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Enhancement in Extractability of Phenolic Compounds and Antioxidant Activity of Rice Straw Fermented using *Aspergillus oryzae*

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Rice straw is an agricultural waste material that is abundantly available in Malaysia. Field burning is the main method used for eliminating rice straw, but this increases air pollution and consequently affects public health. In this study, rice straw was utilized as a substrate for solid state fermentation (SSF) using *Aspergillus oryzae*. SSF was conducted at 30 °C, with 70 % initial moisture content and 10 % v/v of inoculum level. The effects of fermentation duration on the total phenolic content, total flavonoid content and antioxidant activity were investigated. It was found that solid state fermentation enhanced the extractability of the total phenolic content by 104.4 % and the total flavonoid content by 5.32 % after ten days. The antioxidant activity of rice straw increased as fermentation progressed, with an increase of 90.01 % in DPPH radical scavenging activity and 89.24% in the FRAP value. The activity of xylanase and β-glucosidase enzymes produced during SSF was also investigated to study the relationship between polyphenols and the antioxidant activity of fermented rice straw extracts. Both enzymes showed an increase in activity as fermentation progressed proving that the enzymes played a significant role in releasing bound phenolics during SSF.

Key words: Rice straw; solid state fermentation; total phenolic content; antioxidant activity

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Rice is a major food crop, especially in South and Southeast Asia. Nearly 50% or more of non-edible biomass is produced in rice plants as straw, which includes stems, leaf blades, leaf sheaths and post-harvest remains of the panicle. Rice straw is a useful bio-resource with a worldwide annual production of approximately 731 million tons. Traditionally, straw is managed through on-farm burning but this causes air pollution, global warming, plant nutrient losses and environmental damage. The straw is burned during harvesting as it is the cheapest and easiest way to prepare the field for the next cultivation season. Straw burning is mainly practised in areas where crop rotations do not allow sufficient time for decomposing. Burning straw causes air pollution by producing an estimated 13 tons of smoke containing carbon dioxide per hectare. It is reported that burning a ton of straw produces 3 kg of particulate matter, 60 kg of carbon monoxide, 1460 kg of carbon dioxide, 199 kg of dust and 2 kg of sulphur dioxide [2]. These gases affect the atmosphere and environment, leading to global climate change. Therefore, there is an urgent need for finding economically viable, socially acceptable and eco-friendly alternative uses for this biomass. Rice straw, which is composed of cellulose, hemicelluloses and lignin, can be used as a potent feedstock for an environmentally-friendly process called solid state fermentation to produce phenolic compounds that find various applications in the food, cosmetics and healthcare industries.

The extraction of bioactive compounds using conventional organic solvent extraction gives low yields due to bound phenolic compounds, while the utilization of chemical pre-treatment may result in hazards and toxicological effects to the environment and unwanted transformations of the extracted compounds. Microbial fermentation could serve as a potential tool to produce bioactive compounds due to its cost-effectiveness and environmental advantages. Conventional extraction methods using organic solvents also do not allow the complete release of bound phenolic compounds from plant materials. The ability of fermentation to improve the yield and change the profile of phenolic compounds is mainly due to the release of bound phenolic compounds as a consequence of cell wall degradation by microbial enzymes produced during fermentation. The microbial metabolism of phenolic compounds may result in large metabolites through different bioconversion pathways such as glycosylation, deglycosylation or ring cleavage depending on the microbial strains and substrates used [3].

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Solid state fermentation (SSF) can be used to produce some industrially important phenolic compounds, improve the antioxidant potential of solid substrates by increasing the total phenolic content and enhance the bioavailability of phenolic compounds [4]. Previous reports support the idea that SSF is superior in several aspects compared to submerged fermentation because it offers numerous advantages including higher productivity, lower water and energy requirements, easy aeration, lower demand for sterility, a natural habitat for microorganisms, easier downstream processing, utilization of cheaper agro-industrial residues as solid substrates and is more environmentally friendly [5]. Filamentous fungi have great potential to produce bioactive compounds by the SSF process. Therefore, they are the most commonly used microorganisms to achieve this target [6]. One of the best features of the fungus *Aspergillus oryzae* used in the present study is the high activity of hydrolytic enzymes it secretes into the medium during the fermentation. Fast growth and most importantly high resistance to contamination are the main reasons why this fungus is widely used in the soy sauce fermentation industry. Furthermore, *A. oryzae* can be safely used in food production because it lacks expressed sequence tags for genes that promote the production of aflatoxins [7].

MATERIALS AND METHODS

1. Sample Preparation

Rice straw was collected from Tanjung Karang, Selangor during the harvesting period. The samples were dried, blended and kept in tightly capped bottles in the chiller.

2. Inoculum Preparation

The fungus used in this study, *Aspergillus oryzae*, was obtained from the MARDI culture collection, in Serdang, Selangor. The strain was cultured on PDA plates for 4 days in an incubator at 30 ºC. Inoculum preparation was done according to the method from a previous study [8]. Distilled water was measured to 100 mL in a graduated cylinder and poured evenly onto four PDA plates containing the 4 day-old culture. The spores were collected using a hockey stick. Then the suspended fungal cultures were filtered using Whatman filter paper No. 1 into a flask. The filtrate was used as the inoculum. The flask, funnel, filter paper, and distilled water were sterilized prior to use.

3. Solid State Fermentation Conditions

The experiment was conducted in a 250 ml Erlenmeyer flask with 10 g of working volume according to a previous method [9]. A 1 % sucrose and yeast extract solution was added as a supplementary carbon and nitrogen source. Distilled water was added so that the final moisture content of the substrate was 70 %. After medium sterilization, the flask was inoculated with 1 ml of the inoculum and incubated at 30 ºC for 10 days. Sampling was performed once every two days. At the end of fermentation, the biomass was added to 100 ml of distilled water and agitated on a rotary shaker for 1 hour at 180 rpm. The entire contents were centrifuged and then filtered. The clear supernatant was used as the crude extract.

4. Analytical Methods

4.1. Total Phenolic Content (TPC)

The total phenolic content of the samples was determined based on a previous method [10]. The sample (0.1 mL) was mixed with 2 mL of 2 % sodium carbonate (Na_2CO_3) and the mixture was allowed to stand at room temperature for 2 minutes followed by the addition of 0.1 mL of 50 % Folin-Ciocalteu's reagent. The reaction solution was mixed thoroughly and allowed to stand at room temperature for 30 minutes in the dark. The absorbance of the sample was measured at 720 nm using a spectrophotometer. Total phenolic content was expressed as Gallic acid equivalents per gram (GAE/g) of dry sample.

4.2. Total Flavonoid Content (TFC)

The total flavonoid content of the fermented rice straw extracts was determined using the aluminium chloride colorimetric method as described previously [11] with slight modifications. Fermented rice straw extract (1.0 mL), methanol (1.0 mL), 10% aluminium chloride (0.5 mL), and 1 M potassium acetate (0.5 mL) were combined. The mixture was incubated at room temperature for 30 min and the absorbance was measured at 415 nm using a spectrophotometer (Thermo Genesys 20). The total flavonoid concentration was calculated as mg of catechin equivalents (CE)/g sample using an equation obtained from the calibration curve.

4.3. Determination of DPPH Radical Scavenging Activity

The radical scavenging capacity of fermented rice straw extracts was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to a method previously described [12]. Initially, 1 mL of 0.1 mM methanolic solution of DPPH was added to 1 mL of sample. The mixture was shaken well and incubated in the dark for 30 minutes at room temperature. The absorbance of the sample was measured at 517 nm. Ascorbic acid was used as a positive reference and this was diluted in distilled water to obtain different concentrations ranging from 0 to 0.7 mg/mL. At the same time, a control was prepared by mixing 1 mL

of methanol and 1 mL of 0.1 mM DPPH solution. The radical scavenging activity was expressed as a percentage using the following equation:

$$\% \text{ Scavenging activity} = [(A_{control} - A_{sample})/ A_{control}] \times 100\%$$

where $A_{control}$ = absorbance of control (DPPH solution),

A_{sample} = absorbance of the sample solution

4.4. Ferric Reducing Antioxidant Power (FRAP) Analysis

The Ferric Reducing Antioxidant Power (FRAP) assay was performed using a method described previously [13]. FRAP reagent was freshly prepared by mixing 2.5 mL of a 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl with 2.5 mL of 20 mM $FeCl_3.6H_2O$ and 25 mL of 0.3 M acetate buffer (glacial acetic acid 16 mL, sodium acetate trihydrate 3.1 g and distilled water 16 mL) at pH 3.6 with a ratio of 1:1:10 respectively.

The FRAP assay was performed by mixing 0.15 mL of the fermented rice straw extract with 2.85 mL of FRAP reagent. The mixture was incubated at 37 °C for 4 min and the absorbance was determined at 593 nm using a UV spectrophotometer. A calibration curve was prepared using $FeSO_4.7H_2O$ aqueous solutions with concentrations ranging from 0.1 to 1.0 mM and a similar procedure was used with the samples. The FRAP value was calculated by using the equation as follows:

$$FRAP\ value = \frac{raw\ FRAP\ value\ x\ dilution\ factor\ x\ volume\ of\ solvent}{weight\ of\ rice\ straw}$$

5. Enzymes Produced during SSF

5.1. Xylanase Assay

Xylanase activity was determined by adopting a previous method [14] where 0.5 mL of the sample was mixed with 0.5 ml of 1 % Birchwood xylan prepared in 0.05 M citrate buffer (pH 5). The mixture was incubated at 50 °C for 30 min. Then, the reaction was terminated by adding 3 mL DNS reagent and boiling for 10 min. After cooling, the absorbance of the mixture was recorded at 540 nm using a spectrophotometer. The amount of reducing sugars liberated was quantified using a xylose standard curve with concentrations ranging from 0 – 0.8 mg/mL. One unit of xylanase activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugars (xylose) per minute under the assay conditions.

5.2. β-glucosidase Assay

β-glucosidase activity towards p-nitrophenyl-b-D-glucopyranoside (pNPG) was determined with the use of p-nitrophenol (pNP) as a standard based on a method used in a previous study [15]. The reaction mixture contained 0.5 mL of 2 mM pNPG in 50 mM sodium acetate buffer (pH 5) and 0.5 mL of enzyme extract. The mixture was incubated at 45 °C for 10 min and then 1.25 mL of 1 M Na_2CO_3 was added to stop the reaction. A yellow colour developed due to pNP liberation from pNPBG, and this was measured using a spectrophotometer at 410 nm. One unit of β-glucosidase activity was defined as the amount of enzyme required to liberate 1 µmol of pNP per minute under the assay conditions.

5.3. Statistical Analyses

Analyses were performed in triplicate and the results were expressed as an average of the replicates ± standard deviation. The significant differences between the independent variables were analysed by one way analysis of variance (ANOVA) tests followed by Tukey's Multiple Comparison Test. The relationships between TPC, TFC, antioxidant activity and the enzymes produced were investigated by applying Pearson's correlation test.

RESULTS AND DISCUSSION

Total Phenolic Content

In plants, phenolics are usually found in conjugated forms through hydroxyl groups with sugar as glycosides. This condition lowers their antioxidant activity because the free hydroxyl groups on the phenolics are used to convert free radicals. During SSF, different hydrolytic enzymes produced by microorganisms can change the profiles of phenolic compounds, resulting in better extractability and bioavailability of phenolic compounds. Figure 1 shows the total phenolic content of fermented rice straw extracts during the fermentation process.

As shown in Figure 1, the TPC increased up to day 6 of fermentation before slightly decreasing on day 8 and then increasing again on day 10. The results obtained suggest that SSF enhanced the extractability of phenolic compounds in rice straw, with 104.4 % enhancement after 10 days of fermentation. Significant enhancement of the extractable phenolic content clearly shows the positive effects of SSF on rice straw. It was reported that the TPC in rice bran fermented with *Rhizopus oryzae* increased until day 4 of fermentation [16]. The increase of the TPC in this study may be due to the hydrolytic enzymes produced during SSF that can hydrolyse bound phenolics. On day 8 of fermentation, the TPC showed a slight decrease that may have resulted from the degradation of phenolic compounds to aliphatic compounds. On day 10 of fermentation, the TPC further increased to 18.81 mg GAE/g.

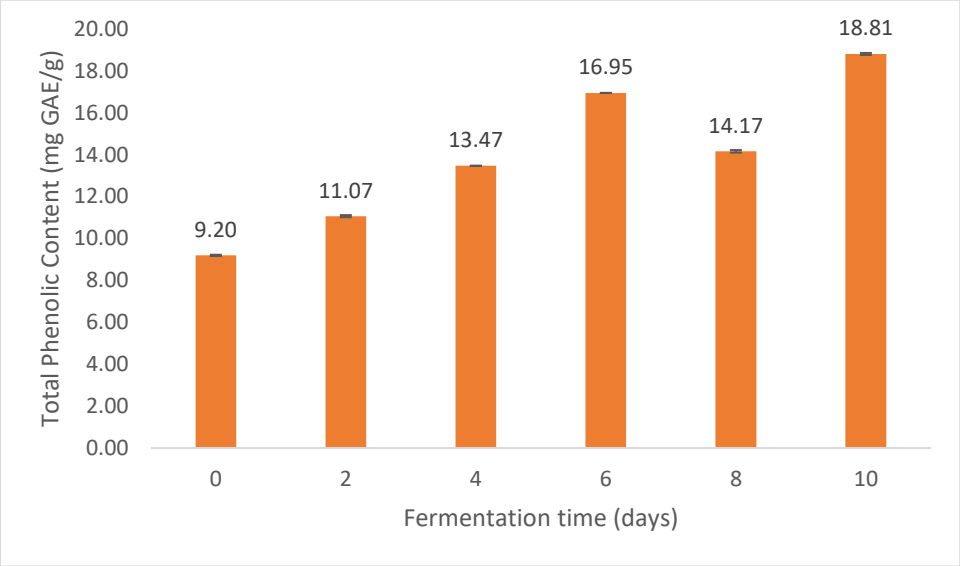

Figure 1. Total phenolic content of fermented rice straw extracts during fermentation

Total Flavonoid Content

The total flavonoid content (TFC) of fermented rice straw during fermentation was illustrated in Figure 2. The results obtained showed that the total flavonoid content increased slightly as fermentation progressed. After 10 days of fermentation, the TFC increased by 5.32%.

The release of bound flavonoids in fermented rice straw may be attributed to the presence of condensed tannins, a group of flavonoid oligomers that are widely distributed in plants, and that are believed to give rice straw its brown colour [17]. In a study conducted in Egypt, rice straw was extracted using ethyl acetate after pre-treatment with sodium carbonate containing 221.6 mg GAE/g total phenolic content and 4.9 mg RE/g extract [18]. By direct comparison, it appears that rice straw extracted using ethyl acetate had a higher TPC, but lower TFC compared to the fermented rice straw extracts obtained in this study. However, other factors may also affect the total phenolic and flavonoid content in rice straw due to the different geographical origins of the samples and the different analysis methods used. Enhancement of phenolic compounds through SSF was reported in wheat koji [19], plum fruits [20], pearl millet [21], rice bran [22], figs [23] and pineapple waste [24].

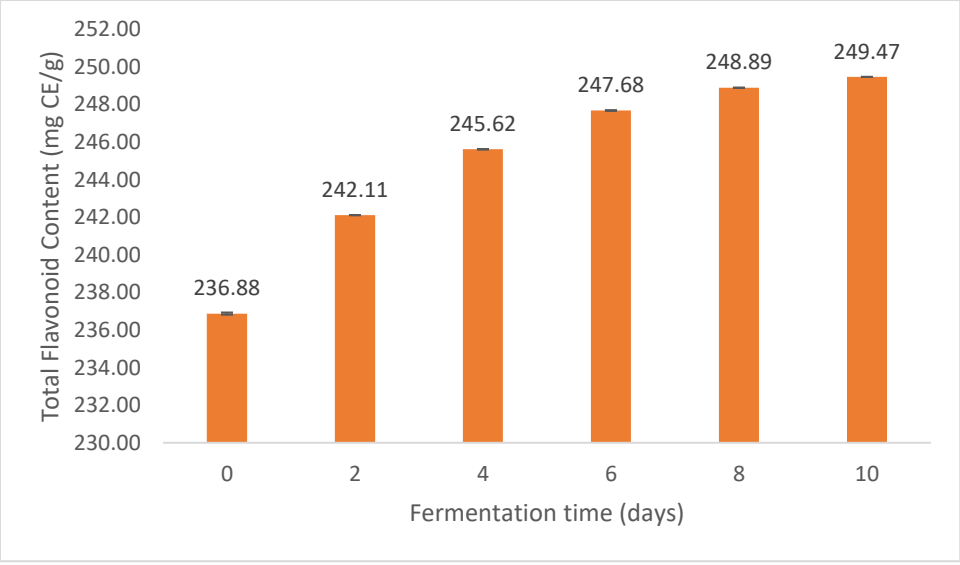

Figure 2. Total flavonoid content of fermented rice straw extracts during fermentation

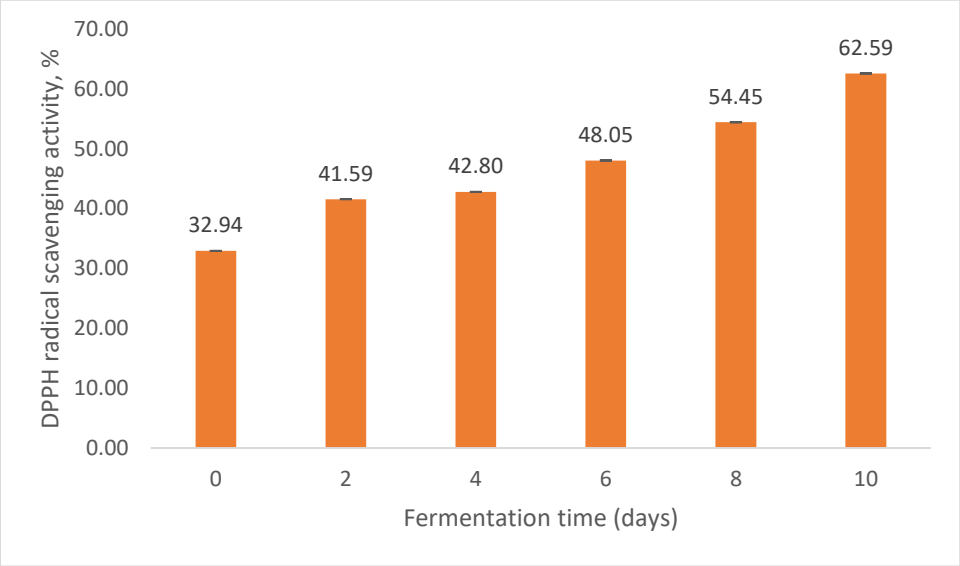

Figure 3. DPPH radical scavenging activity of fermented rice straw extracts during fermentation

DPPH Radical Scavenging Activity

The capacity of the fermented rice straw extracts to transport electrons or hydrogen atoms was measured using the DPPH radical scavenging assay. In comparison to other antioxidant assays, DPPH is a popular choice for assessing the antioxidant capabilities of plant samples in a short period of time. Figure 3 depicts the DPPH radical scavenging activity of fermented rice straw during fermentation.

The results obtained show that DPPH radical scavenging activity increased with fermentation time. DPPH radical scavenging activity increased by 90.01% after 10 days of fermentation, indicating that SSF had a positive effect on the scavenging activity of rice straw. The increase in antioxidant capacity may be due to the increase in total phenolic content during SSF.

FRAP Value

The FRAP assay is inexpensive, uses simple reagents and has highly reproducible results. Figure 4 shows the FRAP values of the fermented rice straw extracts obtained during fermentation.

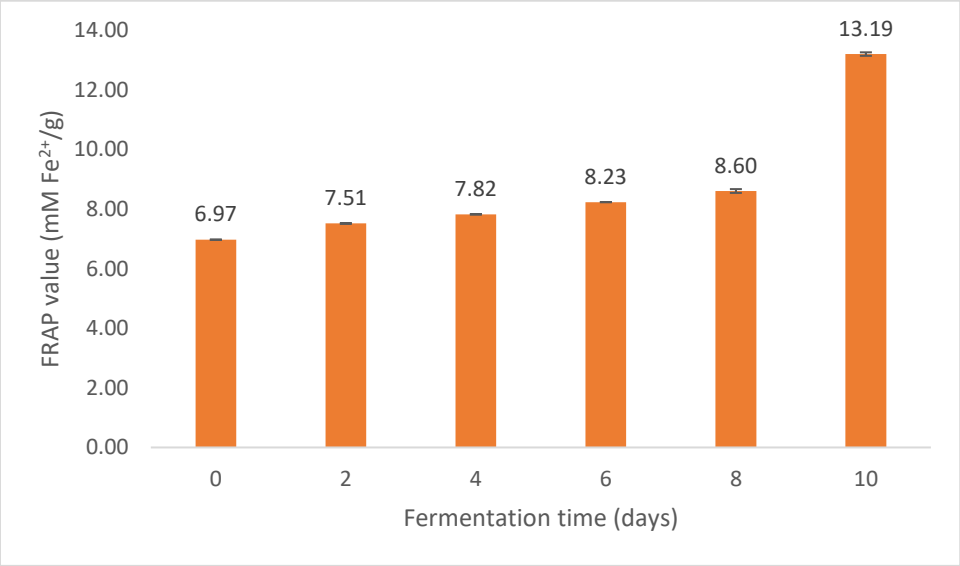

Figure 4. FRAP values of fermented rice straw extracts obtained during fermentation

As shown in Figure 4, the FRAP value of fermented rice straw increased as fermentation progressed, and showed an 89.24% increment after 10 days. The results obtained indicated that SSF enhanced the antioxidant activity of rice straw. The enhancement of antioxidant activity may be related to the TPC and TFC content of rice straw which increased with fermentation. The release of phenolics and flavonoids during the fermentation process enhances the anti-oxidative activity of plant-based foods, which may be a useful method of improving the dietary supply of natural antioxidants.

Xylanase Activity

During SSF, microorganisms were grown on the surface of solid materials with limited amounts of water. Different hydrolytic enzymes produced by microorganisms directly from the solid substrates may change the profiles of phenolic compounds during SSF. These hydrolytic enzymes produced are responsible for the efficient breakdown of the plant wall components and hydrolysis of the ester bond linkages which subsequently assist in releasing the bound phenolics from the plant matrix [25]. Xylanases break down xylan (the main carbohydrate in hemicellulose) into xylose [26]. Figure 5 illustrates the xylanase activity during the solid state fermentation of rice straw.

Figure 5 indicates that more xylanase was produced as fermentation progressed. Xylanase can degrade the cell wall matrix, which enhances the hydrolysis of glycosidic bonds and the release of bound phenolics. Correlation tests conducted showed that strong correlations were observed between the activity of xylanase with TPC (R^2 = 0.799), DPPH (R^2 = 0.852), and TFC (R^2 = 0.915) while there was a moderate correlation with FRAP (R^2 = 0.691).

β-glucosidase Activity

The enzyme β-glucosidase catalyzes the hydrolysis of glycosidic linkages in alkyl or aryl β-glucosides as well as glucosides containing only carbohydrates residues. This enzyme is relatively common in all living organisms but is expressed in high quantities by fungi during the solid state fermentation of lignocellulosic wastes [27]. It is responsible for hydrolysing phenolic glycosides and releasing extractable free aglycones, which potentially enhances antioxidant activity [28]. β-glucosidase activity during the solid state fermentation of rice straw is shown in Figure 6.

Figure 6 shows that the activity of β-glucosidase increased as fermentation progressed. Results obtained showed strong correlations between the activity of β-glucosidase and TPC (R^2 = 0.887), TFC (R^2 = 0.910), FRAP (R^2 = 0.787) and DPPH (R^2 = 0.946) (refer to Table 1). These findings indicate that β-glucosidase played a significant role in the enhancement of extractable phenolics and antioxidant activity of rice straw after SSF.

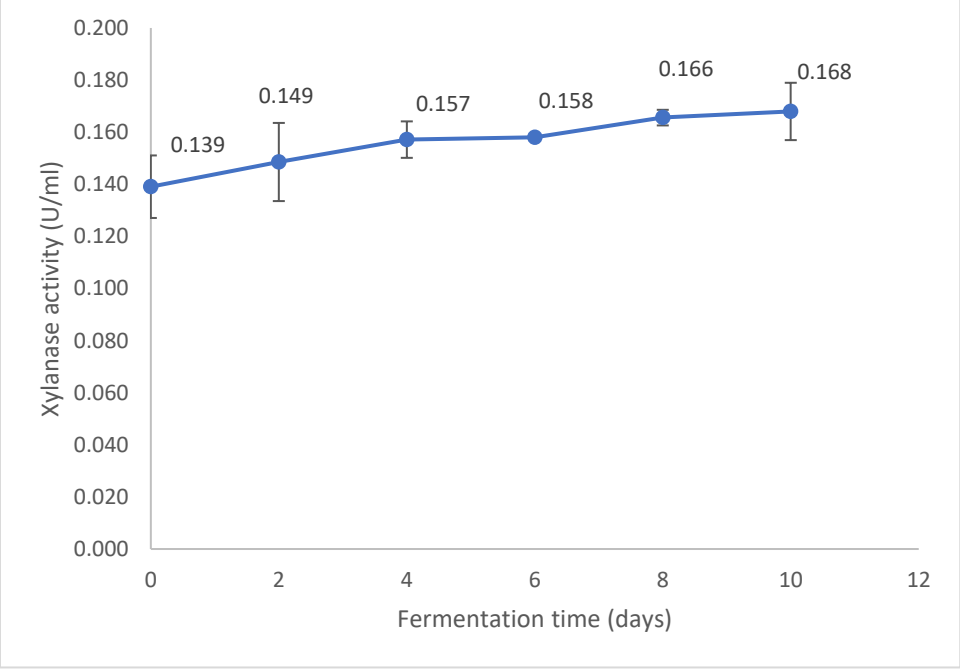

Figure 5. Xylanase activity during solid state fermentation of rice straw

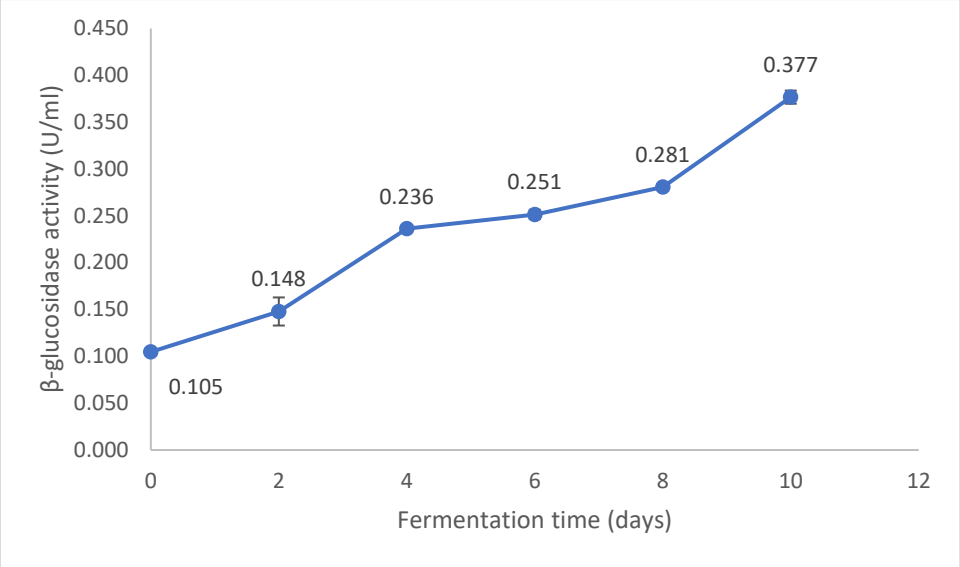

Figure 6. β-glucosidase activity during solid state fermentation of rice straw

Table 1. Correlations between TPC, TFC, antioxidant activity and enzymes produced (xylanase and β-glucosidase) during the SSF of rice straw

		TPC	DPPH	FRAP	TFC	Xylanase	β-glucosidase
TPC	Pearson Correlation	1	.818**	.494*	.831**	.799**	.887**
	Sig. (2-tailed)		.000	.037	.000	.000	.000
DPPH	Pearson Correlation	.818**	1	.824**	.885**	.852**	.946**
	Sig. (2-tailed)	.000		.000	.000	.000	.000
FRAP	Pearson Correlation	.494*	.824**	1	.674**	.691**	.787**
	Sig. (2-tailed)	.037	.000		.002	.001	.000
TFC	Pearson Correlation	.831**	.885**	.674**	1	.915**	.910**
	Sig. (2-tailed)	.000	.000	.002		.000	.000
Xylanase	Pearson Correlation	.799**	.852**	.691**	.915**	1	.910**
	Sig. (2-tailed)	.000	.000	.001	.000		.000
β-glucosidase	Pearson Correlation	.887**	.946**	.787**	.910**	.910**	1
	Sig. (2-tailed)	.000	.000	.000	.000	.000	

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Strong correlations were also observed between xylanase enzymes and TPC (R^2 = 0.799), TFC (R^2 = 0.915) and DPPH (R^2 = 0.852). However, a moderate correlation (R^2 = 0.691) was observed between xylanase and FRAP. These findings suggest that the xylanase enzyme also played a significant role in the enhancement of extractable phenolics and antioxidant activity of rice straw after SSF. The enhancement of phenolic content may be due to the hydrolytic enzymes produced by *A. oryzae*, which catalyzed the release of aglycone from the substrate and thus increased the phenolic content as well as the antioxidant activity. Both phenolic content and antioxidant activity correlated with the increase in β-glucosidase and xylanase activity, suggesting that the enzymes played an important role in releasing bound phenolics from rice straw.

CONCLUSION

In conclusion, SSF enhanced the extractable phenolic content as well as the antioxidant activity of rice straw. The increase in activity of both the β-glucosidase and xylanase enzymes was associated with an increase in phenolic content and antioxidant activity, indicating that these enzymes were involved in the release of bound phenolics from rice straw.

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DECLARATION

The authors declare that they have no conflict of interest.

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