

Antioxidant activities, tyrosinase inhibition activity and bioactive compounds content of broken rice fermented with *Amylomyces rouxii*

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Abstract

Solid state fermentation (SSF) utilizing filamentous fungus *Amylomyces rouxii* was investigated as a bio-processing strategy to enhance the bioactive properties of broken rice. Fermentation was carried out for 18 days and samples were withdrawn at 2-days interval. Established methods were deployed to assess the changes in bioactive properties and compounds content in fermented broken rice. The bioactive properties studied were total phenolic content (TPC), total flavonoid content (TFC), DPPH-radical scavenging activity and ferric-reducing antioxidant power (FRAP). Additionally, tyrosinase inhibition activity, which represents anti-pigmentation/browning property, was evaluated. Free phenolic acids and organic acids content were determined through high performance liquid chromatography (HPLC). The results showed that fermentation significantly increased the total phenolic content of broken rice from 0.03 mg GAE/g sample to 3.94 mg GAE/g sample and total flavonoid content from 0.04 to 1.71 mg QE/g sample. By the end of the fermentation, DPPH-radical scavenging of fermented broken rice was enhanced to 94.22%, compared to 9.03% in the unfermented sample. It was also observed that FRAP and tyrosinase inhibition activity of fermented broken rice were improved up to 39-fold and 50-fold, respectively. Kojic acid, a potent antioxidant and tyrosinase inhibitor, was detected in fermented broken rice, along with oxalic and ascorbic acid. Gallic, protocatechuic and 4-hydroxybenzoic acids were enhanced upon fermentation. This study manifested the positive effect of broken rice after fermentation with *A. rouxii* and thus revealed the potential of fermented broken rice as a promising natural bio-ingredients in food, cosmetics and medicinal products.

1. Introduction

Solid state fermentation (SSF) is a biotechnological process used for bio-compounds production in pharmaceutical, cosmetic, food and other industries. A wide range of substrates and microorganisms that can be utilized in SSF renders it as a versatile and diverse bio-processing technique with extensive applications (Lizardi-Jiménez and Hernández-Martínez, 2017). It is regarded as an environmentally friendly technology - for having low energy and water requirements as well as producing concentrated products (Thomas *et al.*, 2013).

Broken rice is one of the by-products of rice milling industry, and consists of white grains that have been damaged along in processing steps. It is used for livestock feeding, aquaculture as well as the food industry. Broken rice contains many types of bioactive

compounds such as flavonoids, phenolic acids, proanthocyanidins, γ -oryzanol, phytic acid and many more. Despite this, broken rice is undervalued and underutilized. Solid state fermentation can be adapted to enhance the active compounds in broken rice and ultimately increase its value and potentially expanding its usage in many types of industry.

Due to the physiological capabilities and mode of growth on solid substrate, filamentous fungi are the most important group of microorganisms for SSF (Krishna, 2007). *Aspergillus* sp. has become the model microorganism for SSF since the successful production of citric acids by *Aspergillus niger* (Hölker *et al.*, 2004). Many fungi species have been utilized in SSF to produce a variety of products such as *Rhizopus oligosporus* (Ul-Haq *et al.*, 2002), *Aspergillus terreus* (Elinbaum *et al.*, 2002), *Fusarium oxysporum* (Panagiotou *et al.*, 2003)

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and *Rhizopus oryzae* (Christen et al., 2000). Solid state fermentation with fungi is an effective process to elevate the concentration of bioactive compounds, such as phenolic compounds (Lee, Hung and Chou, 2008). In this context, the present investigation was undertaken to study the effect of fermentation by the fungi *Amylomyces rouxii* on the bioactive properties of broken rice.

2. Materials and methods

2.1 Solid state fermentation procedure and extraction process

Fermentation of broken rice was carried out according to modified procedures described by Shankar and Mulimani (2007), using fungal culture of *A. rouxii* F5344 from MARDI's Collection of Functional Food Cultures (CFFC). Broken rice (30 g) was added to Erlenmeyer flasks and autoclaved at 121°C for 15 mins. Approximately, 1% of fungal spores (10^6 spores/mL) and 35 mL sterilized distilled water were mixed with broken rice in each flask. Samples were incubated at 32°C for 18 days and samples were harvested at 2-days interval. All collected samples were oven dried at 50°C for 24 hrs. Unfermented broken rice was also prepared and used as comparison.

The hot water extraction process was done according to Lee, Yang and Mau (2008) with slight modifications. Each 1 g of the collected sample was mixed with 5 mL distilled water and boiled for 15 mins. The samples were centrifuged at 10,000 rpm for 15 mins and the supernatant was filtered using filter paper (Whatman No.1). Collected filtrates were kept at -20°C until further analyses. All experiments were performed in triplicates.

2.2 Determination of total phenolic content (TPC) and total flavonoid content (TFC)

The total phenolic content assay was performed according to a method by Okmen et al. (2009). An aliquot (1 mL) of fermented and unfermented broken rice extracts was combined with 5 mL of Folin-Ciocalteu reagent and 4 mL of 7.5% Na₂CO₃ and allowed to react at room temperature in a dark condition. After 2 hrs, the absorbance of each sample was read using a UV-Vis spectrophotometer at 765 nm. A calibration curve was constructed by using 0 to 200 ppm gallic acid as a standard. Results were expressed as mg/g gallic acid equivalent (GAE).

For total flavonoid content of samples, the assay was conducted as stated by Chang et al. (2002). An aliquot (1 mL) of the sample was added to 0.3 mL 5% NaNO₂ solution and incubated for 5 mins. Then, 0.3 mL of 10% AlCl₃ solution was added to the mixture, followed by

incubation for 6 mins before the addition of 2 mL 1M NaOH solution. By using distilled water, the mixture was brought to 10 mL and incubated for 15 mins. Absorbance was measured at 430 nm using UV-Vis spectrophotometer. The standard used in this assay was quercetin and the concentration used to plot calibration curve was 0 – 100 ppm. The results were expressed as mg/g quercetin equivalent (QE).

2.3 Determination of antioxidant activities (FRAP and DPPH)

The ferric-reducing antioxidant power (FRAP) assay as described by Benzie and Strain (1999) was used in this study. Fresh FRAP working solution was prepared by combining 25 mL of acetate buffer, 2.5 mL TPTZ (2,4,6-tripyridyl-s-triazine) solution and 2.5 mL FeCl₃.6H₂O solution. The mixture was warmed at 37°C prior to use. An aliquot of each sample amounting to 150 µL was mixed with 2850 µL of FRAP working solution. The mixture was allowed to react at room temperature in the dark for 30 mins. Absorbance was measured at 593 nm using a spectrophotometer. A calibration curve was prepared using FeSO₄ solution at the concentration of 0 to 2000 µM. The results were expressed as mM/g ferrous equivalent (FE).

DPPH-radical scavenging antioxidant assay was carried out according to a method as described by Thaipong et al. (2006). Freshly prepared DPPH working solution (2850 µL) was mixed with 150 µL aliquot of sample and the mixture was allowed to react in the dark for 30 mins. The absorbance of mixture at 515 nm was determined using a spectrophotometer. The following equation was used to calculate the percentage of scavenging activity:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$$

2.4 Determination of tyrosinase inhibition activity

Tyrosinase inhibition activity assay was performed to determine the anti-pigmentation or anti-browning potential of the sample. This assay was conducted according to a method as described by Alam et al. (2011). A mixture of sample (40 µL), 0.1 M phosphate buffer pH 6.8 (80 µL) and 31 U/mL mushroom tyrosinase (40 µL) was prepared in a 96-well plate. Sample solution without enzyme as well as blank solutions with and without enzyme was also prepared. Forty µL of the reaction substrate (10 mM L-DOPA solution) was added to all sample and blank solutions. The final mixtures were incubated for 5 mins in the dark at 25°C. The dopachrome produced in the reaction mixture was determined by taking the absorbance

reading at 475 nm using a microplate reader. The reference inhibitor used in this assay was kojic acid at the concentration of 100 µg/mL. Tyrosinase inhibition activity was calculated using the following equation:

$$\% \text{ inhibition} = \{[(A - B) - (C - D)] / (A - B)\} \times 100$$

Where A = absorbance of blank solution with enzyme; B = absorbance of blank solution without enzyme; C = absorbance of sample solution with enzyme and D = absorbance of sample solution without enzyme

2.5 Determination of bioactive compounds (phenolic and organic acids)

The quantification of phenolic acids was carried out according to a method by Aleksandra *et al.* (2011). An HPLC (Waters 2695) with photodiode array detector (Waters 2996) and a reversed-phased analytical column (150 mm x 4.6 mm x Bridge C18, 3.5 µm, Waters) were used in this study. The detector was set at $\lambda=280$ nm, $\lambda=330$ nm and $\lambda=360$ nm. For compounds separation, a mobile phase consisting of 0.1% formic acid and methanol in gradient condition at 40°C was used and the flow rate was set to 0.7 mL/min. Calibration curves were constructed using a known quantity of individual standard compounds, which were gallic, protocatechuic, 4-hydroxybenzoic, vanillic and syringic acids.

A Waters HPLC (2695) was used in the quantification of organic acids, following the method as described by Violeta *et al.* (2010). Separation of organic acids was performed by using an Extrasil ODS 5 µm column (250 mm x 4.6 mm). The detector was set at $\lambda=210$ nm and $\lambda=245$ nm for simultaneous detection. A mobile phase of 50 mM phosphate solution (pH 2.8) in isocratic condition at 30°C was used for compounds separation, with a flow rate set at 0.7 mL/min. Known amounts of individual standard compounds (oxalic, kojic, acetic and ascorbic) were used to construct the calibration curves to determine the quantity of organic acids in the samples.

2.6 Statistical analysis

Data obtained from triplicate experiments were analysed by using Minitab Version 14 software. One-way analysis of variance (ANOVA) was conducted with Tukey's test at a significance level of $p<0.05$. Data were represented as mean \pm standard deviation.

3. Results and discussion

3.1 Total phenolic content (TPC) and total flavonoid content (TFC) of unfermented and fermented broken rice

Table 1 tabulates the total phenolic and total

flavonoid content of unfermented and fermented broken rice. Many studies have reported increase of TPC and TFC after fermentation process (Juan and Chou, 2010; Dulf *et al.*, 2016; Ibarruri *et al.*, 2019).

Table 1. Bioactive component of unfermented and fermented broken rice

Sample	TPC (mg GAE/g sample)	TFC (mg QE/g sample)
Unfermented BR	0.03 \pm 0 ^c	0.04 \pm 0 ^{cd}
Day 0	0.03 \pm 0.02 ^c	0.03 \pm 0 ^d
Day 2	0.88 \pm 0.07 ^d	0.09 \pm 0.01 ^{cd}
Day 4	1.14 \pm 0.09 ^d	0.19 \pm 0.01 ^c
Day 6	2.04 \pm 0.16 ^c	0.43 \pm 0.03 ^b
Fermented BR	1.93 \pm 0.05 ^c	0.41 \pm 0.02 ^b
Day 8	2.13 \pm 0.11 ^c	0.49 \pm 0.02 ^b
Day 10	1.97 \pm 0.25 ^c	0.46 \pm 0.02 ^b
Day 12	2.19 \pm 0.03 ^c	0.49 \pm 0.01 ^b
Day 14	3.46 \pm 0.25 ^b	1.66 \pm 0.13 ^a
Day 16	3.94 \pm 0.06 ^a	1.71 \pm 0.1 ^a
Day 18		

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

Our results showed that the highest TPC and TFC was recorded from the fermented broken rice at the end of the fermentation period. The values were significantly ($p<0.05$) higher than the value at the beginning as well as at the middle of fermentation. The results are in accordance with a study by Choi and Koh (2017) and Xu *et al.* (2018) that reported a higher content of total phenolic and flavonoid content with prolonged fermentation time. Fermentation with *A. rouxii* significantly enhanced phenolics content up to 130-fold and flavonoids up to 43-fold. Our results suggested that *A. rouxii* metabolically regulates the release of bound phenolic compounds in the broken rice. During fermentation, hydrolytic enzymes released by fungi such as β -glucosidase, hydrolyse complexes of conjugated phenolics into free phenolics compounds, and also, according to Ademiluyi and Oboh (2011), other compounds that are simpler and biologically more active. It is interesting to note that increased fermentation time can also decrease the total phenolic content and flavonoids content of fermented substrates, as reported by Adetuyi and Ibrahim (2014) as well as Amarasinghe *et al.* (2018). Aside from fermentation period, the changes of phenolics and flavonoids content can be attributed to many other factors such as the type of substrates and microorganisms used in the process, inoculum size, incubation temperature, moisture content as well as extraction process.

3.2 Antioxidant activities of unfermented and fermented broken rice

Many assays can be employed to determine the antioxidant activities of samples and among them, DPPH-radical scavenging and ferric-reducing antioxidant potential (FRAP) assays are two of the most commonly accepted assays due partly to their simple procedures. Figure 1 exhibits the antioxidant activities of fermented broken rice at different intervals during fermentation.

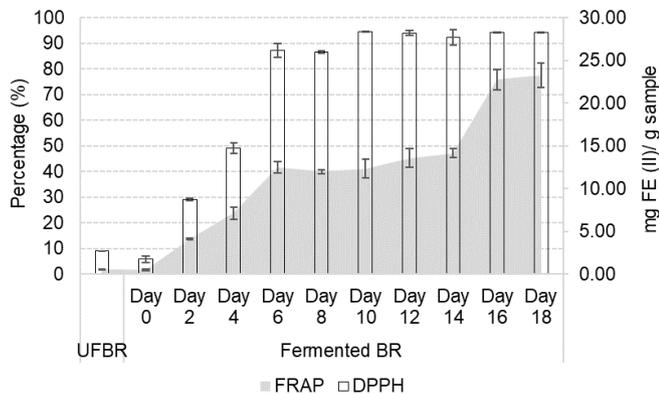


Figure 1. Antioxidant activities of unfermented (UFBR) and fermented broken rice

Similar to the results of TPC and TFC, fermentation with *A. rouxii* improved the antioxidant activities of broken rice, and these changes were dependent on the fermentation time. Kim *et al.* (2012) also reported time-dependent changes of metabolites and biological activities from the fermentation of rice with *Aspergillus* strains. The DPPH-radical scavenging activity of broken rice reached the highest values from day-10 onwards until the end of fermentation, which recorded at 92 to 94%. However, the ferric-reducing antioxidant activity of the fermented broken rice did not follow the same trend as DPPH. The highest FRAP value was observed at day-16 and day-18 of fermentation, with the value of 22.74 and 23.26 mg FE(II)/g sample, respectively. From our observations, the value of FRAP remained constant from day-6 to day-14 of fermentation, similar to TPC and TFC values (Table 1). Due to this similarity, it can be suggested that the ferric-reducing antioxidant activity of the fermented broken rice could be attributed to the presence of phenolic and flavonoid content in the samples. Our results are consistent with a study by Piluzza and Bullitta (2011) which suggested that the phenolic content of plant materials is highly correlated with their antioxidant properties. The ability of fermented broken rice to show a significant increase in antioxidant activities could be due to the synthesis and release of various antioxidant compounds, caused by the structural breakdown of the substrate during fermentation (Hur *et al.*, 2014). These compounds, other than phenolics and flavonoids, also include

anthocyanins, proanthocyanidins, tocopherols, phytic acid, γ -oryzanol and many more. The results indicated that the two antioxidant analyses showed different trends, which can be explained by the different reaction mechanisms of the assays and, therefore, different reaction affinities to the compounds present in the tested samples (de Moraes *et al.*, 2018).

3.3 Tyrosinase inhibition activity of unfermented and fermented broken rice

Tyrosinase is a rate-limiting enzyme involved in a formation of melanin in our skin – a process called melanogenesis as well as enzymatic browning of food such as fruits. Tyrosinase inhibitor compounds or extracts are often sought as an anti-pigmentation or whitening agent in medicinal and cosmetics industries and also as an anti-browning agent in food industries. Inhibitors from natural sources such as plants and fungi are preferred for food, cosmetics and medicinal applications because of their better bioavailability and less toxicity (Zolghadri *et al.*, 2019). Some tyrosinase inhibitors include hydroquinone, vanillin, kojic acid, quercetin, arbutin, kaempferol, cumic acid, *p*-Coumaric acid, resveratrol and others. Many studies have reported the enhancement of tyrosinase inhibition activity of samples through fungal fermentation (Jamaluddin *et al.*, 2014; Wu *et al.*, 2018). Tyrosinase inhibition activity of unfermented and fermented broken rice are shown in Figure 2.

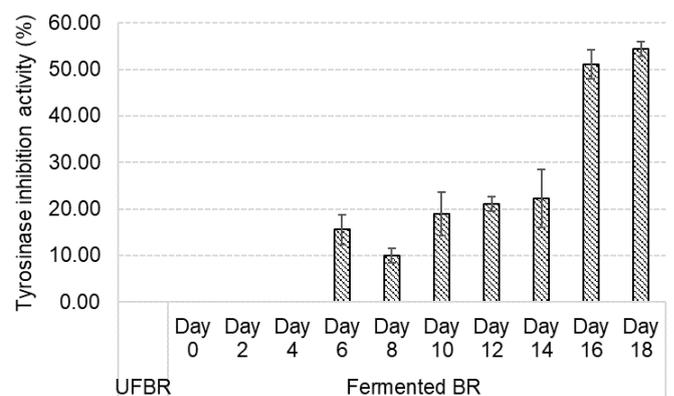


Figure 2. Tyrosinase inhibition activity of unfermented (UFBR) and fermented broken rice

Fermented broken rice started to exhibit the ability to inhibit tyrosinase activity from day-6 of fermentation with the value of 15.56%. The highest activity was recorded on day-18 with the value of $54.44 \pm 1.57\%$, followed by day-16 with 51.11 ± 3.14 . However, these values were lower than that of the reference inhibitor used in this study: kojic acid with 96% activity (data not shown). Tyrosinase inhibition activity of fermented rice was also observed by Saraphanchotiwitthaya and Sripalakit (2019). In their study, rice was fermented with *A. rouxii* and *Aspergillus niger*. Our previous study of

fermentation of broken rice with *Aspergillus oryzae* also resulted in an enhancement of tyrosinase inhibition activity up to 68.7% (Abd. Razak et al., 2018).

3.4 Bioactive compounds content of fermented broken rice

Table 2 tabulates the organic acids and phenolic acids content of fermented broken rice. These bioactive compounds content was determined on samples from day -12 to day-18 of fermentation and unfermented broken rice. There was no presence of the tested bioactive compounds in unfermented broken rice (data not shown), therefore it is apparent that fermentation with *A. rouxii* considerably enhanced the phenolic and organic acids content in broken rice. As elaborated by Martins et al. (2011), important factors that regulate the changes in phenolic acids profile through fermentation are fermentation conditions as well as the type of fungus and substrate used in a study. As explained earlier, these changes usually relate to the action of fungal enzymes such as β -glucosidase, α -amylase and laccase. As shown in Table 2, the highest content of gallic and 4-hydroxybenzoic acids were observed from day-18, however, the value bearing statistically no significant difference ($p>0.05$) between all tested samples.

Kojic acid and gallic acid proved to be an effective inhibitor of tyrosinase activity (Momtaz et al., 2008; Zolghadri et al., 2019) as well as potent antioxidant compounds. Both compounds were detected in all tested samples and their content was not statistically different ($p>0.05$) between day-12, 14, 16 and 18 of fermentation. However, the greatest tyrosinase inhibition activity and antioxidant activities in this study were detected in broken rice fermented for 16 and 18 days. Our findings were contrary to a report by Kim et al. (2012) which found that the tyrosinase inhibition activity was highly correlated with the quantity of kojic acid in the *Aspergillus*-fermented rice. In this study, we used hot

water as the extraction solvent to draw out the compounds from fermented and unfermented broken rice. The yield of compounds is strongly influenced by the type of solvent used in the study, solvent-to-sample ratio, extraction time and temperature. A study by Dey and Kuhad (2014) found that extraction using water at 40°C for 45 mins resulted in the highest phenolic compounds yield from wheat fermented with *R. oryzae*, compared to other solvents such as methanol, ethanol and acetone. In a different study, conducted by Do et al. (2014), 100% ethanol was found to be the best solvent for optimum extraction of phenolics and flavonoids as well as antioxidant activities of a medicinal herb (*Limnophila aromatica*), compared to water, acetone and other solvents used in the study. It is obvious that optimum recovery of compounds is different from one sample to another and also depends on the type of plant.

Based on our results, the antioxidant activities and tyrosinase inhibition activity of our samples can be inferred to be dependent more on the profile of bioactive compounds present in the samples than the total concentration of the individual compounds. However, further investigation on the content of bioactive compounds such as flavonoids should be undertaken to prove this hypothesis. It can also be speculated that these results were the outcome of synergistic, additive and/or antagonistic effects between different antioxidant compounds and tyrosinase inhibitors present in our samples. Wang et al. (2018) has demonstrated synergistic and additive effects of tyrosinase inhibition activity between different antioxidant compounds. Effective concentrations of compounds in samples is also a determining factor for antioxidant and tyrosinase inhibition activities. It is known that some potent antioxidant compounds, such as ferulic acid, possess both antioxidant and prooxidant characteristics and their effects and efficiency are strictly concentration-dependent.

Table 2. Bioactive compounds content of fermented broken rice

Bioactive Compound	Fermented Broken Rice			
	Day 12	Day 14	Day 16	Day 18
Phenolic acids ($\mu\text{g/mL}$):				
Gallic	19.22 \pm 4.1 ^a	19.91 \pm 0.7 ^a	19.18 \pm 0.6 ^a	23.44 \pm 0.8 ^a
Protocatechuic	5.04 \pm 3.1 ^b	3.77 \pm 0.78 ^b	4.73 \pm 0.17 ^b	16.11 \pm 1.28 ^a
4-hydroxybenzoic	2.52 \pm 0.22 ^a	2.15 \pm 1.18 ^a	2.75 \pm 0.11 ^a	5.24 \pm 1.17 ^a
Syringic	nd	nd	nd	nd
Organic acids: ($\mu\text{g/mL}$):				
Oxalic	127.72 \pm 34.7 ^a	172.07 \pm 13.3 ^a	86.15 \pm 9.4 ^a	130.09 \pm 68.6 ^a
Kojic	7.86 \pm 2.88 ^a	12.7 \pm 2.37 ^a	12.29 \pm 0.16 ^a	10.74 \pm 1.07 ^a
Ascorbic	114.94 \pm 12.6 ^a	132.14 \pm 6.5 ^a	98.17 \pm 7.06 ^a	134.98 \pm 15.5 ^a
Acetic	nd	nd	nd	nd

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

4. Conclusion

Solid state fermentation with *A. rouxii* greatly improved the phenolics and flavonoids content as well as antioxidant and tyrosinase inhibition activities of broken rice. The phenolic and organic acids profiles of broken rice were also modified during fermentation, where several strong antioxidant compounds such as kojic, protocatechuic, gallic and ascorbic acids were detected in fermented broken rice. The results of this study demonstrated that fermentation using *A. rouxii* can be successfully used to exploit underutilized broken rice to produce bioactive compounds. However, the optimization of fermentation conditions should be undertaken to achieve maximum enhancement of targeted bioactive compounds and their related biological activities. For optimum recovery of compounds, extraction method should be further studied and optimized. Broken rice fermented with *A. rouxii* may be exploited as a promising source of natural ingredient for products in food, medicinal and cosmetics industries.

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