

## OPTIMIZATION OF SOLID-STATE MIXED FUNGAL FERMENTATION OF TOTAL PHENOLIC COMPOUNDS FROM DEFATTED RICEBERRY BRAN USING SUBCRITICAL WATER EXTRACTION

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### ABSTRACT

The aim of this study was to optimize the proportion of mixed culture fungi: *Aspergillus oryzae*, *Aspergillus awamori* and *Monascus purpureus* for enhancing the total phenolic compounds from defatted Riceberry bran (DRB) via solid-state fermentation (SSF) and employing Response Surface Methodology (RSM) with modified Box Behnken Design. In addition, fermented DRB was extracted the bioactive compounds by using subcritical water extraction at 200°C for 30 minutes. In all experiments, the fungal growth was almost uniform through the DRB at a room temperature for 5 days, 55% moisture content, pH 5.5 and  $6 \times 10^7$  spores/g DRB. The optimum of SSF conditions for mixed cultures of fungi as *A. oryzae* and *M. purpureus* was the proportion of 0.07 to 0.93. *A. awamori* has no significant influent on enhancing active compounds with mixed cultures of fungi. The prediction values of total phenolic content obtained 20.22 mg gallic acid equivalent / g dry DRB, which increased 1.7 times of non-fermented DRB. Thus, the model fitted almost seamlessly to the experimental data.

**KEYWORDS:** solid-state fermentation; defatted Riceberry bran; total phenolic compounds; mixed culture fungi

### 1. Introduction

Riceberry rice is the new variety of purple rice that originated from Thailand between crossing of Dawk Mali 105 and Hom Nin species. It has been a popular brown rice due to its health-promoting properties to help ameliorate food-related chronic diseases like diabetes, heart disease, high blood cholesterol, obesity, and cancers [1-2]. It is rich in anthocyanins and antioxidants such as phenolic compounds, carotenoid,  $\gamma$ -oryzanol, vitamin E, vitamin

B1, zinc, polyphenol,  $\beta$ -carotene, omega3 and dietary fiber [3-5]. DRB still contains high-value nutrients such as fat, protein, carbohydrate, fiber, vitamins, minerals, and antioxidants as shown in some research studies [6-7]. Phenolic compounds are important substances due to their antioxidant activities [8], and offer beneficial effects against cancers, cardiovascular disease, diabetes, and Alzheimer's disease [9]. They were identified from decomposition of rice bran under subcritical water conditions up to 11 phenolic compounds: caffeic ((E)-3- (3, 4-dihydroxyphenyl)-2-propenoic acid), ferulic, gallic, gentisic, p-coumaric, p-hydroxybenzoic (4-hydroxybenzoic acid), syringic, protocatechuic (3, 4-dihydroxybenzoic acid), sinapic, vanillin (4-hydroxy-3-methoxybenzaldehyde) and vanillic acids [10].

Solid-state fermentation (SSF) is defined as the bioprocess carried out in the absence, or near-absence of free water. It is useful in enhancing the value of products such as improved phenolic compounds, proteins, chitosan, pectinase, glucoamylase, antioxidants and tyrosinase inhibition etc. The benefits of itself are small space fermentation, environmentally friendly, high product yield, simple process and cost-effective [11-13]. The general fungi used in SSF to enhance active ingredients such as total phenolic compounds in agricultural residues are *Aspergillus ssp.*, *Monascus ssp.*, *Rhizopus ssp.* or co-culture fungi [14-17]. A study of SSF with *A. awamori* [14] increased the total phenolic compounds 4.5 times of untreated sample by 50% ethanol extraction. The report of SSF with *M. purpureus* [15] showed that total phenolic compounds in fermented rice bran was detected 1.73 mg GAE/g dry sample, which was one fold increasing of the non-fermented sample by water extraction at 100 °C and 15 minutes.

Subcritical water extraction (SWE) is hot water or superheated water that maintains in a liquid state at the temperature between 100 °C to 374 °C (the critical temperature and pressure of water are 374 °C and 22.4 MPa) under pressurized conditions [18]. According to the breakdown of intermolecular hydrogen bonds, the dielectric constant of water decreased to nearly value of organic solvent under subcritical conditions. Therefore, the higher temperature was used for extraction of less polar compounds [19]. There are reported that the viscous of mixture was less and still had toasty aroma at the high temperature above 180 °C and 30 min., and It became more pungent odor and dark color at temperature 220 °C [7]. It was still showed that the highest protein and amino acid were found at 200 °C and 30 minutes [7]. SWE is an eco-friendly, cheap, harmless and safe extraction technique

compared to conventional organic solvent extraction [20-27]. It has been used to extract bioactive phenolic compounds which are value-added products from plants such as *Inga edulis* leaves [20], *Coriandrum sativum* seeds [21], dried red grape [22], black rice bran [23], defatted rice bran [24-26], rambutan peels [27], mango seed kernel [28] and mango peels [29].

Response Surface Method (RSM) is a set of mathematical and statistical tool for empirical modeling that aims to optimize the response (output variables), which are influenced by multiple independent variables (input variables). It has been used to find the interaction of factors that produces an optimum response. Experimental Design for determining the correction of multi-variable factors uses statistical methods. It is based on the selected models such as Central Composite Design (CCD) or Box-Behnken Design (BBK) [14, 30, 31].

The main objective of this work was to investigate effects of SSF with the mixed fungi of *A. oryzae*, *A. awamori* and *M. purpureus* on total phenolic compounds (TPC) from Defatted Riceberry Bran as the substrate. Applying response surface methodology (RSM) approach via modified Box-Behnken model was used as a tool to investigate optimal conditions with the observed response for the highest content of TPC on fermented DRB. In addition, the fermented DRB was extracted total phenolic compounds by using SWE.

## 2. Materials and methodology

### 2.1 Microorganism and inoculum

*Aspergillus oryzae* (TISTR 3082), *Aspergillus awamori* (TISTR 3193) and *Monascus purpureus* (TISTR 3541) were used in all experiments which obtained from Thailand Institute of Scientific and Technological Research, TISTR. on potato dextrose agar media. Three fungi were incubated at 32 °C for 7, 7 and 10 days, respectively. Then, 0.2% (w/v) Tween 80 was used for leaching spores. For each spore solution,  $3.5 \times 10^8$  spores/mL solutions were used for solid-state fermentation. These fungi are safe and widely used in food and cosmetic industries. The mixed cultures of fungi have potential to enhance bioactive ingredients between fungal interactions [7]; preliminary consideration is given to the total phenolic compounds.

## 2.2 Preparation of defatted Riceberry bran

Defatted Riceberry bran was obtained from Nong Sano, Phichit, Thailand. Defatted Riceberry bran was selected using a sieve shaker, the particle size was less than 0.3 mm and sterilized at 121 °C for 15 minutes.

## 2.3 Solid-state fermentation

For fermentation, mixed 70.2 g of defatted Riceberry bran (DRB), 10.5 mL of acid solution (0.01g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 3 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.1g  $\text{K}_2\text{HPO}_4$  in 1N HCl, 1L), 27.3 mL of pH solution (0.1g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 5g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 3 g  $(\text{NH}_4)_2\text{SO}_4$ , 1g  $\text{K}_2\text{HPO}_4$  in 1L distilled water, pH 5.5) and 12 mL of the total spore solution. Then it was incubated at the room temperature for 5 days and 55% moisture was controlled along experiments. In the fermentation process, the spore proportions of fungi were applied by using the modified Box-Behnken design in Table 1. After that, the fermented materials were dried at 50 °C until constant weight for extraction. All the SSF were carried out at least in triplicate.

## 2.4 Subcritical water extraction

The fermented samples were extracted the active ingredients by using subcritical water extraction. 3 g of samples and 30 g of water (1:10 ratio of solid to water) were put into the tube reactor (0.35m long, 0.015m inner diameter and 0.003 m thickness) and put into the heating bath at 200 °C for 30 minutes. At this condition, it was used to extract the total phenolic compounds, proteins and antioxidants etc. as the reported studies above [7, 32]. After extraction, the extracts were filtrated by using filter paper no.4. The extracts were kept at -4 °C for further analysis.

## 2.5 Total phenolic compounds (TPC) analysis

The total phenolic compounds were evaluated by Folin-Ciocaltea reagent according to Tang et al. [33]. The extracts were diluted in a suitable concentration. 0.5 mL of extracts were mixed with 2.5 mL of Folin's reagent (10% v/v) and allowed to react for 5 minutes in the dark. After that, the mixtures were added 2.0 mL of  $\text{Na}_2\text{CO}_3$  (7.5% w/v) and allowed to store further at 45 °C for 15 minutes. The absorbance was measured at 765 nm by using a UV-visible spectrophotometer. The TPC in each extract was determined using a standard

curve which was the equation as  $0.9699x + 0.1269$ , with the  $R^2$  equal to 0.9855. The results were expressed as mg of gallic acid equivalents per gram of dry defatted Riceberry bran (mg GAE/g DRB).

## 2.6 Design of experiments and statistical analysis

The RSM was applied to evaluate the proportions of mixed three fungi: *A. oryzae*, *A. awamori* and *M. purpureus* on TPC from defatted Riceberry bran in Table 1 and to optimize the conditions for various responses using modified Box-Behnken design. A second-order polynomial equation was used to fit the experimental data of the studied variables. The generalized second-order polynomial model used in the response surface analysis as shown in Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j \quad (1)$$

Where Y is the predicted response,  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$ , and  $\beta_{ij}$  are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and  $X_i$ , and  $X_j$  are the independent variables [30-31]. The statistical significance of the terms in the regression equations was examined by ANOVA for each response. The terms statistically found as non-significant were excluded from the initial model and the experimental data were re-fitted only to the significant ( $p < 0.05$ ) parameters using the Solver toolbox.

**Table 1 The Box-Behnken design applied for solid-state mixed fungal fermentation**

Run	Fungal proportion <sup>a</sup>			TPC (mg GAE/g DRB <sup>b</sup> )
	$X_1=A. oryzae$	$X_2=A. awamori$	$X_3=M. purpureus$	
1	-1(0)	-1(0)	0(1)	16.80±0.10
2	-1(0)	1(0.66)	0(0.34)	17.71±0.02
3	1(0.66)	-1(0)	0(0.34)	12.93±0.00
4	1(0.4)	1(0.4)	0(0.2)	14.63±0.06
5	-1(0)	0(1)	-1(0)	17.38±0.04

**Table 1 (continued) The Box-Behnken design applied for solid-state mixed fungal fermentation**

Run	Fungal proportion <sup>a</sup>			TPC (mg GAE/g DRB <sup>b</sup> )
	$X_1=A. \text{oryzae}$	$X_2=A. \text{awamori}$	$X_3=M. \text{purpureus}$	
6	-1(0)	0(0.34)	1(0.66)	19.68±0.02
7	1(0.66)	0(0.34)	-1(0)	15.85±0.02
8	1(0.4)	0(0.2)	1(0.4)	20.91±0.02
9	0(1)	-1(0)	-1(0)	18.80±0.02
10	0(0.34)	-1(0)	1(0.66)	22.08±0.02
11	0(0.34)	1(0.66)	-1(0)	23.54±0.02
12	0(0.2)	1(0.4)	1(0.4)	17.14±0.02
13	0(0.33)	0(0.33)	0(0.33)	20.13±0.00
14	0(0.33)	0(0.33)	0(0.33)	20.17±0.02
15	0(0.33)	0(0.33)	0(0.33)	20.17±0.06
Without SSF	0	0	0	12.01±0.00

<sup>a</sup> 12 mL of total spore solution added.<sup>b</sup> Mean ± standard deviation.

### 3. Results and discussion

#### 3.1 Effects of mixed fungal culture on total phenolic compounds (TPC)

Experimentally obtained values for total phenolic compounds on fermented DRB (Table 1) varied from 12.93 to 23.54 mg GAE/g DRB, while DRB without SSF gave 12.01 mg GAE/g DRB. Thus, solid-state fungal fermentation in all samples enhanced total phenolic compounds in DRB and agreement with Razak et al [15-16]. As a single culture, *A. oryzae* viewed the highest of TPC as 18.80 mg GAE/g DRB, whilst *M. purpureus* showed the least of TPC as 16.80 mg GAE/g DRB. As a mixed culture, it was found to be the maximum at 23.54 mg GAE/g DRB in 11<sup>th</sup> fermentation condition at *A. oryzae*: *A. awamori* as 0.34:0.66 and without *M. purpureus*. In addition, this work used subcritical water extraction to result in

a higher extracted total phenolic compounds than conventional extraction such as maceration and soxhlet [21-28].

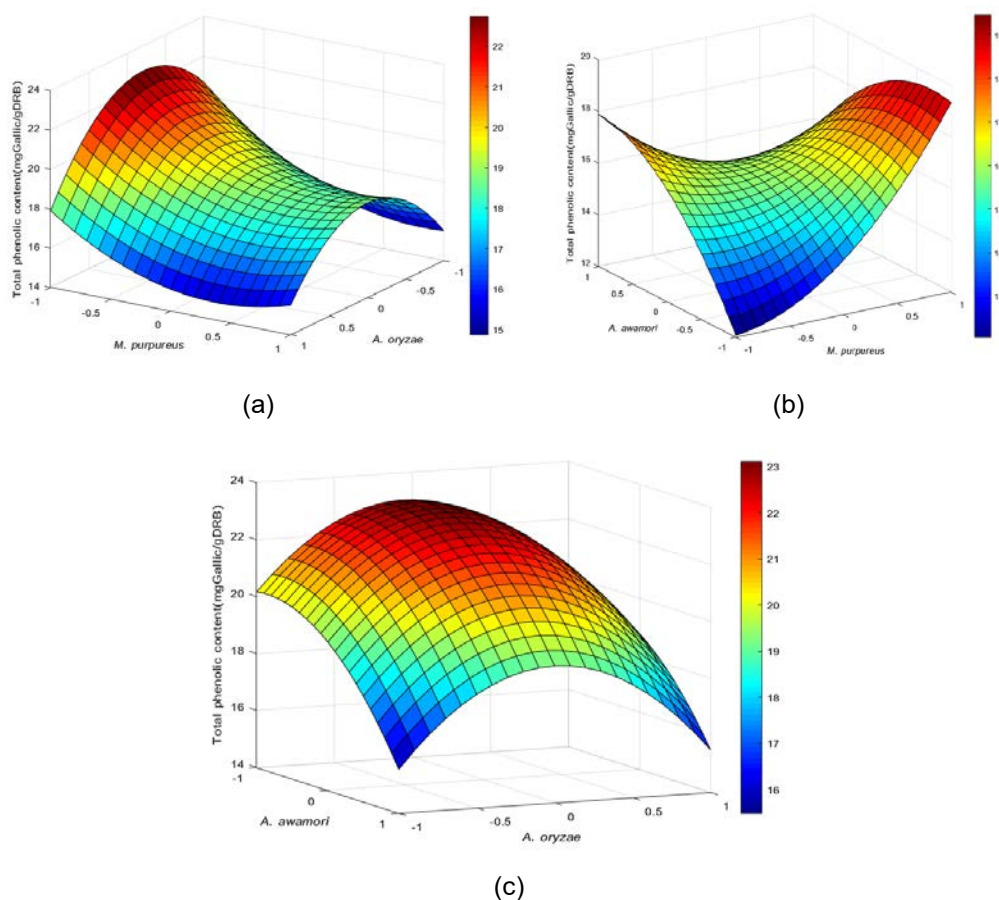
### 3.2 Influence of independent variables on investigated responses

The influence of the proportion of three fungi on TPC was reported through significant ( $p < 0.05$ ) regression coefficients of the second-order polynomial regression equation. The model for TPC on fermented DRB could predict the investigated response:

$$Y = 20.15 - 0.91X_1 + 0.30X_2 + 0.53X_3 - 3.29X_1^2 - 1.53X_2^2 + 1.59X_3^2 - 0.20X_1X_2 + 0.69X_1X_3 - 2.42X_2X_3 \quad (2)$$

Where  $Y$  = total phenolic compounds response,  $X_1$  = proportion of *A. oryzae*,  $X_2$  = proportion of *A. awamori* and  $X_3$  = proportion of *M. purpureus*.

By predicting the optimum condition of the RSM model using Solver analysis, it was found that the optimum condition for SSF was found in the coded values of *A. oryzae*, *A. awamori*, and *M. purpureus* at -0.85: -1: 0.85 (0.08: 0: 0.92 in proportion value) respectively as 20.22 mg GAE/g DRB at 95% confidence interval. The coefficient of multiple determinations for TPC response showed a good regression data of the model ( $R^2=0.82$ ). In case of enhanced TPC on fermented DRB, a linear term of *A. oryzae*, *A. awamori* and *M. purpureus* had no significant influence on the TPC and only quadratic term of had *A. oryzae* had significant influence on TPC ( $p < 0.02$ ), but there was no quadratic term with *A. awamori* and *M. purpureus*. The interaction term of them had significant influence as  $p < 0.05$  only in the *A. awamori* and *M. purpureus*, but there was no interaction in other terms. The impact of proportion of three fungi (*A. oryzae*, *A. awamori*, and *M. purpureus*) of TPC in fermented DRB is shown in Fig. 1(a)-(c).



**Figure 1** The effects of mixed fungi proportions on total phenolic compounds at (a) *A. oryzae* and *M. purpureus* with constant *A. awamori* (at 1), (b) *A. awamori* and *M. purpureus* at constant *A. oryzae* (at 1), (c) *A. oryzae* and *A. awamori* at constant *M. purpureus* (at 1)

From Fig. 1(a), it could be seen that TPC increased when the *A. oryzae* approached to 1, after that it was divergent. The *M. purpureus* decreased TPC from 1 to 0 and increased TPC from 0 to -1 at any values of *A. oryzae*. The maximum of TPC was 21.21 mg GAE/g DRB at the coded values of *A. oryzae*, *A. awamori*, and *M. purpureus* as 0: 0:-1 (0.5: 0.5: 0 in proportion values). In addition, *M. purpureus* was high accumulated of TPC from proportion 0 to 1 with absent of *A. awamori* as shown in Fig. 1(b). The behaviour of *A. awamori* was similarly *M. purpureus*. The total phenolic compounds was found to be the highest of 22.58 and 23.04 mg GAE/g DRB at the coded values of *A. awamori* and *M. purpureus* as 0, 1, -1



(0.5: 1: 0 in proportion values) and 0, -1, 1 (0.5: 0: 1 in proportion values), respectively. As shown in Fig. 1(c), the total phenolic compounds was the highest when the coded value of *A. oryzae* approached the middle values and the coded values of *A. awamori* were less. So, the proportion of *A. oryzae* and *A. awamori* on TPC also was significantly correlated.

### 3.3 Model validation

Experimental data and predicted data of total phenolic compounds showed in Table 2. The optimized condition of *A. oryzae*, *A. awamori*, and *M. purpureus* was in the coded value at -1, 0 and 1, respectively.

**Table 2 Validation of the predicted value and experimental value from the mixed cultures of fungal conditions**

Run	Total phenolic compounds (mg GAE/g DRB)		
	Predicted value	Experimental value	Relative error
<i>A. oryzae</i>	17.14	18.80±0.02	0.10
<i>A. awamori</i>	19.52	17.38±0.04	0.11
<i>M. purpureus</i>	16.32	16.80±0.10	0.03
Optimum condition (0.08: 0: 0.92)	20.22	-	-
DRB without SSF	15.65	12.01±0.00	0.23

Verification experiments were performed with the predicted conditions derived from the RSM model to determine the total phenolic compounds. It demonstrated that the experimental data were in agreement with the predicted data. It can be inferred that the RSM model was effective enough to predict the fermentation conditions (which depend on the proportions of fungi) on the total phenolic compounds that can be applied in the future.

## 4. Conclusion

The proportions of mixed cultures of fungi in the solid-state fermentation were investigated on the total phenolic compounds using RSM to predict the optimum condition.

SSF with mixed cultured of fungi were more effective on the phenolic compounds enhancement than single fungal fermentation and a non-fermented sample about 1.3 times compared to a non-fermented sample. It can be inferred that solid-state fermentation with fungi enhanced the efficiency of total phenolic compounds in the substrates. Solid-state fermentation with the mixed cultures of fungi research for addition of phenolic compounds can be applied in the fields of food and cosmeceutical industries. The predicted value was in good agreement with the experimental value. Using the RSM model is useful for predicting the optimal system conditions for the desired amount of active ingredients, which is convenient and suitable for use ahead. Additional, SWE is a non-chemical method, no residue in the product, which is a green technology.

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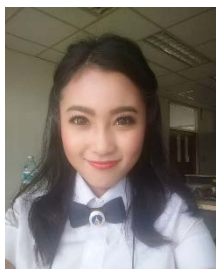
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