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Effect of particle size and ammonium sulfate concentration on rice bran fermentation with the fungus *Rhizopus oryzae*

Cristiano Gautério Schmidt *, Eliana Badiale Furlong

Escola de Química e Alimentos, Universidade Federal do Rio Grande, Rua Engenheiro Alfredo Huck, 475, Rio Grande-RS 96201-460, Brazil

HIGHLIGHTS

- Rice bran was used as substrate for solid-state fermentation.
- The increase in particle size of rice bran favored the *Rhizopus oryzae* biomass.
- Particles of a smaller size provided the highest protein and phenolic contents.
- A higher concentration of ammonium sulfate favored the phenolic content gain.

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ABSTRACT

The effects of rice bran particle size (0.18–0.39 mm) and ammonium sulfate concentration in the nutrient solution (2–8 g/L) on biomass production, protein and phenolic content generated by solid state fermentation with the fungus *Rhizopus oryzae* (CCT 1217) were studied. Particle size had a positive effect on biomass production and a negative effect ($p \leqslant 0.05$) on protein and phenolic contents. Ammonium sulfate concentration had a positive effect ($p \leqslant 0.05$) on biomass and phenolic content gain. Cultivation of fungus in rice bran with particle size of 0.18 mm and in the presence of 8 g/L ammonium sulfate, resulted in protein levels of 20 g/100 g dry wt and phenolics content of 4 mg/g dry wt. These values were 53 and 65% higher than those achieved with unfermented rice bran. The results demonstrate that the fermentation process increased the value of compounds recovered for potential use in food formulations.

1. Introduction

Solid-state fermentation (SSF) refers to the cultivation of microorganisms on a solid matrix, with a water content that insures growth and metabolism of microorganisms but does not exceed the maximum water binding capacity of the solid matrix (Del Bianchi et al., 2001). SSF of agricultural and agro-industrial residues can be increase their value, example, by increasing protein content and nutritional value or by production of useful enzymes (Rudravaram et al., 2006; Ravinder et al., 2003).

Fungi of the genus *Rhizopus* are especially suitable for SSF since they produce highly digestible protein and any toxic substances (Oliveira et al., 2010; Anapuma and Ravindra, 2000). The cultivation of *Rhizopus oryzae* on rice bran resulted in a sharp increase in protein content (Oliveira et al., 2010; Silveira and Furlong, 2007). As seen with *Phanerochaete chrysosporium* growing on green coconut husk, SSF can also increase the content of phenolic compounds from agro-industrial residues (Martins et al., 2011; Barbosa et al., 2008). Such phenolic antioxidants that have the potential to reduce the risk of disease and find applications in the food industry and in health and cosmetics markets (Busat and Siriamornpun, 2010; Pourali et al., 2010).

The performance of organism in SSF depends on the physical properties of the solids, including their crystalline or amorphous nature, accessible area, porosity and, mainly, their particle size (Membrillo et al., 2008). Since nitrogen supplementation of rice bran media has also been found to increases protein yield (Anapuma and Ravindra, 2001). The present study evaluated the effect of the substrate particle size and ammonium sulfate concentration

Abbreviations: SSF, solid-state fermentation; GF, content of the glucosamine in fermented bran; GF_0, content of the glucosamine in unfermented bran; PG, percentage protein gain on dry basis; NF, nitrogen content in fermented bran; NF_0, nitrogen content in unfermented bran; PCG, percentage phenolic content gain of on dry basis; PCF, phenolic content in fermented bran; PCF_0, phenolic content in unfermented bran; PF_0, protein in unfermented rice bran; PF, protein in fermented rice bran.

* Corresponding author. Tel.: +55 53 32338663; fax: +55 53 32338745.
E-mail address: eng_ea@hotmail.com (C.G. Schmidt).

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on growth of biomass, protein content and phenolic compounds production by *R. oryzae* during SSF with a focus on the recovery of these compounds for use in food formulations.

2. Methods

2.1. Inoculum preparation

R. oryzae (CCT 1217) was obtained from the Foundation André Tosello (FAT), Campinas, Brazil. The cultures were maintained at 4 °C on potato dextrose agar (PDA, Acumedia®). Spores from 7-day-old cultures grown at 30 °C were by suspending them in 0.2 mL (v/v) of Tween 80. The concentration of spores in the suspension was estimated by enumeration in a Neubauer chamber.

2.2. Substrate characterization

The rice bran was provided by company located in Rio Grande do Sul, Brazil. The bran was sifted using sieves of 35, 42, 65 and 100 mesh. The bran was divided into three particles sizes with average diameter of 0.18 mm (−35 and +42 mesh), 0.28 mm (−42 and +65 mesh), 0.39 mm (−65 and +100 mesh). The proximal composition of the different rice bran fractions was determined according to AOAC methods (2000) for moisture (no. 934.01), protein (no. 955.04C; conversion factor of 5.7), total fiber (no. 962.09), lipids (no. 920.85) and ash (no. 900.02A). Carbohydrates were estimated by difference. The physical characteristics of the rice bran fractions, surface area, pore volume and average pore diameter were estimated by the nitrogen adsorption isotherms obtained with the BET method (Brunauer et al., 1938) using a Quantachrome instrument. The morphology of the particles was visualized by scanning electron microscopy (SEM) (Supplementary data 1).

2.3. Fermentation

Rice bran was fermented in a semi-solid state system (Oliveira et al., 2011). The rice bran fractions were arranged in 2 cm layers and covered with sterile gauze and cotton to allow aeration and prevent contamination. Trays (6 cm × 6 cm × 5 cm) containing 15 g of rice bran (sterilized at 121 °C for 30 min) were moistened with a nutrient solution (2 g/L KH_2PO_4, 1 g/L $MgSO_4$, and 2–8 g/L $(NH_4)_2SO_4$ in 0.4 N HCl, cold sterilized) at 45%p/v.

R. oryzae spores were added an initial concentration of 4×10^6 - spores/g of bran. Sterile distilled water was added to the medium in order to adjust the humidity to 50%. The trays were placed in a fermentation chamber at 30 °C with controlled humidity. After 96 h of incubation, the fermented biomass was placed in plastic containers and stored at −18 °C. The control (labeled unfermented) was the sterilized rice bran to which nutrient and spore solution were addition and stored immediately at −18 °C.

2.4. Experimental designs

To evaluate the average particle size effects of rice bran and ammonium sulfate concentration in the fermentation process, a 2^2 full factorial design using the software *Statistica 7.0* was employed, with dependent variables being the production biomass, protein and phenolic content gain. The levels for the independent variables (Table 1) were based on conditions utilized previously (Pogaku et al., 2009; Rudravaram et al., 2006; Ravinder et al., 2003). The average particle size and ammonium sulfate concentration effects analyzed by a Pareto chart. Pareto analysis was used to determine the environmental factors that were most significant in biomass production, protein and phenolic contents gain after SSF. Seven fermentation runs were made using different batches of rice bran and different ammonium sulfate concentration in the nutrient solution.

Table 1
Levels of the independent variables and values of average particle size and ammonium sulfate concentration used in experimental design.

Levels	Factor	
	$\Phi_{average}$ (mm)	$[(NH_4)_2SO_4]$ (g/L)
−1	0.18	2
0	0.28	5
1	0.39	8

Where: $\Phi_{average}$ = average diameter.

2.5. Biomass production

The biomass generated during fermentation was indirectly estimated by the glucosamine content (Aidoo et al., 1981) determined by spectrometry at 530 nm using a glucosamine standard curve (Sigma, USA) in water (1–15 mg/mL). To convert the glucosamine content into fungal biomass, a conversion factor of 0.057 $mg_{glucosamine}/mg_{dry}$ yeast was used, obtained from a linear regression (r = 0.99) of glucosamine versus fungal dry mass (obtained in solid state growth conditions). The fungal biomass generated during fermentation was calculated according to Eq. (1):

$$\text{Biomass}(mg/g) = (GF - GF_0)/0.057 \quad (1)$$

where: GF = content of the glucosamine in fermented bran, GF_0 = - content of the glucosamine in unfermented bran (time zero).

2.6. Protein Gain

The total nitrogen concentration in the fermented products was determined by micro-Kjeldahl (AOAC, 2000), and the protein gain after fermentation was calculated according to Eq. (2), taking into account the correction factors of 5.7 for rice bran and of 6.25 for fermented biomass calculations.

$$PG(\%) = \frac{[(NF - NF_0) \times 6.25]}{(NF_0 \times 5.7)} \times 100 \quad (2)$$

where: PG = percentage protein gain on dry basis; NF = nitrogen content in fermented bran, NF_0 = nitrogen content in unfermented bran.

2.7. Phenolic content determination

Fermented rice bran samples (5 g) were subjected to agitation for 2 h with 40 mL of methanol in an orbital shaker (150 rpm) at room temperature (Souza et al., 2010). The agitation was stopped for 15 min, then continued for 1 h after the addition of 10 mL of methanol. The extract was filtered through filter paper (Whatman No 4) into a separating funnel and washed three times with 10 mL of hexane and subjected to clarification by adding 10 ml of 0.1 M barium hydroxide and 10 ml of zinc sulfate 5% (p/v). After 20 min, the extract was filtered through filter paper (Whatman No 4) into a volumetric flask and the volume was adjusted to 100 mL with methanol. Aliquots of 1 mL of the phenolic extract mixed 4.5 mL of an alkaline solution (Na_2CO_3 4%, CuSO4.4H2O 2% and KNaC4H4O6 4%, 100:1:1), were incubated for 15 min at 40 °C. After this, 0.5 mL of Folin–Ciocalteau (Qell, diluted 1:2) was added, left for 10 min at room temperature, and readings were performed at 750 nm using a spectrophotometer. The phenolic content in the samples was estimated using feluric acid (Sigma, Japan) as standard (2–20 μg/ml), and its content increase with the fermentation was calculated according to Eq. (3).

Table 2
Proximal composition of rice bran fractions on dry basis.

Component (g/100 g)	Fractions (mm)		
	0.18	0.28	0.39
Protein	13.2 ± 0.5[a]	13.7 ± 1.2[a]	13.2 ± 0.3[a]
Lipids	18.2 ± 0.7[b]	19.8 ± 0.5[a]	21.1 ± 0.4[a]
Fiber	4.5 ± 0.1[b]	8.3 ± 0.7[a]	8.4 ± 0.4[a]
Ash	12.0 ± 0.1[a]	11.8 ± 0.2[a]	11.1 ± 0.2[b]
Carbohydrate	52.1 ± 1.1[a]	46.5 ± 1.8[b]	46.1 ± 0.7[b]

Values are expressed as means ± sd. The values in each column with the same superscript letter are not significantly different by Tukey test ($p \leqslant 0.05$).

$$\mathrm{PCG}(\%) = \frac{(\mathrm{PCF} - \mathrm{PCF}_0)}{\mathrm{PCF}_0} \times 100 \quad (3)$$

where: PCG = percentage phenolic content gain of on dry basis; PCF = phenolic content in fermented bran; PCF_o = phenolic contend in the unfermented bran (time zero).

3. Results and discussion

3.1. Substrate characterization

The rice bran fractions obtained by sieving were classified into different average particle diameters and their proximal composition was determined (Table 2). Only the protein content was the same ($p \leqslant 0.05$) for the different size fractions. The fraction containing smaller particles (0.18 mm) had lower lipid levels and smaller fiber fractions than the others, while their ash and carbohydrates were higher. The high lipid content of larger diameter of particles was associated with germ particles that were visibly larger in these fractions. The same occurred with the fiber content, where high husk residues content was observed in the fractions of higher diameters.

The ash content, although showing a significant difference ($p \leqslant 0.05$) between the smaller and larger diameter fractions, did not vary more than 1% among the bran samples. The components that varied most with particle size were carbohydrates, likely as a consequence the grain polishing process that generates small particles from the starchy endosperm (United Nations Industrial Development Organization, 1985; Amato et al., 2002). The water content average in all fractions was around 11%. The values found are close to those reported in the literature (Oliveira et al., 2010; Pogaku et al., 2009; Feddern et al., 2007).

The bran percentage retained in each mesh used for rice bran classification is shown in Table 3. The 0.28 mm average diameter particles corresponded to the largest fraction (26.8%), with no significant difference ($p \leqslant 0.05$) with the fraction retained in the 100 mesh-size (23.8%). The fraction with a 0.39 mm average diameter was represented the lowest percentage. These three fractions were used separately as substrate in solid state fermentation to evaluate the particle size effect on the fermentation process. The fraction retained in the 35 mesh sieve was discarded due to the presence of whole grains and rice husks. Particles smaller than 0.15 mm were not used because they decreased the medium porosity below desired levels (data not shown).

The larger diameter particles had a surface area about twice that of the other fractions (Table 3), which justified the higher pore volume of these particles (5.5×10^{-3} cm3/g). However, the pore diameter of these particles was smaller than that of the 0.18 and 0.28 mm particles. Microscopic analysis (see Supplementary data 1) showed the particles had an almost planar shape, with low sphericity. The 0.39 mm particles displayed more surface irregularity than 0.18 mm, leading to a greater surface area. According to Table 3, larger particles showed a more superficial difference in relation to other fractions, which could eventually influence in SSF.

3.2. Biomass, protein and phenolic content gain

The highest biomass production was obtained at the central points of the plan, about 189 mg/g (Table 4). The largest increase in protein content (53.4%) was obtained in the third test, using

Table 3
Physical characteristics of different fractions from rice bran.

Granulometry			
Mesh retained (ty)	Diameter (mm)	$\Phi_{average}$ (mm)	% rice bran[a]
35	0.425	>0.42	18.1 ± 0.6[b]
42	0.355	0.39	12.3 ± 1.7[c]
65	0.212	0.28	26.8 ± 1.5[a]
100	0.150	0.18	23.8 ± 1.3[a]
Bottom	–	<0.15	18.6 ± 2.0[b]
Pore characterization			
$\Phi_{average}$ (mm)	Specific surface area (m^2/g)	Pore volume (cm^3/g)	Pore diameter (Å)
0.18	2.0	2.9×10^{-3}	58.3
0.28	1.5	2.6×10^{-3}	67.5
0.39	4.1	5.5×10^{-3}	54.0

$\Phi_{average}$ = average diameter.
[a] Values are expressed as means ± sd. The values in each column with the same superscript letter are not significantly different by Tukey test ($p \leqslant 0.05$).

Table 4
Biomass production, protein and phenolic content gain in fermented rice bran.

Runs	$\Phi_{average}$ (mm)	$(NH_4)_2SO_4$ g/L	Biomass (mg/g)	PG (%)	PCG (%)
1	0.18	2	122.2 ± 5.4	46.0 ± 5.4	51.8 ± 0.8
2	0.39	2	151.1 ± 10.1	34.9 ± 4.8	19.9 ± 1.5
3	0.18	8	147.2 ± 10.7	53.4 ± 5.8	65.1 ± 4.5
4	0.39	8	150.8 ± 10.4	35.7 ± 3.2	25.5 ± 2.3
5	0.28	5	189.0 ± 9.0	32.9 ± 4.9	25.3 ± 3.6
6	0.28	5	188.0 ± 13.6	35.0 ± 4.9	29.0 ± 2.7
7	0.28	5	185.4 ± 6.5	35.6 ± 0.9	25.6 ± 2.6

Where: $\Phi_{average}$ = average diameter; PG = protein gain; PCG = phenolic content gain. Values are expressed as means ± sd.

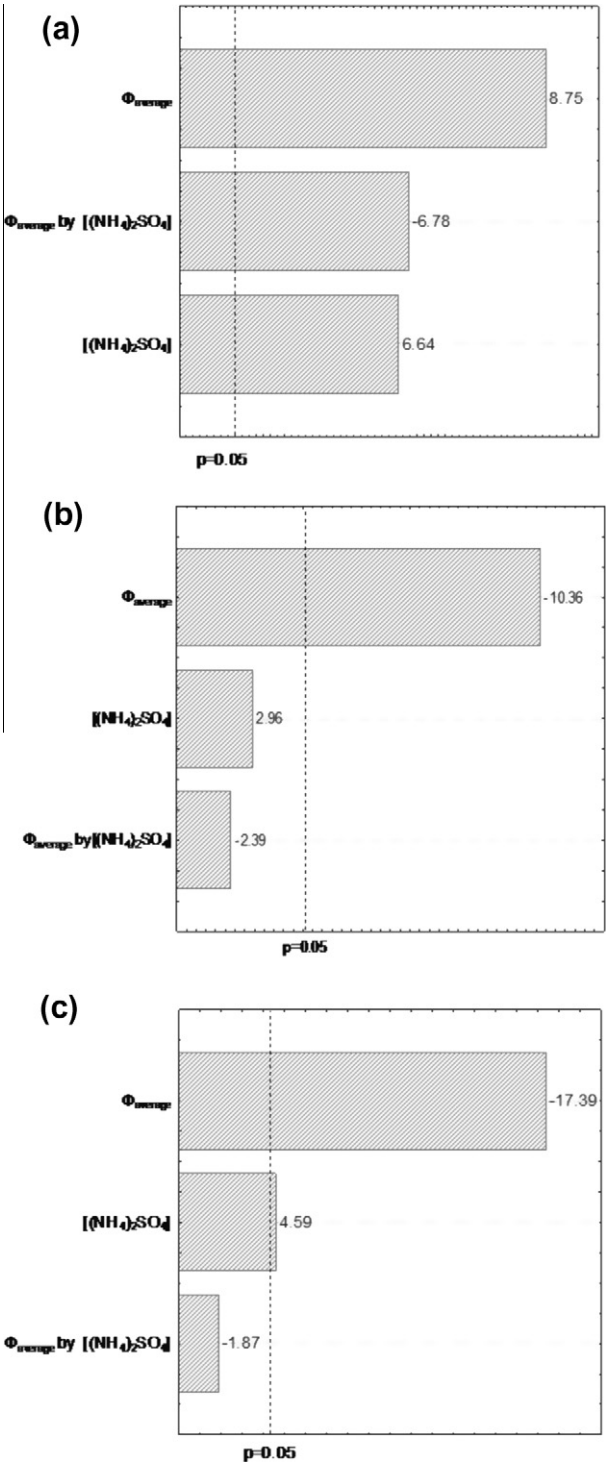

Fig. 1. Pareto charts showing the effects of the particle size ($\Phi_{average}$) and ammonium sulfate concentration ([$(NH4)_2SO_4$]) in biomass (a), protein gain (b) and phenolic content gain.

smaller-sized particles and a higher ammonium sulfate concentration. For the phenolic content, the best condition was the same as that obtained for the highest protein content (65.1%).

The substrate particle size was the parameter that most influenced rice bran fermentation by *R. oryzae* (Fig. 1). To both, the biomass and the protein and the phenolic content gain, this variable had a significant effect ($p \leqslant 0.05$). With respect to ammonium sulfate concentrations, has been reported that exogenous nitrogen sources significantly increase biomass and protein amount (Rudravaram et al., 2006; Wang et al., 2005). This study showed a significant effect ($p \leqslant 0.05$) of ammonium sulfate concentration on biomass production and phenolic content gain and no significant effect on protein production (Fig. 1).

Fig. 2a shows that the highest biomass level as determined by glucosamine content, 17 $mg_{glucosamine}/g_{dry\ weight}$, was obtained by using 0.28 mm average diameter particles and 5 g/L ammonium sulfate in the nutrient solution. It should be noted that glucosamine content increases in old cultivars, mainly due to the biomass proportion represented by the chitin-containing cell wall (Sparringa and Owens, 1999). Thus, the conversion factor between biomass and glucosamine varies with age for many fungi. In this study, it was used a conversion factor derived from the linear regression between dry fungal cell mass, obtained by the overculture system (Ikasari and Mitchell, 2000) at a time corresponding to the late exponential growth phase of the microorganism (2 days), and the glucosamine content.

The larger particles resulted in a positive effect on biomass production (Fig. 1a). According to previous studies (Membrillo et al., 2011, 2008; Valera et al., 2005), substrate particle size can provide two types of opposite effects on solid-state fermentation. Substrates with smaller particles provide a larger contact area between the fungus and the substrate, favoring its growth whereas very small particles are more susceptible to compaction and the formation of agglomerates, resulting in decreased oxygen transfer, affecting respiration and fungal development. The larger particles in the current study had a higher porosity than the smaller particles (Table 3); however, in contrast to findings by others (Membrillo et al., 2011; Valera et al., 2005), particles with a larger diameter also presented a higher surface area (Table 3). Despite the larger surface area and pore volume, 0.39 mm particles had pores that did not exceed 7 nm. Since, the diameter of the hyphae *Rizhopus* is usually greater than 10 nm (Alexopoulos et al., 1996), the fungus would likely grow on the outside of the particles.

The higher porosity of the 0.39 mm particles benefited aeration and dissipation of gases and heat produced during microbial growth. During the filamentous fungi growth on solid substrates, it is generally accepted that there is a limitation in oxygen supply to the cells that are in close contact with the substrate or that penetrate the substrate. For example, in the case of the fungus *Aspergillus oryzae*, oxygen is mainly (70%) absorbed by the aerial hyphae (Asha-Augustine et al., 2006).

A significant influence on biomass production was observed by increasing the ammonium sulfate concentration (Fig. 1a). Anapuma and Ravindra (2001), fermenting defatted rice bran with *Aspergillus niger*, also found that adding an additional nitrogen source resulted in a higher biomass yield.

3.3. Protein enrichment

Several fungal species have been studied aiming at single-cell protein production in a variety of substrates (Oduguwa et al., 2008) and an increase in protein content has been observed when using smaller rice bran particles. In the present study, the increase in particle size caused a negative effect on protein gain (Fig. 1b). Similar results were reported by Membrillo et al. (2011), who found that the physical characteristics of sugar cane bagasse fibers strongly influence solid-state fermentation. The authors found that protein content and biomass were not always proportional. Valera et al. (2005) investigated the effect of wheat bran fractions on solid state fermentation with *Aspergillus flavipes* for the production of lovastatin and found that smaller particles produced the best results. Membrillo et al. (2008) also found more pronounced protein enrichment during *Pleurotus ostreatus* fermentation of sugar cane bagasse with a in smaller particle size.

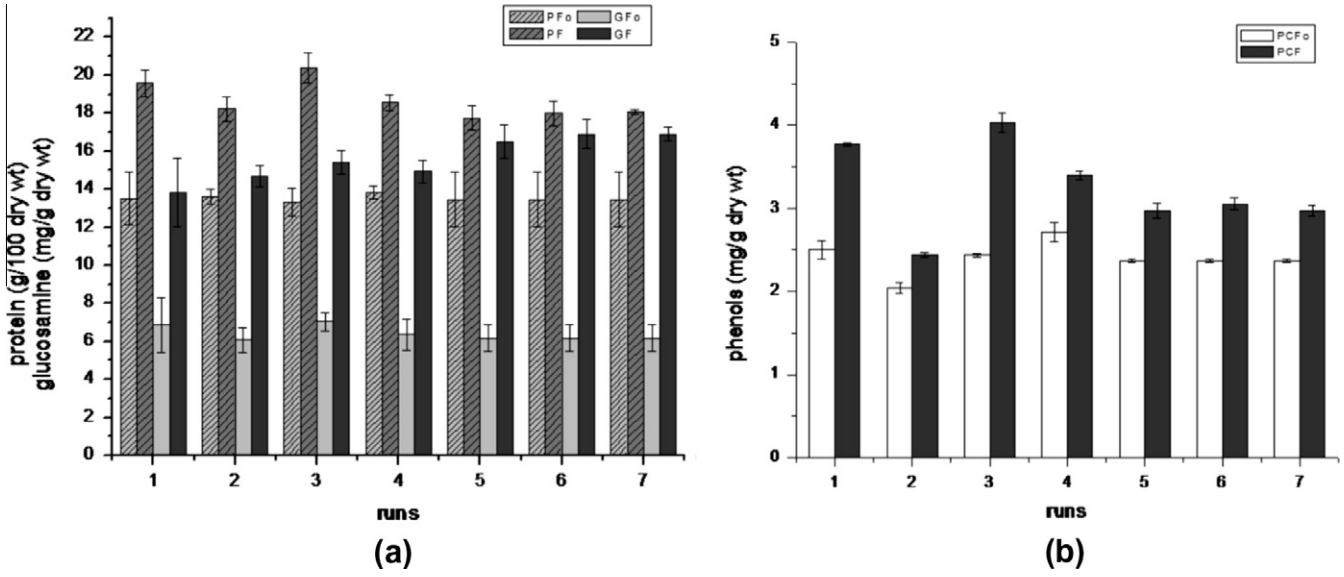

Fig. 2. Glucosamine and protein content (a) and phenols (b) in the different experiments of fermented rice bran and unfermented rice bran. Where: PF_0 = protein in unfermented rice bran; PF = protein in fermented rice bran; GF_0 = glucosamine in unfermented rice bran; GF = glucosamine in fermented rice bran; PCF_0 = phenolic content in unfermented rice bran; PCF = phenolic content in fermented rice bran. Error bars represent standard error with respect to repeated measurements (n = 3).

A significant protein increase can be observed in all the tests performed in the experiment (Fig. 2a), and the third run showed the highest protein content (20.4 g/100 $g_{dry\ wt}$). These results were higher than those found by Pogaku et al. (2009), who studied different fungal strains (*A. oryzae*, *A. niger* and *Trichoderma viride*) cultured in defatted rice bran.

The increase of ammonium sulfate concentration, as well as its interaction with the particle size, did not influence the protein level (Fig. 1b). These results indicate that the substrate used was supplying the necessary nitrogen for the production of fungal protein.

3.4. Phenolic content

The phenolic content increased after 96 h of fermentation. Like protein content and biomass production, the content of phenolic compounds was strongly influenced by the substrate particle size. The ammonium sulfate concentration in fermentation medium also had a significant influence on the phenolic content at the end of the fermentation (Fig. 1c).

Rice phenolic compounds include benzoic and hydroxycinnamic acids, mainly ferulic acid and diferulates, whose concentrations increase from the endosperm to aleurone (Zhou et al., 2004). The most soluble compounds are compartmentalized within the cell vacuoles, and they are in free form or conjugated, whereas insoluble phenols are linked to cell wall structures, esterified with arabinose and galactose residues of pectic or hemicellulosic components (Mira et al., 2009; Adom and Liu, 2002).

The unfermented rice bran showed a mean of 2.4 $mg_{feluric\ acid}/g_{dry\ wt}$ in its phenolic content. Similar values were found by Busat and Siriamornpun (2010), who obtained a total phenolic content ranging from 2.5 to 2.7 mg. Solid-state fermentation has been used to increase the phenolic content in certain food products, thereby enhancing their antioxidant activity (Martins et al., 2011). After fermentation, the free phenolic content reached 4 $mg_{feluric\ acid}/g_{dry\ wt}$ (Fig. 2b), an increase of 65%.

The phenolic compounds could have been produced by cleavage of compounds complexed with lignin (Martins et al., 2011; Sánchez, 2009). Ju et al. (2009) reported that an increase in p-coumaric content was produced possibly due to enzymatic hydrolysis of hydroxycinnamic ester (fertaric) during fermentation. These researchers also reported the oxidation of feluric acid to vanillic acid during fermentation.

Traditionally, the majority of rice bran production is used in fertilizer, animal feed and by the cosmetics industry, but many studies have been conducted to better assess its potential for human consumption (Furlong et al., 2007; Silveira and Furlong, 2007). Rice bran classification into different particle sizes allows the allocation of various sized fractions for different purposes, depending on the product desired. In this study the smaller particles provided the best protein and phenolic content after fermentation, which could eventually be used directly in food product formulation, such as protein concentrates or isolates (Membrillo et al., 2011; Oduguwa et al., 2008), or in the development of new products, such as edible films. In the latter case, both could be used to form a coating matrix; in particular, phenolic compounds could be used in virtue of their antifungal and antioxidant capacity (Souza et al., 2010; Busat and Siriamornpun, 2010) that could decelerate browning reactions and prolong shelf life of minimally processed plant tissues.

4. Conclusion

Rice bran particle size strongly influenced solid-state fermentation with the fungus *R. oryzae*. Smaller particles favored protein and phenolic content production, whereas larger particles benefited fungal biomass production. The addition of ammonium sulfate mainly influenced biomass production. Thus, substrate classification can be used as selection criterion to utilization in SSF, targeting the recovery of protein and phenolic compounds. The use of agro-products as raw material for compounds extraction is interesting as it enables better use of their potential in new food products formulation and/or mechanisms to make best use of the product.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.07.081.

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