Quantification of phenolic compounds changes by *Aspergillus oryzae* on rice bran fermentation

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Abstract

This research highlights the quantification of phenolic compounds of rice by-products (namely rice bran), by solid-state fermentation (SSF) with *Aspergillus oryzae* through assessment of its total phenolic content (TPC) and phenolic acids. The analyses were carried out by comparing fermented extracts of rice bran extracted using water extract according to their fermentation time and uninoculated samples. Alpha-amylase and beta-glucosidase assays were used to identify the differences in their expression levels during fermentation. The results show that fermentation with *A. oryzae* improved the extractable TPC of rice bran by up to 3.8-fold higher in comparison to its unfermented counterpart. A total of three types of phenolic acids (protocatechuic, coumaric and ferulic acids) were detected in unfermented rice bran by HPLC/PDA. SSF with *A. oryzae* on rice bran significantly influenced the free phenolic content i.e. the five phenolic acids p-coumaric, protocatechuic, ferulic, caffeic and sinapic acids detected in the rice bran extracts upon fermentation. The highest concentration of coumaric, ferulic and protocatechuic acids in the fermented extracts increased by up to 3.2-fold, 52-fold and 3.2-fold, respectively, in comparison to its unfermented counterpart obtained at the initial phase of fermentation period on day 8 of fermentation. The present investigation demonstrated that SSF enhanced the rice bran by increasing the free total phenolic content as well as phenolic acids concentration with better nutritional and functional properties.

1. Introduction

Milling of paddy yields 70% of rice (endosperm) as the major product and the by-products consist of 20% rice husk, 8% rice bran, 3% broken rice and % brewer's rice. A large amount of these by-products produced by the rice processing industry, in addition to the loss of valuable materials, raises the issue of management problems, both from the economic and environmental point of view. Their utilisation in the food system is very limited despite them being a good source of secondary metabolites.

In nature, most of the phenolic compounds associated with rice bran are in insoluble forms. Phenolic compounds in insoluble form are covalently bound to cell wall components such as indigestible polysaccharides (hemicellulose and cellulose), and thus should be liberated from the cell wall in order to measure the phenolic contents. Bound forms of phenolic compounds can be released from cell wall structures by acid/alkaline hydrolysis (Nenadis *et al*., 2013), enzymatic treatment (Lei *et al*., 2017) or fermentation (Bhanja *et al.,* 2014). Solid state fermentation (SSF) has been widely used to enhance levels of phenolic compounds. It has the advantages of being environmentally friendly, higher efficiency and lower cost.

The solid-state fermentation uses fungus to increase nutrients and bioactive compounds in rice bran. The fermentation process with different species microorganisms can result in different secretion of enzymes, followed by a diversity in the liberation of phenolic compounds from the cell wall matrix of substrates. *Aspergillus oryzae* has a long historical application as cell factories in the fermented food products and food industries. It is a filamentous fungus commonly used in the fermentation process of Asian foods such as tempeh, soy sauce, miso and rice vinegar. Characteristically, this genus of fungi does not produce mycotoxins and is Generally Recognized as Safe

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(GRAS) by the Food and Drug Administration (FDA). This fungus is also used to produce various hydrolytic enzymes such as α-amylase (Francis *et al.,* 2003) and xylanase (Szendefy *et al.,* 2006).

This study focused on the application of rice by-products rice bran (RB) using bioprocesses technology (i.e. fermentation) as an alternative way to enhance total free phenolic content. The evaluation will be made on how the phenolic compounds are altered by *A. oryzae* fermentation and these changes will be assessed in comparison to the unfermented substrate. During fermentation, *A. oryzae* secretes a variety of extracellular enzymes such as α-amylase, β-glucosidase, and cellulase, leading to the liberation of the insoluble bound phenolic acids. To the best of our knowledge, no study has investigated the effect of SSF on the content of phenolic compounds and to understand the changes of phenolic acid caused by *A. oryzae* during rice bran fermentation. High-performance liquid chromatography (HPLC) with PDA detector was used to identify and quantify phenolic acids changes. The involvement of the enzymatic activities such as α-amylase and β-glucosidase in the changes of phenolic content was also investigated during fermentation.

2. Materials and methods

2.1 Culture preparation

Aspergillus oryzae was obtained from the Collection of Functional Food Cultures (CFFC), MARDI, maintained on potato dextrose agar media (PDA) and kept at 4°C. The same medium was used at their sporulating stage, which was incubated for 7 days at 30°C. The spore suspension was dissolved in Tween 80 (0.01%) and adjusted to approximately 1×10^6 spore/mL in this study.

2.2 Fermentation process

Fermentation was carried out in 250 mL Erlenmeyer flask. Rice bran substrate (30 g) was added to 35 mL of distilled water, covered and sterilized at 120°C for 20 mins. The spore suspension was added into a flask, mixed well using a sterile spatula and incubated at 32°C for 18 days. For enzyme activity assays and extraction of phenolic compounds, the samples were collected every 2 days and dried at 50°C for 24 hrs.

2.3 Glucosamine activity

Dried sample (0.5 g) was hydrolysed in 6 mL of concentrated hydrochloric acid (10N) at 100°C for 16 hrs. Then, it was neutralised with natrium hydroxide (NaOH) to pH 7 and further diluted to 50 mL. Next, 1 mL of the sample was transferred into a test tube and

1 mL acetyl acetone reagent was added before being incubating at 100°C for 20 mins. After cooling down to room temperature, 6 mL of absolute ethanol and 1 mL of Ehrlich reagent were added into 60 mL of the solution. The mixture was incubated at 65°C for 10 mins and the absorbance value was determined at 530 nm.

2.4 α-Amylase activity assay

The crude extract of the sample (0.5 mL) was mixed with 0.5 mL starch solution prepared in 0.02M phosphate buffer (pH 6.9). After 3 mins of incubation, 1 mL of dinitrosalicylic acid (DNS) colour reagent was added to the mixture. The mixture was immersed in a water bath for 5 min, followed by the addition of 10 mL of distilled water. Absorbance was measured at 540 nm.

2.5 β-Glucosidase activity assay

The crude enzyme extract of the sample (0.1 mL) was mixed with 0.1 ml of 9 mM p-nitrophenol β-D glucopyranoside (pNPG) and 0.8 mL of 200 mM sodium acetate buffer (pH 4.6). The mixture was incubated at 50°C for 15 min. Then, the reaction was stopped by adding 1 mL of 0.1M NaOH. Absorbance was measured at 400 nm using a 4-nitrophenol as the standard curve.

2.6 Total reducing sugar assay

Fermented samples were centrifuged at 10,000 rpm, 30°C for 5 mins. A total of 1 mL of the supernatant was aliquoted and mixed with 1 mL DNS and 2 drops of NaOH was added. The mixture was incubated at 90°C in a water bath for 15 mins. The reducing sugar was measured by monitoring absorbance at 540 nm. A standard (glucose) and blank were prepared in the same way as the analysed samples.

2.7 Extraction of phenolic compounds

The extraction of TPC and phenolic acids was carried out using distilled water. The sample and water were mixed at a ratio of 1 g: 5 mL in an incubator shaker at 150 rpm, 30°C for 2 h. The mixture was centrifuged at 10,000 rpm for 20 mins.

2.8 Detection and quantification of phenolic acids using HPLC/PDA

2.8.1 Standard and quantification

The standards with different concentrations were prepared and filtered using 0.2 μm nylon membrane filter. Individual standards of 12 types of phenolic acids (gallic, protocatechuic, 4-hydroxybenzoic, 2,5-dihydroxybenzoic, vanilic, syringic, benzoic, ferulic, caffeic, cinnamic, salycylic and p-coumaric acids) were used. The retention time for each individual standard was

compared with the retention time of the mixed standard solutions for identification purposes. The calibration standard was prepared by injecting different concentrations of mixed standard solutions performed in serial dilution. To determine the contents of the phenolic acids in unfermented and fermented rice bran, the mixed standard solution stock solution was analysed together with the samples. The quantifications of phenolic acids were performed by generating calibration curves, where X = concentration of each standard expressed as microgram per mL samples for phenolic acids; Y = measured peak area of the chromatogram. All the samples were analysed in triplicates.

2.8.2 Chromatographic analysis of phenolic acids

For the identification and quantification of phenolic acids, a reversed phase (4.6 x 100 mm, 3.5μm) column was used and the detector was set at ʎ= 280 nm, and ʎ= 306 nm. The separation of phenolic acid was made in gradient condition at 30°C, using methanol and acid water (0.1% acetic acid) as the mobile phase at a flow rate of 0.7mL/min.

3. Results and discussion

Table 1 shows the rice bran chemical composition before and after the fermentation process by *A. oryzae*. Chemical composition analysis showed that rice bran is particularly rich in carbohydrate. The growth of *A. oryzae* on rice bran can change its chemical composition by the production of enzymes to obtain nutrients. This is an enzyme that breaks down carbohydrate into simple sugar for utilisation by the growth fungus. *A. oryzae* growth caused a decrease in the carbohydrate content and an increase in protein, fat, crude fibre and ash contents. Amylase has been derived from several fungi such as *Aspergillus* and *Rhizopus* species (Pandey *et al*., 2005). The degradation of carbohydrate after fermentation and the increase in total reducing sugar during fermentation could be an indicator of *A. oryzae* growth.

Determination of fungal growth in SSF can be assessed through biochemical methods such as measuring the glucosamine, ergosterol and total sugar
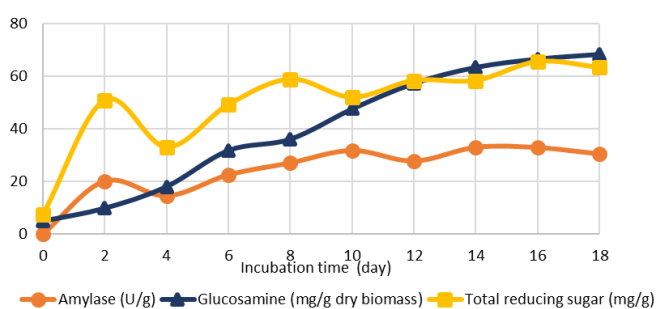
Table 1. Chemical composition of unfermented and fermented rice bran (%)

Analysis	Unfermented rice bran	Fermented rice bran with *A. oryzae*
Moisture	6.83±0.10[a]	4.24±0.77[a]
Protein	13.72±0.03[a]	19.48±0.50[a]
Fat	0.86±0.05[a]	19.78±1.11[b]
Carbohydrate	55.84±0.53[b]	32.85±0.91[a]
Crude fibre	6.43±0.46[a]	13.24±0.69[b]
Ash	7.21±0.13[a]	10.41±0.09[a]

Values followed by the same letter superscript in the same row are not significantly different from each other (p>0.05).

content (Bhargav *et al.,* 2008). The growth of the *A. oryzae* in this study was monitored by the glucosamine content and total reducing sugar. During the fermentation of rice bran by *A. oryzae,* the growth of the fungus was rapid from day 6 of inoculation and efficiently colonised the entire substrate by day 10 (Figure 1). The glucosamine content increased up to 6-fold during the growth phase in comparison to its initial fermentation. α-amylase, which is involved in the formation of simple sugar, is likely to be produced concomitantly in *A. oryzae* growth. *A. oryzae* started to produce α-amylase during the first days of fermentation and prominently after 2 days of incubation time, α-amylase activity and total reducing sugar increased by 614% of the total sugar content from 7.19 mg/g glucose initially, to 50.73 mg/g glucose. Interestingly, α-amylase activity and the total reducing sugar content remained unchanged until after 18 days of incubation. The results showed that *A. oryzae* has the ability to hydrolyse the rice bran carbohydrates to glucose for its own metabolic activity.

β-glucosidase is well known to play an important role in the mobilisation of phenolic compounds during fungal fermentation (Randhir *et al.,* 2004). The changes in total phenolic content due to mobilisation by *A.oryzae* and production of enzyme β-glucosidase on the rice bran are shown in Figure 2. The total soluble phenolic content of fermented rice bran samples increased by 289% from 3.20±0.68 mg gallic acid equivalent (GAE)/g of rice bran samples to 12.46±0.28 mg GAE/g after 10 days of incubation time. However, after 12 days of incubation, the TPC of fermented rice bran decreased to 8.91±0.41 mg GAE/g, a decrease of nearly 28%. The fluctuation in

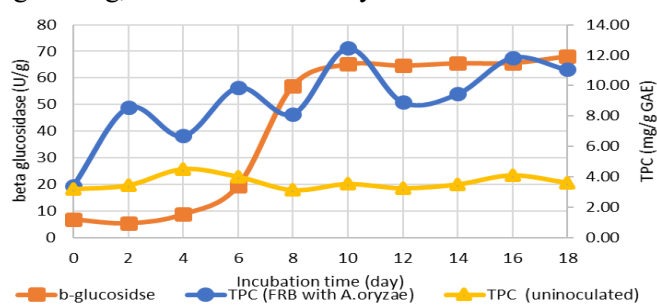Figure 1. Glucosamine activity, α-amylase activity and total reducing sugar of rice bran during fermentation

Figure 2. β-glucosidase activity and total phenolic content of uninoculated and fermented rice bran with *A. oryzae*

Table 2. Concentrations of phenolic acid in unfermented and fermented rice bran with *A. oryzae*

Phenolic acids	Protocatechuic	Caffeic	Coumaric	Ferulic	Sinapic
UNF RB	2.78±0.1[a]	nd	3.99±0.2[a]	0.62±0.1[a]	nd
FRB Day 4	8.83±3.3[b]	3.98±2.5[a]	10.23±1.5[b]	6.70±1.6[a]	5.21±1.2[a]
FRB Day 8	8.84±4.2[b]	9.77±0.3[b]	12.83±0.3[b]	32.62±3.8[b]	13.32±1.7[b]
FRB Day 10	8.08±5.3[b]	6.18±1.0[b]	12.70±0.1[b]	27.49±4.3[b]	8.45±2.6[a]
FRB Day 12	7.99±3.5[b]	2.64±0.1[a]	4.92±0.7[a]	3.02±0.5[a]	nd
FRB Day 14	8.59±2.1[b]	2.65±0.2[a]	5.57±0.1[a]	1.19±0.3[a]	nd
FRB Day 16	7.86±1.3[b]	2.58±0.5[a]	4.63±0.4[a]	2.55±0.9[a]	nd
FRB Day 18	7.60±2.3[b]	2.55±0.3[a]	4.16±0.4[a]	1.04±0.1[a]	nd

Values followed by the same letter within the same column are not significantly different from each other ($p>0.05$). nd = not detected

the TPC suggested that the glucosidase activity may be capable for catalysing the hydrolysis of β-glucosidic linkages, generating high amounts of free phenolics and phenolic polymerisation activities that may aid phenolic degradation.

This study was carried out to study the modification/ bioconversion of phenolic acids profile resulted from the SSF with the *A. oryzae* by HPLC/PDA. Phenolic acids listed in Table 2 were identified based on the retention time of standards. The performed analyses revealed that three types of phenolic acids were detected in the unfermented rice bran (coumaric protocatechuic and ferulic acids) and five types of phenolic acid were identified in fermented rice bran (coumaric, protocatechuic, ferulic, sinapic and caffeic acids). Generally, SSF with *A. oryzae* on rice bran had significantly ($p<0.05$) influenced the evolution of free phenolic acids amounts, by increasing all the phenolic acids concentration on day 8 of fermentation, in comparison to its unfermented counterpart.

It is important to mention that the highest concentration of phenolic acids for rice bran substrate were obtained at the initial phase of fermentation period, particularly for ferulic acid. The highest concentrations for coumaric, ferulic and protocatechuic acids were obtained on the 8th day of the incubation time, with the increase of up to 3.2-fold, 52-fold and 3.2-fold, respectively, in comparison to its unfermented counterpart. Nevertheless, a reduction in these concentrations was observed from the 10th to the 18th day of incubation, indicating that the fermentation period influenced the conversion of the conjugated phenolic acids.

The most significant finding of this research is the presence of caffeic and sinapic acids in all of the extracts upon fermentation, which indicates that the inter-conversion of phenolic acids has occurred (Huynh *et al.*, 2014). During the fermentation processes, enzymes are produced and simultaneously utilised for the release of phenolics from the matrix substrates, along with the production of new phenolic compounds on the rice bran by the secondary metabolism of *A. oryzae* (Bhanja *et al.*, 2014).

4. Conclusion

SSF treatment has a positive effect on the release of free phenolic compounds and the production of new phenolic acids on the rice bran substrate. Fermentation of rice bran with *A. oryzae* is a good strategy to improve the total phenolic content and phenolic concentration, as well as for enhanced functionality value of the rice bran substrate. The present study has provided useful information on the increase of free phenolic acids in rice bran through fermentation with *A. oryzae*. Further studies are necessary to optimise the fermentation process to produce a higher level of free phenolic compounds that are useful for value addition of rice bran.

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