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 ℃, 150 watt 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.

Keywords：Soybean curd residue，*Flammulina velutipes*，Polysaccharides, Macrophages, Antioxidant activities, Immunomodulatory activities

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 provide 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, *et al.*, 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. velutipe*s) 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

68 perceived health benefits, *F. velutipes* has become one of the valuable mushrooms in
69 China.

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

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

84 Up to now, *F. velutipes* polysaccharides are mainly extracted from the fruiting body
85 growing on the solid culture medium. However, the time for the growth of fruiting
86 body is too long and its product quality is difficult to control. Therefore, it deserves
87 investigation to produce polysaccharides from the mycelia of *F. velutipest* by SSC. As
88 SSC can be more commonly applied than liquid-state cultivation (Lekha, & Lonsane,
89 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, *et al.*, 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. (Florida, 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 ℃ 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 ℃. 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 ℃ 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 ℃ for 6 days. Theflask 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 ℃. All of the media were autoclaved at 121℃ 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 modfications (Di*, et al.*, 2011). The fermented SCR was dried in a convection oven at 50 ℃ and ground to a powder. The crushed powder was removed the impurities for 24h 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

156 concentrated in a rotary evaporator under reduced pressure at 50 ℃ and removed
157 free protein layer by the use of method of Sevage. At last, the above extract was
158 subjected to the precipitation with eight-fold volumes of ethanol. The curd
159 polysaccharides were collected by centrifugation, washed with ethanol twice, and
160 then freeze-dried and total polysaccharide was the subtraction of reducing sugar
161 from the total carbohydrate.

162 2.4.2 Determination of total carbohydrate content

163 The carbohydrate contents were determined by the phenol-sulfuric acid method
164 with certain modifications. (Mauro, 2005). The color reaction was initiated by mixing
165 1 mL of polysaccharides solution with 0.5 mL of a 5% phenol solution and 2.5 mL of
166 concentrated sulfuric acid, and the reaction mixture was incubation in a boiling water
167 bath for 15 min. After cooling to the room temperature, the optical density (O.D.) of
168 the mixture was determined at 490 nm and the total carbohydrate content were
169 calculated with D-glucose as a standard. The results were expressed as milligram of
170 D-glucose equivalent per gram of fermented SCR.

171 2.4.3 Determination of reducing sugar

172 Reducing sugar content was analyzed by dinitrosalicylic (DNS) colorimetric
173 method (Miller, 1959), using d-glucose as a standard. For each of the 1 mL of the
174 sample, 2 mL of DNS reagent and 12 mL deionized water were added. The mixture
175 was then heated in boiling water for 5 min until the red brown color was developed.
176 Then, the mixture was cooled to room temperature in a water bath. The absorbance
177 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 ℃ for 3 h in asealed glass tube. The hydrolyzate was repeatedly co-concentrated with methanol to remove the excess acid at 50 ℃, and then the hydrolyzed products were prepared for acetylation. The acetylation was carried out with 10 mg hydroxylamine hydrochloride and 0.5 mL pyridine by heating in a water bath for 30 min at 90 ℃. After incubation, the tubes were removed from the heat block, allowed to cool to room temperature, and then 0.5 mL of acetic anhydride was added and mixed thoroughly by vortexing. The tubes were sealed and incubated in a water bath shaker set at 90 ℃ for 30 min again. After cooling, approximately 0.1 mL of clear supernatant was added to the autosampler vials with inserts for injection into the gas chromatograph on a GCMS-QP2010Plus (SHIMADZU, JAP) instrument equipped with a hydrogen flame ionization detector, using a DB-1 column (30 m × 0.25 mm × 0.25 μm). The following chromatographic conditions were used: high-purity helium was used as the carrier gas at a flow rate of 1 mL/min. The temperature of the injector and detector was 250 ℃. An initial column temperature held at 60 ℃ for 2 min followed by 15 ℃/min to 180 ℃ and then 4 ℃/min to 250 ℃ for 3min. Injections were made in the splitless mode. The temperature of mass spectrometer ion source was 250 ℃. 1 μL sample was injected into the column with the split ratio of 10:1.

2.5 Antioxidant properties

2.5.1 DPPH radical scavenging activity

The free radical scavenging activities of the extracts were measured by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH •) (Shuang li*, et al.*, 2011). Briefly, 0.2 mL of polysaccharides extract at various concentrations was added to 3.3 mL of DPPH solution (25 μg/mL) for 30 min at room temperature in the dark. Methanol was used instead of polysaccharides extract as the control. Then the absorbance was measured at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation: DPPH scavenging effect (%) = $[(A_0 - A_1/A_0) \times 100]$, where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of DPPH scavenging effect against the extract concentration. Ascorbic acid was used as the positive control.

2.5.2 Determination of SOD-like activity

The levels of SOD-like activity in the extracts were measured using the SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular Technologies, Inc. Briefly, in a 96-well plate, 20 μL of sample solution was added to each sample and blank 2 well, and 20 μL of double distilled water was added to each blank 1 and blank 3 well. Then 200 μL of WST working solution was added to each well. After mixing, 20 μL of dilution buffer was added to each blank 2 and blank 3 well, and 20 μL of enzyme working solution (15 μL of enzyme mixted with 2.5 μL dilution buffer) was added to each sample and blank 1 well. The plate was incubated at 37 ℃ for 20 min and the O.D. was determined at 450 nm using a microplate

reader (BIO-RAD Model 550, USA). The SOD-like activity was calculated by the following equation:

SOD activity (inhibition rate %) =

$$\{[(A_{blank1}-A_{blank3})-(A_{sample}-A_{blank2})] / (A_{blank1}-A_{blank3})\} \times 100 \quad (1)$$

Where $A_{blank\ 1}$, $A_{blank\ 2}$, $A_{blank\ 3}$ and A_{sample} were the absorbance of blank 1, blank 2, blank 3 and the sample respectively.

2.6 Cell evaluation

2.6.1 Activation assay

The effect of *F. velutipes* polysaccharides on the proliferation of RAW 264.7 cells was estimated using the Cell Counting Kit-8 (CCK-8). RAW 264.7 cells were cultured in a 96-well plate at a density of 5×10^4 cells/mL at 37 ℃ in a 5% CO_2 atmosphere for 24 h. Next the cells were incubated with various concentrations of polysaccharides (1.25, 2.5, 5, 10, 20 and 40 μg/mL) at 37 ℃ for 24 h. After incubation, 10 μL of CCK-8 solution was added and incubated at 37 ℃ for 4 h. The cell viability was assessed by the O.D. at a wavelength of 450 nm with a microplate reader (BIO-RAD Model 550). The data were expressed as the percentage of the control.

2.6.2 Measurement of the production of the nitric oxide

The nitrite accumulation was measured using Griess reagent and used as an indicator of nitric oxide (NO) production in the medium (Di*, et al.*, 2011; Gamal-Eldeen*, et al.*, 2007). The macrophage cells (1×10^5 cells/mL) were dispensed into a 96-well plate for 24 h. Next the cells were stimulated with LPS (1 μg/mL) and

various concentrations of *F. velutipes* polysaccharides (2.5, 5, 10, 20 and 40 μg/mL) for 24 h. After the incubation, 50 μL of the culture supernatants were mixed with an equal volume of Griess reagent in a 96-well plate and incubated at 25 ℃ for 10 min. The absorbance at 570 nm was measured on a microplate reader. The nitrite concentrations in the culture supernatants were measured to assess the NO production in the RAW 264.7 cells. $NaNO_2$ was used as a standard to calculate the nitrite concentrations.

2.6.3 Phagocytosis assay

The phagocytic ability of the macrophage was measured by the neutral red uptake (Cheng*, et al.*, 2008). The cells were cultured in a 96-well plate at a density of 5×10^4 cells/mL at 37 ℃ in a 5% CO_2 atmosphere for 24 h. Next the cells were incubated with various concentrations of *F. velutipes* polysaccharides (1.25, 2.5, 5, 10, 20 and 40 μg/mL) and LPS (1 μg/mL) at 37 ℃ for 48 h. 100 μL of 0.075% neutral red solution was added and incubated for 1 h. Then the supernatant was discarded and the cells were washed with PBS twice. Then 100 μL of the cell lysate solution (ethanol and 0.01% acetic acid at the ratio of 1:1) was added into a 96-well plate to lyse the cells at the room temperature for 2 h. The optical density at 570 nm was measured by a microplate reader (BIO-RAD Model 550).

2.6.4 Protective activity

The macrophages RAW 264.7 cells were cultured in a 96-well plate at a density of 5×10^4 cells/mL for 24 h at 37 ℃ in a 5% CO_2 atmosphere. Next the cells were incubated with DOX (5 μM) in the presence or absence of various concentrations of *F. velutipes* polysaccharides (2.5, 5, 10 and 20 μg/mL) for 24 h. After the drug exposure,

267 10 μL of the CCK-8 solution was added and incubated at 37 ℃ for 4 h. The cell
268 numbers were quantitated by reading the absorbance at 450 nm. The data were
269 expressed as the percentage of the control.

270 *2.7 Experimental design*

271 2.7.1 The optimization using response surface methodology

272 RSM is a collection of mathematical and statistical technique for building the
273 empirical models. It was applied to evaluate the relative significance of the
274 environmental factors and to determine the optimum conditions under which the
275 mycelia growth is the maximum, within the experimental range of the independent
276 variables (Hwanyoung, Minkyung, & Seokhwan, 2003). In this study, RSM was used
277 to assess the relationship of the radial extension rate to the fermentation conditions
278 (the inoculum size, the moisture content and the C/N ratios).

279 The experiment (Table 1) was based on the central composite in the cube design
280 and consisted of a 3 × 2 the central composite design (CCD) (the inoculum size, the
281 moisture content and the C/N ratio, each at two levels). The ranges of the independent
282 variables were 7.5% to 12.5% of the inoculum size, 60% to 80% of the moisture
283 content and 20 to 40 of the C/N ratios. Each treatment with a center point (i.e., 10% of
284 the inoculum size, 70% of the moisture content and 30 of the C/N ratio) was
285 replicated 5 times as previously described. This type of design was used to minimize
286 the number of the trials needed to obtain statistically the valid results.

287 A sequential procedure of collecting data, estimating polynomials, and checking the
288 adequacy of the model was used. The method of least squares was used to estimate

289 the parameters in the approximating polynomials. For the statistical analysis,
290 Stat-Ease Design-Expert 8.0.5 (Stat-Ease Corporation, USA) was used to establish the
291 experimental design and to test the complex polynomials.

292 2.7.2 The orthogonal array design

293 *F. velutipes* polysaccharides were extracted from fermented SCR by ultrasonic
294 assisted extraction and the extract process was optimized through the orthogonal array
295 design table of $L_9(3^4)$ shown in Table 2 (Xiong, Shouwen, Ming, & Ziniu, 2005).

296 # 3. Results and discussion

297 ## *3.1 Parameters of the solid-state cultivation* of *F. velutipes polysaccharides* using
298 *response surface methodology*

299 Based on the single-factor experiment, three variables (the inoculum size, the
300 moisture content and the C/N ratios) were used to determine the optimum levels of
301 these parameters and their interactions according to 20 days of fermentation time and
302 5.5 of pH value. There was a considerable variation in the yield of polysaccharides
303 depending upon the fermentation conditions, as shown in Table 3. The replication at
304 the center point conditions resulted in a higher yield of polysaccharides than at the
305 other levels. The predicted response Y for the production of polysaccharides was
306 obtained as follows:

307 $$y = -371 + 23.99x_1 + 6.5x_2 + 4.75x_3 - 0.01x_1x_2 + 0.27x_1x_3 + 0.02x_2x_3 - 1.63x_1^2 - 0.05x_2^2 - 0.14x_3^2 \quad (2)$$

308 The statistical significance of Eq. (2) was confirmed by an F-test and the analysis of
309 variance (ANOVA) for the response surface quadratic model summarized in Table 4.

The ANOVA of the quadratic regression model demonstrated that the model was significant, with an F-test of a very low probability value ($P > F$) < 0.0001. The goodness of the model was indicated by the determination coefficient (R^2) and the multiple correlation coefficient (R). The value of R^2 (0.993) for Eq. (2) suggested that 99.3% of the sample variation for *F. velutipes* polysaccharides was attributed to the independent variables, and only 0.7% of the total variation could not be explained by this model (Pujari, & Chandra, 2000). The "Pred R-Squared" of 0.9040 was in reasonable agreement with the "Adj R-Squared" of 0.9839. The insignificant lack-of-fit showed that the polynomial model was satisfactorily accurate for predicting the relevant responses. The adequate precision was used to measure the ratio of signal to noise, which is generally desired to be greater than 4. In the present study, the value of this ratio (32.681) suggested that the polynomial quadratic model was of an adequate signal, which could be used to navigate the design space (Table 4).

The *P*-values were used as a tool to check the significance of the each coefficient, the smaller the value of *P*, the more significant was the corresponding coefficient (Rao , Kim, & Rhee, 2000). As can be seen from Table 4, three linear coefficients (X_1–X_3) and two quadratic coefficients were significant. The insignificant coefficients were still considered in the Eq. (2) because it was a hierarchical model (Wang, & Lu, 2004). The 3D-surface plot and 2D-projection were able to visually show the response over a region of the interesting factor levels, the relationship between the response and the experimental levels of each variable, and the type of the interactions between the test variables to deduce the optimum conditions (Fig.1 (A–F)).

The 3D-surface plot and 2D-projection (Fig.1 (A, B)) depicted the effects of the inoculum size and the moisture content on the yield of *F. velutipes* polysaccharides, whereas the C/N ratio was fixed at its optimal concentration. The 3D-plot showed that the production of *F. velutipes* polysaccharides significantly increased upon increasing the moisture content to approximately 74%, but decreased sharply beyond this duration, reaching a maximum yield at 74.5%. The effect of the inoculum size on the yield of *F. velutipes* polysaccharides was additionally sensitive within the tested range, reaching a maximum yield at approximately 10. The same trends were indicated in Fig. 1(C-F).

By solving the inverse matrix (from Eq. (2)), the optimum values of the test variables were determined to be 74.5% of the moisture content, 9.69% of the inoculum size and 30.27 of the C/N ratio. In this situation, the maximum predicted production of *F. velutipes* polysaccharides were 59.40 mg/g. Cultivated with the optimized conditions, *F. velutipes* polysaccharides of fermented SCR (59.15 ± 1.47 mg/g) were accumulated and as much as sevenfold compared with unfermented SCR (8.01 ± 0.54 mg/g).

3.2 The optimization of F. velutipes polysaccharides extraction

There were many factors affecting *F. velutipes* polysaccharides by ultrasonic assisted extraction, such as extracted time, extracted temperature, the ultrasonic power and the ratio of liquid to solid. Based on the orthogonal experiment design $L_9(3^4)$, a total of four factors above, each with three different levels (Table 2), were selected in this study. The results and the effects of those factors on *F. velutipes* polysaccharides

354 extraction were additionally showed in Table 2. Based on the magnitude order of R,
355 the effects of the factors on the extraction of *F. velutipes* polysaccharides decreased in
356 the following order: extracted temperature (B), the ratio of liquid to solid (D),
357 extracted time (A), the ultrasonic power (C). Based on the magnitude order of K
358 (Table 2), the optimal combination was $A_3B_3C_3D_2$, namely 30 min of the extracted
359 time, 80 ℃ temperature, 110 watt of the power and 20:1 of the ratio of liquid to solid.
360 The results of the orthogonal experiment additionally showed that all of the single
361 factor effects on the yield of *F. velutipes* polysaccharides were significant ($P < 0.01$)
362 (Table 5). The mean yield of *F. velutipes* polysaccharides under the optimum
363 extracted conditions was 106.74 ± 1.73 mg/g. *F. velutipes* polysaccharides yield
364 increased approximately 70% against the process parameters before the optimization.
365 *3.3 Monosaccharide composition of F. velutipes polysaccharides*
366 The compositions of the crude *F. velutipes polysaccharides* have been assessed,
367 which contained 88.74% carbohydrates and 0.92% protein. Furthermore, there was
368 0.32% reducing sugar in the extracts, therefore, the percentage of *F. velutipes*
369 polysaccharides was 88.42%.
370 Usually, GC analysis could give the accurate content of sugars in the
371 polysaccharides. The experimental results from GC showed that *F. velutipes*
372 polysaccharides was a typical heteropolysaccharide charide, and the retention times
373 were 11.21, 11.39, 11.52, 13.73, 13.83, 14.08 and 15.04 min for rhamnose, arabinose,
374 xylose, mannose, glucose, galactose and myo-inositol, respectively. After
375 identification and quantitation, GC chromatogram showed six monosaccharides

including rhamnose, arabinose, xylose, mannose, glucose and galactose to be present in *F. velutipes* polysaccharides at a molar ratio of 0.42: 0.37: 1.3: 1.79: 3.38: 0.46, respectively.

3.4 Antioxidant activities of F. velutipes polysaccharides

3.4.1 Scavenging activity of DPPH radicals

ROS produced the in vivo including superoxide radical, hydrogen peroxide and hypochlorous acid. Hydrogen peroxide and superoxide can interact in the presence of certain transition metal ions to yield a highly-reactive oxidising species, the hydroxyl radical (Halliwell, & Gutteridge, 1985). The antioxidants react with the stable free radical DPPH (deep violet color) and convert it to 1,1-diphenyl-2-picryl hydrazine with decoloration. The scavenging effects of *F. velutipes* polysaccharides increased with the concentrations, around 90% at 2.5 mg/mL of the concentration with 418.31 μg/mL of an IC_{50} value, compared with ascorbic acid (293.23 μg/mL) (Fig. 2). Previous reports indicated that ascorbic acid and polyphenols exhibited potent DPPH radical scavenging activity, those conjugated with polyphenols such as ferulic acid, have been shown to possess such activity. However, *F. velutipes* polysaccharides extracted with fermented SCR, showed a strong DPPH radical scavenging activity, moreover, its radical free scavenging capacity was superior to those found for several other edible mushroom methanolic extracts (Ferreira, Baptista, Vilas-Boas, & Barros, 2007). The results of Rao *et al*. that showed acidic sugars (galacturonic and glucuronic acids), and a hydroxyl group of acidic polysaccharides possessed radical scavenging activity (Rao, & Muralikrishna, 2006). Therefore, the effect observed in

this study was likely to be related to the acidic sugar present in *F. velutipes* polysaccharides.

3.4.2 SOD-like activity

All living bodies have a complex antioxidant defense system that includes various antioxidant enzymes, such as superoxide dismutase and catalase. A rapid and facile method for the assay of SOD-like activity, based on the ability to inhibit the auto-oxidation of pyrogallol, is widely used to predict antioxidant capability. SOD is an antioxidant enzyme that catalyzes the dismutation of superoxide anions into O_2 and H_2O_2 (Mates, Perez-Gomez, & Nunez de Castro, 1999). In the present study, we found that SOD-like activity increased with the concentrations of *F. velutipes* polysaccharides, and treated with *F. velutipes* polysaccharides at the concentration of 5 mg/mL, SOD-like activity was 82.48% (Fig. 3).

3.5 Evaluation of macrophage RAW 264.7 cells

3.5.1 The effect of on the proliferation of the macrophage

The stimulatory effect of *F. velutipes* polysaccharides extracted from fermented SCR on the proliferation of the macrophage was tested. The results showed that, exposure to *F. velutipes* polysaccharides activated the proliferation of the macrophage (Fig. 4). In the range of 1.25-40 μg/mL, polysaccharides stimulated the proliferation of RAW 264.7 cells in a dose-dependent manner. At the concentration of 20 μg/mL, the stimulatory effect reached a maximum, was 175.36%. Furthermore, the high concentrations (20-40 μg/mL) were tested on the macrophage. Although the survival rate of the cells decreased dose-dependently at high concentration, this may be related

420 with the immunological paralysis caused by the high dosage (Jinwei*, et al.*, 2011), the
421 survival rate of RAW 264.7 cells at 40 μg/mL was still higher than the control, was
422 106.15%. It was suggested that *F. velutipes* polysaccharides possessed a stimulatory
423 effect on the proliferation of the macrophage RAW 264.7 cells with low cytotoxicity.
424 3.5.2 The effect of *F. velutipes* polysaccharides on the production of the nitric oxide
425 It was reported that LPS showed strong immunomodulating activity (Avni, Ernst,
426 Philosoph, & Zor, 2010). This compound stimulates the macrophages to produce
427 pro-inflammatory cytokines and secondary mediator, such as NO which is a gaseous
428 molecule synthesized from L-arginine by nitric oxide synthase (NOS). It is a highly
429 reactive free radical that can form a number of oxidation products such as NO_2, NO_2^-,
430 N_2O_3 and S-nitrosothiols. NO participates in the physiology and pathophysiology of
431 many systems (Diouf, Stevanovic, & Boutin, 2009). It is an important mediator of the
432 non-specific host defense against invading microbes and tumors. Thus NO can be
433 used as a quantitative index of the macrophage activation. The results of the
434 stimulatory effect of polysaccharides on the NO production of the macrophage were
435 showed in Figure 5. The data showed that the incubation with polysaccharides
436 stimulated the NO production in RAW 264.7 cells in a dose-dependent manner.
437 Treatment with polysaccharides at a concentration of 40 μg/mL significantly
438 stimulated the NO production (29.11 μM) in comparison with the control (6.32 μM)
439 ($p<0.01$), and was higher than 20.52 μM of 1 μg/mL LPS (the positive control)
440 produced.
441 3.5.3 Effect of *F. velutipes* polysaccharides on the phagocytosis activity

Because the macrophages play an important role in the host defense that phagocytize the pathogens (Zhao, Dong, Chen, & Hu, 2010). Thus phagocytosis is an important indicator of the macrophage effector activity (Yu, *et al.*, 2008) and it represents the final and the most indispensable step of the immunological defense system (Campelo, *et al.*, 2002). The phagocytic activity of the macrophage was monitored by measuring the amount of neutral red internalized in the macrophage. The results were shown in Figure 6. *F. velutipes* polysaccharides significantly and dose-dependently increased the phagocytosis of RAW 264.7 cells in comparison with the control ($p<0.01$). Moreover, the O.D. value of treatment with *F. velutipes* polysaccharides at 5 μg/mL was higher than that of the positive control, which was treated with LPS at 1 μg/mL. The results demonstrated that the administration of *F. velutipes* polysaccharides might result in the initiation of the immune reaction against the foreign materials such as pathogen and tumors (WX Chen, Zhang, Shen, & Wang, 2010).

3.5.4 the protective effect of *F. velutipes* polysaccharides on DOX-induced macrophage viability

Doxorubicin (DOX) is a drug used in cancer chemotherapy. It is an anthracycline antibiotic, closely related to the natural product daunomycin, and like all anthracyclines, it works by intercalating DNA. Treatment with DOX resulted in a decrease of the macrophage survival rate, which was 58.45% (Figure 7). However in the presence of *F. velutipes* polysaccharides, the viability of macrophages was significantly higher than that of the incubation with DOX. For example, the

incubation with 20 μg/mL of *F. velutipes* polysaccharides, the cell survival rate (93.48%) was significantly higher than the negative control (exposure to 5 μM DOX). When incubated DOX-induced macrophages with the various concentrations of *F. velutipes* polysaccharides, the cells survival rate increased in a dose-dependent manner.

4. Conclusions

The production of polysaccharide from *Flammulina velutipes* reusing soybean curd residue was investigated in the solid-state cultures and the optimized culture conditions for polysaccharides were obtained by response surface methodology. In addition, the yield of *F. velutipes* polysaccharides was improved by ultrasonic assisted extraction. Moreover, the extracted *F. velutipes* polysaccharide from fermented SCR showed a strong DPPH radical scavenging activity, SOD-like activity, stimulatory effect on the proliferation of the cells, the NO production, phagocytosis and the protection of the macrophages from DOX damage. These finding indicated that fermented SCR by *F. velutipes* could be a potential and nutritious ecologic feed and a functional food material. Further works are in progress on the isolation, purification, characterization and functional effects of polysaccharide from *F. velutipes* generated by reusing SCR.

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Figure caption

Fig.1 Response surface 3D-surface plot and 2D-projection showing the effects of the inoculum size, the moisture content and the C/N ratio on the yield of *F. velutipes* polysaccharides.

Fig.2 DPPH radical scavenging activity of *F. velutipes* polysaccharides. The data were expressed as means ± S.D. of triplicate determinations. Ascorbic acid was used as the positive control.

Fig.3 SOD-like activity of *F. velutipes* polysaccharides. The data were expressed as means ± S.D. of triplicate determinations.

Fig.4 The effect of *F. velutipes* polysaccharides from fermented soybean curd residue on the proliferation of the macrophage RAW 264.7 cells.
The cells were incubated with various concentrations of *F. velutipes* polysaccharides (1.25, 2.5, 5, 10, 20 and 40 μg/mL). The data were expressed as means ± S.D. of three independent experiments. (*p<0.05, **p<0.01 in comparison with the control).

Fig.5 The effect of F. velutipes polysaccharides from fermented soybean curd residue on the nitric oxide production of the macrophage RAW 264.7 cells.
The cells were incubated with various concentrations of *F. velutipes* polysaccharides (2.5, 5,10, 20 and 40 μg/mL) and 1 μg/mL of LPS for 24 h. LPS was the positive control. The data were expressed as means ± S.D. (n=3). (**p<0.01 in comparison with the control).

Fig.6 The effect of treatment with *F. velutipes* polysaccharides for 48 h on phagocytosis of the macrophage RAW 264.7 cells.
The cells were incubated with various concentrations of *F. velutipes* polysaccharides (1.25, 2.5, 5, 10, 20 and 40 μg/mL) and 1 μg/mL of LPS for 48 h. The data were expressed as means ± S.D. (n=3), (**p<0.01 in comparison with the control).

Fig.7 The effect of *F. velutipes* polysaccharides from fermented soybean curd residue on DOX-induced the macrophage RAW 264.7 cells survival rate.
The macrophage RAW 264.7 cells were incubated with DOX (5 μM) in the presence or absence of various concentrations of *F. velutipes* polysaccharides (2.5, 5, 10 and 20 μg/mL) for 24 h. The data were expressed as means ± S.D. (n=3). (* p<0.05, **p<0.01 in comparison with DOX).

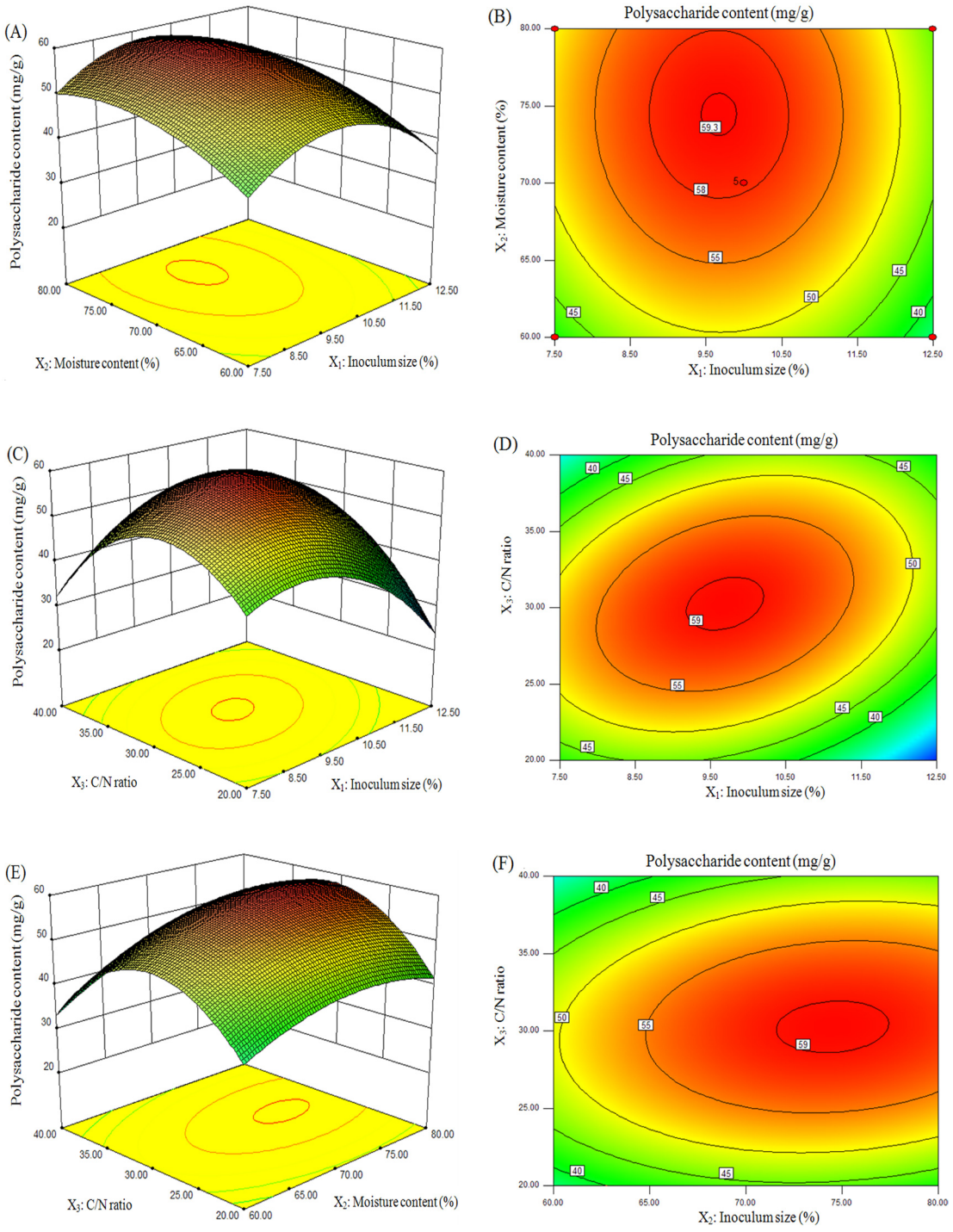
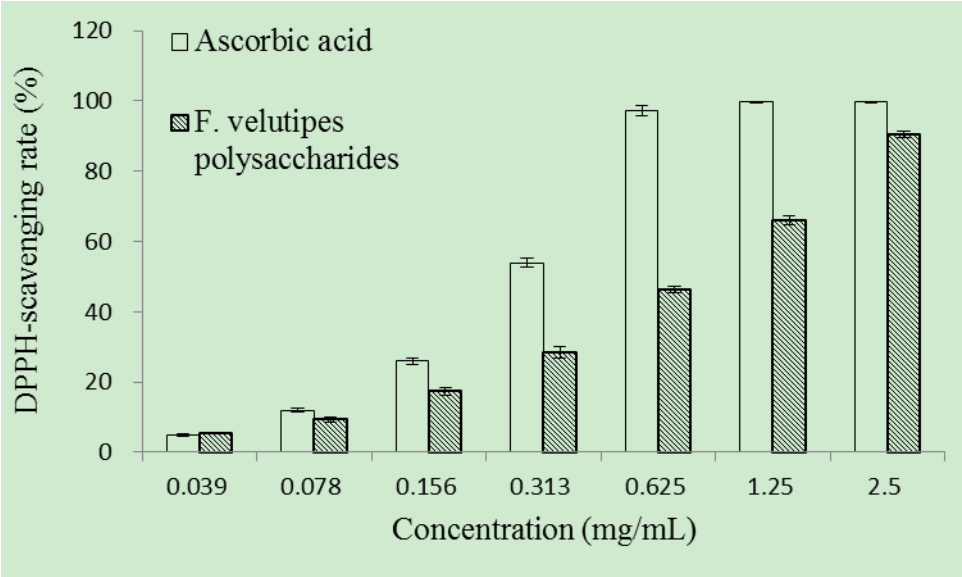


Fig. 1 Shi etal.



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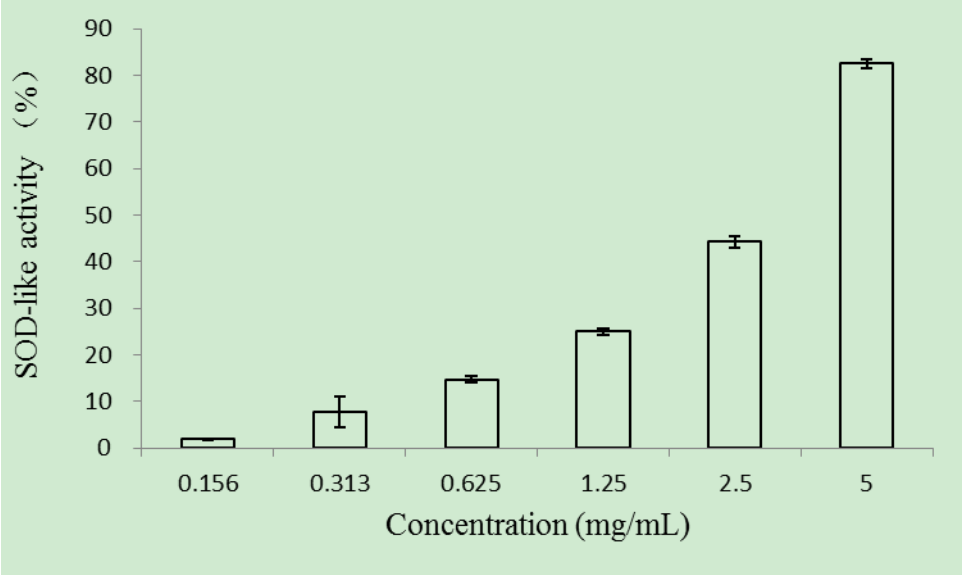

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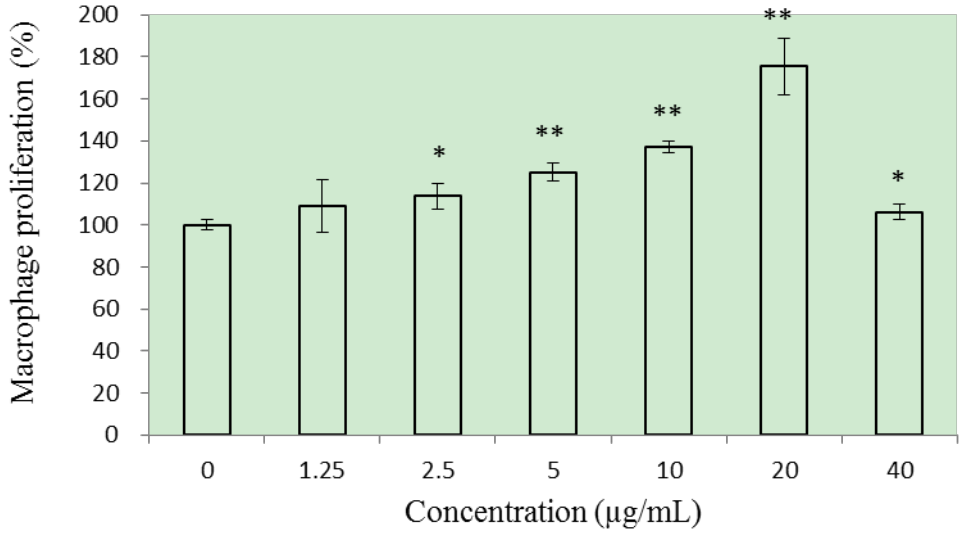

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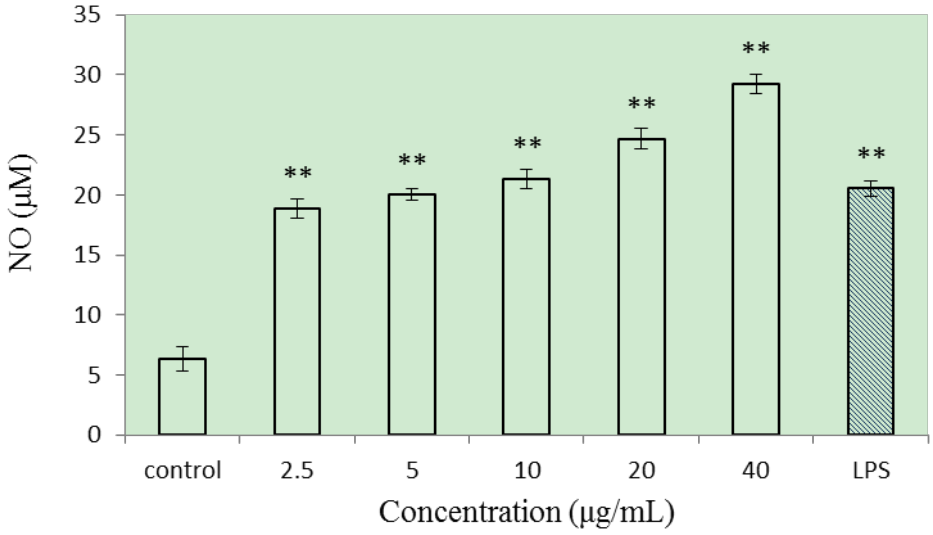

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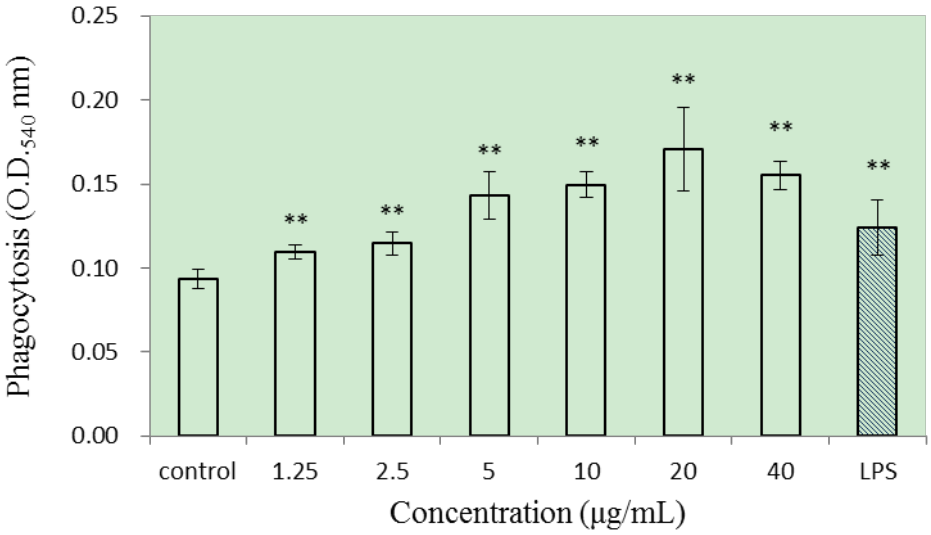

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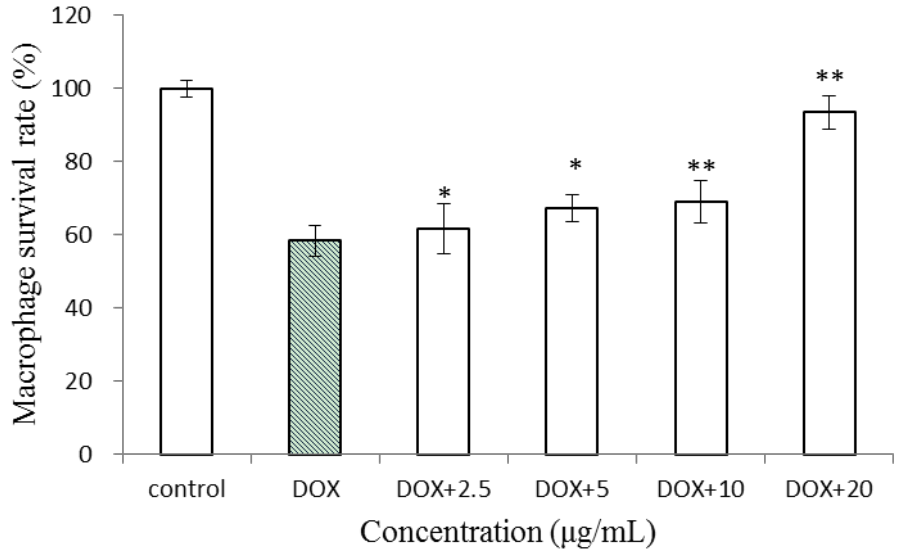

Fig. 7 Shi etal.

Table 1. Independent variable values of the process and their corresponding levels

Independent variable	Unit	Coded variables levels		
		-1	0	1
Inoculum size	%	7.5	10	12.5
Moisture content	%	60	70	80
C/N ratio	—	20	30	40

Table 2. 4-Factor, 3-level orthogonal array used in the original series of the extractions, plus the polysaccharides content of fermented SCR extracts measured by the phenol-sulfuric acid method

Run No.	Factor A Time (min)	Factor B Temp (℃)	Factor C Power (W)	Factor D Liquid: Solid [a]	Polysaccharide (mg/g)
1	10	30	50	10:1	31.89 ± 0.92 [b]
2	10	50	80	20:1	43.18 ± 0.84
3	10	80	110	30:1	61.40 ± 3.15
4	20	30	80	30:1	26.68 ± 1.06
5	20	50	110	10:1	39.04 ± 1.14
6	20	80	50	20:1	49.26 ± 1.72
7	30	30	110	20:1	34.64 ± 0.49
8	30	50	50	30:1	40.29 ± 2.15
9	30	80	80	10:1	82.48 ± 2.18
K_1 [c]	61.10 ± 3.53	63.90 ± 3.85	60.12 ± 3.12	63.59 ± 4.05	
K_2	67.28 ± 3.97	58.01± 3.32	69.71 ± 4.51	72.24 ± 4.66	
K_3	73.01 ± 4.81	79.47 ± 5.15	71.55 ± 4.94	60.55 ± 3.55	
R [d]	11.92 ± 1.16	21.46 ± 1.34	11.43 ± 0.93	16.69 ± 1.25	
Optimal level	3	3	3	2	

[a] Liquid: Solid was Liquid (mL): Solid (g)

[b] Values were mean of three determinations with the standard deviation (±).

[c] $K_i^A = \sum$ polysaccharide yield at A_i. Values were mean of three determinations with the standard deviation (±).

[d] $R^A = \max\{K_i^A\} - \min\{K_i^A\}$. Values were mean of three determinations with the standard deviation (±).

Table 3. Central composite experimental design matrix, as well as the responses and the predicted values for the polysaccharides content

Run	Independent variable			Polysaccharides content (Y) (mg /g)		
	Inoculum size (%)	Moisture content (%)	C/N ratio	Experimental (Y_0)	Predicted (Y_i)	Y_0-Y_i
1	7.5	60	30	41.42	41.70	-0.28
2	12.5	60	30	37.87	36.71	1.16
3	7.5	80	30	49.10	50.33	-1.23
4	12.5	80	30	45.00	44.80	0.20
5	7.5	70	20	42.30	42.23	0.07
6	12.5	70	20	22.33	23.69	-1.36
7	7.5	70	40	31.90	30.47	1.43
8	10	70	40	44.37	44.67	-0.30
9	10	60	20	36.29	36.12	0.17
10	10	80	20	41.91	40.79	1.12
11	10	60	40	32.90	33.95	-1.05
12	10	80	40	45.90	45.99	-0.09
13	10	70	30	58.40	58.28	0.12
14	10	70	30	57.09	58.28	-1.19
15	10	70	30	58.85	58.28	0.57
16	10	70	30	58.61	58.28	0.33
17	10	70	30	57.61	58.28	-0.67

With 20 days of the fermentation time and 5.5of pH value, the experiment was repeated four times and the response represents average values.

Table 4. Analysis of variance (ANOVA) for the regression equation

Term	degree of freedom	sum of squares	F value	Prob >F	significant
Model	9	1882.79	109.84	< 0.0001	**
X_1	1	38.91	20.43	0.0027	**
X_2	1	139.69	73.35	< 0.0001	**
X_3	1	3.85	2.02	0.1979	
X_1X_2	1	0.074	0.039	0.8489	
X_1X_3	1	95.56	50.18	0.0002	**
X_2X_3	1	13.60	7.14	0.0319	*
X_1^2	1	346.61	181.99	< 0.0001	**
X_2^2	1	83.43	43.81	0.0003	**
X_3^2	1	802.60	421.41	< 0.0001	**
Residual	7	13.33			
Lack of fit	3	9.35	3.14	0.1492	
Pure Error	4	3.98			
Cor Total	16	1896.12			
R^2		0.9930			
Adj R^2		0.9839			
Pred R^2		0.9040			
Adequate Precision		32.681			

With 20 days of the fermentation time and 5.5 of pH value, the experiment was repeated four times and the response represents average values. p* < 0.05, ** p < 0.01

Table 5. Analysis of variance for the orthogonal experiment

Variation source	d.f.	Sum of squares	F value	*P* value
Time (min)	2	638.01	159.23	< 0.01
Temp (℃)	2	2214.03	552.56	< 0.01
Power (W)	2	678.21	169.26	< 0.01
Liquid (mL): Solid (g)	2	1423.16	355.18	< 0.01
Error	2	32.05		
Sum	16	5007.20		

d.f. : degree of freedom