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Optimum Fermentation Condition of Soybean Curd Residue and Rice Bran by *Preussia aemulans* using Solid-State Fermentation Method

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Abstract

An environmental method for using soybean curd residue (SCR) and rice bran (RB) was developed in this study. SCR and RB were utilized as growth medium for *Preussia aemulans,* a new fungus isolated from *Cordyceps sinensis* fruiting body. According to Orthogonal test and Duncan's multiple range test, the optimum fermentation condition of fermented SCR and RB for producing polysaccharide, adenosine and ergosterol were summarized. Under the optimum fermentation condition of SCR, the polysaccharide, adenosine and ergosterol contents were reached to 39.18 ± 1.06 mg/g dry matter, 127.94 ± 1.82 mg/100g dry matter and 37.53 ± 0.11 mg/100g dry matter, respectively. And under the optimum fermentation condition of RB, the content of polysaccharide, adenosine and ergosterol were also enhanced 3-fold, 10-fold and 10-fold, respectively. Therefore, the fermented SCR and RB could be utilized as nutritious functional food or food additives in the future.

Keywoeds: *Cordyceps sinensis*, *Preussia aemulans*, soybean curd residue, rice bran, solid-state fermentation

1. Introduction

In recent years, due to the serious economic and environmental concerns, the utilization of food by-products is unprecedentedly expected to increase and become more efficient. Thus, reduce, reuse, and recycle (3R) of by-products is getting more and more important in food industries (Wang & Nishino, 2008).

Soybean curd residue (SCR), is produced from the tofu industry in China and Japan. It was once consumed as a traditional food, but modernization and urbanization in the lifestyle has reduced its status to that of a mere industrial waste, which is now mainly incinerated like other industrial wastes (Ohno, Ano, & Shoda, 1996; O'Toole, 1999). The main disadvantage of SCR is natural spoilage when storage is not under refrigeration. In Japan, 0.7 million tons of SCR is disposed annually, mostly by incineration which has caused severe environmental pollution (Mizumoto, Hirai, & Shoda, 2006). In fact, SCR is rich in carbohydrate, protein and many other nutrients, suggesting that it is a potential source of low cost medium for the growth of mycelia. Many researchers have investigated the possibility of bioconversion of the residues by submerged and solid-state cultivation (Yokoi, Maki, Hirose, & Hayashi, 2002; Shi, Yang, Li, Wang, & Zhang, 2011).

Rice bran (RB), which includes the pericarp, the aleurone and sub-aleurone layers, parts of the germ and the embryo as well as small portions of the starchy endosperm (Jiamyangyuen, Srijesdaruk, & Harper, 2005), is a valuable milling by-product. However, RB contains enzyme lipase, which rapidly degrades the oil making the bran rancid and inedible, therefore 63 to 76 million tons of rice bran is produced in the world and more than 90% of RB is sold cheaply as animal feed each year. Actually, RB contains 12-22% oil, 11-17% protein, 6-14% fiber, 10-15% moisture and 8-17% ash and also rich in vitamins, minerals, amino acids and essential fatty acids (Hernandez, Rodriguez, Gonzalez, & Lopez, 2000; Jiang & Wang, 2005; Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000). RB is highly nutritious and hence used as a food additive (Nagendra, Sanjay, Shravya, Vismaya, & Nanjunda, 2011).

Cordyceps sinensis (Berk.) Sacc. is a parasitic fungus and has long been used to treat multitude of ailments, promote longevity, increase athletic power and improve quality of life. The physiological activators of *Cordyceps sinensis* have been detected, including adenosine, cordycepin, cordycepic acid, d-mannitol, polysaccharides,

vitamins and trace elements, etc. (Kumara et al., 2011). Polysaccharide exhibited antioxidative and antitumor activities, and regulating immune functions (Paterson, 2008). Adenosine is an endogenous purine nucleoside that modulates many physiological processes, and it has been used as a marker for the quality control of *C. sinensis* in Chinese Pharmacopoeia (Li, Yang, & Tsim, 2006). Ergosterol have multiple pharmacological activities, such as cytotoxic activity (Bok, Lermer, Chilton, Klingeman, & Towers, 1999) and antiviral activity (Lindequist, Lesnau, Teuscher, & Pilgrim, 1989). Furthermore, according to previous researches, 572 species fungi (*Preussia intermedia, Penicillium boreae* etc.) were isolated from different parts (stromata, sclerotia, and external mycelial cortices) of natural *Cordyceps sinensis* fruiting body, and all of the isolated fungus had the similar metabolites and exhibited the similar pharmacological activities as *Cordyceps sinensis* (Zhang et al., 2010).

For reuse of SCR and RB, reduce of waste and recycle of organic material, according to the nutrition profile of SCR and RB, it could be considered as a growth medium for fungi. In this study, the SCR and RB were used as a culture medium for *Preussia aemulans* (*P. aemulans*) which was isolated from *Cordyceps sinensis* fruiting body. The objective of this research was to find out the optimum fermentation condition, which maximize quantity of polysaccharide, ergosterol and adenosine using SCR and RB, respectively. Fermented SCR and RB were detected to produce potential functional animal feed to substitute the antibiotic added to the feed, and improve the safety of food.

2. Materials and Methods

2.1 Chemicals and Reagents

D-glucose, sucrose, peptone, KH2PO4, MgSO4, Na2CO3, NaOH, potato extract, yeast extract, agar, ethanol, sulfuric acid, phenol were obtained from Wako Pure Chemical Industries, Ltd, Osaka, Japan. adenosine and ergosterol were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). All other chemical reagents were of analytical grade.

2.2 Isolation and Cultivation of P. aemulans

The fruiting body of *Cordyceps sinensis* was purchased from Qinghai, China, and the isolated *P. aemulans* mycelium (SIID11759-01) was identified by Techno Suruga laboratory co., ltd, Japan. The stroma of *Cordyceps sinensis* fruiting body was sterilized with ethanol three times, air-dried, cut into small segment and transferred to slant tube fermentor to incubate for 7 days, at room temperature. The white mycelium appeared on the surface during slant fermentation. Then, mycelium was transferred to agar medium, which contained (per liter): 20 g of sucrose, 10 g of peptone, 20 g of agar powder, 1.5 g of $MgSO_4$, 3 g of KH_2PO_4. After 7 days of the culture, when white mycelium appeared on the surface of the medium, the mycelium was transferred into the liquid medium, which was containing (per liter): 20 g of sucrose, 10 g of peptone, 4 g of potato powder, 1.5 g of $MgSO_4$, 3 g of KH_2PO_4. The *Cordyceps sinensis* mycelium was incubated in a 200 mL of flask with 100 mL of PDA liquid medium, and the mixture was stationary cultured for 7 days. After the stationary culture, the *P. aemulans* mycelium was inoculated to SCR and RB followed by the orthogonal test design.

2.3 Orthogonal Test Design

SCR was obtained from the inamoto toufu factory in tsukuba, Japan. The carbon nitrogen ratio, moisture content, and pH value of SCR were 10.8, 80% and 5.5, respectively. According to the initial conditions, the fermentation conditions were designed to investigate the optimum condition for yield of polysaccharide, ergosterol and adenosine by solid-state fermentation. The carbon resources, nitrogen resources (3% w/w), adding dosage of carbon sources and the fermentation time, were regarded as correlated factors of culture condition. The optimum fermentation condition was obtained by an orthogonal layout $L_9(3^4)$ in a 200 mL flask with 20 g of SCR. The level of factor is shown in Table 1. The inoculum size of *P. aemulans* mycelium (liquid medium) was 20 % (v/w). After fermentation, the fermented SCR mycelia mixture was dried and grounded into powder for further experiment.

RB was collected from Automatic rice-polishing machine in tsukuba, Japan. The carbon nitrogen ratio and moisture content of RB were 12 and 10%, respectively. According to the initial conditions, the fermentation conditions were designed to investigate the optimum condition for yield of polysaccharide, ergosterol and adenosine by solid-state fermentation. The carbon resources, nitrogen resources (3% w/w), adding dosage of carbon and nitrogen sources, moisture content and the fermentation time, were regarded as correlated factors of culture condition. The optimum fermentation condition was obtained by an orthogonal layout $L_{16}(4^5)$ in a 500 mL flask with 20 g various moisture content of RB. The level of factor is shown in Table 3. The inoculum size of *P. aemulans* mycelium (liquid medium) was 20 % (v/w). After fermentation, the fermented RB mycelia mixture was dried and grounded into powder for further experiment.

Table 1. L_9 (3^4) orthogonal layout and results of fermented SCR by *P. aemulans*

Experimental group	CS	NS (3% w/w)	ADCS (% w/w)	FT (day)	Polysaccharide content (mg/g dry matter)	Ergosterol content (mg/100g dry matter)	Adenosine Content (mg/100g dry matter)
1	Glucose	Peptone	5%	10	22.74 ± 0.66	11.90 ± 1.22	40.95 ± 1.62
2	Glucose	Beef extract	10%	15	27.25 ± 1.77	35.65 ± 2.76	99.62 ± 1.89
3	Glucose	Yeast extract	15%	20	21.43 ± 1.50	11.99 ± 1.89	101.99 ± 1.74
4	Sucrose	Peptone	10%	20	19.21 ± 1.08	10.60 ± 0.31	45.32 ± 1.88
5	Sucrose	Beef extract	15%	10	31.43 ± 0.37	26.19 ± 1.39	69.92 ± 1.43
6	Sucrose	Yeast extract	5%	15	25.06 ± 1.60	13.64 ± 1.08	117.96 ± 1.24
7	Fructose	Peptone	15%	15	21.07 ± 2.41	14.79 ± 1.92	41.66 ± 1.58
8	Fructose	Beef extract	5%	20	22.39 ± 1.86	20.11 ± 1.87	82.74 ± 1.63
9	Fructose	Yeast extract	10%	10	20.32 ± 1.67	9.81 ± 1.03	100.63 ± 1.33

*Note: CS, carbon source; NS, nitrogen source; ADCS, adding dosage of carbon source; FT, fermentation time. Mean values were mean of three determinations with standard deviation (±).

2.4 Determination of Polysaccharide Content

The fermented SCR and RB dried powder was extracted with boiling water for two hours. The water-soluble polysaccharide was precipitated by adding eight volumes of 99.5% ethanol and stored at 4℃ overnight. The precipitated polysaccharide was collected by centrifuging at 7000 rpm for 30 min. Then the precipitate was dissolved in 10 mL of distilled water. The total polysaccharide was determined by the phenol-sulfuric acid method with some modifications (Li, Ding & Ding, 2007). The color reaction was initiated by mixing 1 mL of the polysaccharide solution with 0.5 mL of 5% phenol solution and 2.5 mL of concentrated sulfuric acid, and the reaction mixture was incubated in a boiling water bath for 15 min. After cooling it to room temperature, the optical density (OD) of the mixture was determined at 490 nm and the polysaccharide content was calculated with D-glucose as the standard. The results were expressed as milligram of glucose equivalent per gram of the fermented SCR and RB.

2.5 Determination of Ergosterol Content

The fermented SCR and RB dried powder were extracted with a mixture of methanol and dichloromethane in the ratio of 75/25 (v/v) and the solid-to-liquid ratio was 1/10 (w/v) using ultrasonic-assisted extract method for 1 h (50 W) at ambient temperature. Then, the supernatant was collected and filtered by filter (0.45 μm) for HPLC determination. The samples were analyzed by the HPLC (JASCO International Co., Ltd) with a reverse-phase Capcell-Pak C_{18} column (4.6 mm I.D. × 150 mm, particle size of 5 μm Nacalai Tesque, Inc. Japan) in a flow rate of 1.0 mL/min, the column temperature was set at 30°C and the UV detection was operated at 254 nm. The mobile phase was methanol (99.5%), and the concentration of ergosterol was calculated by comparing peak areas with appropriate standards.

2.6 Determination of Adenosine Content

The fermented SCR and RB dried powder were extracted with deionized water (1/10 w/v) by using ultrasonic-assisted extract method for 1 h (50 W) at ambient temperature. Then, the supernatant was collected and filtered by filter (0.45 μm) for HPLC determination. The samples were analyzed by the HPLC (JASCO International Co., Ltd) with a reverse-phase Capcell-Pak C_{18} column (4.6 mm I.D. × 150 mm, particle size of 5 μm Nacalai Tesque, Inc. Japan) in a flow rate of 1.0 mL/min, the column temperature was set at 30°C and the UV detection was operated at 260 nm. The mobile phase was a mixture of acetonitrile and water (5:95, v/v). And the concentration of adenosine was calculated by comparing peak areas with appropriate standards.

2.7 Statistical Analysis

Experimental results were means ± standard deviation (SD) of triple determinations. The data were analyzed by one-way analysis of variance (ANOVA). Tests of significant differences were determined by Student's t-test analysis at $P = 0.05$ or independent sample t-test ($P = 0.05$).

3. Results and Discussion

3.1 Orthogonal Test Results of Fermented SCR

The yield of polysaccharide, ergosterol and adenosine from fermented SCR were shown in Table 1, the optimum fermentation conditions and the significant levels were shown in Table 2.

The highest mean yield of polysaccharide was 31.43 ± 0.37 mg/g dry matter. The optimum levels of factors were sucrose as carbon source, beef extract as the nitrogen source, 15% of adding dosage of carbon source, and 10 days of fermentation time, respectively. The *R* value of various factors indicated that the nitrogen source was the highest among these factors. And the significant levels indicated that all of the factors significantly related with the yield of polysaccharide.

About ergosterol, the highest mean yield of ergosterol was 35.65 ± 2.76 mg/100g dry matter. The optimum levels of factors were glucose, beef extract, 10% of adding dosage of carbon source, and 15 days of fermentation time, respectively. The *R* value of various factors indicated that the nitrogen source was the highest among these factors. And the significant levels showed that the ergosterol yield of the fermented SCR was significantly related to all of the factors.

The highest mean yield of adenosine was 117.96 ± 1.24 mg/100 g dry matter. The optimum levels of factors were glucose, yeast extract, 10% of adding dosage of carbon source, and 15 days of fermentation time, respectively. The *R* value of various factors indicated that the nitrogen source was the highest among these factors. And the significant levels revealed that the adenosine content of the fermented SCR was significantly related to all of the factors.

Further, in order to evaluate the fermented SCR, the solid-state fermentation was enlarged by using 500 mL flask with 50 g SCR, under optimum conditions. The polysaccharide yield of the fermented SCR was reached to 43.49 ± 2.48 mg/g dry matter. Compared with the unfermented SCR (12.91 ± 0.39 mg/g dry matter), the polysaccharide content was 4-fold improvement during the fermentation by *P. aemulans* under the optimum fermentation conditions of polysaccharide yield (OPCPS-SCR). The ergosterol yield of the fermented SCR was reached to 37.53 ± 1.34 mg/100 g dry matter. In contrast with the unfermented SCR (3.13 ± 0.26 mg/100 g dry matter), the ergosterol content was enhanced about 10-fold during the fermentation by *P. aemulans* under the optimum fermentation conditions of ergosterol yield (OFCER-SCR). According to previous reports, the ergosterol content of the fermented SCR as much as cultured *C. sinensis* of Wanfong (38 mg/100 g dry matter) (Li, Yang & Tsim, 2006). And the adenosine yield of the fermented SCR was reached to 148.32 ± 4.21 mg/g dry matter. Compared with the unfermented SCR (12.68 ± 1.36 mg/100g dry matter), the adenosine content was increased by 10-fold under the optimum fermentation conditions of adenosine yield (OFCAD-SCR). And on basis of the previous reports, the adenosine content of fermented SCR was 5-fold higher than that of nature *C. sinensis* (Tibet and Qinghai) (Li, Yang & Tsim, 2006).

Table 2. Range and variance analysis of L_9 (3^4) orthogonal experiment results

	Polysaccharide content				Ergosterol content				Adenosine content			
	CS	NS	ADCS	FT	CS	NS	ADCS	FT	CS	NS	ADCS	FT
I_j	214.24	189.05	210.54	223.43	178.61	111.89	136.96	143.69	727.67	383.76	724.95	634.49
II_j	227.04	243.16	200.34	220.14	151.30	245.84	168.17	192.23	699.60	756.87	736.70	777.72
III_j	191.34	200.41	221.75	189.05	134.12	106.29	158.89	128.10	675.08	961.72	640.70	690.14
R	35.70	54.11	21.41	34.38	44.49	139.55	31.22	64.13	52.59	577.96	96.00	143.23
Optimum level	2	2	3	1	1	2	2	2	1	3	2	2

		Polysaccharide content				Ergosterol content				Adenosine content			
Factor	Y	SS	MS	*F* ratio	SL	SS	MS	*F* ratio	SL	SS	MS	*F* ratio	SL
CS	2	72.69	36.35	15.01	***	111.87	55.93	14.94	***	153.90	76.95	29.76	***
NS	2	180.94	90.47	37.37	***	1386.85	693.42	185.19	***	19081.91	9540.95	3689.41	***
ADCS	2	25.50	12.75	5.27	**	57.10	28.55	7.62	***	609.33	304.66	117.81	***
FT	2	79.96	39.98	16.51	***	248.61	124.30	14.94	***	1158.53	579.26	224.00	***
e	18	43.58	2.42			111.87	55.93			46.55	2.59		

*Note: I_j, II_j, III_j, were sum of the polysaccharide, ergosterol and adenosine contents from fermented SCR of level 1, level 2 and level 3; *R* means the maximum of I_j, II_j and III_j minus the minimum of I_j, II_j and III_j; CS, carbon source; NS, nitrogen source; ADCS, adding dosage of carbon source; FT, fermentation time. *F* 0.10 (2, 18) = 2.78; *F* 0.05 (2, 18) = 3.55; *F* 0.01 (2, 18) = 6.01; * *F* ratio > *F*0.1; ** *F* 0.01 > *F* ratio > *F*0.05; *** *F* ratio > *F*0.01. υ: Degree of freedom; e: error., SS: Sum of square deviation., MS: Mean square., SL: Significance level.

3.2 The Optimum Fermentation Condition of Fermented SCR

According to the results of orthogonal test, the optimum fermentation condition of polysaccharide, ergosterol and adenosine were different. Therefore, it was necessary to discuss the integrated optimum fermentation condition. The polysaccharide contents of OFCPS-SCR (43.49 ± 1.48 mg/g dry matter), OFCER-SCR (39.18 ± 1.06 mg/g dry matter) and OFCAD-SCR (35.83 ± 1.24 mg/g dry matter) were shown in Figure 1 a. According to Duncan's multiple range test, the polysaccharide content of OFCPS-SCR was significantly higher than that of OFCER-SCR and OFCAD-SCR. The ergosterol contents of OPCPS-SCR, OPCER-SCR and OPCAD-SCR were 22.06 ± 0.16, 37.53 ± 0.11 and 16.62 ± 0.62 mg/100 g dry matter, respectively (Figure 1 b). The adenosine contents of OPCPS-SCR, OPCER-SCR and OPCAD-SCR were 124.59 ± 1.53, 127.94 ± 1.82 and 148.32 ±1.61 mg/100 g dry matter, respectively (Figure 1 c). As the results, the significant levels of OPCER-SCR ($^{AB, A, B}$) were higher than those of OPCPS-SCR ($^{A, B, B}$) and OPCAD-SCR ($^{BC, C, A}$), thus OPCER-SCR was the optimum fermentation condition for producing polysaccharide, ergosterol and adenosine.

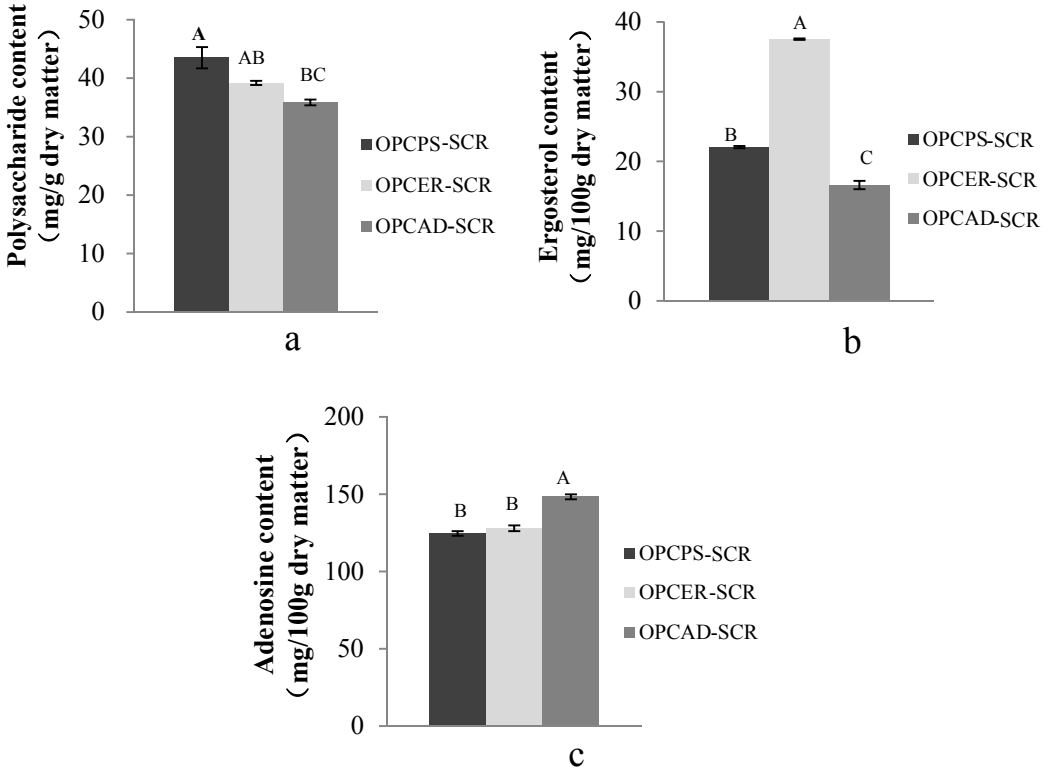

Figure. 1 The optimum fermentation condition of fermented SCR

(a), (b) and (c) were the polysaccharide, ergosterol, adenosine content of three optimum fermentation conditions for SCR (OFCPS-SCR, OPCER-SCR, OFCAD-SCR), respectively ($^{A. B. C.}$ $p < 0.01$, Data were expressed as means ± S.D. n=3 using Duncan's multiple range test).

3.3 Orthogonal Test Results of Fermented RB

The polysaccharide, ergosterol and adenosine yield of fermented RB were shown in Table 3, the optimum fermentation conditions and the significant levels were shown in Table 4.

The highest mean yield of polysaccharide in the orthogonal experiment was 70.02 ± 1.94 mg/g dry matter. The optimum levels of factors were maltose of carbon source, yeast extract of the nitrogen source, 10% of adding dosage of carbon source, 60% of moisture content and 15 days of fermentation time, respectively. The *R* value of various factors indicated that the nitrogen source was the highest among these factors. And the significant levels indicated that all of the factors significantly related with the yield of polysaccharide.

Table 3. L_{16} (4^5) orthogonal layout and results of fermented RB by *P. Aemulans*

Experimental group	CS	NS (3% w/w)	ADCS (% w/w)	MC (%)	FT (day)	Polysaccharide content (mg/g dry matter)	Adenosine Content (mg/100g dry matter)	Ergosterol content (mg/100g dry matter)
1	Glucose	Peptone	5%	60%	5	57.60 ± 1.35	55.65 ± 0.68	57.69 ± 2.65
2	Glucose	Beef extract	10%	70%	10	50.23 ± 2.24	81.61 ± 1.32	41.96 ± 2.99
3	Glucose	Yeast extract	15%	80%	15	46.79 ± 1.06	213.86 ± 2.06	ND
4	Glucose	Ammonium sulfate	20%	90%	20	22.96 ± 1.39	273.48 ± 1.89	10.20 ± 0.32
5	Sucrose	Peptone	10%	80%	20	41.74 ± 1.58	104.30 ± 1.22	76.79 ± 3.36
6	Sucrose	Beef extract	5%	90%	15	56.85 ± 1.96	281.31 ± 2.12	86.47 ± 1.76
7	Sucrose	Yeast extract	20%	60%	10	38.12 ± 1.33	108.72 ± 1.61	42.33 ± 1.88
8	Sucrose	Ammonium sulfate	15%	70%	5	46.58 ± 2.64	38.63 ± 0.33	ND
9	Fructose	Peptone	15%	90%	10	29.03 ± 0.85	30.57 ± 0.58	40.88 ± 0.97
10	Fructose	Beef extract	20%	80%	5	31.49 ± 0.97	50.84 ± 0.63	ND
11	Fructose	Yeast extract	5%	70%	20	50.13 ± 1.81	83.92 ± 0.51	66.03 ± 2.05
12	Fructose	Ammonium sulfate	10%	60%	15	70.02 ± 1.94	104.87 ± 1.06	ND
13	Maltose	Peptone	20%	70%	15	43.47 ± 2.44	29.04 ± 0.22	45.55 ± 1.38
14	Maltose	Beef extract	15%	60%	20	55.88 ± 2.13	81.04 ± 0.72	ND
15	Maltose	Yeast extract	10%	90%	5	66.50 ± 2.06	228.00 ± 1.35	47.19 ± 0.78
16	Maltose	Ammonium sulfate	5%	80%	10	27.60 ± 0.43	59.24 ± 0.68	68.22 ± 1.53

*Note: CS, carbon source; NS, nitrogen source; ADCS, adding dosage of carbon source; MC, moisture content; FT, fermentation time. Mean values were mean of three determinations with standard deviation (±). ND means not detected.

Table 4. Range and variance analysis of l_{16} (4^5) orthogonal experiment results

	Polysaccharide content					Adenosine content				
	CS	NS	ADCS	MC	FT	CS	NS	ADCS	MC	FT
I_j	532.71	515.50	576.55	664.87	606.50	1873.81	658.70	1427.24	1050.83	1119.35
II_j	549.91	583.35	685.48	571.24	434.95	1585.74	1471.27	1556.30	699.60	840.40
III_j	542.02	604.64	534.85	442.84	651.40	810.59	1903.47	1092.30	1284.70	1874.11
IVj	580.35	501.50	408.11	526.03	512.14	1191.95	1428.64	1386.26	2426.96	1628.23
R	30.44	103.14	277.37	222.03	216.45	1063.22	1244.77	464.00	1727.36	1033.71
Optimum level	4	3	2	1	3	1	3	2	4	3

		Polysaccharide content				Adenosine content			
Factor	Y	SS	MS	*F* ratio	SL	SS	MS	*F* ratio	SL
CS	3	106.48	35.49	3.89	**	53744.30	17914.77	1460.34	**
NS	3	636.19	212.06	23.26	***	67012.91	22337.64	1820.88	***
ADCS	3	3284.68	1094.89	120.07	***	9607.12	3202.37	261.05	***
MC	3	2141.59	713.86	78.29	***	139639.20	46546.40	3794.28	***
FT	3	2344.85	781.62	85.72	***	55336.07	18445.36	1503.59	***
e	32	291.80	9.12			392.56	12.27		

*Note: I_j, II_j, III_j and IVj were sum of the polysaccharide and adenosine contents from fermented RB of level 1, level 2, level 3 and level 4; *R* means the maximum of I_j, II_j, III_j and IVj minus the minimum of I_j, II_j, III_j and IVj; CS, carbon source; NS, nitrogen source; ADCS, adding dosage of carbon source; MC, moisture content; FT, fermentation time. *F*0 .10(3, 32) = 2 .28; *F*0 .05(3, 32) = 2 .90; *F*0 .01(3, 32) = 4 .46; **F* ratio > F0 .1; ** *F* 0.01 > *F* ratio > *F*0.05; ****F* ratio > *F*0 .01; CS: carbon Source; NS: nitrogen source; ADCS: adding dosage of carbon source; FT: fermentation time; υ: Degree of freedom; e: error., SS: Sum of square deviation., MS: Mean square., SL: Significance level.

The highest mean yield of ergosterol was 86.47 ± 1.76 mg/100g dry matter (Table 3). Because the contents of several samples were extremely low, the range and variance analysis could not be used in this experiment. Therefore, the optimum levels of factors were sucrose, beef extract, 5% of adding dosage of carbon source, 90% moisture content and 15 days of fermentation time, respectively.

The highest mean yield of adenosine was 281.31 ± 2.12 mg/100 g dry matter. The optimum levels of factors were glucose, yeast extract, 10% of adding dosage of carbon source, 90% moisture content and 15 days of fermentation time, respectively. The *R* value of various factors indicated that the moisture content was the highest among these factors. And the significant levels were indicated that the adenosine content of the fermented RB was significantly related to all of the factors.

Further, in order to evaluate the fermented RB, the solid-state fermentation was demonstrated by using 500 mL flask with 50 g RB, under optimum conditions. The mean polysaccharide content of the fermented RB was reached to 71.16 ± 2.63 mg/g dry matter. Compared with the unfermented RB (19.80 ± 1.23 mg/g dry matter), the polysaccharide content was increased to almost 4-fold during the fermentation by *P. aemulans* under the optimum fermentation conditions of polysaccharide content (OPCPS-RB). The ergosterol content was reached to 88.04 ± 0.36 mg/100 g dry matter, enhanced about 10-fold during the fermentation by *P. aemulans* under the optimum fermentation conditions of ergosterol content (OFCER-RB). The mean adenosine content of the fermented RB was also enhanced to 282.25 ± 1.83 mg/g dry matter. Contrast with the unfermented RB (30.13 ± 1.53 mg/100g dry matter), the adenosine content was increased by 10-fold during the fermentation by *P. aemulans* under the optimum fermentation conditions of adenosine content (OFCAD-RB).

3.4 The Optimum Fermentation Condition of Fermented RB

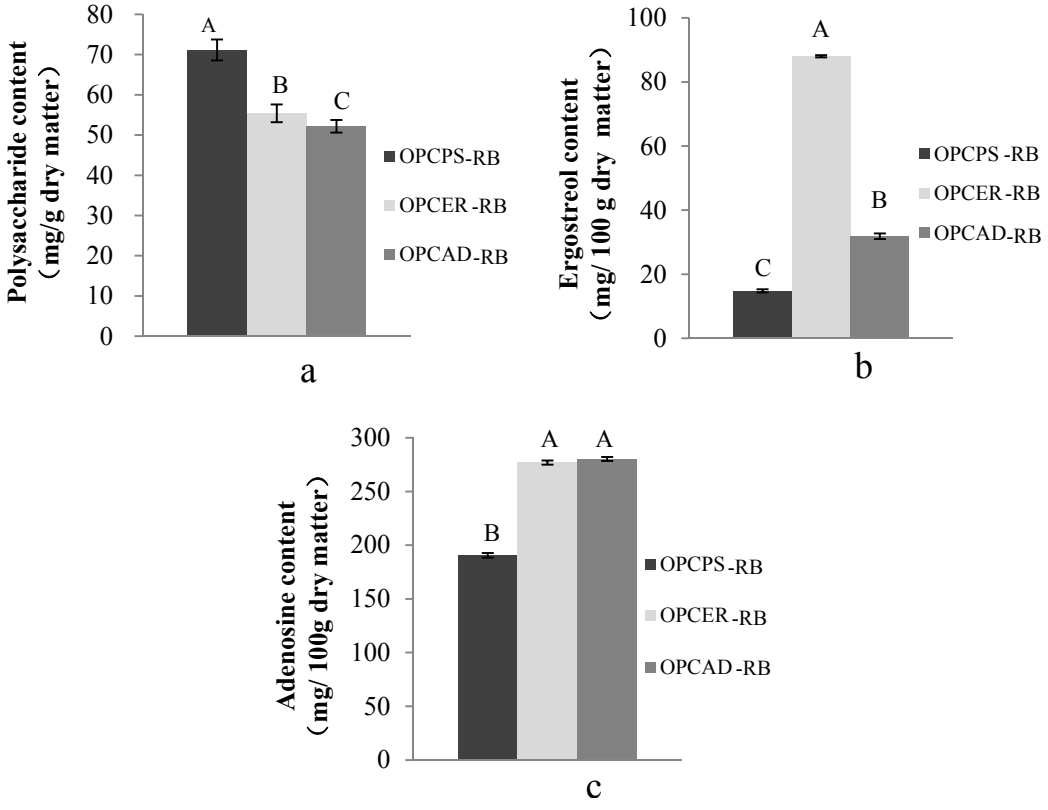

Figure 2. The optimum fermentation condition of fermented RB

(a), (b) and (c) were the polysaccharide, ergosterol, adenosine content of three optimum fermentation conditions for RB (OFCPS-RB, OPCER-RB, OFCAD-RB), respectively ($^{A.\ B.\ C.}$ $p < 0.01$, Data were expressed as means ± S.D. n=3 using Duncan's multiple range test).

According to the results of orthogonal test, the optimum fermentation condition of polysaccharide, ergosterol and adenosine were different. Therefore, it was necessary to discuss the integrated optimum fermentation condition.

The polysaccharide contents of OFCPS-RB (71.16 ± 2.63 mg/g dry matter), OFCER-RB (55.40 ± 2.29 mg/g dry matter) and OFCAD-RB (52.16 ± 1.58 mg/g dry matter) were shown in Figure 2 a. According to Duncan's multiple range test, the polysaccharide content of OFCPS-RB was significantly higher than that of OFCER-RB and OFCAD-RB. The ergosterol contents of OPCPS-RB, OPCER-RB and OPCAD-RB were 14.79 ± 0.48, 88.04 ± 0.36 and 31.85 ± 0.87 mg/100 g dry matter, respectively (Figure 2 b). The adenosine contents of OPCPS-RB, OPCER-RB and OPCAD-RB were 190.57 ± 2.11, 276.94 ± 1.96 and 282.25 ± 1.83 mg/100 g dry matter, respectively (Figure 2 c). As the results, the significant levels of OPCER-RB ($^{B, A, A}$) were higher than those of OPCPS-RB ($^{A, C, B}$) and OPCAD-RB ($^{C, B, A}$), thus OPCER-RB was the optimum fermentation condition for producing polysaccharide, ergosterol and adenosine.

4. Conclusions

The optimum fermentation conditions of SCR were: glucose, beef extract, 10% of adding dosage of carbon source, and 15 days of fermentation time, respectively. Under the optimum fermentation conditions, the polysaccharide, ergosterol and adenosine content were 39.18 ± 1.06 mg/g, 37.53 ± 0.11 mg/100 g dry matter and 127.94 ± 1.82 mg/100 g dry matter, respectively.

The optimum fermentation conditions of RB were: sucrose, beef extract, 5% of adding dosage of carbon source, 90% moisture content and 15 days of fermentation time, respectively. Under the optimum fermentation conditions, the polysaccharide, ergosterol and adenosine content were 55.40 ± 2.29 mg/g dry matter, 88.04 ± 0.36 and 276.94 ± 1.96 mg/100 g dry matter, respectively.

The results indicated that the polysaccharide, ergosterol and adenosine content of SCR and RB were improved by solid-state fermentation using *P. aemulans*. The effective utilization of such agricultural waste not only solves environmental problems, but also promotes the economic value of the agricultural products. The fermented SCR and RB were rich in physiological active substances, low in cost, could be explored as ecological feed or functional food material in the further.

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