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EFFECTS OF SOLID STATE FERMENTATION BY *Monascus purpureus* ON PHENOLIC CONTENT AND BIOLOGICAL ACTIVITIES OF COCONUT TESTA AND RICE BRAN

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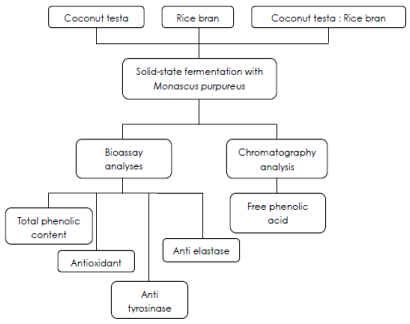
Graphical abstract



Abstract

Solid-state fermentation (SSF) is an alternative low cost useful process that has many important applications in the field of biotechnology. In this study, SSF has been employed as a process for the production of value-added agricultural by-product using coconut testa (CT), rice bran (RB) and the combination of both substrates (CT-RB). The effect of SSF by *Monascus purpureus* on total phenolic content (TPC), antioxidant, anti-tyrosinase and anti-elastase of the substrates were studied and compared with its non-fermented counterparts. The results showed that the SSF has improved the TPC up to three-fold higher in the studied substrates. Antioxidant potential evaluated using FRAP analysis also exhibited an enhancement in fermented substrates with the values ranging from 23.70 to 63.15 mg AAE/g sample. On the other hand, the radical scavenging activity evaluated using DPPH assay showed a different trend in comparison to the TPC and FRAP analyses. In another two analyses, tyrosinase and elastase inhibition activities were also enhanced in most substrates upon the fermentation. The changes in free phenolic acids content (p-coumaric, caffeic, ferulic, sinapic, vanillic, protocatechuic, gallic and 4-hydroxybenzoic and syringic acid) of the substrates after fungal fermentation was also examined through high performance liquid chromatography (HPLC) analysis. In summary, SSF offers a tool to further increase the bioactive potential of the studied substrate.

Keywords: Coconut testa; rice bran; solid-state fermentation; antioxidant; anti-tyrosinase; anti-elastase

1.0 INTRODUCTION

The agro-industrial processing and production have always resulted in large volumes of waste. This has caused waste disposal problems especially in countries where their economy rely heavily on agricultural activities. From the economic and environmental point of views, with the large availability and the composition that always rich in bioactive compounds, reutilization of these wastes for the production of beneficial products would be effective in terms of cost and environmental pollution [1].

Microbial technology has appeared with new potential in the development of value-added product through utilization of agro-industrial by-products. In recent years, solid-state fermentation (SSF) has built up its credibility due to its potential application in producing biologically active compounds aside from other biotechnology based products [2,3]. SSF is defined as a condition that consists of microbial

growth and biomolecules manufacturing process on solid support without free flowing water. However in SSF process, substrate contains sufficient moisture to allow microorganism growth and metabolism [2]. Numerous studies have been carried out and proved that functionalities of various agro by-products were enhanced by SSF and many beneficial compounds have been produced through SSF, such as organic acids [6,7], enzymes [4,5] aromatic and flavor compounds as well as bioactive compounds [10,11].

Coconut testa (CT) and rice bran (RB) are agricultural by-products from coconut and rice processing industry respectively. In Malaysia, the CT has only one principle use which is as an animal feed. Its utilization is very limited despite being a good source of secondary metabolites [12]. CT was reported to contain natural antioxidants such as phenolic compounds, tocopherol and tocotrienols, which may provide health benefits to human [12]. Meanwhile, RB is known for containing high amount of functional compounds and antioxidants [13]. Most RB production was used in the production of fertilizers, animal feed and cosmetics [14].

In this investigation, the SSF has been utilized to enhance the value of these agricultural by-products using *Monascus purpureus*. The total phenolic content (TPC), antioxidant, anti-elastase as well as anti-tyrosinase activities of water extracts from fermented CT, RB and CT-RB mixed substrate are compared with their non-fermented counterparts. In addition, the high performance liquid chromatography (HPLC) analysis was also carried out to observe the changes in free phenolic acids concentration in the studied substrates upon the fungal fermentation.

2.0 METHODOLOGY

The SSF using CT, RB and CT-RB mixed substrate (50:50; w/w) were carried out using *M. purpureus* (strain F0061) from Collection of Functional Food Culture (CFFC) of Malaysian Agricultural and Research Development Institute (MARDI). Thirty grams of CT, RB and CT-RB mixed substrate was weighed into 250ml Erlenmeyer flasks and 35ml of distilled water was added into each flask. The substrates were then autoclaved (121°C; 20min). A known amount of fungal spores (approx.~10^6/ml) was added into each flask, mixed well and incubated at 32°C for 12 days. The samples were then harvested and dried at 50°C for 24 hours. Non-fermented substrates with no microbial spores added were used as the control group. For sample extraction, 1g of both non-fermented and fermented samples was extracted using 10 ml of hot distilled water (100°C) for 15 minutes. After centrifuge (10 000 rpm) for 15 minutes, the supernatant was filtered using Whatman No. 1 filter paper. The filtrates were used for further analysis.

The Folin-Ciocalteu methodology was used to determine TPC in each sample [15] and this assay is regularly used to measure total phenolics in variety of fruits and vegetables [16]. It has been suggested that the phenolic content of plant materials is commonly correlated with their antioxidant activities [17]. Hence, the extracts of both non-fermented and fermented CT, RB and CT-RB mixed substrate were assessed for TPC as well as for antioxidant. The antioxidant potential of these extracts was measured by ferric reducing antioxidant potential (FRAP) and DPPH free radical scavenging method [15].

Tyrosinase inhibition is the most common approach to find out skin lightening agent as this enzyme catalyses the rate-limiting step of pigmentation [18]. Therefore, the anti-tyrosinase assay was performed to investigate the potential of these fermented substrates as anti-pigmentation/skin whitening agent. The tyrosinase inhibitory activity was determined using the dopachrome method with L-DOPA as the substrate and the amount of dopachrome was measured at 475 nm using the microplate reader. Analysis on the ability of the fermented and non-fermented extracts to inhibit the elastase enzyme activity was also carried out. Elastin is an extracellular matrix protein that has an influence on skin elasticity while elastase is the proteinase enzyme capable of degrading elastin which can lead to ageing process [19]. Therefore, inhibition of the elastase activity could be used as a way to protect against skin aging [20]. The elastase inhibition activity was measured with EnzChek Elastase Assay Kit (Invitrogen Life Technologies Inc.USA) according to the manufacturer's recommendations.

Modification of free phenolic acids content in fermented substrates were also determined using HPLC Alliance Separation Module (Waters 2695), equipped with a photodiode array detector (Waters, 2996) (Table 3) [21]. Samples were separated using a reverse-phase analytical column (150mm x 4.6mm XBridge C18, 3.5um, Waters). Peak identification was made by comparing retention times and UV spectra at 280nm and 325nm with authentic compounds. Quantification of phenolic acid content was made using calibration curves obtained by injecting known amounts of pure compounds as external standards. All data in this study were expressed as mean ± standard deviation. Analysis of variance (ANOVA) was done using Minitab 17 Statistical Software and $P<0.05$ was considered statistically significant.

3.0 RESULTS AND DISCUSSION

3.1 Total Phenolic Content And Antioxidant Activity

The use of *M. purpureus* (strain F0061) in SSF has successfully enhanced the antioxidant activity in red fermented rice [22]. Therefore, this starter microorganism might similarly able to mobilize phenolic compounds and enhance the antioxidant activity in the studied substrates during similar bioprocess. As shown in Table 1, the TPC was significantly enhanced ($p<0.05$) in all fermented substrates with an exception for RB. Up to three-fold

higher TPC value was exhibited by mixed substrate compared to its non-fermented counterpart. The enhanced phenolic content can be explained by the fact that bioactive compounds level can be modified during fermentation by the hydrolytic enzymes activity of microbes. In plant, phenolic compounds are often found in conjugated forms through hydroxyl groups with glycosides and sugar [23]. *M. purpureus* is known to be capable of producing β-glucosidase, which can catalyze the bioconversion of the conjugated forms of phenolic compounds into phenolic aglycone during fermentation, potentially leading to an increase in the content of phenolic compounds.

Phenolic compounds are always known to be responsible for the free radical scavenging and antioxidant activities in plants. They have numerous biological effects, mainly attributed to their antioxidant potential [24]. It is crucial to evaluate the antioxidant potential of the studied extracts using more than one method due to the complex nature of phenolic phytochemicals. In this present study, the antioxidant activity of the bioprocessed substrates was measured using ferric reducing antioxidant potential (FRAP) and DPPH free radical scavenging method. Similar trend as TPC was observed for FRAP assay where the fermentation process was significantly enhanced (p<0.05) the antioxidant potential in all fermented substrates (Table 2). Meanwhile insignificant increase in TPC was observed in fermented RB, in which the antioxidant potential of this extract was significantly improved (p<0.05). This scenario could be attributed to some individual phenolic compound with high antioxidant activity or some other non-phenolic compounds such as α-tocopherol and γ-oryzanol which were also reported to have antioxidant activities [25]. In concomitant with the TPC, mixed substrate fermentation also exhibited the highest antioxidant activity in comparison to individual substrates. Synergistic effect among compounds in both substrates suggests the highest antioxidant capacity detected in this substrate.

However, a contradictory result was obtained in free radical scavenging analysis which exhibited a reduced antioxidant activity in fermented RB and CT-RB mixed substrate. Meanwhile, the radical scavenging activity was significantly improved in fermented CT (Table 1). The discrepancy in the antioxidant activities indicates that both DPPH and FRAP assay determine different aspects of antioxidant capacity. The difference might be attributed to different mechanism involved in the radical-antioxidant reactions compared to the FRAP assay mechanism [26]. Besides, the difference in stoichiometry of reactions between the DPPH radical and the antioxidant compounds of the extracts also usually used to explain the variation in the scavenging potential of some compounds [27].

Table 1 TPC, antioxidant potential (FRAP) and radical-scavenging activities (DPPH) of water extracts of fermented CT, RB and CT-RB mixed substrate

Substrate/ Analysis	Treatment	Total phenolic content (mg GAE/g sample)	Antioxidant potential (mg AAE/g sample)	Radical scavenging activity (%)
Coconut testa	Unfermented	0.90 ± 0.06^{c}	14.95 ± 1.60^{c}	50.29 ± 0.41^{d}
	Fermented	1.73 ± 0.03^{b}	23.71 ± 2.36^{b}	69.48 ± 0.38^{c}
Rice bran	Unfermented	1.66 ± 0.61^{bc}	30.22 ± 9.57^{b}	87.82 ± 2.41^{b}
	Fermented	1.73 ± 0.03^{b}	61.21 ± 4.50^{a}	85.14 ± 0.12^{b}
Coconut testa : Rice bran	Unfermented	1.20 ± 0.32^{bc}	22.15 ± 4.98^{bc}	92.29 ± 0.45^{a}
	Fermented	3.90 ± 0.20^{a}	63.15 ± 4.07^{a}	86.87 ± 0.13^{b}

[1] ANOVA analyses were performed using Minitab 17 Statistical Software. Each value is expressed as the mean ± sd. The values in each column with the same letter are not significantly different at the level of 0.05 (p>0.05).

3.2 Tyrosinase And Elastase Inhibitory Activity

Melanogenesis is a pathway that responsible for the melanin production in epidermal layers of the skin and tyrosinase is the important rate limiting enzyme. The abnormal secretion of melanin leads to hyperpigmentation of the skin. Therefore, the anti-tyrosinase assay was performed to investigate the potential of these fermented substrates as skin lightening agent. As shown in Table 2, the tyrosinase inhibitory activity was exhibited by all non-fermented substrates. The *M. purpureus*-treated substrates demonstrated a significant enhancement (p<0.05) in tyrosinase inhibition activity except for that of CT. The fermented mixed substrate demonstrated the highest tyrosinase inhibition activity with 22.06% inhibition compared to individual substrates of RB and CT.

Assessment on the ability of the non-fermented and fermented extracts to inhibit the elastase enzyme activity was also carried out. The mechanical properties of connective tissues are determined by the insoluble elastic fibrous protein together with the collagen. Meanwhile, elastase is the proteinase enzyme that capable to degrade elastin which could lead to skin ageing [28]. Therefore, inhibition of the elastase activity could be used as a way to protect against skin ageing [19]. For non-fermented substrates, the elastase inhibition activity was detected only for RB (9.16%) but not for the other substrate being studied. However, the fermentation process has improved the

elastase inhibitory ability of CT and CT-RB mixed substrate (Table 2). The changes in these bioactivities might be explained by biochemical changes that occur during fermentation process. The changes might lead to ratio alteration of anti nutritive and nutritive components which consequently affects the product properties such as bioactivity and digestibility [29].

Table 2 Tyrosinase and elastase inhibition activity of water extracts of fermented CT, RB and CT-RB mixed substrate

Substrate/ Analysis	Treatment	Tyrosinase inhibition activity (%)	Elastase inhibition activity (%)
Coconut testa	Unfermented	5.56 ± 1.96^{cd}	-
	Fermented	3.21 ± 0.91^{de}	6.80 ± 3.16^{a}
Rice bran	Unfermented	1.69 ± 0.79^{e}	9.16 ± 0.54^{a}
	Fermented	6.74 ± 0.00^{c}	-
Coconut testa : Rice bran	Unfermented	13.73 ± 0.85^{b}	-
	Fermented	22.06 ± 1.47^{a}	7.04 ± 0.47^{a}

[1] ANOVA analyses were performed using Minitab 17 Statistical Software. Each value is expressed as the mean ± sd. The values in each column with the same letter are not significantly different at the level of 0.05 (p>0.05)

3.3 Free Phenolic Acids Composition

Modification of bioactive phenolic compounds is commonly associated to the hydrolytic enzymes (β-glucosidase, esterase, amylase, xylanase, etc.) produced during SSF [31, 32, 33]. To determine the changes in soluble free phenolic acid compositions during *M. purpureus*-fermentation, nine phenolic acids (gallic, protocatechuic, p-hydroxybenzoic, vanillic, and syringic as hydroxybenzoic acid derivatives; caffeic, p-coumaric, sinapic, and ferulic acid as hydrocinnamic acid derivatives) were analyzed and quantified using HPLC. Results shown in Table 3 indicate that fungal fermentation has altered the phenolic acid composition in the substrates.

Results in Table 3 show that for non-fermented CT, only protocatechuic acid and p-hydroxybenzoic were detected with the values of 12.08 and 4.44 ug/ml extract respectively. Fermentation with *M. purpureus* demonstrated the presence of vanillic acid and gallic acid components in fermented CT. On the other hand, a slight decrease in the concentrations of protocatechuic acid and p-hydroxybenzoic acid was detected after the fermentation process. Meanwhile for RB, significant enhancement (p<0.05) in phenolic acid content was found particularly for ferulic, sinapic and syringic acid. Moreover, caffeic acid and vanillic acid were also detected in the fermented RB. This indicates that hydrolytic enzymes produced by *M. purpureus* capable to release the soluble conjugated or insoluble bound phenolic acids from these substrates.

On the other hand, for the CT-RB mixed substrate, a few of the phenolic acids were not detected after the fermentation process (Table 3). The protocatechuic acid content was increased about one-fold after the fermentation while a slight decrease was detected for p-coumaric acid. A significant amount of vanillic acid was also identified along with gallic acid and p-hydroxybenzoic acid in the fermented CT-RB mixed substrate. The conversion of ferulic acid to vanillic acid via β-oxidation during the fermentation process might partially contribute to the presence of vanillic acid in the fermented mixed substrate [30]. The detection of some phenolic acids after bioprocessing procedure also could be due to fermentation-induced structural breakdown of the substrates cell walls that occurred which lead to the liberation and/or synthesis of various bioactive compounds [34]. The decrease in phenolic acid content in fermented substrates can be attributed to either microbial degradation, reduction, or the oxidation of the phenolic compounds by the fermenting microbes [28]. Moreover, bioconversion by enzymatic reactions that occurred during the SSF also might result in the decrease of certain phenolic acid composition.

Table 3 Phenolic acids content in non-fermented and fermented CT, RB and CT-RB mixed substrate (mg/ml)

Phenolic acids/ Substrates	Coconut testa		Rice bran		Coconut testa : rice bran	
	Non-fermented	Fermented	Non-fermented	Fermented	Non-fermented	Fermented
Ferulic acid	nd	nd	1.88 ± 0.14^{bc}	9.72 ± 0.93^{a}	2.26 ± 0.78^{b}	nd
p-coumaric acid	nd	nd	7.33 ± 0.99^{a}	7.01 ± 0.06^{a}	4.22 ± 0.01^{b}	3.20 ± 0.05^{c}
Sinapic acid	nd	nd	2.52 ± 0.10^{b}	4.68 ±1.26^{a}	1.17 ± 0.01^{bc}	nd
Caffeic acid	nd	nd	nd	4.86 ± 0.14^{a}	nd	
Vanillic acid	nd	16.41 ± 0.43^{c}	nd	16.41 ± 0.43^{b}	nd	31.70 ± 0.59^{a}
Syringic acid	nd	nd	6.04 ± 0.69^{b}	10.40 ±1.51^{a}	1.59 ± 0.08^{c}	nd
Protocatechuic acid	12.08 ± 0.49^{a}	11.16 ± 1.43^{ab}	nd	nd	7.88 ± 0.23^{c}	9.33 ± 0.35^{bc}
Gallic acid	nd	10.37± 1.51^{a}	nd	nd	nd	10.10 ± 0.05^{a}
p-hydroxybenzoic	4.44 ± 0.21^{a}	4.21 ± 0.67^{a}	nd	nd	nd	5.26 ± 0.15^{a}

[1] ANOVA analyses were performed using Minitab 17 Statistical Software. Each value is expressed as the mean ± sd. The values in each row with the same letter are not significantly different at the level of 0.05 (p>0.05). nd = not detected

4.0 CONCLUSIONS

This investigation was carried out to observe the effects of SSF using *M. purpureus* on phenolic content and biological activities of CT and RB. Results obtained demonstrated that fermentation with *M. purpureus* could enhance the content of total phenolics, antioxidant potential as well as tyrosinase and elastase inhibitory activities in CT and RB. Different trend in radical scavenging activity shows the importance of using more than one method in analyzing antioxidants. This is due to the complex nature of phytochemicals that might have different antioxidant mechanisms. HPLC analysis also demonstrated a concomitant result with TPC where the total free phenolic acid content was also improved upon fermentation. It shows that the hydrolytic enzymes produced by *M. purpureus* play an important role in the release of phenolic aglycones in the studied substrates which lead to the enhancement of antioxidant potential as well as tyrosinase and elastase inhibitory activities. Therefore, fermentation with *M. purpureus* (F0061) can be applied as a tool to develop CT and RB as a functional food or functional food ingredient with multiple functionalities. Furthermore, the findings in this study contribute additional information on the potential of underutilized CT.

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