การเพาะเลี้ยงเส้นใยเห็ดแครงในอาหารเหลวและคุณสมบัติของเส้นใยที่ผลิตได้

Mycelial fermentation in submerged culture of *Schizophyllum commune* and its properties

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บทคัดย่อ

การผลิตเส้นใยเห็ดแครงและพอลิแซกคาไรด์ด้วยกระบวนการเพาะเลี้ยงแบบกะในอาหารเหลว potato dextrose broth (PDB) ที่มีการเติม 2% malt extract พบว่าที่ระยะเวลาการเพาะเลี้ยง 6 วัน ได้ผลผลิตของเส้นใยเห็ดเท่ากับ 8.65 กรัมต่อลิตร และที่ระยะการเวลาการเพาะเลี้ยง 12 วัน ได้ผลผลิตของพอลิแซกคาไรด์รวมเท่ากับ 1.98 กรัมต่อลิตร ปริมาณของสารประกอบฟีนอลิกโดยวิธี Folin-Ciocalteu reagent ได้ค่า total phenolic compound เท่ากับ 8.54 มิลลิกรัมสมมูลกรดแกลิคต่อกรัมของสารสกัด การทดสอบความสามารถในการต้านอนุมูลอิสระพบว่าสารสกัดจากเส้นใย ที่ความเข้มข้น 2.5 มิลลิกรัมต่อมิลลิลิตร มีค่า reducing power เท่ากับ 0.684 และมีกิจกรรมในการจับอนุมูลอิสระ 0.2 mM DPPH มีค่าเท่ากับ 70.89 เปอร์เซ็นต์ สำหรับเส้นใยที่ได้จากการเพาะเลี้ยง สามารถยับยั้งการเจริญของเชื้อ *Bacillus cerues* และ *Staphylococcus aureus* นอกจากนี้การหมักน้ำองุ่นด้วยเส้นใยเห็ดแครง สามารถผลิตแอลกอฮอล์ได้ความเข้มข้นถึง 6.09 เปอร์เซนต์ ที่ระยะเวลาการหมัก 24 วัน จากผลการทดลองที่ได้แสดงให้เห็นว่าเส้นใยเห็ดแครงมีศักยภาพในการเป็นสารต้านอนุมูลอิสระและสามารถใช้ในการหมักเครื่องดื่มแอลกอฮอล์แทนยีสต์ได้

คำสำคัญ: การเพาะเลี้ยงเส้นใยเห็ด พอลิแซกคาไรด์ สารต้านอนุมูลอิสระ การยับยั้งแบคทีเรีย เห็ดแครง

Abstract

The production of mycelia and polysaccharides from *Schizophyllum commune* was carried out in a potato dextrose broth containing 2% malt extract. The results showed that at 6 days of cultivation the highest dried mycelia was 8.65 g/L and the highest polysaccharides content was 1.98 g/L at 12 days of cultivation. The total phenolic content determined by Folin-Ciocalteu method was 8.54 mg GAEs/g extract. The reducing power of ethanolic extract of mycelia increased when the concentration of extract was increased. At 2.5 mg/ml of ethanolic extract, the reducing power was 0.684 and the radical scavenging activity of mycelia ethanolic extract on 0.2 mM of 1, 1-Diphynyl-2-picrylhydrazyl (DPPH) was 70.89%. Mycelia froma submerged culture showed inhibition activity on the growth of *Bacillus cerues* and *Staphylococcus aureus*. Fermentation of grape juice by *S. commune* mycelia produced approximately 6.09% (v/v) alcohol within 24 days. These results suggested that mycelia of *S. commune* have a high potential to be used as natural antioxidant agent, food supplement and alcoholic beverage producing microorganism instead of ethanol-producing yeast.

Keywords: mushroom mycelia cultivation, polysaccharides, antioxidant, antibacteria, *Schizophyllum commune*

Introduction

Mushrooms have long been used as food and food flavoring because of their unique and fine flavor. Nowadays mushrooms have attracted a great deal of interest in many areas such as functional food, natural antioxidant, antimicrobial agent and are regarded as an effective medicinal used to treat human diseases.[1,2] *Schizophyllum commune* is an edible mushroom highly tasty with nu-

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tritional value.[3] It is extremely important in the field of pharmacology, because it produces the polysaccharides (schizophyllan), which show considerable anti-cancer activity in xenograft and clinical practice.[4] Moreover a phenolic compound has been found in mushroom and mycelia extract which correlates with the ability of antioxidants[5,6,7] and some research has reported that *S. commune* possessed antimicrobial activity.[8] The cultivation of mushrooms usually takes a long period to producea fruiting body, but growing mushrooms mycelia in submerged fermentation can shorten the time to obtain biomass and polysaccharides[9,10,11,12]. Therefore, submerged fermentation was used in this study. The aims of this work were to produce mycelia and polysaccharides, and to investigate the mycelia properties of *Schizophyllum commune*.

Materials and Methods

Mushroom and bacteria strains

The strain of *Schizophyllum commune* was purchased from the Center of Mushroom Collection, Department of Agriculture, Ministry of Agricultural, Thailand. It was grown on potato dextrose agar (PDA) plates and incubated at 30°C for 7 days. This culture was used as inoculum for shake flask culture experiment.

Four strains of bacteria namely *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp. obtained from Thailand Institute of Scientific and Technological Research (TISTR) were used for antibacterial test.

All strains were cultured in nutrient broth (NB) at 110 rpm, 37 °C for 24 hr before used in the manipulations.

Shake-flask culture

The experiments were performed in 250-ml flasks containing 100 ml of medium. The cultivation medium was PDB containing 2% malt extract (w/v) adjusting the pH to 5.50[14,15] and autoclaving at 121 °C for 15 min. After that it was inoculated with 10 pieces of mycelia grown on a PDA plate (using cork borer diameter 9 mm). The culture was incubated on an incubator shaker at 150 rpm, 30°C and the samples were taken every 3-days until the end of cultivation (15 days).

Analytical methods

The mycelia were determined in dry weight using the modified method from Lee *et al*., 2003.[16]The samples were subjected to centrifuge (RC-5C, Sorvall instruments) at 8,000 rpm for 20 min and the supernatant was collected. The resulting pellet was washed twice with distilled water and then lyophilized (LYPH.LOCK, Labconco).

Crude exo-polysaccharide (EPS) was determined by a modified method from Lee *et al*., 2003[16]. The mycelia-free supernatant was precipitated with 95% ethanol (4 volumes of supernatant), stirred and let to stand at 4 °C overnight. The precipitated exo-polysaccharide was recovered by centrifugation at 8,000 rpm for 15 min and the supernatant was discarded. The precipitated crude exo-polysaccharide was washed with ethanol and then the crude exo-polysaccharide was lyophilized.

The crude intracellular polysaccharide (IPS) was determined by modified method from Lee *et al*., 2007[17]. Ten grams of dried mycelium powder was washed with 80% ethanol, and then centrifuged at 8,000 rpm for 10 min, and supernatant was discarded. The precipitate was suspended with distilled water, autoclaved at 121 °C for 45 min. After that, the sample was centrifuged at 8,000 rpm for 20 min and the supernatant was collected. The supernatant was precipitated by adding 95% ethanol (4 volumes of supernatant), stirred and left at 4 °C overnight. Crude IPS was recovered using the same method as for recovering crude EPS.

Reducing sugar was analyzed by Somogyi-Nelson method.[18,19] The phenol-sulfuric acid method with a slight modification from Dbois *et al*., 1956[20] was used for total sugar analyzed and D-glucose was used as standard sugar.

Determination of total phenolic compound and antioxidant assay

The lyophilized mycelia were extracted which modified method from Elmastas *et al*., 2007.[21] Briefly, 10 g of dried mycelium powder was mixed with 100 ml of 80% ethanol (v/v). The mixture was shaken at 150 rpm for 24 hr at room temperature. Then, the supernatant was collected by centrifugation at 8,000 rpm for 15 min. The

residue was re-extracted twice under the same condition. Each supernatant was pooled and filtered using Whatman No.1. The ethanol was removed by rotary evaporator (R3, BUCHI) at 40 °C. The final concentrate extract was kept at -20 °C for assays.

Total phenolic compound of mycelia ethanolic extracted was determined by Folin-Ciocalteu reagent method which was modified method from Barros *et al*., 2007.[22] The gallic acid was used as standard. The total phenolic content of sample was expressed as gallic acid equivalents (GAE)/g mycelia.

The reducing power was determined using the modified method from Oyaizu 1986.[23] The 2.5 ml of each extract (0.10 – 2.5 mg/ml) in de-ionized water was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After that 2.5 ml of 10% trichloroacetic acid (w/v) was added and centrifuged at 2,000 rpm for 10 min. Then 2.5 ml of upper layer was mixed with 2.5 ml of de-ionized water and 0.5 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm (Shimadzu (UV-160A), using butylated hydroxyl toluene, BHT (Sigma) as standard. A higher absorbance indicated a higher reducing power.

The scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma) radicals was determined according to the modified method of Elmastas *et al*., 2007.[21] 2 ml of each extract (0- 2.5 mg/ml) in de-ionized water was mixed with 2 ml of 0.2 mM DPPH in ethanol. The mixture was let to stand for 30 min in the dark then the absorbance was measured at 517 nm. The scavenging effect on DPPH free radicals was calculated as follows:

Inhibition (%) = $(A_{Control} - A_{sample})/ A_{Control} \times 100)$.

Antimicrobial activity using agar well diffusion assay

The agar well diffusion assay was modified from the method of Yaltirak *et al*., 2009.[7] Four strains of bacteria, including Gram positive (*Bacillus cereus*, *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli*, *Salmonella* sp.) were cultured in nutrient broth at 37 °C in shake flasks for 24 hr. Each cultured flask was suspended in normal saline to McFarland No.1, and 100 µl was pipetted for spread plate. After that, wells (7 mm) were made by cork borer. One hundred milligrams of wet mycelia and 100 µl of mycelia free fermented broth were dropped in the wells, and incubated at 37 °C for 24 hr. The well of control was filled with liquid medium. The inhibit zone was measured and reported in millimeter.

Production of wine from mushroom mycelia

Mycelia of *S. commune* was used for wine production instead of yeast. Grape juice was adjusted to obtain the final total soluble solid (TSS) at 22 °Brix with syrup, and pH at 5.80. Then, the sample was autoclaved at 110°C for 10 min. Thirty grams of wet mycelia were added to 300 ml of grape juice in 500 ml flask, and incubated at ambient temperature for 28 days. The ethanol concentration was analyzed by Gas Chromatography (Shimadzu model GC-2014) FID Detector, Rtx-Wax column. The TSS was measured by Hand refractometer (ATAGO, Japan).

Results and Discussion

Shake-flask culture

The results of submerged culture are shown in Table 1. Biomass of mycelia increased rapidly during 3 to 9 days of fermentation and it slightly decreased at the end of fermentation, 15 days. The highest concentration of dry weight was 8.71 g/L at 9 days with no significance among those in day 6, 12, and 15. The result is similar to that of Pandee *et al.,* 2008[24] who reported the maximum growth (8.93 g/L) of *S. commune* BL23 in submerged culture conditions for the production of a fibrinolytic enzyme after 7 days of cultivation. Whereas, Bolla *et al.,* 2008[4] reported the highest biomass (8.2 g Dw/L) of *S. commune* at 14 days.

Table 1 Mycelia dry weight, reducing sugar, total sugar, crude exo-polysaccharide and pH value during fermentation process of *S. commune*

Time (days)	Biomass (g/L)	Residual sugar (g/L)		Polysaccharides (g/L)		Final pH
		Reducing sugar	Total sugar	EPS	IPS	
0	0.57±0.00[d]	30.68±0.80	44.13±0.98	nd	nd	5.50±0.00
3	4.25±0.11[c]	21.07±1.38	34.52±0.90	0.71±0.05[d]	0.26±0.01[b]	4.56±0.06
6	8.65±0.07[a,b]	6.34±0.55	19.17±0.72	0.94±0.04[c]	0.52±0.01[a]	5.05±0.07
9	8.71±0.26[a]	5.06±0.44	18.04±2.05	1.25±0.06[b]	0.53±0.02[a]	5.02±0.12
12	8.64±0.14[a,b]	3.78±0.25	12.18±2.32	1.46±0.03[a]	0.52±0.01[a]	5.35±0.04
15	8.23±0.02[b]	2.49±0.39	7.36±0.77	1.52±0.05[a]	0.50±0.01[a]	4.72±0.09

* Mycelia dry weight nd = not determined

[a ,b,c,d] Means with different letters within a column are significantly different (p<0.05).

The biomass of *S.commune* was sharply increased and highes (8.71±0.26 g/L) at a day wich not different with that in 6 day and 12 day. The crude exo-polysaccharide slightly increased and reached the highest concentration (1.52± 0.05 g/L) at 15 days (Table 1) which is not significantly different with that in day 12. The highest polysaccharides content (EPS with IPS) was 2.02 g/L at days 15, followed by 1.98 g/L at day 12. These results suggest that the suitable time for mycelia production is around 6 to 9 days and 12 to 15 days for polysaccharides production. The pH of culture broth was decreased to the range of 4.56 to 5.35 during fermentation process due to organic acid production which was correlated to other works[25,26]

The initial reducing sugar was 30.68±0.80 g/L (44.13±0.98 g/L of total sugar). The concentration of sugar was reduced while the biomass production was increased (Figure 1 & Table 1) during fermentation. Sugar was used for mycelia growth and polysaccharide production

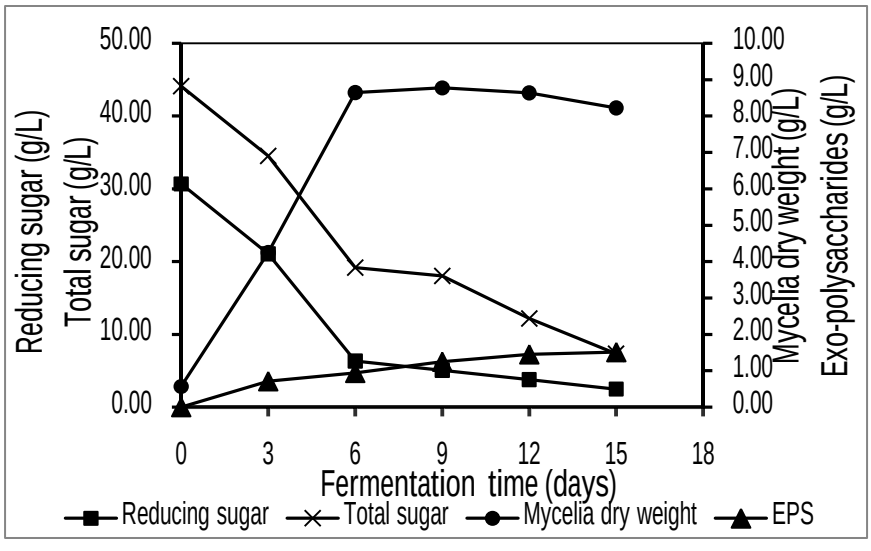


Figure 1 The profile of mycelia dry weight, reducing sugar, total sugar, the crude exo-polysaccharide of *S. commune* during fermentation for 15 days in shake flask culture.

Totalphenolic compound

The total phenolic compound of *S. commune* ethanolic mycelium extract was 8.54 mg GAE/g extract. Yaltirak *et al*., 2009[7] reported the maximum total phenolic content in *Russula delica* Fr. ethanolic extract at 6.23 mg GAE/g extract.

Reducing power ability

The reducing power of ethanol extract from *S. commune* mycelia increased when the concentration of extract was applied. At 2.5 mg/ml of ethanolic extract, the reducing power was 0.684 (Table 2).

Table 2 Reducing power of ethanolic mycelium extract of *S. commune*.

Conc. of extract (mg/ml)	$OD_{700\ nm}$	Equivalent with BHT (ppm)
0.1	0.020	0.067
0.5	0.115	6.902
1.0	0.228	15.007
2.5	0.684	47.837

Scavenging ability on DPPH radicals

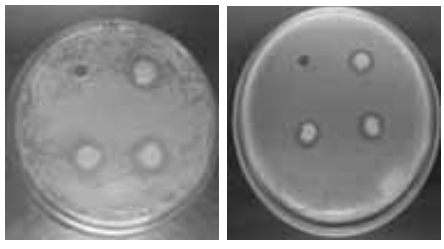
The ethanolic mycelium extract of *S. commune* at 2.5 mg/ml exhibited the strongest radical scavenging activity (70.89%) on 0.2 mM DPPH (Table 3).

Table 3 The scavenging ability on DPPH radicals of ethanol mycelium extract of *S.commune*.

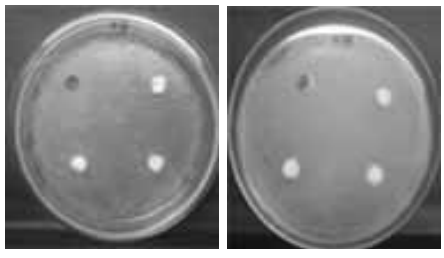
Conc. of extract (mg/ml)	**scavenging ability (%)**
0.0	0.00
0.5	19.38
1.0	35.98
2.5	70.89

Agar well diffusion assay

This experiment aimed to investigate antibacterial properties of fresh mycelia and mycelia-free broth on some Gram-positive and Gram-negative bacteria. The result is shown in Table 4. Fresh mycelia exhibited an inhibition activity against *S. aureus* and *B. cereus*, but not on *Salmonella* sp. and *E. coli* (Fig.2). The range of inhibition zone was from 8.30 to 10.30 mm. Yaltirak *et al*., 2009[7] stated that ethanolic fruiting body extract of *Russula delica* Fr. gave an inhibition zone against all tested bacteria in the range 7-17 mm. Fresh mycelia did not inhibit the growth of Gram-negative bacteria in this experiment. It may be due to the complex structure of cell wall in Gram-negative that made these tested strains more resistant, whereas less complex structure of the cell wall in Gram-positive bacteria could make it more permeable to the antimicrobial compound (Papadopoulou*et et al.,* 2005).[27]



a) *Staphylococcus aureus* b) *Bacillus cereus*



c) *Escherichia coli* d) *Salmonella* spp.

Figure 2 Antimicrobial ability of *S. commune* mycelia against tested bacteria, Figure a) and b) show inhibition zone against *Staphylococcus aureus* and *Bacillus cereus*.

Table 4 The inhibition zone of mycelia-free broth and fresh mycelia against tested bacteria.

	Microorganisms Inhibition zone (mm)		
	Mycelia-free broth	**Mycelia**	**Control**
Staphylococcus aureus	None	8.30 ± 0.58	None
*Bacillus cere*us	None	10.30 ± 0.58	None
Salmonella sp.	None	None	None
Escherichia coli	None	None	None

The values are mean ± SD

Wine brewing and alcohol content

Grape juice was used as substrate for alcoholic fermentation. The juice was adjusted to an initial sugar concentration at 22±0.00 °Brix, pH 5.8. The initial reducing sugar and total sugar were 184.4±3.02 g/L and 269.59 ± 6.91 g/L respectively (data not show). The fermentation process in aerobic condition was operated by hand-shaking once a day (1 min.). The highest alcohol concentration was 6.18 ± 0.11 % (v/v) at 28 days of fermentation, with no difference compared at day 24 of fermentation, at 6.09 ± 0.23 % (v/v) (Fig. 3). Sugar concentration remained about 13.00 °Brix after 28 days of fermentation (Fig.3). Okamura *et al*. 2001[14] reported that alcohol concentration produced by *P. ostreatus* was 12.2%.The different in alcohol concentration is probably due to the difference in varieties of mushroom mycelia.

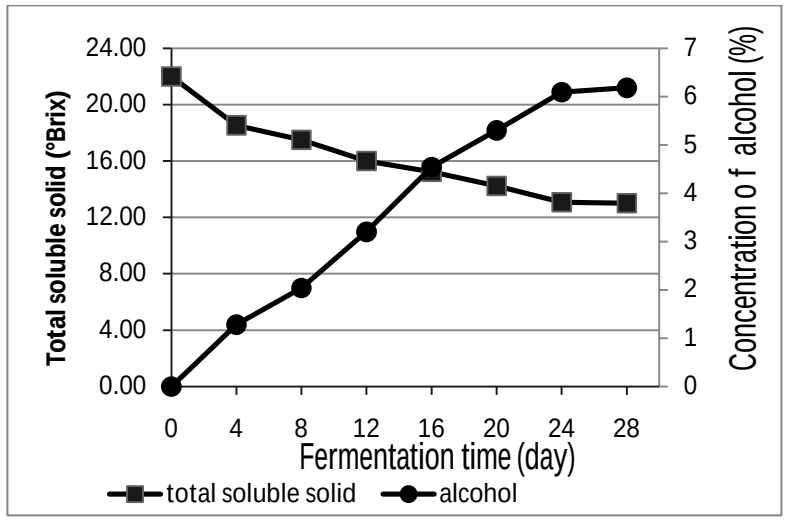


Figure 3 Alcohol production by *S. commune* mycelia and residual sugar (°Brix) at ambient temperature for 28 days of fermentation.

Conclusions

Mycelia of *S. commune* obtained from a submerged culture contained phenolic compounds. They exhibited the reducing power and scavenging on 0.2 mM of 1, 1-Diphynyl-2-picrylhydrazyl (DPPH). The results from this study indicated that *S. commune* had good potential to be used as natural antioxidant agent. The fresh mycelia in submerged culture could inhibit the growth of some Gram-positive bacteria. Moreover, it had ability to produce alcohol, when grape juice was used as substrate. Therefore, using *S. commune* mycelia instead of yeast can be a good alternative for alcoholic beverage fermentation.

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