Production of Phenolic Compounds in Rice Bran and Defatted Rice Bran by Solid-state Fungal Fermentation using Subcritical Water/Ethanol Extraction

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Abstract

The aims of this study were to evaluate total phenolic compounds (TPC) in rice bran (RB) and defatted rice bran (DRB) which was fermented with *Aspergillus Oryzae* fungus by using Solid-State Fermentation (SSF) and investigated the optimum conditions of subcritical water extraction (SWE) for hydrolysis RB and DRB as a novel extraction technique. Response surface methodology (RSM) with Box-Behnken design was used to optimize SWE and SSF. For experiments of TPC in RB using SWE, they were performed in a batch stainless steel reactor at different temperatures ranging from 180 to 220 °C, extraction time of 15 to 45 min and concentration of ethanol 5 to 95% (v/v). The optimal conditions derived from RSM for TPC from RB and DRB using SWE were: ethanol 95 % (v/v), extraction time 30 min and temperature at 220 °C and the maximum content of TPC in RB was 62.72 mg GAE/g dry matter. Both temperature and concentration of ethanol increased TPC in RB dramatically. Results indicated that the novel extraction technique (SWE) is an appropriate technique for TPC extraction by giving a higher yield of TPC in RB and shorter extraction time than conventional technique (soxhlet). For optimization experiments of TPC in fermented DRB using SSF, they were fermented with *A. oryzae* at humidity ranging from 45 to 65 %, fermentation time of 3 to 7 days and pH for fermentation at 4 to 7. As a result, the optimal conditions for enhancement of TPC in DRB with SSF were found to be the highest as 35.56 mg GAE/g dry matter for 7 days fermentation, adjust humidity 65% and pH 5.5 which increased by more than three times with non-fermentation DRB. In conclusion, the SWE and SSF with *A. oryzae* can be an effective strategy to improve bioactive compounds in RB and DRB and the extracts from these solutions can mix and modify to macronutrients into the functionality of healthy drinks.

Keywords**:** *solid-state fermentations* (*SSF*), *Aspergillus oryzae, total phenolic compounds, subcritical water extraction, defatted rice bran (DRB), rice bran (RB)*

1. Introduction

Rice is one of the world's most important food and the staple food of more than half of the world's population. It consists of the 20% rice hull, 69.5% white rice and 10.5% rice bran (Lerma-Garcia, Herrero-Martinez, Simo-Alfonso, Mendonca, & Ramis-Ramos, 2009). The Thai community who plant rice has a problem with the cheap price of rice and rice bran. According to rice bran, it is a brown layer presented between rice grains and the outer husk of paddy, which is rich in proteins, oil and other nutrients. Rice bran was sold as an animal feed and available for the rice bran oil industry. Also, this industry has a lot of waste from the extraction process and called defatted rice bran (DRB). Rice bran has light brown color, cheap only 6-8 THB per kilogram and considered a low-price waste. DRB remained a source of nutrients such as protein and important active ingredients (Sereewatthanawut et al., 2008). These compounds in RB and DRB are valuable for health, can reduce free radicals and cholesterol in the body, prevent oxidation, reduce incidence of chronic diseases caused by free radicals and high cholesterol in the body, such as cancer, coronary heart disease (Lerma-Garcia et al., 2009; Juliano, Cossu, Alamanni, & Piu, 2005). Therefore, RB and DRB are interesting to use as raw materials and use new technology to enhance beneficial substances for health. It will be good for value added of raw materials and will be increased revenue for the community and industry.

Solid-state fermentation (SSF) by filamentous fungi is a biotechnological strategy that has enhanced plant substrates for beneficial bioactive compounds (Mc Cue & Kalidas, 2005). It is a way of providing a higher content of phenolic compounds from agro-industrial residues (Martins et al., 2011). Phenolic compounds are found in plants as defense mechanisms and with other biological functions, including metal chelation, the inhibition of pro-oxidant enzyme and antioxidant activity (Nara, Miyoshi, Honma, & Koga, 2006). They are commonly extracted from wheat bran by alkaline hydrolysis (Stalikas, 2007), acidic and enzymatic hydrolysis (Kim, Tsao, Yang, & Cui, 2006; Stalikas, 2007). SSF increases the

nutrient availability and improves their characteristics of a raw material by giving a highly effective yield, having a simple process, and increasing cost-effectiveness. Many people used SSF to produce various types of products, which increase the nutrient availability and improve their characteristics of the product. It is worth in economics because it uses the remaining waste from industry and reducing the environmental impact (Hölker & Lenz, 2005; Krishna, 2008; Shurtleff & Aoyagi, 2012). SSF with fungi will be enhanced the valuable substances that are beneficial to the body, such as pectinase, protein content, phenolic compound, antioxidants, glucoamylase and inhibition of tyrosinase activity (Sereewatthanawut et al., 2008; Baladhandyutham & Thangavelu, 2011; Schmidt, Gonçalves, Prietto, Hackbart, & Furlong, 2014; Abd Razak et al., 2017; Zambare, 2010; Martins et al., 2011; Oliveira, Cipolatti, Furlong, & Soares, 2012; Ravinder, Venkateshwar, & Ravindra, 2003; Yoswathana & Eshtiaghi, 2013). The genus of *Aspergillus* spp. has been used to the production of a traditional product that high of nutrient. This fungi known for their ability to produce a product of total phenolic compounds, antioxidant (Schmidt et al., 2014) and can increase the protein content of raw materials of low nutritional value such as in DRB (Sereewatthanawut et al., 2008). *A. oryzae* has been used to increase active ingredient in a substrate such as rice bran, corn or DRB and characterized with extending, which appear as light green and fluffy strands appearance on the substrate that the fungi inhabit. These fungi can apply in the many fields of biotechnology (Machida, Yamada, O., & Gomi, 2008). Many researchers have studied the SSF and used DRB or agro-industrial residues as a raw material. Silveira and Badiale-Furlong (2009) studied the rice bran and wheat bran used as substrate were incubated at 30°C after 3 days of fermentation, approximately 69% of protein were increased after fermentation. Zambare (2010) found glucoamylase 1271 U/gdfs can produce on rice bran used as a substrate at pH7, 30°C for 5 days of incubation. In the last few years, Sawangwan and Saman (2016) studied the amount of α-glycosidase activity and total reducing sugar incubated 7 days on rice and rice bran at 1:2 w/w, the results show the highest yield is 4.49 Unit/ml and 16.84 g/L, respectively were determined.

Subcritical water extraction (SWE) is liquid water under the critical temperature of 374 °C, but above the boiling point of 100 °C. At this state, it is enough pressure to maintain water in the liquid state (Ramos, Kristenson, & Brinkman, 2002). It increases the extraction yield effectively, decreases extraction time, gives a high quality of extract, lowers costs of the extracting agent and is technically compatible with the environment when compared to the traditional extraction technique (Yoswathana & Eshtiaghi, 2013; Teo, Tan, Yong, Hew, & Ong, 2010). The applications of subcritical fluid extraction can be divided into two main categories: extraction and conversion of biomass. Many researchers have applied this technique to extract rice bran or DRB at temperatures ranging from 180-300 °C with pressurization and viewed that it can be used to extract the active ingredient in the material, for value-added protein and amino acid in DRB. Wiboonsirikul, Hata, Tsuno, Kimura, & Adachi (2007) found that the extraction yield increased with increasing temperature and suitable conditions for extraction rice bran was evaluated at 200°C. Subsequently, Hata, Wiboonsirikul, Maeda, Kimura, & Adachi (2008) studied DRB was extracted with subcritical water at a temperature range between 180–280 ◦C for 5 min. The result showed that the higher temperature for DRB extraction, the higher amount of protein concentration and radical scavenging activity gave the highest extracts at 200 °C. Chiou, Neoh, Kobayashi, & Adachi (2011) demonstrated that the amount of antioxidant and protein increased with increasing the temperature. Watchararuji, Goto, Sasaki, & Shotipruk (2008) investigated the effect of the temperature for rice bran and soybean extraction in range of 200–220 °C and reaction time 10–30 min. It was found that the suitable temperature and time for the production of protein and amino acids were 220 °C for 30 min.

The objectives of this work were divided into 2 parts; the extraction and solid state fermentation. The first part was a comparison of total phenolic compounds (TPC) in RB and DRB extracts by using SWE and conventional methods. The optimization of SWE using Response Surface Methodology (RSM) was designed to study TPC extraction parameters as the concentration of ethanol, temperature and extraction time. The second part optimized the solid state fermentation of DRB using *Aspergillus* ssp. (*A. oryzae*) with fermentation parameters on the DRB as follows: fermentation time, humidity and pH. Then the fermented DRB was extracted by using SWE technique to extract active ingredients in the DRB. Lastly, the extracts were compared as TPC from RB, DRB before and after SSF with *A. oryzae*. The agricultural waste from the rice bran oil industry is DRB, which is interesting to use as raw material to enhance substances for health by using SWE and SSF, it will be an advantage for value added of raw material and generate an income for the community and industry.

2. Objectives

1. To investigate the optimal condition for total phenolic compounds in rice bran using SWE.
2. To study the optimal condition for Solid-state Fermentations in defatted rice bran with fungal.

3. Materials and Methods

Folin - Ciocalteu's phenol reagent, Gallic acid, Sodium carbonate, Potato dextrose agar, Bovine serum albumin (BSA), Tween 80, Sodium potassium tartarate, Copper Sulfate, Sodium acetate trihydrate were analytical grade (AR grade) and purchased from Sigma Chemical Co., Ltd. St. Louise, USA. Methanol and Ethanol were food grade and purchased from Apex Alco co., Ltd. Bangkok, Thailand.

The equipment for these experiments consist of the Subcritical water apparatus was designed in Department of Chemical Engineering of Mahidol University, UV-visible spectrophotometer (model T60 U, PG Instruments Limited)

3.1 Preparation of material

Rice brans (RB) were taken from Nakhon Pathom, Thailand. They were distributed at the same size using a sieve shaker, and then autoclaved for 15 min at 121°C. *Aspergillus oryzae* (TISTR 3082) From Thailand Institute of Scientific and Technological Research. They were cultured on potato dextrose agar (PDA) containing 1% bacteriological agar and incubated at 30 °C for 7 days.

3.2 Soxhlet

Soxhlet extractions using 15 g (dry weight) of RB with 300 ml of ethanol and hexane. RB was extracted for 3 h and temperature during extraction was set at 70-80°C. The filtrate extracts were evaporated by using a rotary evaporator.

3.3 Subcritical water extraction (SWE)

RB was extracted by SWE apparatus were designed in Department of Chemical Engineering of Mahidol University. Extraction process in a stainless steel pot with oil, heating with LPG by using stainless steel reactor tube (1.6 cm. diameter, 30 cm. length, 0.3 cm. thickness and 40 ml volume) and using a thermocouple to control temperature.

For SWE method bring 7 g (dry weight) of the sample was filled into the reactor tube and then added 35 ml of solvent to varying water-ethanol concentration. The vessel was placed on a gas burner to maintain an operating temperature. RSM with Box-Behnken design was used to determine the optimum conditions for subcritical extraction as shown in Table 1. The studied parameter ranges were: Temperature (X_1) at 180, 200, 220 °C, Ethanol concentration (X_2) at 5, 50, 95 % (v/v) and Extraction time (X_3) at 15, 30, 45 min. After extraction, the vessel was cooled down to room temperature and the extracts were filtrated through filter paper (No.4, a pore size of paper 20-25 μm). The filtrate was purred in Pyrex glass and kept in a freezer until use.

Table 1 Box-Behnken design of subcritical water extraction

Number of sample	X_1 = Temperature (°C)	X_2 = EtOH % v/v	X_3 = Time (min)
1	180	50	15
2	180	95	30
3	180	5	30
4	180	50	45
5	200	5	15
6	200	5	45
7	200	95	15
8	200	95	45
9	220	50	15
10	220	5	30
11	220	95	30
12	220	50	45
13	200	50	30
14	200	50	30
15	200	50	30

3.4 Solid-state Fermentation (SSF)

The SSF process was carried out in a laboratory-built equipment and fermented in the generated operating cabinet. The suitable conditions of fermented DRB by using RSM with Box-Behnken design consisted of 15 conditions as shown in Table 2. The studied parameter ranges were as follows: fermentation times (X_1) at 3, 5, 7 day, pH (X_2) at 4, 5.5, 7 and % fermented humidity (X_3) at 45, 55, 65.

Table 2 Box-Behnken design of solid-state fermentation

Number of sample	X_1 = Day	X_2 = pH	X_3 = % Humidity
1	3	4	55
2	5	4	65
3	5	4	45
4	7	4	55
5	3	5.5	45
6	7	5.5	45
7	3	5.5	65
8	7	5.5	65
9	3	7	55
10	5	7	45
11	5	7	65
12	7	7	55
13	5	5.5	55
14	5	5.5	55
15	5	5.5	55

In the fermentation process, twenty gram of DRB was mixed with 10 ml distilled water, autoclaved (121^{o}C, 15 min) and subsequently cooled to ambient temperature. Fungal spore (*A. oryzae)* suspensions (6×10^8 spores/gram sample) onto the surface of steamed DRB. Then adjust the percentage of humidity with distilled water at 45, 55, 65 base on dry basis, and adjust pH 4, 5.5 and 7. The SSF was carried out in stainless screen trays (size 35 cm × 35 cm) with a 1 cm thickness layer of sample and incubated at temperature 32°C in the cabinet oven. The unfermented DRB (control) was prepared without addition of spore suspension. After fermented DRB, the sample was autoclaved (121^{o}C, 15 min) and dried until the weight of sample constant. Then, it was kept in a plastic bag until extraction.

3.5 Total Phenolic Compounds (TPC)

The total phenolic content (TPC) of extracts was determined using Folin-Ciocalteu's phenol reagent (Modified from He and Xia (2006)). The SCW extracts were diluted thirtyfold with distilled water because of excessive total phenolic contents. The diluted 0.5ml aliquots from extracts were mixed with 2.5 ml Folin–Ciocalteu reagent (10%v). Then 2 ml of aqueous sodium carbonate solution (7.5% w) was added, mixed properly and incubated into an oven at 45 °C for 15 min. The samples were diluted with 5 ml of distilled water and absorbance was recorded at 725 nm against a blank. The amount of total phenolic was calculated as gallic acid equivalent (GAE) from the standard calibration curve of gallic acid and expressed as mg GAE/ g grain. Preparation of Folin-Ciocaltea's Phenol Reagent) 10%v and Na_2CO_3, 7.5%w bring 0.5 ml aliquots from standard solution with 2.5 ml Folin-Ciocaltea's Phenol Reagent mixed together and waiting 5 min after that added Na_2CO_3, 2 ml mixed together , and then heated at 45°C for 15 min. The absorbance of sample extracts and a prepared blank were measured at 765 nm using a spectrophotometer (UV detector 1000/P2000, Thermo Separation Products, California, USA). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry weight.

4. Results and Discussion

4.1 Optimization for Subcritical Water Extraction (SWE)

The effect of temperature, time and concentration of ethanol on the amount of TPC were significantly shown in Figure 1. The parameter in this study are temperature (X_1) at 180, 200, 220 °C, concentration of ethanol (X_2) at 5, 50, 95 % (v/v) mixture and extraction time (X_3) at 15, 30, 45 min. The experimental design by RSM using Box-Behnken can calculate equations to estimate TPC from RB. From

experimental data after SWE were used to calculate the coefficients of the second-order polynomial shown in equations (1). The coefficient of determining (R^2) was 0.945, indicating adequate accuracy.

$$Y = -552.854 + 4.720X_1 - 130.398X_2 + 4.180X_3 - 0.009X_{12} - 3.546X_{22} + 0.010X_{32} + 0.708X_1X_2 - 0.023X_2X_3 + 0.205X_1X_3 \quad (1)$$

Effects of temperature (°C), concentration of ethanol and extraction time on amount of TPC as shown in Figure 1.

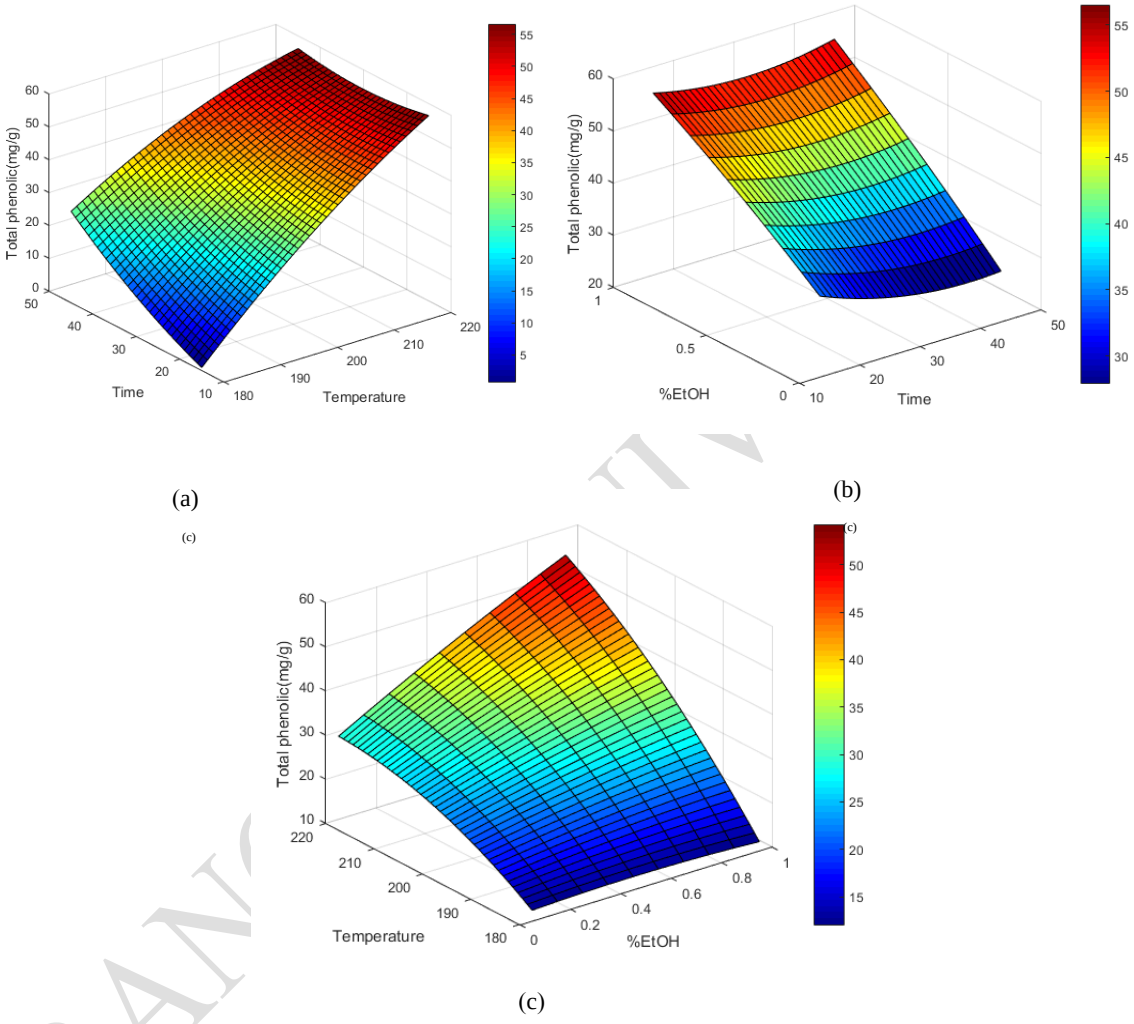

Figure 1 Amount of total phenolic compounds in rice bran by using subcritical water extraction at different condition for extract (a) at concentration of ethanol 95% (v/v), (b) at temperature 200 °C, and (c) at time 30 min

Figure 1(a) depicted response surface of the effects of the two variables, extraction time and temperature on RB extracts. The extraction temperature was a major important factor that could influence the TPC extraction content. It had shown that a higher temperature favored the TPC extraction from plants (Yoswathana & Eshtiaghi, 2013; Tunchaiyaphum, Eshtiaghi, & Yoswathana, 2013). When the extraction temperature at 220°C was showed the maximum yield of TPC at all the extraction times. As shown in Figure 1(b), the higher concentration of ethanol and longer extraction times displayed a positive effect on the TPC content. From Figure 1(c), the increased TPC content was likely due to the rising extraction temperature and high concentration of ethanol which could increase the diffusion rate from the solid phase

to the liquid phase and improve cell wall damage and decompose cell wall material (Carr, Mammucari, & Foster, 2011).

According to the result from other researchers (Chantaravichit & Ongard, 2015), it showed the ethanolic solvent 95% v/v had the highest TPC extracts. From this experiment, the optimal conditions for RB extraction were ethanol concentration 95% (v/v) and 220°C and 30 min and gave the maximum content of TPC 62.72 mg GAE/g dry matter.

4.2 Comparison of total phenolic compounds (TPC) from various extraction methods

The TPC on RB was extracted at different extraction methods as follows: the conventional method using soxhlet with hexane and ethanol 95% (v/v) and the novel method as subcrition water/ethanol extraction and presented in Figure 2.

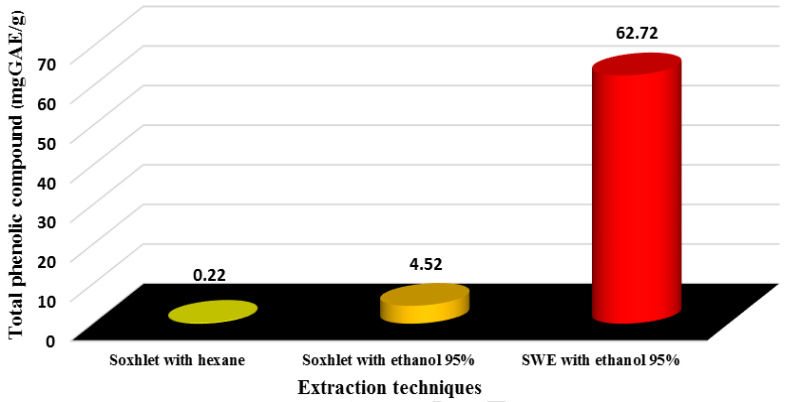

Figure 2 Comparison of total phenolic compounds from different extraction techniques

From Figure 2, the extracts TPC in RB at different extraction techniques as follows: SWE (220°C, 30 min), soxhlet with hexane (70°C, 3 h) and soxhlet with ethanol 95% (v/v) (70°C, 3 h) were compared and demonstrated that TPC content were 62.72, 0.22 and 4.52 mg GAE/g dry matter, respectively. From the results showed TPC from SWE higher than from soxhlet extraction. SWE might be an alternative green technology give the high yield efficiency of TPC extraction, which substitute the conventional methods (soxhlet).

4.3 Optimization for Solid-state fermentation (SSF)

The effect of time for fermentation, humidity and pH were relative significant as shown in Figure 3. Response surface methodology was (RSM) a good tool for optimization of extraction conditions (Zhu, Heo, & Row, 2010; Shi et al., 2003; Karacabey & Mazza, 2010). The experimental design data of TPC in SSF with DRB using SWE (220°C, 30 min, and concentration of ethanol 95% (v/v) were used to calculate the coefficients of the second-order polynomial in equations (2). The coefficient of determining (R^2) was 0.920 and indicated adequate accuracy.

$$Y = -80.345 - 11.190X_1 + 32.845X_2 + 1.691X_3 + 0.432X_{12} - 2.573X_{22} - 0.011X_{33} + 0.587X_1X_2 + 0.073X_1X_3 - 0.142X_2X_3 \quad (2)$$

The times of fermentation (days), pH values and % humidity viewed effects on the amount of TPC on RB as shown in Figure 3.

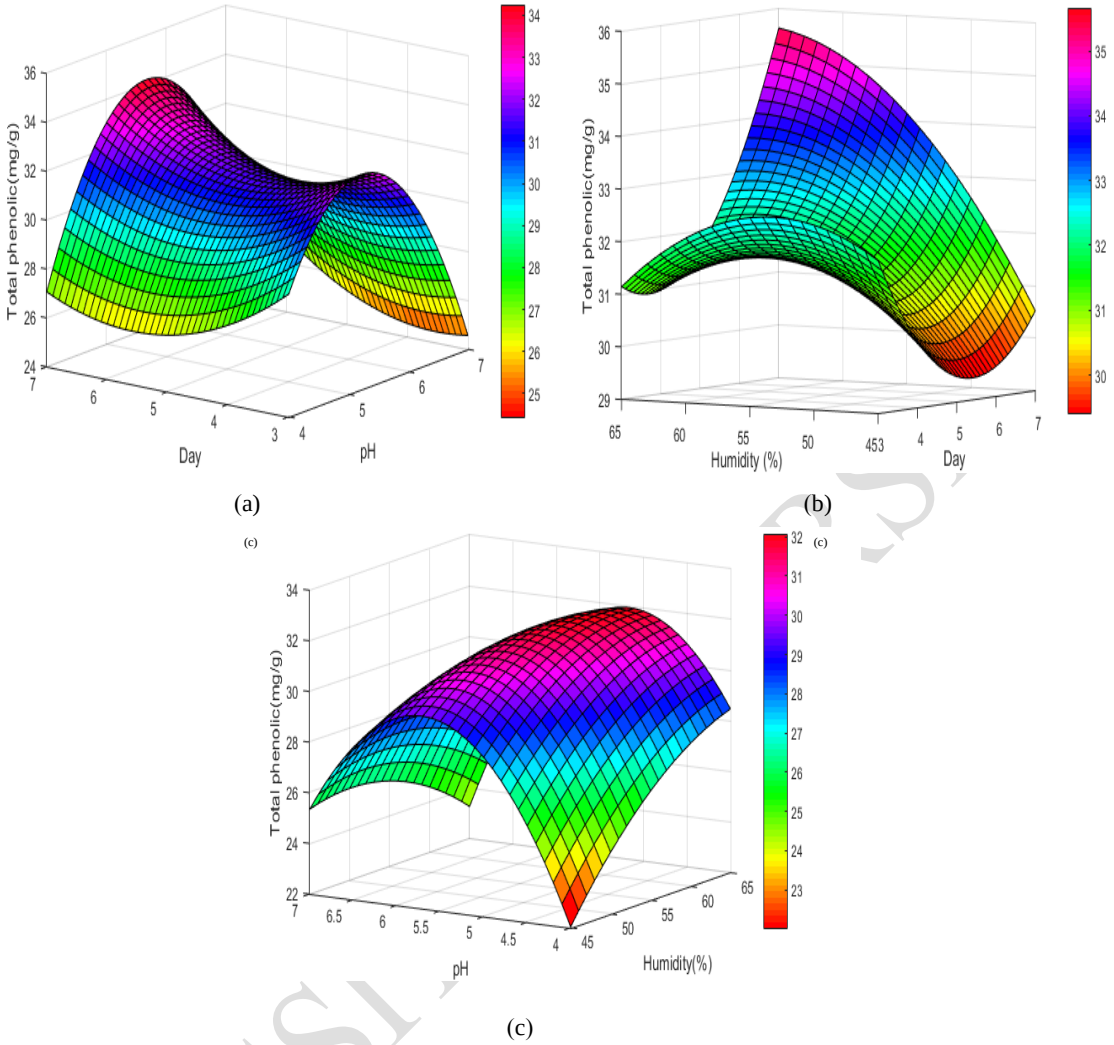

Figure 3 Amount of total phenolic compounds in fermented defatted rice bran at different condition for fermentation (a) at 55% humidity, (b) at pH 5, and (c) at fermentation time 5 days

The effect of fermentation time and pH on TPC of RB extracts was shown in Figure 3(a), the pH value displayed a quadratic response on TPC content at maximum of pH 5.5 at all fermented times. At constant fermentation time, the TPC content increased with increasing pH value until pH 5.5 and turnover plots with decreasing TPC content. From Figure 3(b), the fermented times in the range between 3-5 days had given TPC maximum content at humidity 55%. After 5 days of fermented time, the fungal growth rate increased with increasing the humidity. In general, the increasing fermentation time can increase the rate of fungi growth and give TPC content too. Hence, the highest content of TPC occurred within 7 days of fermentation and adjust 65% of humidity. As shown in Figure 3(c), the suitably condition of pH 5.5 at any humidity reached the maximum of TPC content, the less or higher pH 5.5 indicated a descending tendency subsequently. The equation (2) can predict the optimal conditions for TPC maximum content from SSF with *A. oryzae* on surface of DRB and give TPC content as 35.67 mg GAE/g dry matter with the fermented time for 7 days, 65% of humidity and pH 5.5. Meanwhile the experiment obtained TPC content as 35.56 mg GAE/g dry matter. As a result, it showed the amount of TPC from the equation and the experiment was not significantly different.

4.4 Comparative total phenolic compounds from different raw material using subcritical water extraction

The amount of TPC in the RB, non-fermented DRB, fermented DRB and overall TPC in RB and DRB with the optimum condition for SWE using the ratio 1:5 (g of sample: ml of solution) at a temperature of 220 °C for 30 min were compared in Figure 4. The DRB was fermented with the optimum conditions (*A. oryzae* 6.8×10^8 spores / g material, fermentation time of 7 days, 65% humidity and adjust the pH 5.5).

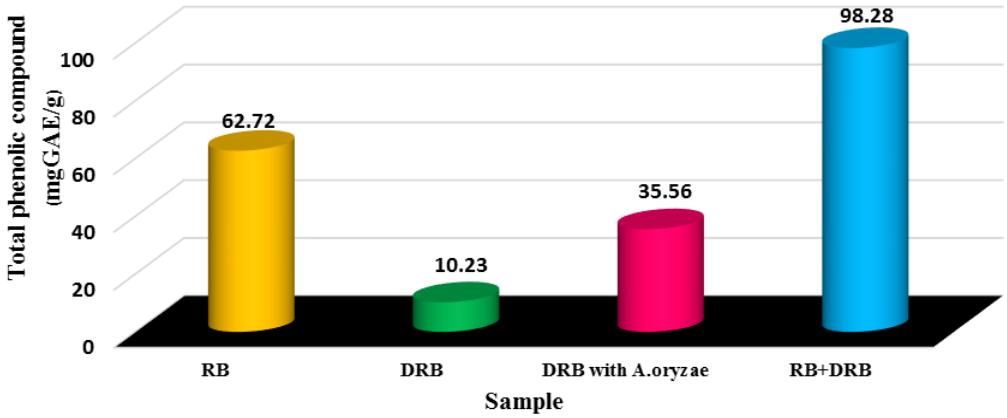

Figure 4 Total phenolic compound of subcritical water extraction from rice bran, non-fermented defatted rice bran, defatted rice bran fermented with *A. oryzae* and overall total phenolic compounds from novel extraction techniques

As shown in Figure 4, TPC content from RB using subcritical water/ethanol extraction obtained 62.72 mg GAE/g dry matter. After the removing oil from RB, it still remained TPC on DRB as a source of carbon suitable for fungi growth and gave high efficiency for enhanced TPC. The solid state fermentation of DRB with *A. oryzae* can increase the amount of TPC on DRB from 10.23 to 35.56 g GAE/g dry matter. *A. oryzae* used substrate in DRB to produce many enzymes and cut the molecule of substrate for digestion and enhanced TPC content after fermentation. This suggestion is supported by other studies (Schmidt et al., 2014) which reported TPC of RB was also modified during fermentation with fungi which increased with increasing the fermented time. From this work, the overall TPC extracts from subcritical water/ethanol extraction technique from RB and fermented DRB were 98.28 mgGAE/g dry matter.

5. Conclusion

The subcritical water/ethanol extraction as an alternative technique successfully extracted total phenolic compounds (TPC) on RB and fermented DRB, which give a higher yield and shorter extraction time than conventional techniques. The response surface methodology with Box-Behnken design was used and proved to be useful for the optimization of the TPC extraction from RB and fermented DRB with *A. Orysae*. The experimental TPC values agreed with the prediction TPC values. The solid state fermentation process of DRB with *A. Orysae* can increase TPC three times in DRB. It is therefore recommended that further purification, characterization, the action of active ingredient extracts for potential of functional food or cosmetic and also cost analysis for economics could be studied.

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