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Solid-State Fermentation for the Enrichment and Extraction of Proteins and Antioxidant Compounds in Rice Bran by *Rhizopus oryzae*

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ABSTRACT

The objective of this work was to evaluate the solid-state fermentation with Rhizopus oryzae CCT 7560 of rice bran for the enrichment of proteins and the antioxidant compounds in the fermented biomass. Fermentation was performed in tray bioreactors at 30ºC for 120 h. Protein extraction was done at alkaline pH, followed by precipitation with acetone. Phenolic compounds were extracted with cold methanol. The maximum protein was recovered from after 120 h (26.6%). The content of total phenolic compounds increased during the fermentation and was maximum after 96 h, which inhibited the DPPH radical by 87%. The promising characteristics of the protein and phenolic extracts of the biomass suggested the application in the coating composition for vegetal tissues preservation.

Key words: Protein extract, Phenolic extract, Fermentation

INTRODUCTION

The availability of nutrients is one of the most frequent criticism about the use of cereal bran in the diets, because in the outer layers of the grains, the proteins and other micronutrients are strongly linked to cellulose, hemicellulose and to some minerals that hamper the whole use of nutrients in the digestive processes of monogastric animals (Zdradeck 2001). The use of microorganisms to change several substrates during their metabolic activity is among the preferred ways to increase the availability of nutrients in the raw materials and rejects (Pelizer et al. 2007). Solid-state fermentation (SSF) has been adopted by the biotechnological industry due the potential for the production of secondary metabolites to the ration, fuel, food, chemical and pharmaceutical industries, and to aggregate value to non or sub-used residues (Singhania et al. 2009).

SSF presents some advantages in relation to submerged fermentation, such as it requires less space for fermenters and is proper for small effluent quantities; it uses simple substrates and at low cost, without the need of strict control of standards during the process (Pandey et al. 2000). Several raw materials, mainly agro industrial co-products, can be applied in the SSF, and the choice depends on the final product, being primarily used cellulose, hemicellulose and lignin from vegetal biomass; wheat bran and wheat straw; rice bran among others.

According to United State Department of Agriculture (USDA), the world production of rice in 2011/2012 would be 456.3 million tons (USDA 2011), generating large quantity of agro-industrial

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co-products, including rice bran, which possesses unfavorable sensory character, such as odor and color, which limit its use. However, it has a high content of carbohydrates (49.5%), proteins (14%) and lipids (22%), which show its potential for alternative application. One of the ways to employ these by-products could be to use them as substrate in bioprocesses and change their properties by nutritional enrichment. Microbial fermentation could result an increase in the availability of functional compounds (Kester and Fennema 1986). According to Oliveria et al. (2010), after 96 h of rice bran fermentation with *Rhizopus oryzae*, there was increase in the protein content (43%), and also an availability of phenolic compounds with high antioxidant activity from the breaking down of lignin in the substrate cell wall.
The objective of this work was to evaluate the effect of fermentation with *Rhizopus oryzae* CCT 7560 on the composition of rice bran and also to establish the best way to recover the proteins and the antioxidant compounds from the fermented biomass, aiming its application for food supply.

MATERIAL AND METHODS

Rice Bran

Whole rice bran was supplied by IRGOVEL (Indústria Riograndense de Óleos Vegetais Ltda), located in Rio Grande do Sul, Brazil. It was stored at -10°C until use. It has particles between 0.35 and 0.60 mm.

Solid-State Fermentation (SSF)

SSF was performed in tray bioreactors (29 x 17 x 5.5 cm^3,). The rice bran (100g) was placed in bioreactor in the form of a fine layer of ~ 2 cm and autoclaved. After the substrate homogenization with 45 mL of the nutrient solution (KH_2PO_4 2g/L, $MgSO_4$ 1g/L, NH_2CONH_2 1.8g/L in HCl 0.4N), humidity was corrected to 50% with sterile water, followed by inoculation with a suspension of spores of *Rhizopus oryzae* CCT 7560 containing 4.0 x 10^6 spores.g $medium^{-1}$ (Oliveira et al. 2010). The bioreactors were covered with sterile gauze to allow ventilation and the incubated at 30ºC. Samples were taken out in the beginning of the process and at every 24 h during five days.

Physicochemical Characterization

The AOAC methods (2000) were applied to determine the proximate composition of the unfermented and fermented bran. Humidity and ash were assayed by the drying at 105°C (nr. 935.29) and incinerating at 550°C (nr. 923.03), respectively. The lipids were extracted with petroleum ether (nr. 920.85) and the proteins were determined with determining the total nitrogen by of micro-Kjeldahl method (nr. 920.87), applying 6.25 as conversion factor for protein. Fibers were analysed by the gravimetric determination of the residue of acid and basic digestion of the samples (Cientec 1991).

Protein Recovery

For protein recovery, rice bran and fermented bran were primarily defatted with petroleum ether in the proportion 1:7 (w/v) under agitation in orbital shaker for 1h with three repetitions. Protein extraction was performed in alkaline medium (pH 9.5), adding NaOH 0.02 M in the proportion 1:7 (w/v) and further homogenization in orbital shaker for 30 min. Solution was filtered with glass wool and centrifuged at 4ºC and 2240 x g for 20 min. The supernatant was then divided in two aliquots. In the first one, the pH of the was adjusted to 4.5 (isoeletric point) with HCl 1 M (Adebiyi et al. 2008) and the precipitate was dissolved in NaOH (0.02 M). The protein fractions were quantified by Lowry (1951) method using an albumin curve. The protein from the second aliquot was precipitated with acetone in a ratio 1:3 (v/v). The protein content of the supernatant and of the dissolved precipitate in NaOH (0.02 M) was quantified by Lowry (1951) method and the yields were compared using Equations 1 and 2.

$$R_{sol.} = \frac{\text{mg of protein}_{soluble}}{\text{mg of protein}_{bran}} x100 \quad (1)$$

$$R_{ppt.} = \frac{\text{mg of protein}_{precipitat\,e}}{\text{mg of protein}_{solúble}} x100 \quad (2)$$

Where:
R_{sol}: yield of solubilization
R_{ppt}: yield of precipitation

Phenolic Compounds Recovery

The total phenolic compounds (TPC) were cold extracted from the rice bran and fermented bran with methanol in the proportion of 1:5 (w/v), under orbital agitation at 160 rpm for 3h at 25°C, followed by the partition with hexane. Methanolic

extracts were concentrated in rotary evaporator and re-suspended in 70 mL of water, filtered and clarified with 15 mL of barium hydroxide (0.1 M) and 15 mL of zinc sulfate (5 %) before quantification. The quantification was done by UV/VIS spectrometer using Folin-Ciocalteau reagent, which consisted of taking 500 µL aliquots of each extract, shaking with 500 µL of distilled water, addition of 4.5 mL of solution (Na_2CO_3 2 %, $CuSO_4$ 2 % and double tartrate and of sodium and potassium 4 %, in the proportions 100:1:1) for 1 min, and placing in water bath at 40°C for 15 min. The mixtures were agitated in ultrasound bath with 500 µL Folin-Ciocalteau reagent diluted 1:2 with distilled water. After 10 min, the solutions absorbance was measured at 750 nm, interpolating the values of absorbance of the samples against a ferulic acid analytical curve (1.7 to 12.2 µg/mL). The total phenolic compounds of the samples were expressed as $mg_{TPC}.g_{db}^{-1}$ (Sousa et al. 2007).

Antioxidant Activity of the Phenolic Extracts

The antioxidant activity of the phenolic extracts was determined by the method of the DDPH (1,1 diphenyl-2-picryhydrazyl) free radical scavenger. The stock solution of DPPH was prepared weighing 0.01 g and transferring to a 50 mL flask, filling up the volume with methanol. The extract from the biomass was diluted in the proportion of 1:10, and 0.2 mL of the extracts were added to 3.8 mL of DPPH solution in methanol (5 x 10^{-5}M); after hand agitation, tubes were left at rest at dark. At the end of 15, 30, 45 and 60 minutes, the absorbance was measured at 515 nm and the ability of scavenging the radical was expressed as inhibition percentage according to the Equation 3 (Souza et al. 2009).

$$\% \text{ inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}}{\text{Abs}_{\text{control}}} \quad (3)$$

Where:

$Abs_{control}$: absorbance of the DPPH solution without antioxidant extract

RESULTS AND DISCUSSION

Table 1 presents the variation in rice bran composition during the fermentation with *R. oryzae* CCT 7560

The variation in the contents of ashes in un-fermented and fermented biomass was similar to that reported by Feddern et al. (2007), which after 6 h of fermentation with *Saccharomyces cerevisiae* obtained 10.5% of ashes. In this work, the maximum ash content was after 72 h fermentation (14.7%). The lipid content of rice bran was 15.2% and after 48 h of fermentation, there was a decrease due its use for the mycelial synthesis. The same behavior was mentioned by Oliveira et al. (2010) using rice bran as substrate during SSF with *R. oryzae* and by Silveira and Badiale-Furlong (2007), using defatted rice bran and wheat bran as substrates for *Rhizopus sp* and *Aspergillus oryzae* cultivation. According to Griffin (1993), nutrients in the substrate are used for the fungus cell wall production; consequently there is a decrease in polysaccharides in the substrate. This was shown by the increase in fibers content in the biomass and the maximum was after 96 h, showing 58% compared to the unfermented rice bran.

Table 1 - Proximate composition in dry basis of the bran and of the biomasses at distinct fermentation periods.

Sample	Moisture (%)	Ash (%)	Lipids (%)	Fiber (%)	Protein (%)
Bran	12.0 ± 0.1^{b}	9.4 ± 0.1^{d}	15.2 ± 0.1^{e}	8.1 ± 0.4^{e}	14.8 ± 0.2^{a}
FB 0 h	48.0 ± 0.2^{d}	9.5 ± 1.5^{d}	15.4 ± 0.1^{e}	7.2 ± 0.1^{e}	15.7 ± 0.4^{a}
FB 24 h	49.8 ± 2.8^{a}	11.4 ± 0.2^{cd}	15.7 ± 0.0^{e}	9.5 ± 0.1^{b}	19.0 ± 1.0^{c}
FB 48 h	39.5 ± 0.1^{c}	12.1 ± 1.5^{bc}	11.7 ± 0.0^{a}	10.5 ± 0.1^{d}	18.8 ± 0.2^{c}
FB 72 h	40.1 ± 0.0^{c}	14.7 ± 2.2^{a}	10.9 ± 0.1^{b}	11.2 ± 0.0^{cd}	21.6 ± 1.2^{b}
FB 96 h	47.8 ± 0.3^{d}	13.7 ± 0.0^{ac}	8.7 ± 0.5^{c}	12.8 ± 0.3^{a}	21.3 ± 0.5^{bc}
FB 120 h	43.4 ± 0.3^{d}	13.9 ± 0.0^{ab}	7.4 ± 0.4^{d}	11.7 ± 0.7^{c}	22.1 ± 1.0^{b}

FB 0: fermented bran at 0 h; FB 24: fermented bran at 24 h; FB 48: fermented bran at 48 h; FB 72: fermented bran at 72 h; FB 96: fermented bran at 96 h; FB 120: fermented bran at 120 h. Mean ± standard deviation. Different letters indicate significant difference ($p < 0.05$).* dry basis.

The changes from the carbohydrates into proteins by fungal species have been explored in several areas. The agro-industrial raw material bioconversion is a potential process for the

proteins production, mainly for the nutritional supplementation in animal feed. Some fungi have been reported to contribute to protein contents increase in agro-industrial raw materials, such as *Aspergillus niger* AS 101 in corn cobs, *Trichoderma reesei* in beet-pulp and white rot fungi in sugarcane bagasse (Anupama and Ravindra 2000). In this work, the fungal biomass development brought an increase in the protein content, which was maximum after 120 h (22.1%). This increase was 30% in the first 24 h, which remained constant till 48 h, which further increased, reaching 49% at 120 h. This was higher than found by Oliveira et al. (2010), who applied the same fermentation conditions and achieved a 42.8% protein after 96 h. Silveira and Badiale-Furlong (2007) evaluated the rice bran fermentation with *Rhizopus* sp and *Aspergillus oryzae* and also obtained an increase in the protein content higher than the one presented in this work (69%). This difference could be related to the fact that the authors used defatted rice bran as substrate. The relevant increase in protein content and in the phenolic compounds antioxidant activity during fermentation have also been reported by Silveira and Badiale-Furlong (2007) and Oliveira et al. (2010), guiding the interest in extracting both the components to apply in food formulations.

Table 2 presents the protein solubilization and precipitation percentage from bran and fermented bran in the isoelectric point and with acetone (1:3).

Table 2 – Yields in percentages of solubilization and precipitation of proteins from the fermented bran.

Samples	Solubilization (%)	Precipitation (%)	
		I.P	Acetone
Bran	80.5 ± 10.6^{a}	24.7 ± 3.9^{b}	45.4 ± 6.0^{a}
FB 0 h	23.7 ± 0.6^{c}	15.6 ± 0.4^{c}	16.1 ± 0.4^{cd}
FB 24 h	20.3 ± 0.5^{c}	37.0 ± 1.7^{a}	10.1 ± 0.2^{c}
FB 48 h	54.0 ± 0.7^{d}	1.4 ± 0.1^{d}	16.8 ± 0.2^{d}
FB 72 h	53.5 ± 1.8^{d}	1.4 ± 0.1^{d}	18.2 ± 0.6^{d}
FB 96 h	66.4 ± 1.8^{b}	2.0 ± 0.1^{d}	20.1 ± 0.5^{d}
FB 120 h	62.5 ± 1.3^{bd}	1.6 ± 0.1^{d}	26.6 ± 0.6^{b}

FB 0 h: fermented bran at 0 h; FB 24 h: fermented bran at 24 h; FB 48 h: fermented bran at 48 h; FB 72 h: fermented bran at 72 h; FB 96 h: fermented bran at 96 h; FB 120 h: fermented bran at 120 h. I.P- isoeletric point. Mean ± standard deviation. Different letters indicate significant difference ($p < 0.05$).

In an alkaline medium, 80.5% of the rice bran proteins were solubilized and an increase of 180% during the fermentation was obtained, reaching up the maximum 66.4% after 96 h, corresponding to 499 $mg_{protein}.g_{db}^{-1}$. The difference between the rice bran and fermented bran at 0 h could be explained due the presence of salt solution in fermented bran, causing a decrease in protein extraction. There was a decrease in the precipitation of proteins at the isoelectric point (pH 4.5) throughout the fermentation, which suggested that the proteins from the produced biomass were more soluble in nature, possibly due to the reduction in the rice bran protein chain or fungal proteins production.

The employment of acetone was more efficient for protein precipitation from the fermented bran, increasing in up to 16 times the yield during precipitation, and the biomass from 120 h had the highest yield (26.6 %). Therefore, for that protein source, the precipitation with acetone was more promising for protein recovery from the fermented biomass. Besides the highest yield, the acetone applied can be easily removed from the protein fraction by using mild temperatures. The biomass obtained at 120 h had higher yield in precipitation, but at 96 h, there was a higher yield of solubilized protein, thus the choice of fermentation time will depend on the kind of application to be given to the protein.

The results from the phenolic compounds quantification as well as the ability of DPPH radical scavenger exhibited by the extracts are shown in Table 3 and in Figure 1, respectively.

Phenolic acids are hydorxyanalogues of the benzoic and cinnamic acids. The hydroxycinnamic acids are more common than the hydroxybenzoic acids, which are mainly originated from p-coumaric, caffeic, ferulic and synaptic acids. Several of these phenolic acids are also found in the products from the grain and ferulic acid is the most abundant and concentrated in the outer part of the grain. They have been considered to offer potential protection against cancer and heart

diseases (Mattila et al. 2005), which are determined by their antioxidant power. In cereals, phenolic compounds are bound or linked with sugar, fatty acids or proteins (Kahkonen et al. 1999) and can be available during fermentation.

Table 3 - Content of total phenolic compounds during fermentation.

Samples	TPC ($mg_{TPC}. g_{db}^{-1}$)
Bran	$0,3\pm0,0^{c}$
FB 0 h	$0,4\pm0,0^{b}$
FB 24 h	$1,5\pm0,0^{e}$
FB 48 h	$1,3\pm0,1^{d}$
FB 72 h	$1,1\pm0,0^{a}$
FB 96 h	$1,3\pm0,0^{de}$
FB 120 h	$1,4\pm0,0^{e}$

FB 0 h: fermented bran at 0 h; FB 24 h: fermented bran at 24 h; FB 48 h: fermented bran at 48 h; FB 72 h: fermented bran at 72 h; FB 96 h: fermented bran at 96 h; FB 120 h: fermented bran at 120 h. TPC- total phenolic compounds. Mean ± standard deviation. db- dry basis. Different letters indicate significant difference ($p < 0.05$)

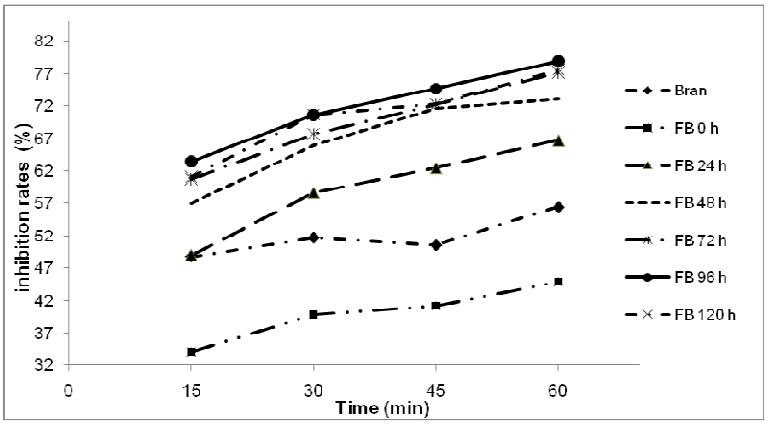

Figure 1- Inhibition percentage of DPPH done by the phenolic compounds both from the bran and from the biomasses generated at different fermentation intervals.

In rice bran, the content of phenolic compounds was 0.3 $mg_{TPC}. g_{db}^{-1}$, which was higher than the one found by Oliveira et al.(2005) in the rice (0.04 $mg_{TPC}. g_{db}^{-1}$). This could be related to the protecting function assigned to compounds justifying the abundance in the outer parts of the vegetal tissues (Rassoli and Abyaneh 2004; Vázquez 2001). A relevant increase in TPC content was observed during the fermentation, being the highest production after 24 h (1.5 $mg_{TPC}. g_{db}^{-1}$). It could be inferred these compounds were formed during the fermentation process by the breaking down of the lignin present in the substrate cell wall.

The study on the ability to scavenger DPPH free radical showed that all the extracts acted as scavengers of the radicals, but due to the complexity of the lignin structure, phenolics in the extracts biomass presented distinct potentials to donate electrons at each interval of collection. Coumaric and ferulic acid were the prominent compounds, which have been considered as the most active antioxidants due to the double link in the molecule, taking part in the stability of the radical by resonance shift of the unpaired electron (Wanasundara 1994). Figure 2 proved this behavior, as despite of the biomass fermented for 24 h showed the highest content of phenolic compounds, the biomass extract at 96 and 120 h presented the largest antioxidant activity. The ability of scavenging radicals from distinct extracts showed a linear and increasing relation with the time of reaction, because the inhibition percentage of them was higher in 60 minutes. The most constant ability of oxidation inhibition along the 60 minutes of reaction was observed in the extract obtained from the biomass produced after 96 h. In this case, the behavior, similar to the proteins, showed that this duration could be appropriate to the type of application intended in the food formulation.

CONCLUSION

The lipid content of rice bran was reduced to 49% during the fermentation and an increase of 48, 43 and 49% in the content of ashes, fibers and proteins respectively were observed. The proteins recovery from the fermented bran, employing the alkaline solubilization and precipitation with acetone was 16 times higher than precipitation at the isoelectric point. During the fermentation, antioxidant compounds were also produced. The biomass after 96 h fermentation was the most promising protein and antioxidant source and could be employed in food formulations, especially if the intention was to prevent oxidative processes at a larger interval.

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