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# Bioactivity of the crude polysaccharides from fermented soybean curd residue by *Flammulina velutipes*

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ABSTRACT

The solid-state fermentation, reusing soybean curd residue (SCR) as a solid substrate, was conducted for producing polysaccharides by *Flammulina velutipes* (*F. velutipes*). The optimal fermentation conditions were 74.5% of moisture content, 9.69 of inoculum size and 30.27 of C/N ratio by response surface methodology. 59.15 mg/g of polysaccharides were obtained. *F. velutipes* polysaccharides were subsequently extracted from fermented SCR by ultrasonic assisted extraction. The optimal extract conditions were 30 min, 80 °C, 150 W and 20 of water to solid ratio and 106.74 mg/g of polysaccharides were obtained. Furthermore, the antioxidant and the immunomodulatory activities of polysaccharides were assessed. The results showed that polysaccharides exhibited a strong DPPH radical scavenging activity, SOD-like activity, stimulatory the proliferation of the macrophage, the production of nitric oxide, phagocytosis and the protection on Doxorubicin damage. These could lay the foundation for changing SCR into a nutritious functional food or a food additive.



## 1. Introduction

In recent years, there has been an unprecedented increase in interest in the more efficient utilization of the agro-industrial residues because their application provides an alternative way to reduce the production costs and solve many environmental hazards (Qijun et al., 2008). Soybean curd residue (SCR), a by-product of tofu, soymilk or soy protein manufacturing, is discharged as an agro-industrial waste. 0.7 million tons of SCR is disposed in Japan annually, and the most of SCR was incineration, which has caused severe environmental pollution. In fact, SCR contains the protein up to 25.4–28.4% (dry basis) with the high nutritive quality and a superior protein efficiency ratio, suggesting that SCR is a potential source of low-cost vegetable protein for human consumption (Kasai, Murata, Inui, Sakamoto, & Kahn, 2004; O'Toole, 1999). In addition to, this material was rich in the fat, the starch and the sugar, which could allow them to be potentially utilized as a high quality media for the microbial fermentation. Many researchers have investigated the possibility of bioconversion of the residues by submerged and solid-state cultivation (SSC) (Adams, Eiteman, & Hanel, 2002; Holker & Lenz, 2005; Pandey, Soccol, Nigam, & Soccol, 2000; Yokoi, Maki, Hirose, & Hayashi, 2002).

*Flammulina velutipes* (*F. velutipes*) is a cultivated mushroom. Few studies, however, have been conducted on this species. An alkaline protease and the antitumor activities have been reported from this mushroom (Cui et al., 2006; Wang, Hu, Liang, & Yeh, 2005). Both methanolic and ethyl acetate extracts of this mushroom exhibited anti-hyperlipidemic and antioxidant activities (Hu et al., 2006). As a result of its perceived health benefits, *F. velutipes* has become one of the valuable mushrooms in China.

The low immune function of an organism may not only result in the generation and development of a tumor, but may also be one of the most important factors that prevent the tumor patient's recovery. Immunomodulation through natural or synthetic substances may be considered an alternative for the prevention and cure of diseases.

Macrophages play a significant role in the host defense mechanism. When activated, they activate phagocytic activity, produce and release reactive oxygen species (ROS) and the nitric oxide (NO) in response to the stimulation with various agents and can inhibit the growth of a wide variety of tumor cells and micro-organisms (Schepetkin et al., 2008). Moreover, the immunomodulatory activity not only involves effects on macrophage activation but also on cell proliferation and differentiation (Schepetkin & Quinn, 2006). Papers report that polysaccharide from the mushroom can enhance and activate the macrophage immune responses, leading to immunomodulation, anti-tumor activity, wound-healing and other therapeutic effects (Berner, Sura, Alves, & Hunter, 2005; Sakurai, Kaise, Yadomae, & Matsubara, 1997).

Up to now, *F. velutipes* polysaccharides are mainly extracted from the fruiting body growing on the solid culture medium. However, the time for the growth of fruiting body is too long and its

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product quality is difficult to control. Therefore, it deserves investigation to produce polysaccharides from the mycelia of *F. velutipest* by SSC. As SSC can be more commonly applied than liquid-state cultivation (Lekha & Lonsane, 1994). SSC has also been frequently utilized in the preliminary tests for cultivating the microorganisms under the experimental conditions because it requires less time and less labor intensive than liquid-state cultivation.

To achieve higher polysaccharides yield in a SSC, it is a prerequisite to design an optimal production conditions. The single-factor at a time, the most widely used optimization method, does not account for the combined effects of all the influential factors since other factors are maintained arbitrarily at a constant level. In addition, it is time consuming and requires a large number of experiments to determine the optimum levels of the production conditions (Qijun et al., 2008). However, such drawbacks of the single-factor optimization method can be overcome by statistical optimization techniques (Chen, Zhao, Chen, & Li, 2008). Response surface techniques of central composite design (CCD) is an important statistical optimization method which many factors can be optimized simultaneously and much quantitative information can be extracted by only a few experimental trials (Li et al., 2011). This method has been successfully applied to the improvement of the culture media or the production of primary and secondary metabolites in the cultivation process of many edible and medicinal mushrooms (Chang, Tsai, & Houng, 2006; Chen et al., 2008). However, there is still a lack of knowledge concerning SSC conditions for the production of polysaccharides from *F. velutipes* by statistical optimization techniques.

Therefore, the objective of this study is to estimate the optimum technology of the fermentation of *F. velutipes* polysaccharides by response surface technology. *F. velutipes* polysaccharides of fermented SCR were subsequently extracted by the ultrasonic assisted technology to investigate the antioxidant activities and the immunomodulatory activities on macrophage RAW 264.7 cells.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Minimum Essential Medium Eagle (MEM) medium, fetal bovine serum (FBS), were purchased from Sigma–Aldrich, Inc. (Saint Louis, MO, USA). Acetic anhydride, pyridine hydroxylamine hydrochloride, trifluoroacetic acid lipopolysaccharide (LPS) from *E. coli* 055 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan), Doxorubicin (DOX) was purchased from TopoGEN, Inc. (FL, USA).

### 2.2. Cell lines

The murine macrophage cell line, RAW 264.7 was obtained from the Riken Cell Bank (Tsukuba, Japan) and maintained in MEM medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL of streptomycin at 37 °C in a humidified 5% $CO_2$ atmosphere (ESPEC $CO_2$ Incubator). The cells were cultured for 2–3 days to reach the logarithmic phase and then used for experiments.

### 2.3. Microorganism and culture conditions

The A16 strain of *F. velutipes* was purchased from agriculture and forestry strains Kaishas, Japan. D-Glucose, sucrose, peptone, $KH_2PO_4$, $MgSO_4$, potato extract, agar were obtained from Wako Pure Chemical, Osaka, Japan.

The strain was maintained on potato dextrose agar (PDA) at 4 °C. To maintain the strain activity, a mycelium square of size 5 mm × 5 mm was transferred to a fresh PDA agar every 3 months. The activation medium (Yang & Liau, 1998) consisted of the following components: 2% glucose, 2% peptone, 0.4% potato extract, 0.3% $KH_2PO_4$, 0.15% $MgSO_4$, 2% agar. The initial pH was not adjusted (5.0–5.5). The mycelial agar petri dish was incubated at 25 °C for 7 days. The 15-mL liquid culture was performed in a 50-mL flask containing one unit of mycelial agar, which was a 5 mm × 5 mm square that was obtained using a self-designed cutter on a rotary shaker at 100 rpm and 25 °C for 6 days. The flask of the liquid culture medium was composed of the following components: 2% sucrose, 2% yeast extract, 0.4% potato extract, 0.1% NaCl, 0.3% $KH_2PO_4$, 0.15% $MgSO_4$. The initial pH was from 5.0 to 5.5. The seed for the solid culture was from the liquid culture. The solid-state culture experiment was performed in a 200-mL flask with the wet SCR in the different culture conditions and incubated at 25 °C. All of the media were autoclaved at 121 °C for 30 min.

### 2.4. The polysaccharides determination

#### 2.4.1. Crude polysaccharides extraction

The treatment of the crude *F. velutipes* polysaccharides was according to a literature procedure with a few modifications (Di, Zhenya, Yingnan, Guoqing, & Jiqiang, 2011). The fermented SCR was dried in a convection oven at 50 °C and ground to a powder. The crushed powder was removed the impurities for 24 h with 80% ethanol at room temperature. The extract was discarded and the residue was further extracted with the optimal conditions of ultrasonic assisted extraction. Then, the extract was filtered and centrifuged at 7500 rpm for 30 min at room temperature. The supernatant was concentrated in a rotary evaporator under reduced pressure at 50 °C and removed free protein layer by the use of method of Sevage. At last, the above extract was subjected to the precipitation with eight-fold volumes of ethanol. The curd polysaccharides were collected by centrifugation, washed with ethanol twice, and then freeze-dried and total polysaccharide was the subtraction of reducing sugar from the total carbohydrate.

#### 2.4.2. Determination of total carbohydrate content

The carbohydrate contents were determined by the phenol–sulfuric acid method with certain modifications (Mauro, 2005). The color reaction was initiated by mixing 1 mL of polysaccharides solution with 0.5 mL of a 5% phenol solution and 2.5 mL of concentrated sulfuric acid, and the reaction mixture was incubation in a boiling water bath for 15 min. After cooling to the room temperature, the optical density (O.D.) of the mixture was determined at 490 nm and the total carbohydrate content were calculated with D-glucose as a standard. The results were expressed as milligram of D-glucose equivalent per gram of fermented SCR.

#### 2.4.3. Determination of reducing sugar

Reducing sugar content was analyzed by dinitrosalicylic (DNS) colorimetric method (Miller, 1959), using D-glucose as a standard. For each of the 1 mL of the sample, 2 mL of DNS reagent and 12 mL deionized water were added. The mixture was then heated in boiling water for 5 min until the red brown color was developed. Then, the mixture was cooled to room temperature in a water bath. The absorbance was then measured at 540 nm. The concentration of total reducing sugars was calculated based on a standard curve obtained with D-glucose.

#### 2.4.4. Monosaccharide analysis

The crude polysaccharides (10 mg), dissolved in 2 M trifluoroacetic acid (TFA, 2 mL), was hydrolyzed at 120 °C for 3 h in a sealed glass tube. The hydrolyzate was repeatedly co-concentrated with methanol to remove the excess acid at 50 °C, and then the