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Profile of Phenolic Compounds, DPPH-Scavenging and Anti α-Amylase Activity of Black Rice Bran Fermented with *Rhizopus oligosporus*

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ABSTRACT

Fermentation by the solid state fermentation (SSF) technique is an alternative method that may increase bioactive compounds and their functionality due to enzymatic activities. This study evaluated the effect of the fermentation time of black rice bran on the bioactive compound profile and the antioxidant and anti-α-amylase activities of the compounds. Fermentation was performed by using *Rhizopus oligosporus* (*R. oligosporus*) for 0, 24, 48, 72 and 96 hours. Fermented rice bran samples were collected every 24 h. The results showed that the bioactive compounds in black rice bran significantly increased ($p < 0.05$) during fermentation. Further significant increases were found for DPPH free radical scavenging activity (76.91 ± 0.06%) and α-amylase inhibition (73.05 ± 0.25%) in fermented (96 h) black rice bran compared to those in non-fermented bran (0 h). It was confirmed that fermentation had a significant effect on increasing the amount and activity of bioactive compounds in black rice bran.

Keywords: Anti α-amylase, antioxidant, bioactive compound, fermented bran, *Rhizopus oligosporus*

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INTRODUCTION

Bran is a by-product of rice milling that contains high nutrient contents such as oil (12-22%), protein (11-17%), food fibre (6-14%), water (10-15%), and ash (8-17%). It also contains bioactive compounds such as

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phenolic acids, flavonoids, anthocyanins, γ-oryzanol, tocopherols, phytic acid, and others (Sharif et al., 2014). These bioactive compounds are known to have antioxidant activity that can scavenge free radicals, enhance the immune system of the body, prevent high blood pressure, hyperlipidaemia, and hyperglycaemia and reduce the risk of cancer (Nagendra et al., 2011; Zhang et al., 2010).

The content of rice bran bioactive compounds varies depending on the type of rice, climatic conditions and processing methods (Gul et al., 2015). The highest total amounts of phenolic and flavonoid compounds are found in black rice bran (Chen et al., 2017). According to Tuarita (2017), the IC_{50} value of the methanol extract of black rice variety Cempo Ireng (67.58 µg/ml) was lower than that of red and white rice (82.50 µg/mL and 410.02 µg/mL, respectively). In general, these compounds are intricately bonded to polysaccharides, lignocelluloses, fat or protein matrices via ester bonds (Cheng et al., 2016; Oliveira et al., 2012; Rashid et al., 2015). Phenolic compounds with ester bonds have a complex structure and a large molecular weight, there the compounds cannot be absorbed by humans; their utilisation in the body is also not optimal. Several methods can be used to increase the concentration of bioactive compounds in bran, one of which is fermentation. In fermentation, microorganisms produce enzymes that hydrolyse the complex of bioactive compounds to result in more active free bioactive compounds (Oliveira et al., 2012).

Fermentation with the solid state fermentation (SSF) method is one of the fermentation techniques that can produce better product characteristics, such as improving the nutritional value and bioactive compounds and their functionality which can increase the value of rice bran (Pourali et al., 2010; Razak et al., 2015). The SSF of rice bran using various microorganisms with different fermentation times has been reported, such as *Rhizopus oryzae* for 120 h and 96 h (Oliveira et al., 2012; Schmidt & Furlong, 2012, Schmidt et al., 2014), *R. oryzae* and *R. oligosporus* for 96 h (Zulfafamy et al., 2018), *R. oligosporus* and *Monascus purpureus* for 12 days (Razak et al., 2015), *Saccharomyces boulardii* (Ryan et al., 2011), *Aspergillus oryzae* and *R. oryzae* for 12 days (Razak et al., 2017) and *Saccharomyces cerevisiae* with 24 h of fermentation (Chaiyasut et al., 2017). These studies reported that fermentation time of 96 h can increase the bioactive compounds and bran fermentation activity.

The utilisation of fungi from the genus *Rhizopus* sp. is highly effective in the SSF process due to its capability of producing enzymes that can degrade lignocellulose and polysaccharide matrices, increase the chemical content and bioactivity, and increase the availability of bioactive compounds in the bran (Oliveira et al., 2012; Schmidt & Furlong, 2012). The utilisation of *Rhizopus* sp. also improves digestibility and specific catalytic activity without producing toxins or toxic substances in the environment during the controlled fermentation process (Oliveira et al., 2012; Schmidt et al., 2014; Razak et al., 2015).

The *R. oligosporus* is able to provide good results in the bran fermentation process for 12 days (Razak et al., 2015), 72 h and 96 h (Zulfafamy et al., 2018). To date, this study used *R. oligosporus* with fermentation time of 0, 24, 48, 72, and 96 hours, as it has not yet been extensively explored.

The condition of diabetes or hyperglycaemia encourages free radical formation by accelerating reactive oxygen species (ROS) formation that triggers a state of oxidative stress. Oxidative stress causes decreased insulin secretion, inhibits glucose uptake in muscle, and contributes to complications from diabetes, such as heart attacks and hypertension (Hasim et al., 2017). Therefore, antioxidants-rich food intake can be an alternative way to prevent complications from diabetes. The increased bioactivity in rice bran through fermentation is expected to be used in diabetes management. The α-amylase inhibitory activity is a measure that can be analysed as an approach to evaluate the antidiabetic potential of fermented bran. The purpose of this research is to determine the effect of the fermentation time of black rice bran with *R. oligosporus* mould using the SSF technique on the bioactive compound profile, antioxidant activity and α-amylase inhibition of the bran.

MATERIALS AND METHODS

Sample Preparation

The black rice bran sample from the Indonesian varieties of Cempo Ireng was prepared as follows: dry grains of black rice were first removed using a Rice Machine (Satake, Japan) to obtain black brown rice. The skin of the rice was broken and then the rice was milled with a Grain Testing Mill (Satake, Japan). The obtained bran was then sieved with a 40-mesh sieve to separate the chaff and groats. Next the bran samples were packaged in plastic bags of high-density polypropylene (HDPE) and stored in a freezer at -20^{o}C for further analysis.

Preparation of the Culture

The culture preparation was performed according to Razak et al. (2015). The *R. oligosporus* was obtained from the Indonesian Institute of Science Research Center. The *R. oligosporus* isolate was prepared in potato dextrose agar (PDA) medium and incubated for 7 days at 30°C. The mould inoculum was then suspended in 10 ml of sterile distilled water. The suspension was calculated as the number of spores up to 10^6 spores/mL. The spore suspension solution was ready to be inoculated into the rice bran for the fermentation process.

The Fermentation Process

The bran fermentation process was performed according to the procedure of Zulfafamy et al. (2018). The fermentation process was initiated with the addition of water to the substrate at as much as 50% of the weight of the substrate (100 g), which aimed to create a moist condition so that mould could subsist. Subsequently, the bran substrate was sterilised at 121°C for 15 min by autoclaving. After that, the sterile culture suspension 15% (v/w) of *R. oligosporus*

(0.15×10^6 spores/g bran) was added to the sterile substrate, mixed well. The plastic container used (22 x 20 cm^2) as a fermenter was then perforated (0.2 mm/1 cm^2) to create aerobic conditions. The fermentation process was carried out at a temperature of 30°C for 24, 48, 72, and 96 hours, hereafter referred to as fermented rice bran (FRB). Non-fermented rice bran (NFRB), which was sterilised under the same conditions served as control (Webber et al., 2014). Next, NFRB and FRB were dried at 50°C for 4 h and stored in a freezer at -18°C until analysed.

Sample Extraction

Each 5 g of sample was extracted with 50 mL of 70% ethanol at room temperature by using a shaker for 30 min and then centrifuged for 15 min at 1207 × *g* (3000 rpm). Then the extract was evaporated (50°C for 1 h) until all the solvents were removed and used for analysis (Cheng et al., 2016).

Total Phenol Analysis

The total phenol analysis was based on Slinkard and Singleton (1977) by the Folin-Ciocalteu method. Gallic acid was used as the standard. A 1 mL sample was mixed with 5 mL of Folin-Ciocalteu (5 min), and then 5% sodium carbonate (1 mL) was added. The sample solution was vortexed and incubated in a dark room for 2 h at room temperature. The sample absorbance was measured by using a UV spectrophotometer at 725 nm. The obtained results were expressed in milligrams of gallic acid equivalent per 100 gramme of dried sample (mg GAE/100 g db).

Total Flavonoid Analysis

The total flavonoid analysis was performed by the colorimetric method of aluminium chloride as described by Chang et al. (2002). The extract 3 mL was added to 7 mL of distilled water. After that it was reacted with 0.1 mL of $AlCl_3$ and 0.1 mL of potassium acetate. The sample absorbance was measured by using a UV-VIS spectrophotometer at 432 nm. Quercetin was used as the standard. The obtained results were expressed in milligrams of quercetin equivalent per 100 gramme of dried sample (mg QE/100 g db).

Total Anthocyanin Analysis

The total anthocyanin content in the fermented bran was measured by the pH difference based on the method described by Lee et al. (2005). The sample was dissolved in KCl buffer with a pH of 1.0 and sodium acetate buffer with a pH of 4.5, and the absorbance of the sample was measured at 510 and 700 nm. The absorbance value was calculated as A = [(A510-A700) at pH 1 - (A510-A700) at pH 4.5]. The results were expressed as mg anthocyanin (cyanidin-3-glucoside) per g of dried sample using 26.9 as the molar coefficient and the molecular weight (BM) of 449.

Analysis of γ-Oryzanol

The γ-oryzanol analysis was conducted by using high-performance liquid chromatography (HPLC) (Sabir et al., 2017). A mixture of methanol and acetonitrile (35:65) was used as the mobile phase with a C-18 column (Zorbax Eclipse XDB

C-18 column 4.6 X 150 nm) and 1 mL/min flow rate. The separation process was carried out with an HPLC system (Agilent Technologies) and UV-VIS detector at 325 nm wavelength. Quantification was done using the γ-oryzanol standard curve, and the results were expressed as milligrams per gramme of dried sample (mg/g db).

Analysis of α-Tocopherol

The analysis of α-tocopherol was performed following the official Association of Official Analytical Chemists (AOAC) (2007) method 940.28. The sample (20 µL) was injected into an HPLC (Agilent Technologies) system with a UV-VIS detector at a 280 nm wavelength, a C-18 column (Zorbax Eclipse XDB C-18 column 4.6 X 150 nm), methanol and isopropanol (98:2) as the mobile phase, and a 1 mL/min flow rate. The α-tocopherol standard was used for standard curve, and the results were expressed as milligrams per gramme of dried sample (mg/g db).

The Ability to Scavenge DPPH Free Radicals

The ability to scavenge DPPH was evaluated to assess antioxidant activity (Kubo et al., 2002). A mixture of 0.1 mL of DPPH, 1.87 mL of methanol and 1 mL of acetate buffer (pH 5.5) was vortexed. Then, 0.03 mL of the extracted sample was added. The sample was vortexed and then incubated for 20 min in the dark room. The sample absorbance was measured by using a UV-VIS spectrophotometer at a wavelength 517 nm. DPPH radical scavenging was calculated as (1-absorbance of sample/absorbance of blank) x 100. Ascorbic acid was used as the standard.

Analysis of α-Amylase Inhibition

The analysis of α-amylase inhibition was based on Thalapaneni et al. (2008). A sample of 125 µL was reacted with 125 µL of pancreatic amylase enzyme solution and was incubated for 10 min at 37°C. Then, 125 µL of starch solution (1%) was added to the sample solution and was incubated again for 10 min at 37°C. Next, 96 µL of 3.5-dinitrosalicylate (DNS) reagent was added and the sample was incubated for 5 min in boiling water. As much as 5 mL of distilled water was added to the sample, and its absorbance was measured using UV-VIS spectrophotometry at a wavelength of 540 nm.

Statistical Analysis

The data were analysed by one-way analysis of variance (ANOVA) using Statistical Package for the Social Science (SPSS) version 20. If a significant difference was obtained at the treatment level, the data were then analysed with Duncan's multiple range test at $\alpha = 0.05$. The data are displayed as the mean±SD (n=3).

RESULTS AND DISCUSSION

Total Phenols, Flavonoids and Anthocyanins

The phenolic content of NFRB was 193.47 mg GAE/100 g, significantly increased ($p < 0.05$) to 304.50 mg GAE/100 g after

four days of fermentation (Table 1). In this study, the total phenolics increased by as much as 57.39% after fermentation. This result was in accordance with that by Oliveira et al. (2012), who stated that the SSF technique improved the total phenolic content in rice bran. Rice bran (variety BR-IRGA 417) fermentation with the use of *R. oryzae* (Schmidt et al., 2014) doubled the total phenol content at 48 h. However, rice bran (Thailand varieties) fermentation with the use of *S. cerevisiae* did not significantly increase the amount of total phenolics. It was suspected that *S. cerevisiae* activity did not affect the phytochemical content in rice bran for 24 h (Chaiyasut et al., 2017). These results show that the phenolic profile changes through fermentation are strongly influenced by substrate types, microorganisms, and fermentation conditions (Martins et al., 2011).

Total flavonoid content at 0 h (NFRB) was about 168.08±0.22 mg QE/100 g, and slightly decreased at the 24 h of fermentation (165.95±1.59 mg QE/100 g). After 24 h of fermentation, the flavonoid levels significantly increased ($p < 0.05$) to 184.54±0.79 mg QE/100 g at the 96 h of fermentation (Table 1). Flavonoids are the most widespread group of phenolic compounds. Fungal genera such as *Rhizopus, Aspergillus, Monascus* and *Penicillium* are known to divide the heterocyclic C-ring flavonoids (Das & Rosazza, 2006).

The amount of anthocyanin obtained in this study significantly increased ($p < 0.05$) during fermentation, at the beginning (0 h) 26.43±0.03 mg/g sample increased to 96.38±0.42 mg/g sample on the fourth day (96 h) of fermentation. Anthocyanin is a glycoside compound and belongs to the class of flavonoids that are mostly contained in black rice bran (Zhang et al., 2010). The presence of a high amount of anthocyanins in FRB is particularly useful because anthocyanins have anticancer, antioxidant properties (Pengkumsri et al., 2015), and antidiabetic properties (Jayaprakasam et al., 2005).

The phenolic compounds can be classified into free and bound phenolic compounds. Fermentation process can

Table 1
Phenolic compounds of non-fermented and fermented rice bran (db)

Fermentation time (hours)	Total phenolics (mg/100g)	Total flavonoids (mg/100g)	Total anthocyanins (mg/g)	γ-Oryzanol (mg/g)	α-Tocopherol (mg/g)
0	193.47±0.21^{a}	168.08±0.22^{b}	26.43±0.03^{a}	12.58±0.49^{a}	0.04±0.00^{a}
24	258.57±0.93^{b}	165.94±1.59^{a}	51.55±0.18^{b}	17.29±0.61^{b}	0.72±0.01^{c}
48	298.39±1.16^{c}	170.99±0.86^{c}	58.74±0.36^{c}	20.03±0.32^{c}	0.56±0.03^{b}
72	300.44±2.04^{c}	174.87±1.59^{d}	69.48±0.26^{d}	22.44±0.46^{d}	0.77±0.03^{d}
96	304.50±1.75^{d}	184.54±0.79^{e}	96.38±0.42^{d}	23.48±0.65^{e}	1.51±0.03^{e}

Values are mean ± SD (n = 3). Different letters in the same column are significantly different by Duncan's Multiple Range Test ($p < 0.05$)

enhance the release of bound phenolic compounds to free phenolics were due to extracellular enzymatic hydrolytic activity of mould in the rice bran. Free phenolic aglycones that contribute to the high antioxidant activity. According to Dey and Kuhad (2014), the main enzyme produced by *R. oligosporus* is β-glucosidase. This enzyme may increase the availability of free hydroxyl groups in phenolic structures by hydrolysing glycosidic bonds between carbohydrates or between carbohydrates and non-carbohydrates and acting as a catalyst in the process of hydrolysing glycosidic bonds in alkyl and aryl β-D-glucoside groups. Compared to phenolic glycosides, phenolic aglycones have better bioactivity and bioavailability in the human digestive system because they are more easily absorbed by the small intestine (Celep et al., 2014; Huynh et al., 2014). The β-glucosidase enzyme is also capable of converting anthocyanins into aglycone forms that have better antioxidant properties than the glycoside forms (Kong et al., 2003). In addition to the β-glucosidase enzyme *R. oligosporus* also produces enzymes such as β-glucuronidase, α-amylase, xylanase, proteases, laccase, phenolic esterase, and lipases that help break down the phenolic compounds attached to cell wall compounds, such as cellulose and proteins in conjugated forms (Huynh et al., 2014; Razak et al., 2015). According to Schmidt et al. (2014), the laccase enzyme is able to degrade the phenyl ring during fermentation so that the phenolic compounds increase.

The Compounds of γ-Oryzanol and α-Tocopherol

The enzymatic hydrolysis process that occurs during fermentation also increased the contents of γ-oryzanol and α-tocopherol in the rice bran (Table 1). The NFRB content of γ-oryzanol (13.11 mg/g) increased to 23.78 mg/g on the 4th day of fermentation. The increase was approximately 81.39%. These results were similar to those reported by Massarolo et al. (2017) that showed that control rice bran had 13.54 mg/g γ-oryzanol which increased to 20.52 mg/g after the fermentation process with *R. oryzae*. During fermentation mould activity produces several enzymes that can degrade cell walls, producing intracellular compounds that may contribute to the increase in oryzanol (Massarolo et al., 2017).

The content of α-tocopherol also significantly increased ($p < 0.05$) during the fermentation from only 0.04 mg/g dried sample to 1.51 mg/g dried sample on the 4th fermentation day. This increase was due to the enzymatic reactions occur during fermentation that break the phytochemical bonds of the toxoid-bound forms to produce free forms with a high bioavailability (Belobrajdic & Bird, 2013; Gani et al., 2012). According to Chen and Bergman (2005) the content of γ-oryzanol was 10-20 times greater than that of total tocopherol in rice bran, which was similar to the results in this study.

Scavenging Activity of DPPH Free Radicals

Antioxidant activity is related to the activity of compounds that maintain or protect the biological system from oxidation reactions or processes involving ROS. The most common method to measure antioxidant activity is by using radical compounds, such as DPPH compounds to simulate ROS (Razak et al., 2017).

The DPPH free radical scavenging activity in NFRB was 26.28±4.16%, which significantly increased ($p < 0.05$) to 76.91±0.06% after 96 h of fermentation (Figure 1). The DPPH free radical scavenging activity increased approximately three times after 96 h of fermentation compared to that of NFRB. These results were different from those reported by Oliveira et al. (2012), in which rice bran fermented with *R. oryzae* for 96 h showed antioxidant activity of 59±1.7%. Razak et al. (2015) showed that rice bran fermented with *R. oligosporus* for 12 days had DPPH free radical scavenging activity of 90.19±0.77%. The differences in the results were due to different types of microbes, types of substrate and fermentation time used in the experiment.

According to Sompong et al. (2011), pigmented rice bran is rich in sources of phenolic antioxidants. An increase in the flavonoid content during fermentation may contribute to antioxidant activity (Cheng et al., 2016). But on this studied, increased amounts of phenols, flavonoids, and anthocyanins that contributed to the increased antioxidant activity of the fermented rice bran. This result supported by the correlation result obtained which showed that there was a positive correlation between the scavenging activity of DPPH and total phenols, flavonoids and anthocyanins, i.e $r = 0.95$, $r = 0.61$, and $r = 0.90$ (respectively). According to Heim et al. (2002), the phenolic aglycones obtained by fermentation have the higher antioxidant activity than the glycoside forms and are effectively absorbed by the gut. The hydroxyl group of phenolic compounds becomes a hydrogen donor for

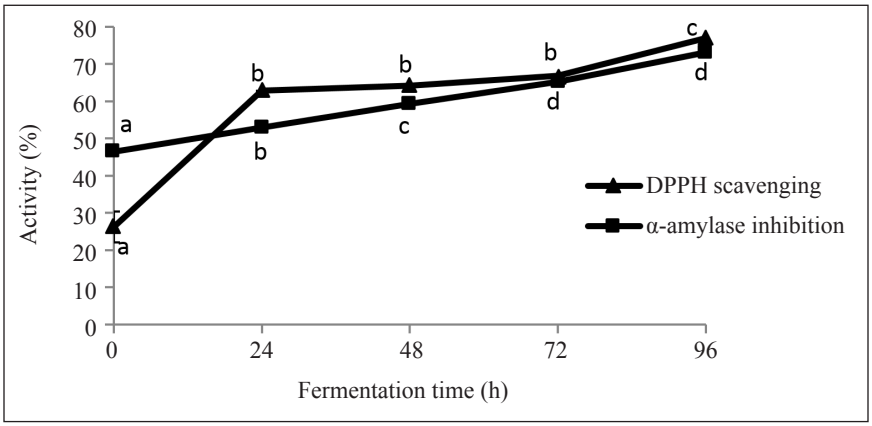

Figure 1. DPPH radical scavenging activity and inhibition of α-amylase by NFRB and FRB. The points in each line with different letters are significantly different at $p < 0.05$ by Duncan's Multiple Range Test

DPPH free radicals, thereby it increases the antioxidant activity of fermented rice bran (Dey & Kuhad, 2014; Razak et al., 2015). The processes of free radical scavenging, ion metal chelation and pro-oxidant enzymes are possible mechanisms of this change in antioxidant activity (Amorati et al., 2006). According to Kong et al. (2003), anthocyanins also have an antioxidant effect through several mechanisms: hydrogen donation, metal binding and protein binding. Its antioxidant activity is attributed to the glycosilated B-ring structure (Khoo et al., 2017).

Rashid et al. (2015) reported that the bioactive compounds in rice bran strongly contributed not only to the phenolic content but also to the antioxidant capacity. This statement is supported by the positive correlation between the scavenging activity of DPPH free radicals and γ-orizanol (r = 0.93) and α-tocopherol (r = 0.88). The γ-oryzanol compound is a mixture of ferulic acid esters of sterols (cholesterol, stigmasterol and beta-sitosterol) and triterpenes of alcohol (cycloartanol, 24-methylenecycloartanol and cyclobranol) (Nagendra et al., 2011). The ferulate in the molecular structure of γ-oryzanol is known to be a potent antioxidant that is stable at high temperatures. The γ-oryzanol has antioxidant activity that is four times more effective than that of the compounds of vitamin E (α-tocopherol, β-tocopherol, α-tocotrienol, and β-tocotrienol) in inhibiting oxidation (Nagendra et al., 2011). In addition to γ-oryzanol, the antioxidant ability of rice bran is also due to the availability of other antioxidant compounds, such as tocopherols, tocotrienols, and ferulic acids that can act as good radical scavengers against DPPH radicals (Arab et al., 2011; Gul et al., 2015; Razak et al., 2015).

α-Amylase Inhibition Activity

The inhibitions of α-amylase activity by NFRB and FRB extracts are presented in Figure 1. The percentage of rice bran inhibition against α-amylase significantly increased from 46.45% for NFRB to 73.05% after 96 h of fermentation. The inhibition of α-amylase is highly effective in delaying glucose absorption, thus preventing increased postprandial blood glucose, and may act as one of the therapeutic agents to prevent diabetes. According to Randhir and Shetty (2007), fermentation can increase the amount of free phenolics and antioxidant activity. The antioxidant in fermented rice bran contributes to the inhibition of α-amylase. Other researchers also reported that the phenolic phytochemicals foods had inhibitory activity against α-amylase (Yilmazer-Musa et al., 2012). In addition, it is known that flavonoids such as luteolin, quercetin, myricetin and cyanidin may act as α-amylase inhibitors and are claimed to be useful for regulating type 2 diabetes (McCue et al., 2004; Tadera et al., 2006). In this study, there was a positive correlation between the anti-amylase activity of the fermented rice bran and phenolic compounds (r = 0.87), total flavonoids (r = 0.92) and anthocyanins (r = 0.98). Jung et al. (2015) also reported that γ-oryzanol-containing granules might be anti-hyperglycaemic agents by

stimulating the activity of peroxisome proliferator-activated receptor-gamma (PPAR-γ), which was an important receptor in lipid metabolism and glucose balance. The γ-oryzanol also increased glucose digestion by stimulating the translocation of glucose transporter type 4 (GLUT-4) from the cytosol to the cell surface. A positive correlation was also obtained from the results of this study between the α-amylase inhibition activity and γ-oryzanol ($r = 0.96$) and α-tocopherol ($r = 0.91$).

CONCLUSIONS

The 96 h fermentation process with the use of the SSF method and *R. oligosporus* significantly increased total phenolics, total flavonoids, total anthocyanins, γ-oryzanol and α-tocopherol contents in black rice bran. The increases in the bioactive compounds in fermented bran contributed to the increased in DPPH free radical scavenging activity and α-amylase inhibition. It is suggested that the fermented rice bran contains bioactives and antioxidative activities, which could be potentially explored for the development of functional foods.

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