPB 79*	12.10 ± 1.30
Aspergillus sp.*	14.83 ± 0.02

* Selected strains. Growth conditions: *Rhizopus*-temperature 32°C, pH 6.0, 32 h; *P. chrysosporium* temperature 35°C, pH 4.5, 92 h; *Aspergillus* sp. temperature 28°C, pH 4.5, 92 h.

3.2. Strains selection

The results obtained in biomass production for all 14 fungal strains are shown in Table 2. The spreading rate of *Rhizopus* strains ranged from 0.94 mm/h to 2.19 mm/h (data not shown here). It was found that the strains of *Rhizopus*, which produced a higher quantity of biomass, corresponded to those showing higher spreading rate. Among the *Phanerochaete* strains, *P. chrysosporium* BK showed better performance. Although the strain of *Aspergillus* sp. showed lower mycelial spreading rate (data not shown here), its overall growth was best among all the strains tested. Based

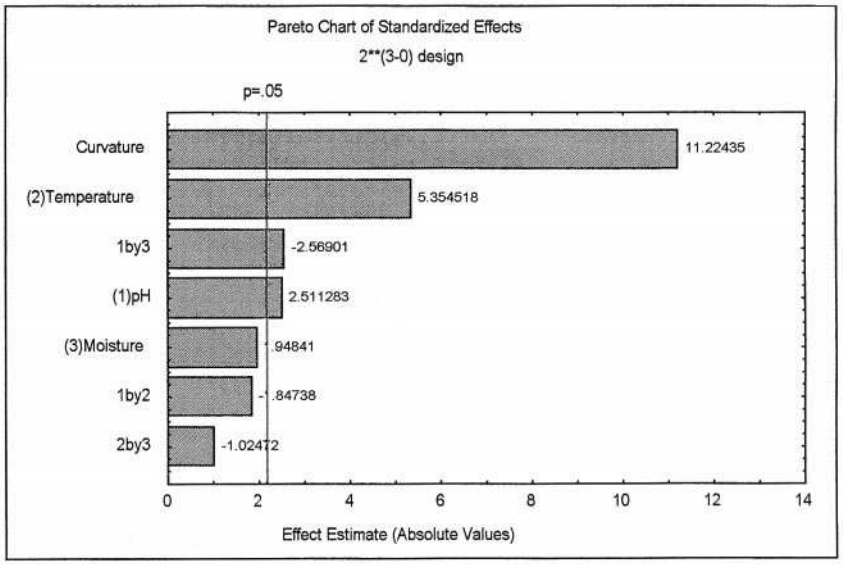

Fig. 1. Pareto chart of effects for 2^{3-0} experimental design for SSF of coffee husk by *Aspergillus* sp.

on best biomass production, three strains: *R. arrhizus* LPB-79, *P. chrysosporium* BK, and *Aspergillus* sp. were chosen for fermentation studies.

3.3. SSF using R. arrhizus LPB-79

During SSF using *R. arrhizus*, it was observed that the pH was the parameter with most influence on the metabolism of the mold (data not shown here). Substrate moisture in the experimental range had little influence on detoxification. The best results on the degradation of caffeine (87%) and tannins (65%) were obtained with pH 6.0 and moisture 60% for 6 days (data not shown here). Soccol [23], while studying production of fumaric acid from cassava wastes, found that a pH of 6.0 was most suitable for the activity of this strain. Coffee husk binds a larger amount of moisture than starchy materials, and therefore needs more water to allow the level of available water required for the mold growth. [21]. In the present study, supplementation of the fermentation medium with nutrient solution showed neither any influence on the detoxification rate of caffeine and/or tannins, nor better growth of the fungal culture (data not shown here). This showed that it was not necessary to enrich the coffee husk medium with such nutrients. This could be perhaps due to the fact that coffee husk itself contains several minerals as indicated by its high ash content. In view of the earlier results [31], describing that the addition of nitrogen to a coffee pulp medium interfered with caffeine metabolism by the fungal culture, we did not provide any additional nitrogen source in the medium

3.4. SSF using P. chrysosporium BK

During SSF using *P. chrysosporium* it was found that alhough both the pH and moisture content affected the degradation rate of caffeine and tannins, the pH had more effect (data not shown here). Best results on the degradation of caffeine (70.8%) and tannins (45%) were obtained with

Table 3
ANOVA for the experimental responses of detoxification of coffee husk (factorial design 1) by *Aspergillus* sp.

Effect	Sum of squares	Degrees of freedom	Mean square	F ratio	*p* value
Curvature	1512.061	1	1512.061	125.986	0
(1) pH	75.69	1	75.69	6.3065	0.02892
(2) Temperature	344.103	1	344.103	28.6709	0.000232
(3) Initial moisture	45.563	1	45.563	3.7963	0.077327
1 By 2	40.96	1	40.96	3.4128	0.091741
1 By 3	79.21	1	79.21	6.5998	0.026096
2 By 3	12.602	1	12.602	1.05	0.327503
Lack of fit	12.96	1	12.96	1.0798	0.321037
Pure error	132.02	11	12.002	—	—
Total	2255.168	19			

$R^2 = 0.93571$

Table 4
ANOVA for the experimental responses of detoxification of coffee husk (factorial design 2) by *Aspergillus* sp.

Effect	Sum of squares	Degrees of freedom	Mean square	F ratio	*p* value
(1) pH	306.2433	2	153.1217	3.417174	0.078685
(2) Temperature	100.27	2	50.135	1.118849	0.368166
Lack of fit	647.1667	14	161.7917	3.61066	0.050778
Pure error	403.285	9	44.80944		
Total	1456.965	17			

$R^2 = 0.90211$

substrate having 65% moisture and 5.5 pH in 14 days. Jeffries et al. [32] reported pH 5.5 as the optimum for the growth of *P. chrysosporium*. The culture, however, took a longer time to achieve the best degradation level. Supplementation of fermentation medium with nutrients did not improve the results.

3.5. SSF using Aspergillus *sp*

The first step of SSF using *Aspergillus* sp. for the detoxification of coffee husk consisted in the use of a 2^{3-0} factorial design with three factors studied: pH and moisture of the substrate and temperature of fermentation. Results (Fig. 1) were submitted to analysis of variance (ANOVA, see Table 3) and by eliminating no significant effects; the data obtained for detoxification of coffee husk showed that the significant factors at the confidence level of 5% were the pH of the substrate and the temperature of fermentation. The best pH and temperature were 4.5 and 26°C, respectively, resulting in about 89% degradation of caffeine and 56% of tannins.

In the second step of optimization, only two factors varied: the pH of the substrate (3.0–5.0) and the temperature of fermentation (26–30°C). The results were submitted to ANOVA (see Table 4). Variation of the pH led to an increase in the degradation rate at level of 5%. Fig. 2 shows the surface response for the degradation of caffeine and tannins as a function of pH of the substrate and temperature of fermentation. Apparently variation of experimental temperature range used in this step did not cause a significant effect on degradation rate, although best results were obtained at 28°C. The pH of the substrate showed its positive influence on degradation of these compounds. Best results on the degradation of caffeine (91%) and tannins (70%) were obtained at pH 4.0 and 28°C.

Kinetic studies of SSF at the best conditions of pH and temperature showed that as the pH increased, the caffeine and tannin contents decreased, possibly due to degradation (Fig. 3). There are a few reports describing the metabolic pathway of caffeine degradation by bacterial and fungal cultures, with urea as the final product [33,34]. In fungal metabolism, the intermediates formed were theobromine, paraxanthine, and 3-methyl xanthine [34], which were different from those of bacterial fermentation [33]. In present studies, SSF was characterized by a lag phase of 18 h. Then the pH started to increase as the degradation of caffeine and tannins began. It was observed that the rate of the degradation of tannins was slower than that for caffeine. After 54 h,

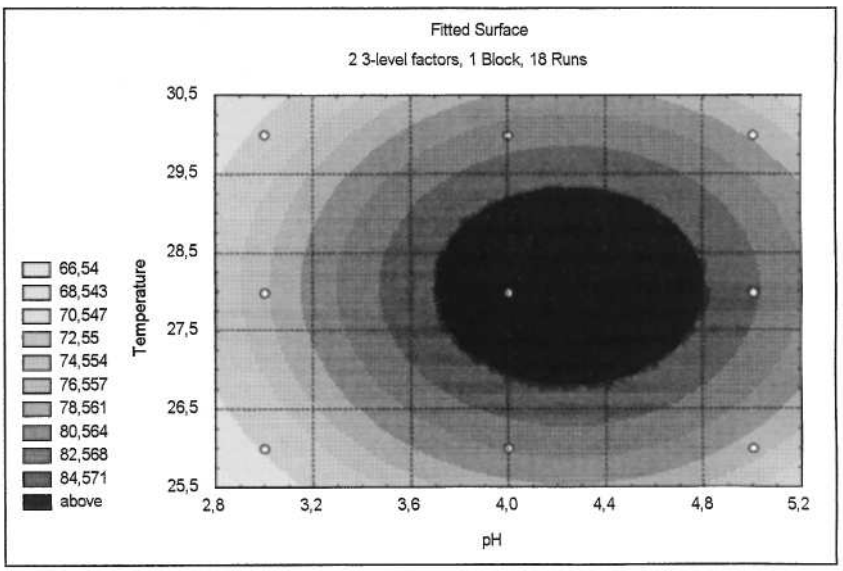

Fig. 2. Surface response for 3^{2-0} experimental design for SSF of coffee husk by *Aspergillus* sp.

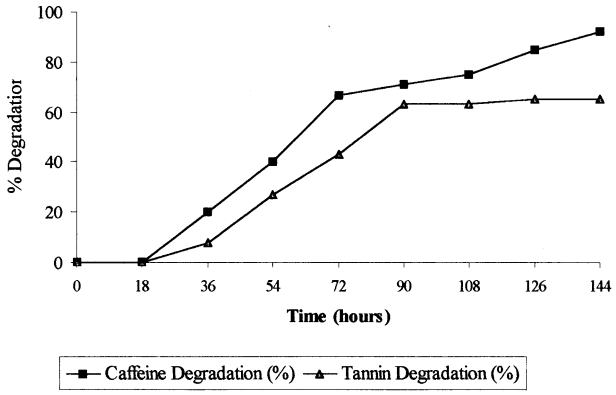

Fig. 3. Kinetics of caffeine and tannin degradation by *Aspergillus* sp.

the pH reached 4.8 and the degradation of caffeine and tannins was 40% and 27%, respectively. After this period, the pH started to increase and was 7.85 after 108 h. At this time, the degradation of tannins had almost stopped, at about 65%. However, the caffeine degradation still continued until 144 h to a maximum of 92%. The pH decreased to 7.3.

4. Conclusions

All 14 strains belonging of *Rhizopus*, *Phanerochaete*, and *Aspergillus* sp. were capable of growing in coffee husk extract-agar medium, showing their resistance to toxic factors present in it. SSF, using three selected strains, showed best growth on CHA medium, and showed partial degradation of caffeine and tannins present in the coffee husk. These experiments were realized without nutrient supplements, showing that the micro-organisms were able to use the components present in coffee husk itself by degrading the toxic constituents of the coffee husk. The strain of *Aspergillus* sp., which showed the highest biomass formation on CHA medium, resulted in 92% caffeine degradation, showing high efficiency.

The results demonstrated the potential of the selected fungal strains for detoxification of coffee husk with the objective of its use in animal feed, or as fermentation substrate.

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