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Short communication

Development of optimal culture method of *Sparassis crispa* mycelia and a new extraction method of antineoplastic constituent

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Abstract

The optimal culture condition of basidiomycete *Sparassis crispa* mycelia was determined for producing a large amount of antineoplastic constituent, i.e. β-1,3-glucan. Furthermore, a new extraction method with steam explosion as a pretreatment was attempted to increase the amount of antineoplastic constituent extracted from *S. crispa* mycelia. The shake-flask liquid culture of fungal mycelia could shorten a culture time up to about 1/3 compared with the conventional stationary liquid culture. The productivity of *S. crispa* mycelia reached its maximum at an initial glucose concentration of 30 g/L, a culture temperature of 25–30 °C, and pH 5. Steam explosion at a steam temperature of 225 °C for 5 min not only enhanced the amount of antineoplastic constituent extracted from the mycelia but also shortened the extraction time significantly.
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1. Introduction

In recent years, mushroom has been attractive as a healthy food source and medicine [1–4]. Lentinun (β-1,3-glucan) from *Lentinus edodes* and Sonifilan from *Schizophyllum commune* have been used in cancer chemotherapies [5,6]. A basidiomycete *Sparassis crispa*, an edible mushroom, can be cultivated comparatively easily and it has a large amount of β-1,3-glucan, i.e. about 43.6% of its dry weight [7]. Furthermore, β-1,3-glucan from fruit body of *S. crispa* shows anti-tumor activity [8]. Mostly, polysaccharide extracts whose primary binding pattern is β-1,3-linkage are obtained by various extraction methods, i.e. hot water extraction, cold alkali extraction, and hot alkali extraction [9]. The basidiomycete fungi can take various forms according to a living environment. However, any of those cell walls are covered by fibrous chitin that is fundamentally a β-1,4-linkage polymer composed of *N*-acetylglucosamine. The primary constituents of cell walls are amorphous polysaccharides that consists of *R*-glucan and *S*-glucan. *R*-Glucan is composed of both β-1,3-linkage and β-1,6-linkage of glucose, whereas *S*-glucan is composed of only β-1,3-linkage of glucose [10–13]. In the cultivation of basidiomycetes, since the culture condition of fruit body is more complex and delicate than that of fungal mycelia and required too much culture time, it is desired for producing β-1,3-glucan rapidly and efficiently to develop the effective culture method of fungal mycelia.

In this work, the optimal culture method to obtain a large amount of *S. crispa* mycelia was investigated. Furthermore, the steam explosion that degraded and depolymerized the cell wall was used as a pretreatment for the development of a new efficient extraction method of β-1,3-glucan from the *S. crispa* mycelia.

2. Material and methods

2.1. Microorganism

S. crispa mycelia kindly supplied by Oze Shockin Ltd. was used as a basidiomycete for producing the antineoplastic constituent, β-1,3-glucan.

2.2. Culture media and cultivation

Fungi were incubated in a medium containing the following ingredients per liter: 0.2 g yeast extract, 0.2 g polypeptone,

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10–70 g glucose, and 1 mL Kirk salt [14] at 15–40 °C. Media for submerged cultures were buffered with 0.4 M phthalic acid (pH 4–6) or 0.4 M phosphoric acid (pH 7). Incubation experiments were carried out in 300 mL Erlenmeyer flasks containing 60 mL of medium on stationary state or shaken at 100 rpm.

Dry cell weight was determined by freeze-drying method. The glucose concentration was measured by the mutarotase GOD method (Glucose *C*-Test; Wako Pure Chemicals, Osaka, Japan).

2.3. Pretreatment

Homogenizer (Model PT2100, Kinematica AG, Lucerne, Switzerland), ultrasonic homogenizer (Model 250 Sonifer, Branson Ultrasonics Co. Ltd., Danbury, USA), and the steam explosion (Japan Chemical Engineering and Machinery Co. Ltd., Osaka, Japan) [15] were used as pretreatment methods for increasing the amount of antineoplastic constituent extracted from fungal mycelia. Using 1 g fungal mycelia in 100 mL distilled water the homogenizer treatment was done for 5 min at 15,000 rpm and the ultrasonic homogenizer treatment was carried out for 20 Hz and 20 min at 80 W. Furthermore, the steam explosion treatment was done using 1 g fungal mycelia for 5 min at a steam temperature of 225 °C.

2.4. Extraction of antineoplastic constituent

Extraction method for obtaining the antineoplastic constituent from the pretreated fungal mycelia consisted of three stages, i.e. hot water extraction, cold alkali extraction, and then hot alkali extraction as shown in Fig. 1. Initially, 1 g freeze-dried and powdered *S. crispa* was extracted by hot water at 98 °C for 4 h. The resulting residue was further extracted with cold alkali (1N, NaOH) at 25 °C for 4 h, and then hot alkali of the same concentration at 98 °C for 4 h. The antineoplastic constituent concentration of each filtrate was measured by HPLC (Shimadzu LC-9A, Shimadzu Co. Ltd., Kyoto, Japan) with a column (Ultron PS-80P; Shinwa Chemicals Industries Ltd., Kyoto, Japan). All data were the mean values and standard deviations corresponding to three times experiment.

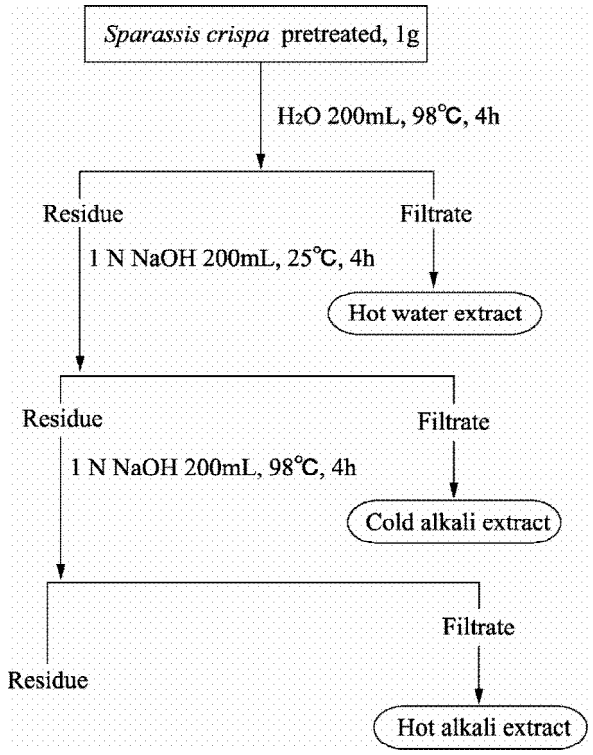


Fig. 1. Flow chart of extraction method for obtaining antineoplastic constituent from *S. crispa* pretreated.

3. Results and discussion

3.1. Comparison of culture methods

Fig. 2 shows time courses of dry cell weight and glucose concentration in the shake-flask liquid culture and the stationary liquid culture at a glucose concentration of 50 g/L, pH 5, and 25 °C. The error bounds of data were also shown. The dry cell weight increased gradually with the consumption of glucose. In the stationary liquid culture that was often used for fungal incubation, glucose hardly decreased until 4 days, whereas in the shake-flask liquid culture, glucose decreased from 2 days. Furthermore, the complete consumption of glucose was observed after 23 days in the stationary liquid culture while 16 days in shake-flask liquid culture. This means that the growth form of fungal mycelia in stationary liquid culture was different with that in the shake-flask liquid culture. The growth of *S. crispa* in the stationary liquid culture was evidenced by the spread of mycelia, covering the surface of the medium. However, in the shake-flask liquid culture, the fungus became a pellet mass in the medium. Since there was enough concentration of dissolved oxygen in the shake-flask liquid culture medium, the fungus could grow up well. However, in case of the stationary liquid culture, only the fungus in the vicinity of the medium surface

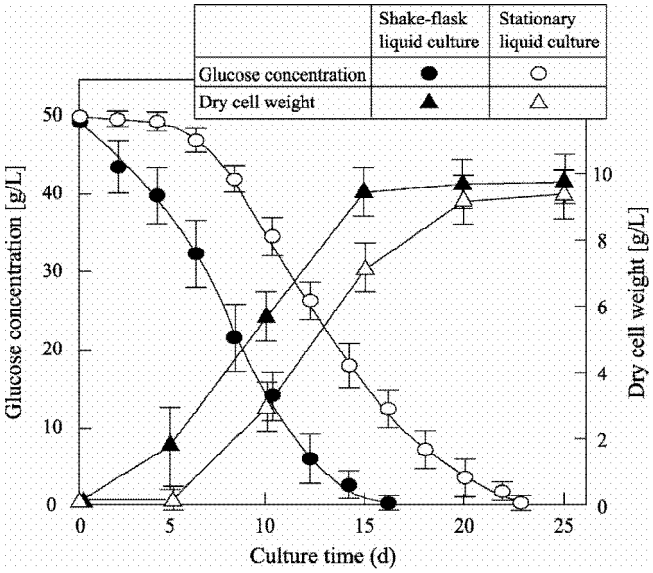


Fig. 2. Time courses of dry cell weight and glucose concentration in shake-flask liquid culture and stationary liquid culture of *S. crispa* at 50 g/L glucose, pH 5, and 25 °C.

Table 1
Effect of pH on growth properties of *S. crispa* in the incubation at 50 g/L glucose and 25 °C

pH	Time required to consume glucose completely [days]	Maximum dry cell weight [g/L]	Productivity [g/L/day]
4	26	8.9	0.34
4.5	20	9.16	0.46
5	16	9.92	0.62
6	18	9.82	0.55
7	23	7.43	0.32
9	–	–	–

could grow up due to insufficient dissolved oxygen in the inner part of medium, causing the delay in glucose consumption. However, no significant difference of maximum dry cell weight, i.e. 9.5–10 g/L, was observed in these cultures. Therefore, it seems that the shake-flask liquid culture method is more suitable for incubating *S. crispa* mycelia because it can shorten the culture time up to about 2/3 of the stationary liquid culture.

3.2. Effect of culture conditions on fungal growth

Table 1 shows the effect of pH value on the time required to consume glucose completely, the maximum dry cell weight, and the productivity defined as the quotient of maximum dry cell weight to time required to consume glucose completely in the incubation of *S. crispa* mycelia at a glucose concentration of 50 g/L and 25 °C. The time required to consume glucose completely decreased with the increase of pH value reaching its minimum value of 16 days and then increased while the maximum cell dry weight and the productivity increased with the increase of pH value reaching its maximum values of 9.92 g/L and 0.62 g/L/day, respectively, and then decreased. On the other hand, no growth of fungal mycelia and glucose consumption was observed beyond pH 9. Since the maximum productivity was obtained at pH 5, in the following experiments the pH value was adjusted at 5.

Table 2 shows the effect of initial glucose concentration on the time required to consume glucose completely, the maximum dry cell weight, the cell yield, and the productivity in the incubation of *S. crispa* mycelia at pH 5 and 25 °C. An initial glucose concentration of 20 g/L gave the highest cell yield of 0.32 but the highest productivity of 0.88 g/L/day was obtained at an initial glucose concentration of 30 g/L. Beyond an initial glucose concentration of 30 g/L, the cell yield remained as about 0.2 but the productivity decreased rapidly. This reason seems that dissolved oxygen necessary to cell growth decreased significantly due to a high level of mycelial growth resulting in a longer culture time. Also, the production of organic acids and secondary mycelial metabolites such as enzymes might decrease the mycelial growth

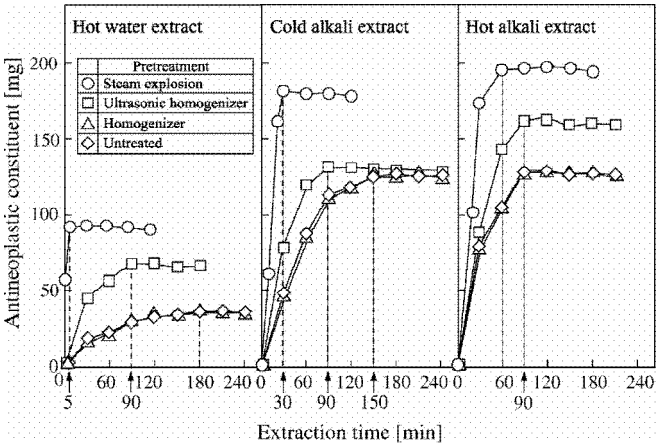


Fig. 3. Time courses of amount of antineoplastic constituent from 1 g *S. crispa* pretreated by various extraction methods. The numerical values accompanied with arrows and broken lines represent the time required to reach the highest amount of antineoplastic constituent.

Table 2
Effect of glucose concentration on growth properties of *S. crispa* in the incubation at pH 5 and 25 °C

Initial glucose concentration [g/L]	Time required to consume glucose completely [days]	Maximum dry cell weight [g/L]	Cell yield	Productivity [g/L/day]
10	5	2.96	0.30	0.59
20	8	6.32	0.32	0.79
30	9	7.95	0.27	0.88
40	12	8.41	0.21	0.70
50	16	9.92	0.20	0.62
60	20	11.2	0.19	0.56
70	25	13.7	0.20	0.54

Table 3
Effect of temperature on growth properties of *S. crispa* in the incubation at 30 g/L glucose and pH 5

Temperature [°C]	Time required to consume glucose completely [days]	Maximum dry cell weight [g/L]	Productivity [g/L/day]
15	34	6.45	0.19
20	15	7.18	0.48
25	9	7.95	0.88
30	9	7.98	0.89
40	–	–	–

Table 4
Amount of antineoplastic constituent in various extracts and optimal extraction time for obtaining antineoplastic constituent from 1 g *S. crispa* mycelia pretreated by various methods

	Steam explosion treatment		Ultrasonic homogenizer treatment		Homogenizer treatment		Untreated	
	Antineoplastic constituent [mg]	Optimal extraction time [min]	Antineoplastic constituent [mg]	Optimal extraction time [min]	Antineoplastic constituent [mg]	Optimal extraction time [min]	Antineoplastic constituent [mg]	Optimal extraction time [min]
Hot water extract	93 ± 8	5	68 ± 6	90	36 ± 6	180	35 ± 4	180
Cold alkali extract	182 ± 5	30	132 ± 5	90	127 ± 4	150	126 ± 8	150
Hot alkali extract	196 ± 7	60	162 ± 5	90	133 ± 7	90	137 ± 4	90
Total extract	471 ± 20	95	362 ± 16	270	296 ± 17	420	298 ± 16	420

due to end-product inhibition. Therefore, it was found that the initial glucose concentration should be 30 g/L in order to obtain a large amount of mycelia at a short incubation time.

Table 3 shows the effect of temperature on the time required to consume glucose completely, the maximum dry cell weight, and the productivity in the incubation of *S. crispa* mycelia at a glucose concentration of 30 g/L and pH 5. The productivity reached its highest value at 30 °C but there was no significant difference between 25 and 30 °C. Though even below 20 °C fungal growth occurred, the glucose consumption was slow and the productivity was very low. On the other hand, no cell growth and glucose consumption was observed at 40 °C. As a result, optimal temperature range for mycelial growth was found to be 25–30°C. The reason why the optimal temperature was comparatively low depended on the fact that *S. crispa* grows up mainly in higher mountains.

3.3. Extraction of antineoplastic constituent

Fig. 3 shows the time courses of amount of extracted antineoplastic constituent from 1 g dry fungal mycelia by various extraction methods. The numerical values accompanied with arrows and broken lines in this figure represent the time required to reach the highest amount of antineoplastic constituent. The amounts of antineoplastic constituent increased rapidly with the increase of extraction time reaching their maximum values. The extraction time required to reach the maximum value, i.e. the optimal extraction time, varied significantly with the extraction methods.

Table 4 shows the amount of extract, i.e. antineoplastic constituent, from 1 g dry fungal mycelia and the optimal extraction time using various pretreatment methods. The optimal extraction time is the time required to reach the highest amount of extract. The homogenizer treatment showed no differences in both the extraction time and the amount of extract as compared to untreated sample. It seems that the homogenizer treatment was merely a mechanical pretreatment that could not hydrolyze polysaccharides in the cell walls and increase the amount of extract. The amount of extract by the ultrasonic homogenizer treatment was 1.2 times more than that of the untreated sample and its total extraction time was shortened to 3/5 of the untreated sample. It suggests that the polysaccharides in the cell walls were hydrolyzed by reaction with hydroxyl radical produced by the ultrasonic waves. In the hot water extraction, the amount of the extract was about two times more than that of untreated sample but no remarkable increase was observed in the cold and hot alkali extractions. It is thought that hot water extraction was more efficient because the hydrolysis of polysaccharides in the cell wall and the extraction of antineoplastic constituent progressed at the same time by the ultrasonic homogenizer treatment. Total extract of 471 ± 20 mg was obtained from 1 g dry fungal mycelia pretreated by steam explosion because the polysaccharides in the cell wall were degraded and depolymerized by the hydrolysis reaction with high temperature and pressure steam followed by the sudden reduction of the pressure. This amount is about 1.6 times more than that of untreated sample and its total extraction time was shortened up to below 1/4. Since the cell wall was highly destroyed due to the exposure to high temperature and pressure steam, i.e. the polysaccharides combined with the chitin were separated and depolymerized, the amount of extract increased and the extraction time was shortened. Furthermore, it seems that the remarkable increase in the amount of the extract is attributed to the solubilization of polysaccharides into low molecular weight constituents.

4. Conclusions

The shake-flask liquid culture obtained the highest productivity of *S. crispa* mycelia, i.e. 0.89 g/L/day, at 30 g/L glucose, pH 5, and 25–30 °C. The steam explosion for 5 min at a steam pressure of 25 atm was a very effective pretreatment for the extraction of antineoplastic constituent from *S. crispa*: this method increased by about 1.6 times of antineoplastic constituent compared with the untreated sample and made an extraction time shorter up to below 1/4. Furthermore, these methods will be applied to mass production and extraction of antineoplastic constituent form not only *S. crispa* but also other basidiomycetes.

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