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Production of β-Carotene-Enriched Rice Bran Using Solid-State Fermentation of *Rhodotorula glutinis*

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This work was aimed at utilizing rice bran as a substrate for β-carotene production by *Rhodotorula glutinis* DM 28 under optimized conditions of solid-state fermentation. The biomass and β-carotene content of *Rhodotorula glutinis* DM 28 grown on rice bran as a sole substrate under solid-state fermentation were 54 g/kg rice bran and 1.65 mg/kg rice bran, respectively. Its biomass and β-carotene content, however, could be improved by 60% and 30%, respectively, using the Central Composite Design for the optimization of its cultivation conditions. The optimized conditions obtained were a pH of 5, a moisture content of 70% (w/w), and a carbon-to-nitrogen ratio of 4. Under these conditions, rice bran containing *R. glutinis* DM 28 had nutritional values of β-carotene, protein, and fat higher than those of rice bran alone. Yeast-grown rice bran could be suitable, therefore, to use as a β-carotene-enriched supplement in animal feeds.

Keywords: β-Carotene, *Rhodotorula glutinis*, rice bran, animal feed, optimization

β-Carotene is an orange-yellow pigment that can be used as a coloring agent, a source of provitamin A, and as an antioxidant agent [3]. It is used commercially in the food, feed, cosmetic, and pharmaceutical industries [18]. In the animal feed industry, β-carotene is employed as a feed additive for fish and laying hens to enhance the color of their flesh and egg yolk, respectively. At present, however, the pigment used in these industries is mostly chemically synthesized. Natural β-carotene is becoming increasingly attractive in these industries because of consumer demand. It can be found in fruits, flowers, vegetables, and animal tissues and it is produced by algae (*i.e.*, *Dunaliella bardawil* and *Murielopsis* sp. [13]), fungi (*i.e.*, *Blakeslea trispora* [12]), and yeast (*i.e.*, *Rhodotorula glutinis* [5, 16, 24, 31]). Among microbial sources, *Rhodotorula glutinis* is widely known to produce β-carotene at a relatively high growth rate and is a good source of proteins, lipids, and vitamins [5]. This yeast is suitable, therefore, for use as a feed additive.

Rice bran is a by-product of the rice-milling industry. Approximately 1–1.5 million tons per year are generated in Thailand. It is a low-cost source of high contents of fat, protein, carbohydrate, and vitamins [27]. It generally is used, therefore, as an animal feed additive and as a source of cooking-oil and vitamin E. It is also popularly employed as a substrate for microbial enzyme production using solid-state fermentation. Solid-state fermentation is a simple technique for microbial growth and its metabolite production of single-cell proteins, ethanol, enzymes, organic acids, and antibiotics [23]. The technique has an advantage over other fermentation methods since it does not require complex equipment and sophisticated control systems. This results in lower production costs.

Animal feeds are normally composed of agricultural wastes such as rice bran, corn, soybean meal, and fish meal or feather meal as complex nutrients. Responding to consumer demand for appealing food products, aquaculture and chicken feeds usually contain β-carotene. The addition of this feed supplement, however, creates an added expense for farmers. Adding safe and nontoxic *Rhodotorula* cell mass to animal feeds can increase the β-carotene level in the feeds [10]. This requires a large cultivation of yeast, however, which could be costly and complicated. A possible alternative approach is to cultivate yeast-producing β-carotene *Rhodotorula* sp. directly on rice bran serving as a substrate for yeast growth and a raw material for animal feed. With yeast biomass incorporated in the rice bran, this could also increase protein, lipid, and vitamin contents in the animal feed.

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Response Surface Methodology (RSM) is a powerful statistical technique generally applied to improve carotenoid yields by microorganisms under optimum conditions [7, 25, 32]. The optimization study of β-carotene production by *R. glutinis* was successfully performed using concentrated rectified grape must and sugar cane molasses as a main component in a medium [6, 24]. Using rice bran as a substrate, however, has not been previously reported.

The purpose of this work was therefore to produce rice bran containing increased levels of β-carotene produced by *R. glutinis* DM 28 under optimized conditions of yeast fermentation and to evaluate the nutritional values of the yeast-grown rice bran.

Materials and Methods

Microorganism, Medium, and Substrate

Rhodotorula glutinis DM 28 was used from a microbial culture collection of the Department of Microbiology, KMUTT. It was isolated from fermented vegetable brine and was found to produce β-carotene [17, 31]. The yeast was maintained on yeast and malt extract agar slants (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l glucose, and 15 g/l agar) at 4°C until used.

Rice bran was obtained from rice (*Oryza sativa* Linn.) kindly provided by the Royal Chitralada Projects and kept at 4°C until used.

Yeast Inoculum Preparation

One loopful of a 24-h-old slant culture was inoculated into 250-ml Erlenmeyer flasks containing 50 ml of yeast and malt extract medium. The flasks were incubated at 30°C on a rotary shaker operated at 150 rpm for 18 h. After incubation, yeast cells were centrifuged at 6,000 ×*g*, 4°C for 10 min, washed with sterile distilled water, and collected by centrifugation. The washing procedure was done twice. The cells were resuspended in sterile distilled water to make a final cell concentration of 10^8 cells/ml.

Rice Bran Composition

Rice bran was dried at 70°C for 2 h and cooled in a desiccator. Moisture, pH, and ash, protein, nitrogen, total carbon, fat, and fiber contents in the rice bran were analyzed according to the procedure described in AOAC [2].

Growth of *R. glutinis* DM 28 on Rice Bran and Its β-Carotene Production

One ml of freshly prepared *R. glutinis* DM 28 (10^8 cells) was transferred to each of the 250-ml Erlenmeyer flasks containing 5 g of sterile rice bran with an added 3.35 g of sterile distilled water to adjust the initial moisture content of the rice bran to 50% (w/w). The flasks were then shaken well and incubated at 30°C for 12 days. During incubation, the flasks were manually shaken twice a day. Samples were removed periodically for yeast growth and β-carotene determination.

Growth of *R. glutinis* DM 28 on Rice Bran and Its β-Carotene Production Using Various Ratios of Carbon to Nitrogen

Growth of *R. glutinis* DM 28 on rice bran and its production of β-carotene were investigated by varying the ratios of carbon to nitrogen (4, 6, 10, and 15) by adding suitable amounts of sucrose and ammonium sulfate as the carbon and nitrogen sources, respectively. Rice bran having various ratios of carbon to nitrogen was autoclaved at 121°C for 15 min and given an initial moisture content of 50% (w/w) by appropiate additions of sterile distilled water and inoculum. The rice bran was inoculated with 1 ml of yeast cell suspension (10^8 cells) and incubated at 30°C for 7 days. Samples were taken at days 4 and 7 for yeast growth and β-carotene analyses.

Optimization of Cultivation Conditions for the Growth of *R. glutinis* DM 28 on Rice Bran and Its β-Carotene Production

Studies on the optimization for growth of *R. glutinis* DM 28 and its production of β-carotene were designed by the Central Composite Design (CCD) [20] and data were analyzed by the Response Surface Methodology (RSM) method using the Minitab software (Minitab Inc., State College, PA, U.S.A.). Five levels of three important factors (x), including pH of rice bran (x_1), moisture content of rice bran (x_2), and the ratio of carbon to nitrogen in rice bran (x_3), were evaluated for their effects on cell dry weight and β-carotene production (responses, y). A total of 20 experiments designed from the CCD is shown in Table 1. Other parameters were controlled as mentioned in the previous section. All experiments were carried out at 30°C for 7 days. After data had been analyzed and an optimum condition was reached by an assumption of a regression model, the actual experiments were performed to confirm the predicted results.

Nutritional Values of Yeast-Grown Rice Bran

R. glutinis DM 28 was cultivated on rice bran under the optimum conditions for 7 days. Yeast-grown rice bran was analyzed for ash, protein, fat, fiber, and phosphorus contents according to the AOAC methods [2] and for β-carotene content.

Chemical and Statistical Analyses

Yeast growth was determined by total plate count on yeast malt extract agar. The cell numbers were then transformed into biomass as dry cell weight using a calibration curve (R^2=0.98) obtained by growing yeast cells on yeast malt extract medium. Calculation of yeast cell dry weight was done by centrifuging samples at 6,000 ×*g* for 10 min at room temperature, washing twice with distilled water, and drying the cell pellet at 85°C for 24 h or until constant weight.

β-Carotene extraction from yeast biomass grown on rice bran was performed by a modified method of Calo *et al.* [9] using dimethyl sulfoxide (DMSO) for cell wall disruption, and petroleum ether and acetone. Briefly, rice bran with the biomass was dried in flasks at 45°C for 24 h. Dried rice bran and biomass were suspended in 15 ml of DMSO (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 15 ml of acetone (Mallinckrogt Baker, Philipsburg, NJ, U.S.A.). The suspension was agitated on a rotary shaker operated at 250 rpm and at 40°C for 2 h and was then filtered through glass wool to remove cell discard and rice bran. The clear filtrate was mixed with 10 ml of petroleum ether (Mallinckrogt Baker, Philipsburg, NJ, U.S.A.). The organic phase was separated by centrifugation for 5 min [28] and the pigment-containing phase was recovered. Finally, the samples were filtered through Scheringer membranes with 0.45 mm pore size and stored at −20°C until analysis.

β-Carotene was analyzed by HPLC [21] with a modification using an ODS Hypersil column (5 μm, 125×4 mm) (Hewlett Packard, Waldbronn, Germany) and a mobile phase of acetonitrite:methanol (10:90 v/v; Merck, Darmstadt, Germany) with a flow rate of 0.5 ml/min. The eluant was monitored at 450 nm. β-Carotene purchased from Sigma-

Table 1. Actual and predicted values of yeast growth and β-carotene production from *Rhodotorula glutinis* DM28 on rice bran on day 7, obtained by the Central Composite Design.

Experiment	Actual value			Response (actual)		Response (predicted)	
	pH[a] (x_1)	Moisture[b] (%, x_2)	C:N ratio[c] (x_3)	Dry weight (g/kg rice bran)	β-Carotene (mg/kg rice bran)	Dry weight (g/kg rice bran)	β-Carotene (mg/kg rice bran)
1	6	40	6	70.2	1.71	69.6	1.69
2	8	40	6	26.6	1.23	37.1	1.30
3	6	60	6	57.9	1.61	53.2	1.66
4	8	60	6	26.3	1.25	20.7	1.27
5	6	40	10	28.5	1.27	32.7	1.31
6	8	40	10	56.7	1.58	54.9	1.56
7	6	60	10	22.4	1.16	16.3	1.12
8	8	60	10	28.4	1.28	38.5	1.37
9	5	50	8	32.4	1.37	36.8	1.38
10	9	50	8	32.5	1.31	26.6	1.25
11	7	30	8	80.2	1.68	74.8	1.67
12	7	70	8	38.1	1.48	42.0	1.44
13	7	50	4	38.3	1.46	39.2	1.42
14	7	50	12	22.5	1.16	20.1	1.15
15	7	50	8	40.5	1.50	41.7	1.49
16	7	50	8	54.1	1.55	41.7	1.49
17	7	50	8	34.4	1.41	41.7	1.49
18	7	50	8	42.2	1.52	41.7	1.49
19	7	50	8	38.3	1.47	41.7	1.49
20	7	50	8	42.2	1.51	41.7	1.49
Optimum	5	70	4	88.2	2.12	89.3	2.02

[a]Adjusted by using 0.1 N NaOH or 0.1 M HCl.
[b]Adjusted by adding an appropiate sterile distilled water.
[c]Adjusted by using sucrose or ammonium sulfate.

Aldrich (St. Louis, MO, U.S.A.) was used as an external standard. The β-carotene content in yeast, calculated by excluding the β-carotene content in rice bran, was reported. Total carotenoid concentrations in the pigmented layer were also quantified spectroscopically at an optical density of 450 nm, using an $E^{1\%}_{1cm}$ value of 2,590 [26]

Triplicate experiments were performed and the mean results were reported. The ANOVA and the Least Statistical Difference (LSD) tests were performed according to Montgomery [20] using the SPSS software (SPSS Inc., Chicago, IL, U.S.A.) in order to determine significant differences between the treatments.

RESULTS AND DISCUSSION

Rice Bran Composition

Rice bran in the current study was a fine dried powder that was slightly acidic (pH of 6.2). It contained 0.3% (w/w) moisture, 8% (w/w) ash, 11.4% (w/w) protein, 6.1% (w/w) fiber, 17.8% (w/w) fat, 1.9% (w/w) phosphorus, and a small amount of β-carotene (Table 2). Its main components were comparable to those of other reports [4, 27]. In general, the differing composition of rice bran from various sources may be due to differences in rice types, milling processes, processing time, and storage time. Nevertheless, the investigated rice bran contained nutrients that could be used as a substrate for *R. glutinis* DM 28.

Growth of *R. glutinis* DM 28 on Rice Bran and Its β-Carotene Production

R. glutinis DM 28 grew rapidly during the first 3 days and maximally on day 6 in rice bran having a yeast biomass of

Table 2. Compositions of rice bran and *R. glutinis* DM 28-grown rice bran.

Composition	Rice bran (g/kg rice bran)	Rice bran containing *R. glutinis* DM 28 (g/kg rice bran)
Protein	114.2[a]	116.2[b]
Fat	177.5[a]	178.9[b]
Fiber	61.0[a]	61.1[a]
Ash	80.2[b]	76.1[a]
Phosphorus	19.2[a]	19.2[a]
β-Carotene (mg/kg)	0.26[a]	2.12[b]

Different letters (a, b) in the same rows indicate the significant differences ($p<0.05$)

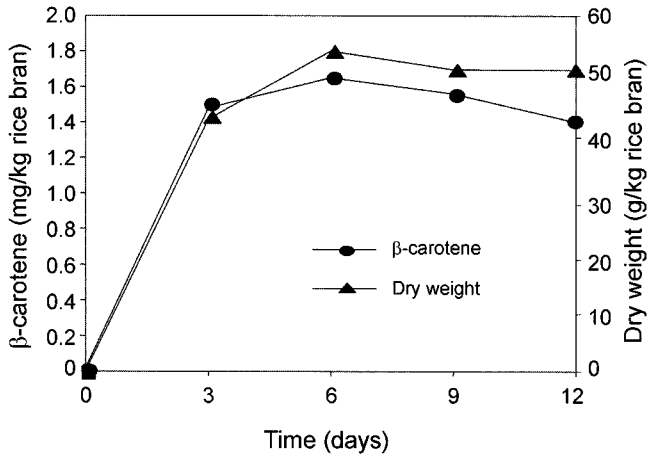

Fig. 1. Growth of *Rhodotorula glutinis* DM 28 grown on rice bran and its β-carotene production.

54 g/kg rice bran (Fig. 1). After that, the growth rate declined slightly owing to a reduction in moisture content and the presence of nutrients in the rice bran that were not conducive to yeast growth.

The β-carotene content increased on day 6 of the incubation in rice bran, having a maximum content of 1.65 mg/kg rice bran (Fig. 1) and declined thereafter. It seems that β-carotene was produced during the period of yeast growth and reached a maximum at the late log phase, as had been previously reported [5, 16]. Interestingly, β-carotene produced by *R. glutinis* DM 28 accounted for 60% of the total carotenoid production. The β-carotene production by this strain was relatively much higher than that of other wild strains as reported by Bhosale and Gadre [5]. Most other *Rhodotorula glutinis* strains typically produced higher amounts of torulene and torularhodin, with smaller amounts of β-carotene [5, 8].

To our knowledge, this study is the first to demonstrate that β-carotene could be produced by a species of *R. glutinis* with rice bran as the sole substrate. It was observed, however, that owing to a limitation of growth factors such as pH, moisture content, the ratio of carbon to nitrogen, mixing, aeration, and oxygen transfer on solid-state fermentation, its yield (30.6 μg β-carotene/g biomass) was not comparable to that reported in the literature for submerged fermentation using low-cost substrates from agricultural sources and food-processing wastes such as sugar cane juice [18], peat extract [19], whey [1, 11], sauerkraut brine [29], grape must, beet molasses, soybean and corn flour extracts [8], sugar cane, and molasses [1, 5, 24]. The selected factors therefore were investigated to improve its production.

Growth of *R. glutinis* DM 28 on Rice Bran and Its β-Carotene Production Using Various Ratios of Carbon to Nitrogen

The ratio of carbon to nitrogen was reported to be the most important factor affecting *R. glutinis* growth and β-carotene production in a medium containing molasses, urea, and KH_2PO_4 as main components [24]. A study on various ratios of carbon to nitrogen in rice bran was carried out. The rice bran had a total carbon content of 13.9% (w/w) and a nitrogen content of 2.5% (w/w). The ratio of carbon to nitrogen in the rice bran used in this study equaled 5.5. Sucrose and ammonium sulfate were added to the rice bran because all yeasts can utilize sucrose and ammonium sulfate as carbon and nitrogen sources, respectively [26].

Table 3 shows the results of yeast growth and β-carotene production in rice bran for various ratios of carbon to nitrogen on days 4 and 7. On day 4, maximum growth and β-carotene production occurred with carbon-to-nitrogen ratios of 6 and 10, with a cell biomass of 47.8–48.3 g/kg rice bran and β-carotene production of 1.55–1.59 mg/kg rice bran, whereas on day 7, the optimum ratio was at 6 with a dried cell weight of 54.2 g/kg rice bran and β-carotene production of 1.61 mg/kg rice bran. In a previous report, however, a carbon-to-nitrogen ratio of 8 in lettuce brine yielded the maximum β-carotene production by *R. glutinis* [31]. Additionally, a carbon to nitrogen ratio of 10 was found to provide the maximum β-carotene production by *R. glacilis* in semidefined minimal salt media [30].

The results indicated that differing ratios of carbon to nitrogen influenced *R. glutinis* growth and its β-carotene production. The ratios of 6 and 10, however, showed no significant difference in *R. glutinis* growth and β-carotene production on days 4 and 7 (Table 3). A ratio of 8, as a

Table 3. Effect of the ratio of carbon to nitrogen on yeast growth and β-carotene production on rice bran.

C:N ratio	4 Days		7 Days	
	Dry weight (g/kg rice bran)	β-Carotene (mg/kg rice bran)	Dry weight (g/kg rice bran)	β-Carotene (mg/kg rice bran)
4	44.4^{b}	1.46^{b}	48.3^{c}	1.57^{a}
6	47.8^{a}	1.59^{a}	54.2^{a}	1.61^{a}
10	48.3^{a}	1.55^{a}	52.5^{b}	1.59^{a}
15	42.0^{c}	1.45^{b}	52.9^{b}	1.46^{b}

Different letters (a, b, and c) in the same columns show the significant differences ($p<0.05$).

midpoint between 6 and 10, was therefore selected for the design of the CCD experiments (Table 1).

Optimization of Cultivation Conditions for the Growth of *R. glutinis* DM 28 on Rice Bran and Its β-Carotene Production

Apart from the ratio of carbon to nitrogen, pH and moisture were selected to improve the growth and β-carotene production of *R. glutinis* DM 28 on rice bran because both factors are essential for microbial growth by solid-state fermentation [23]. The results obtained from the CCD experiments are shown in Table 1. At a low carbon-to-nitrogen ratio, 6, increasing the pH reduced growth and β-carotene production. In contrast, at the higher carbon-to-nitrogen ratios, 8 and 10, increasing the pH enhanced growth and β-carotene production. Moreover, increasing both the carbon-to-nitrogen ratios and the moisture content resulted in decreased growth and β-carotene production. The results suggested that these three factors and their interactions affected yeast growth and β-carotene production.

Response Surface Methodology was employed for statistical analysis, and the results revealed that pH (x_1), moisture content (x_2), and carbon-to-nitrogen ratio (x_3) affected the growth of *R. glutinis* DM 28 on rice bran and its β-carotene production, as shown in quadratic regression model Eqs. (1) and (2). Additionally, the carbon-to-nitrogen ratios and pH interaction also influenced the growth of *R. glutinis* DM 28, as described in Eq. (1) and Fig. 2A. Furthermore, the three variables and their interactions affected the β-carotene production of *R. glutinis* DM 28 [Eq. (2), Figs. 2B and 2C].

$$\text{Dry weight}=41.70-2.55x_1-8.20x_2-4.79x_3-2.50x_1^2 +4.18x_2^2-3.01x_3^2+13.68x_1x_3 \quad (1)$$

$$\beta\text{-Carotene}=1.4-0.033x_1-0.056x_2-0.069x_3-0.042x_1^2 +0.0.18x_2^2-0.05x_3^2+0.16x_1x_3-0.041x_2x_3 \quad (2)$$

The same optimum conditions predicted by Eqs. (1) and (2) for the growth of *R. glutinis* DM 28 on rice bran and its β-carotene production were found at a pH of 5.0, a moisture content of 70% (w/w), and a carbon-to-nitrogen ratio of 4, with the predicted values of 89.3 g/kg rice bran and 2.02 mg/kg rice bran, respectively (Table 1). The optimum condition at pH 5 produced a high growth rate of *R. glutinis* and its β-carotene production because enzymes associated with nutrient transportation were active [26]. This result was in accordance with a report of Martin *et al.* [19] that *R. glutinis* showed high β-carotene production at pH 4.0–4.7. Moisture content in a substrate is also one of the critical factors for microbial growth and production on solid substrates. In general, bacteria and yeast can grow on solid substrates at the 40–70% (w/w) moisture levels with a free water requirement [23].

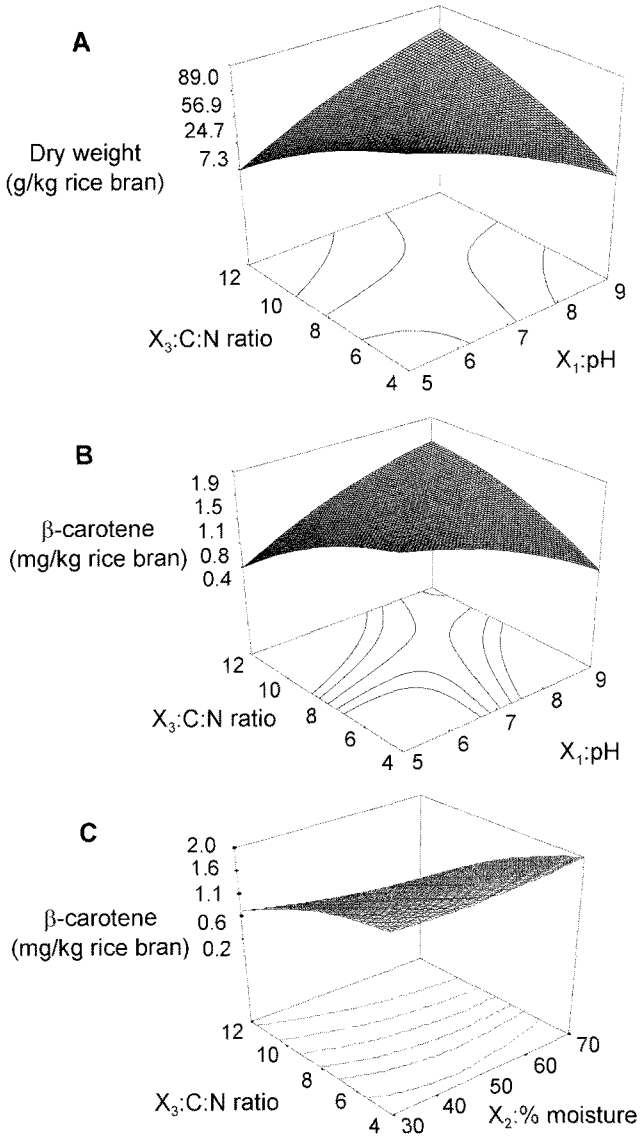

Fig. 2. Response surface of variables on growth (**A**) and β-carotene production (**B** and **C**) by *Rhodotorula glutinis* DM 28 on rice bran.

Under the optimum condition, the actual growth and β-carotene production from experiments were in good agreement with their predictions, as shown in Table 1. The results indicated that, under this condition, the yeast yielded 60% greater growth and 30% higher β-carotene production than those obtained from the initial condition.

Nutritional Values of Yeast-Grown Rice Bran

Table 2 reveals the composition of rice bran alone and rice bran containing *R. glutinis* DM 28. The latter contained amounts of β-carotene, protein, fat, and ash significantly different from that of the former, whereas the fiber and phosphorus contents were the same. The β-carotene content in yeast-cultured rice bran showed an increase approximately

8 times greater than that in rice bran alone. The increase in protein and fat was due to yeast biomass containing 45–50% (w/w) protein and 4–7% (w/w) fat [14]. *R. glutinis* DM 28-grown rice bran would also provide better protein quality than rice bran alone. The yeast contains several essential amino acids such as lysine [2.3% (w/w)] [26], whereas rice bran alone has only 0.5–0.6% (w/w) lysine [4, 27]. In addition, rice bran combined with yeast would yield an improved vitamin content, because yeast biomass is a good source of vitamins [14]. The fiber content in both samples, however, was similar owing to the fact that only a trace amount of fiber is found in yeast biomass. The yeast also consumes nutrients in rice bran, resulting in a lower amount of ash as compared with the yeast-free rice bran.

R. glutinis DM 28 cultivated in rice bran did not reduce the rice bran quality. It improved certain nutritional values in the rice bran, particularly β-carotene, protein, fat, and vitamins. The concentration of β-carotene present in *R. glutinis* DM 28-grown rice bran (2.12 mg/kg rice bran) would be suitable for some animal feeds since the β-carotene concentration generally required in poultry diets is 2.4 mg/kg feed [15, 22]. The use of *R. glutinis* DM 28-grown rice bran would offer an advantage over the addition of a typical yeast into animal diets, such as *S. cerevisiae*, in that *S. cerevisiae* provides only protein and vitamins. It would also have more benefit than the direct addition of *R. glutinis* DM 28 biomass into animal feeds, because large-scale yeast fermentation and biomass recovery would be costly. Rice bran cultured with *R. glutinis* DM 28 would be, therefore, a high-nutritional supplement for animal feeds.

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