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Physiological Active Substance Changes of Soybean Curd Residue Fermented by *Preussia aemulans* and its Bioactivity

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Abstract: A new species of fungus *Preussia aemulans* isolated from the *Cordyceps sinensis* fruiting body was cultured by soybean curd residue using solid-state fermentation method. The content of polysaccharide, amino acid, protein, phenolics and nucleoside were massively improved under the optimum conditions, including 10% adding dosage of glucose, 3% adding dosage of beef extract, 15 days of fermentation time. The crude Polysaccharide (CPS) fraction indicated significant immunomodulatory activity on macrophage RAW 264.7 cells and the Ethyl Acetate Extract (EAE) fraction revealed the moderate anti-proliferation ability on human colon cancer cell DLD-1 at high concentration. Both of CPS and EAE showed excellent antioxidant activities properties. The results were demonstrated that the fermented Soybean Curd Residue (SCR) could be utilized as nontoxic, safe and functional food, food additive and feed.

Keywords: Soybean Curd Residue, *Preussia aemulans*, Antioxidative Property, Immunomodulatory Activity, Anti-Tumor Activity

Introduction

Soybean Curd Residue (SCR), a by-product of tofu, soymilk or soy protein processing, is discharged as an agro-industrial waste and has caused severe environmental pollution (Mizumoto *et al*., 2006). In fact, dried SCR is quite rich in proteins (20-30%), lipids (9-20%), carbohydrates (more than 50%) and fibres (9-20%) (Surel and Couplet, 2005). Several studies have recently investigated SCR as a nitrogen source was used for the solid-state fermentation of a microorganism (Li *et al*., 2014) and to produce polysaccharide by *Flammulina velutipes* and *Ganoderma lucidum* (Shi *et al*., 2012; 2013).

Cordyceps sinensis (C. sinensis) (Berk.) Sacc is a parasitic fungus used to treat multitude of ailments, promote longevity, increase athletic power and improve quality of life. Recent studies have demonstrated its multiple pharmacological actions in potentiating the immune system (Meng *et al*., 2013), the antitumor activity (Li *et al*., 2006) and the anti-oxidation activity (Wang *et al*., 2015). 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 *C. sinensis* fruiting body and all of the isolated fungus had the similar metabolites and exhibited the similar pharmacological activities as *C. sinensis* (Zhang *et al*., 2010). Previous studies have revealed that the *Cordyceps* and its anamorph possessed variety of biologically active substances, such as adenosine and ergosterol, especially polysaccharides which exhibited antioxidative and antitumor activities and regulation of immune functions (Paterson, 2008).

Many studies have shown that Reactive Oxygen Species (ROS) may be responsible for or contribute to human diseases (Wu and Hansen, 2008). Antioxidants, such as polysaccharide, phenolic compounds, flavonoids compounds can scavenge free radicals and protect against diseases. Macrophages have a significant role in host defense mechanisms. Phagocytic activity produces ROS and Nitric Oxide (NO) in response to stimulation from a variety of agents and it can inhibit the growth of a wide variety of tumor cells and micro-organisms. Moreover, the immunomodulatory activity not only involves macrophage activation but also proliferation and differentiation of these cells (Schepetkin and Quinn, 2006). Cancer is one of the most prevalent diseases

worldwide. Papers reported that high ROS levels could induce apoptosis and necrosis, which were both evidenced to be related to cancer and neurodegenerative disorders (Kregel and Zhang, 2007). Therefore, antioxidants, which could prevent the high level of ROS, were considered as anti-cancer agents.

Up to now, the new species fungus *Preussia aemulans* (*P. aemulans*) which isolated from *C. sinensis* fruiting body has not been reported in any other research. Therefore, the purpose of this study was investigated the physiological active substance content and the bioactivity of the *P. aemulans* fungus using SCR by solid-state fermentation.

Materials and Methods

Isolation and Cultivation of Preussia Aemulans

The fruiting body of *C. 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 the *C. sinensis* fruiting body was sterilized with ethanol three times, air-dried, cut into small segments and transferred to a slant tube fermentor to incubate for 7 days at room temperature.

A white mycelium appeared on the surface during slant fermentation. Then, this mycelium was transferred to agar medium, that contained (per liter): 20 g of sucrose, 10 g of peptone, 20 g of agar powder, 1.5 g of $MgSO_4$ and 3 g of KH_2PO_4. After 7 days incubation, the mycelium was transferred into the liquid medium, that contained (per liter): 20 g of sucrose, 10 g of peptone, 4 g of potato powder, 1.5 g of $MgSO_4$ and 3 g of KH_2PO_4. The 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 stationary culture, the *P. aemulans* mycelium was inoculated onto SCR following the orthogonal experiment design which revealed in our previous research (Li *et al*., 2015) and the optimum fermentation conditions were: 10% (w/v) of glucose, 3% (w/v) of beef extract and 15 days of fermentation time.

Separation of the Fermented SCR

As shown in Fig. 1, the fermented SCR was separated into five fractions, Petroleum Ether Extract (PEE), Ethyl Acetate Extract (EAE), 1-Butanol Extract (BE), Water Extract (WE) and Crude Polysaccharide (CPS), respectively. After the separation, each fraction was collected and lyophilized for the further analysis.

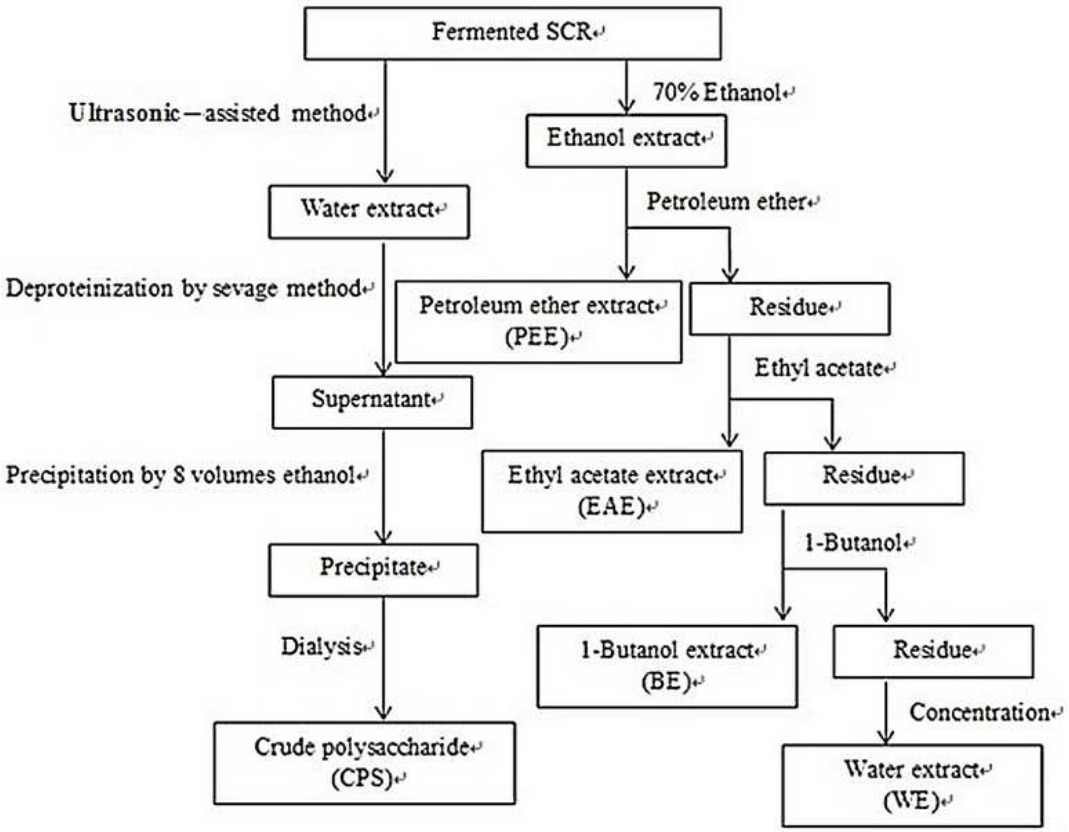


Fig. 1. Extraction flow chart of fermented SCR

Determination of Polysaccharide Content

The color reaction was initiated by mixing 1 mL of sample with 0.5 mL of phenol solution and 2.5 mL of 95% sulfuric acid and the mixture was kept in a boiling water bath for 15 min (Masuko *et al*., 2005). After it cooling to room temperature, the O.D. was determined at 490 nm and the polysaccharide content was calculated with D-glucose as the standard.

Determination of Total Phenolics Content

The total phenolics content was estimated by the Folin-Ciocalteu colorimetric method with some modifications (Hu *et al*., 2009). The sample (0.5 mL) was mixed with 0.5 mL of the Folin-Ciocalteu reagent. Three minutes later, 0.5 mL of 20% Na_2CO_3 was added and the mixture was made up to 5 mL with distilled water. After being kept in dark for 90 min, the O.D. of the mixture was read at 725 nm. The total phenolics content was calculated with gallic acid as the standard and expressed as milligram gallic acid equivalent (mg GAE/g extract).

Determination of Total Free Amino Acid Content

The free amino acid content was determined using a previously reported method (Shi *et al*., 2011). The fermented SCR powder was extracted by 80% ethanol in 80°C water bath for 20 min and the step was repeated twice. Then all of the collected supernatant was centrifuged and filtered. The supernatant was evaporated to dryness and dissolved with distilled water. The solution was mixed with TCA solution at the ratio of 4:1 and placing at 4°C for 10 min. The pH was adjusted with NaOH and HCl at the range of 2-3. Finally the supernatant was filtered by 0.45 μm filter and assessed by an auto amino acid analyzer (JLC - 500/V2, Jeol Ltd., Tokyo, Japan) in Chemical Analysis Center (university of Tsukuba).

Determination of Protein Content

The protein content was determined using the Protein Quantification Kit-Rapid (Wako Pure Chemical, Osaka) (Shi *et al*., 2011). Briefly, 6 μL of fermented broth and 300 μL of Coomassie Brilliant Blue (CBB) were added into a 96-well plate separately, then the O.D. of the mixture was read at 595 nm and the protein content was calculated using a Bovine Serum Albumin (BSA) solution as the standard. The results were expressed as milligram of protein per gram of the fermented SCR.

Determination of Nucleosides Content

The fermented SCR was extracted with deionized water by ultrasonic-assisted extract method for 1 h (50 w). Then, the supernatant was collected and filtered (0.45μm) for HPLC determination. The samples were analyzed by the HPLC with Capcell-Pak C_{18} column (4.6 mm I.D. ×150 mm, particle size of 5 μm) 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. For the determination of adenosine, guanosine and thymidine, the mobile phase was a mixture of acetonitrile-water (5:95, v/v). For the determination of uridine, the mobile phase was a mixture of acetonitrile-water (2:98, v/v) and the determination of cytidine, 0.01M KH_2PO_4 was used as the mobile phase and the concentration of adenosine, guanosine, uridine, thymidine and cytidine was calculated by comparing peak areas with appropriate standards.

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 was added to each sample and blank 1 well. The plate was incubated at 37°C for 20 min and the Optical Density (OD) was determined at 450 nm using a microplate reader (BIO-RAD Model 550, California, USA). The SOD-like activity was calculated by the following Equation 1:

$$\text{SOD activity}\left(\text{inhibition rate}\%\right) = \left\{\left[\begin{matrix}\left(A_{blank1}-A_{blank3}\right)\\ -\left(A_{sample}-A_{blank2}\right)\end{matrix}\right] / \left(A_{blank1}-A_{blank3}\right)\right\} \times 100\% \quad (1)$$

where, A_{blank1}, A_{blank2}, A_{blank3} and A_{sample} were the absorbance of blank 1, blank 2, blank 3 and the sample, respectively. 1 Unit of SOD activity was defined as the amount of enzyme having a 50% inhibitory effect on WST-1.

DPPH Radical-Scavenging Properties

The DPPH radical-scavenging property was measured according to previous report (Nakajima *et al*., 2007). 0.5 mL of various concentrations of samples were mixed with 2 mL (25 μg mL^{-1}) of a Methanol solution of DPPH. The mixture was shaked immediately after adding DPPH and then kept stand in the dark for 30 min at room temperature. The absorbance was measured at 517 nm. Ascorbic Acid was used as the positive control. DPPH free radical-scavenging property was calculated according to the following Equation 2:

$$\text{DPPH radical} - \text{scavenging activity}\,(\%) = \left[A_0 - A_1 / A_0\right] \times 100\% \quad (2)$$

where, A_0 represented the absorbance without samples and A_1 was the absorbance in the presence of samples.

Scavenging Ability on Hydroxyl Radicals

The HO• scavenging activity was measured according to previous report with some modifications (Liu *et al*., 2010). The reaction mixture (250 μL) contained 50 μL of $FeSO_4$ (1.5 mM), 35 μL of H_2O_2 (6 mM), 15 μL of sodium salicylate (20 mM) and 100 μL of different concentrations of samples. Ascorbic Acid was used as the positive control. After reaction for 1 h at 37°C, the absorbance of the hydroxylated salicylate mixture was determined at 560 nm, by a microplate spectrophotometer (BIO-RAD Model 550, USA). The percentage scavenging effect was calculated as Equation 3:

$$\text{Scavenging activity}(\%) = \left[1 - (A_1 - A2) / A_0\right] \times 100\% \quad (3)$$

where, A_0 represented the absorbance of the solvent control, A_1 was the absorbance of sample or ascorbic acid and A_2 was absorbance of the reagent blank without sodium salicylate.

FT-IR Spectrum of CPS

The Infrared (IR) spectrum of the CPS was determined using a Fourier transform infrared spectrophotometer (Herscel FT/IR-300, Jasco, Japan). The CPS was ground with spectroscopic-grade potassium bromide (KBr) powder and then pressed into 1 mm pellets for IR measurement in the 4000-400 cm^{-1} frequency range (Chen *et al*., 2011).

Cell Culture

The murine macrophage cell line, RAW 264.7 was obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in MEM medium containing 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} of streptomycin at 37°C in a humidified 5% CO_2 atmosphere (ESPEC CO_2 Incubator). Cells were cultured for 2-3 days to reach the logarithmic phase and used for further analysis.

DLD-1 human colon cancer cell line was obtained from the Cell Resource Center for Biomedical Research, Aging and Cancer, Tohoku University (Japan). It was grown in RPMI1640 medium containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Culture was maintained at 37°C in a humidified 5% CO_2 atmosphere (ESPEC CO_2 Incubator). Cells were cultured for 2-3 days to reach the logarithmic phase and used for further analysis.

The Effect of CPS on Macrophage Cell Proliferation

The effect of CPS on the proliferation of RAW 264.7 cells was estimated using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). RAW 264.7 cells were cultured in a 96-well plate at a density of 5×10^4 cells/mL at 37°C in a 5% CO_2 atmosphere for 24 h. Next the cells were incubated with various concentrations of CPS at 37°C for 24 h. After incubation, 10 μL of CCK-8 solution was added and incubated at 37°C for 4 h. The cell viability was determined by the O.D. at the wavelength of 450 nm with a microplate reader (BIO-RAD Model 550, USA). The data were expressed as percentages of the control.

Measurement of the Production of the Nitric Oxide

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^{-1}) and various concentrations of CPS for 24 h. After incubation, 50 μL of the culture supernatants were mixed with 50 μL of Griess reagent in a 96-well plate and incubated at 25°C for 10 min. The absorbance at 570 nm was measured on a microplate reader. The nitrite accumulation was measured to assess the NO production in the RAW 264.7 cells. $NaNO_2$ was used as standard to calculate the nitrite concentrations.

Inhibitory Effect of EAE on DLD-1 Cells

DLD-1 cells were grown in RPMI-1640 medium at 37°C in a 5% CO_2 atmosphere to logarithmic phase. Cells were harvested and an aliquot (100 μL) of DLD-1 cells suspension (5×10^4 cells/mL) were dispensed into a 96-well plate and pre-incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Then cells were exposed to various concentrations of extracts (5, 10, 20 and 40 μg mL^{-1}) for 24 h. After EAE fraction exposure, 10 μL of CCK-8 solution was added and incubated at 37°C for 4 h. Cell numbers were quantitated by reading the OD at 450 nm with a microplate reader (BIO-RAD Model 550, California, USA).

Statistical Analysis

Statistical analyses were done using the DPS statistical analysis (DPS, version 13.5) software package (Hangzhou city, Zhejiang province, China). The results were presented as means of three determinations±SD (standard deviation).

Results

Physiological Active Substance Content in Fermented and Unfermented SCR

The results of physiological active substance content in fermented and unfermented SCR were shown in Table 1.

Table 1. Physiological active substances contents of unfermented and fermented SCR

Physiological active substances		Unfermented SCR	Fermented SCR
Phenolics (mg GAE/g dry matter)		12.21±0.56	48.44±1.29
Protein (mg/g dry matter)		217.56±14.7	192.20±10.28
Nucleoside (mg/100 g dry matter)	Guanosine	26.21±0.37	83.51±4.62
	Uridine	250.11±14.28	369.79±12.14
	Thymidine	0.28±0.01	9.51±0.56
	Cytidine	14.18±0.61	68.56±5.57
Amino acid (μmol/g dry matter)	Totoal amino acid	19.15±0.76	189.14±4.32
Essential amino acid	Threonine	0.39±0.01	3.07±0.10
	Valine	0.95±0.01	8.66±0.23
	Methionine	0.14±0.01	1.04±0.06
	Leucine	0.89±0.03	8.16±0.12
	Tyrosine	0.41±0.01	6.86±0.18
	Lysine	0.14±0.02	2.85±0.16
	Isoleucine	0.43±0.04	5.32±0.24
	Histidine	0.09±0.01	0.65±0.01
	Phenylalanine	0.92±0.04	11.91±0.31
Nonessential amino acid	Alanine	2.53±0.13	8.34±0.28
	Cysteine	ND	0.27±0.01
	Asparagines	1.24±0.09	3.41±0.11
	Glutamic acid	0.81±0.07	13.80±0.72
	Glycine	0.38±0.01	2.85±0.25
	Arginine	0.18±0.01	1.14±0.06
	Serine	0.46±0.01	1.85±0.07
	Proline	0.53±0.02	8.46±0.48
	γ-Aminobutyric acid	0.63±0.04	2.55±0.13

*Note: ND means not detected

The polysaccharide content of the fermented SCR was increased from 12.91 ± 0.39 mg g^{-1} dry matter to 43.49 ± 1.48 mg g^{-1} dry matter. The total phenolics content of fermented SCR was increased by 4-fold after fermentation, from 14.16 ± 0.53 to 89.83 ± 4.26 mg GAE g^{-1} dry matter. For the protein content, the fermented SCR was 192.20 ± 10.28 mg g^{-1} dry matter. In contrast to the unfermented SCR, the total free amino acid content of fermented SCR was 189.14 ± 4.32 μmol g^{-1}, exhibiting an increase of 10-fold. For the essential amino acid, all of the amino acids of fermented SCR were increased significantly during fermentation time. Lysine was enhanced an increase of 20-fold which was the maximum increase of these essential amino acids. For the nonessential amino acid, all of the amino acids were increased. Especially, the glutamic acid was increased the most remarkably (17-fold). Cysteine, the one species of nonessential amino acid does not exist in unfermented SCR and it reached to 0.27 ± 0.01 μmol g^{-1}. Γ-Aminobutyric acid was increased 4-fold. Compared with the unfermented SCR, guanosine, uridine, thymidine and cytidine were improved by 3-fold, 1.5-fold, 34-fold and 5-fold, respectively.

Antioxidant Properties

For the results of DPPH radical-scavenging activities, the EC_{50} of PEE, EAE, BE, WE and CPS were shown in Table 2. It was indicated that all of the isolated fractions and ascorbic acid (positive control) were found to be effective scavenger against DPPH radical. Moreover, the CPS indicated the most excellent antioxidative property with 0.48 mg mL^{-1} of the EC_{50}.

All of the isolated fractions and ascorbic acid (positive control) exhibited remarkable hydroxyl radical removal activity (Table 2). Moreover, among the isolated fractions, CPS indicated the highest radical scavenge activity with 0.32 mg mL^{-1} of the EC_{50} and EAE (0.32 mg mL^{-1} of EC_{50}) showed the similar property as CPS.

Components Analysis of CPS and EAE

As shown in Table 3, the polysaccharide, phenolics, protein content and SOD-like activity of CPS and EAE were determined. The polysaccharide content of CPS was 602.42 ± 4.35 mg g^{-1} extract, which was much higher than other compounds. The SOD-like activity of EAE was 74.63 ± 0.72 U mg^{-1}.

FT-IR Spectrum of CPS

As shown in Fig. 2, the largest absorption band at 3380 cm^{-1} is assigned to the hydroxyl group and the small band at 2921 cm^{-1} are due to C–H-stretching vibration. The absorption bands at 1640 and 1542 cm^{-1} are attributed to the stretching vibration of the carbonyl bond, C–O of the amide group and the bending vibration of the N–H bond in peptides or proteins (Leung *et al*., 2009). The

stretching peak at 1089 and 1026 cm^{-1} are suggestive of a C–O bond (Yan *et al*., 2010). Moreover, characteristic absorptions at 889 cm^{-1} in the FT-IR spectrum indicated a β-anomeric configuration in CPS (Pan and Mei, 2010; Kozarski *et al*., 2012).

Effect of CPS on Macrophages Proliferation and Nitric Oxide Production

It was interesting to note that in the range of 5-20 μg mL^{-1}, CPS stimulated RAW 264.7 cells proliferation in a dose-dependent manner (Fig. 3). At the concentration of 20 μg mL^{-1}, the stimulation effect reached maximum, which was 169.55%. Though the concentration increased to 40 μg mL^{-1}, the cells survival rate decreased and the RAW 264.7 cells survival rate (160.55%) was still much higher than control. The positive control, LPS (1μg mL^{-1}) was showed the higher stimulation effect with the stimulation effect of 196.06%.

As shown in Fig. 3, the NO production indicated that incubation with CPS stimulated RAW 264.7 cells to produce NO in a dose-dependent manner at 5-20 μg mL^{-1} and the NO production was decreased at 40 μg mL^{-1}, with 12.24 μM. At the concentration of 20 μg mL^{-1}, the maximum NO production was observed (14.27 μM), in contrast with the control (4.63 μM) ($p<0.01$). The positive control, LPS (1 μg mL^{-1}) was 20.52 μM of NO production.

Inhibitory Effect of EAE on DLD-1 Cells

EAE fractions were used for the anti-proliferation on DLD-1 cells (Fig. 4). In this experiment various concentrations of extracts (5, 10, 20 and 40 μg mL^{-1}) were used. The results showed that, the EAE revealed the moderate anti-proliferation ability on human colon cancer cell DLD-1 and the cell survival rates were decreased with increase of the concentration of extracts. While the treatment at the concentration of 40 μg mL^{-1}, EAE revealed the strongest inhibitory effect on DLD-1 cells proliferation, which was 19.21%.

Table 2. EC50 of various antioxidative activities

	EC_{50} mg/mL					
	PEE	EAE	BE	WE	CPS	Ascorbic acid
DPPH radical -scavenging activities	11.73	1.44	1.83	1.69	0.48	0.04
Scavenging activities of hydroxyl radical	1.20	0.33	0.46	0.61	0.32	0.05

Table 3. Compounds analysis of CPS and EAE

	CPS	EAE
Polysaccharide content (mg/g extract)	602.42±4.35	-
Phenolics content (mg GAE/g extract)	26.71±0.25	42.52±1.50
Protein content (mg/g extract)	33.04±1.46	-
SOD-like activity (U/mg)	8.06±0.18	74.63±0.72

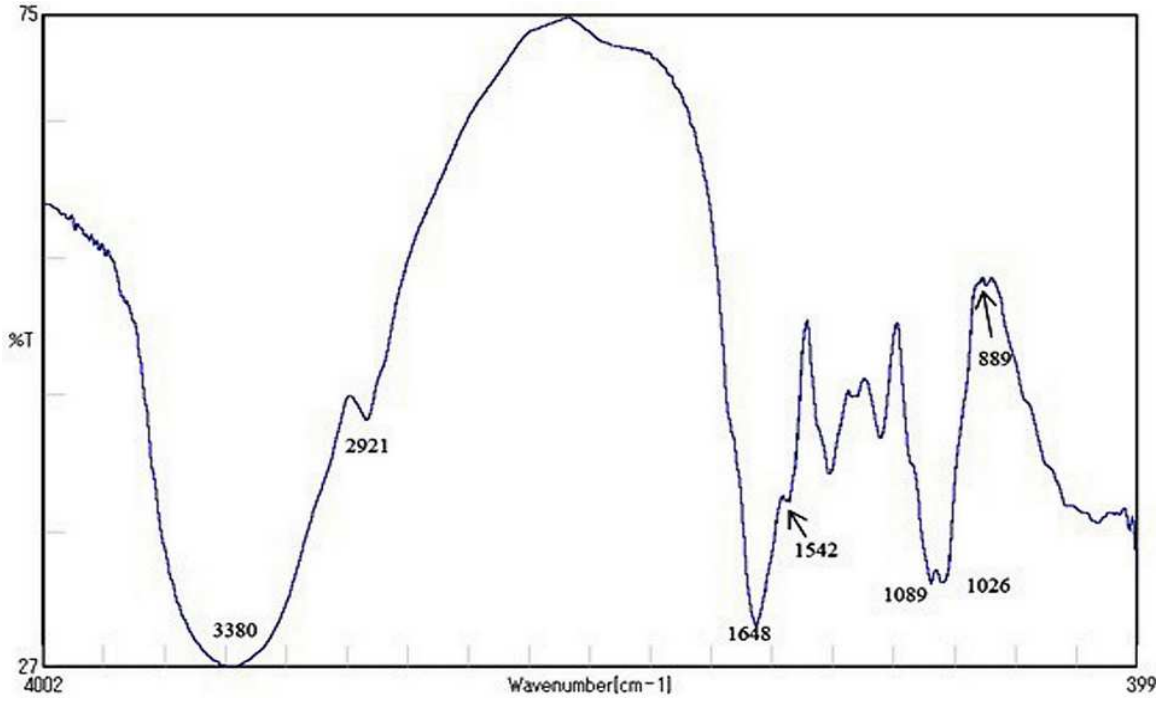
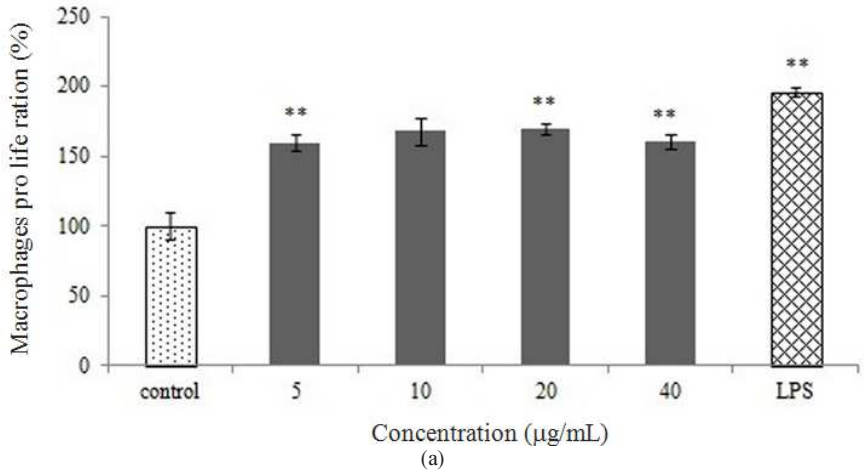
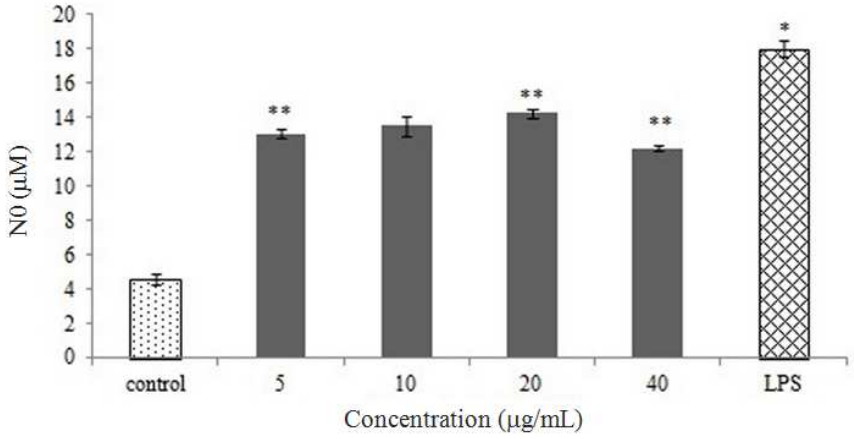


Fig. 2. IR spectrum of CPS



(a)



(b)

Fig. 3. Effect of CPS on macrophage RAW 264.7, (A) Cells proliferation; (B) NO production LPS was positive control, at a concentration of 1 µg mL^{-1}. Data are expressed as means ± S.D. of three independent experiments ($^{*}p<0.05$ and $^{**}p<0.01$ in comparison with control)

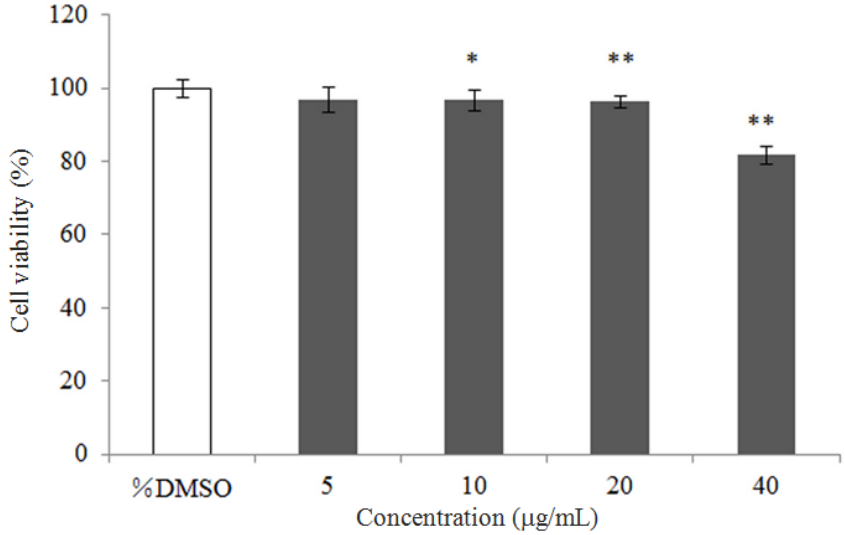


Fig. 4. Anti-proliferative effect of EAE on DLD-1 cells data are expressed as means±S.D. of three independent experiments (*p<0.05 and **p<0.01 in comparison with control)

Discussion

After fermentation, the polysaccharide, total phenolics, protein, amino acids and nucleosides contents were increased significantly. Polysaccharides have been identified as the major active components of *C. sinensis* with a wide range of bioactivities including immunomodulation, antitumour, antioxidation and hypoglycemic effects (Yan *et al*., 2014). Mushrooms could accumulate a variety of secondary metabolites, such as phenolic compounds, polyketides, terpenes and steroids (Cheung *et al*., 2003). Among of them, phenolics are suggested to be the major bioactive compounds for health benefits and found to be associated with the inhibition of atherosclerosis and cancer (Puttaraju *et al*., 2006; Klimczak *et al*., 2007). In this study, the protein content was decreased after fermentation, however the protein of SCR is hydrolysed to produce the peptide which takes on good solubility, the low viscosity (Shi *et al*., 2011). Thus, it is assimilated easily in vivo digestion to be suitable for children and old people. In free amino acids, lysine is an important essential amino acid for people and is beneficial for the physical growth and intelligence of children (Shi *et al*., 2013). Glutamic acid could exhibit protective effects on the heart muscle in human with heart disease. For γ-Aminobutyric acid, a non-protein amino acid that is widely distributed in nature from microorganisms to plants and animals (Li *et al*., 2010) and it acts as the major inhibitory neurotransmitter in the mammalian central nervous system. In addition, γ-Aminobutyric acid also has hypotensive, tranquilizing and diuretic effects and can prevent diabetes (Wong *et al*., 2003). For Nucleosides, guanosine has many trophic effects in the central nervous system, including the stimulation of neurotrophic factor synthesis and release by astrocytes, which protect neurons against excitotoxic death (DiIorio *et al*., 2004). Uridine, is an essential component of RNA synthesis and plays an important role in the synthesis of glycogen (Yamamoto *et al*., 2011). In humans, uridine is administered to reduce the adverse effects of cancer chemotherapy, such as bone marrow and gastrointestinal toxicity (Yamamoto *et al*., 2002). Cytidine plays key roles in phosphoinositide signaling and the synthesis of lipids (Helmink and Friesen, 2004). In humans, thymine also plays an important role in the synthesis of DNA. In sum, the quantity and verities of active substances in SCR were raised obviously after fermentation. These indicated that the fermented SCR could be a potential and nutritious ecologic feed.

In the antioxidant assay, the EAE and CPS fractions showed the significant activity. According to the components analysis result, the polysaccharide content of CPS was much higher than other compounds. Polysaccharides have been demonstrated to play an important role as dietary free radical scavenger for the prevention of oxidative damage (Mohsen *et al*., 2009). Thus, the antioxidative property of CPS could be due to the high content of polysaccharide. SOD, which catalyzes the dismutation of the superoxide anion (O^{2-}) into hydrogen peroxide and molecular oxygen, is one of the most important enzymes in the front line of defense against oxidative stress (Jiang and Patrice, 2006). Therefore the strong antioxidative property of EAE could be related with the higher SOD-like activity.

For the bioactivity, the CPS fraction showed the significant immunomodulatory activity on macrophage RAW 264.7 cells and the EAE fraction revealed the moderate anti-proliferation ability on human colon cancer cell DLD-1 at high concentration. Immunomodulation is the most notable biological function of natural polysaccharides, which is associated with their putative role as biological response modifiers (Moradali *et al*., 2007) and previous literature has been reported that the β-glycosidic linkage was the essential structural feature for immunostimulatory and antitumoral effects (Hung *et al*., 2008; Song *et al*., 2012). Therefore, the immunomodulatory activity of CPS fraction may be connected with the β-anomeric configuration. Reactive Oxygen Species (ROSs) exist in various forms, including free radicals such as superoxide ions, hydroxyl radicals and peroxyl, as well as non-free-radical species such as hydrogen peroxide (Squadriato and Pryor, 1998; Waris and Ahsan, 2006) These ROSs could induce various diseases especially cancer. Thus, the moderate anti-proliferation ability of EAE fraction on human colon cancer cell DLD-1 could be related with its significant antioxidant activity.

Conclusion

The annual production of SCR reaches as high as 20 million tons in China, with 0.7-0.8 million tons in Japan. In Japan, most SCR is incinerated or landfilled directly and the disposal cost of SCR is 10,000–20,000 yen per ton. In this study, SCR was utilized as a media by the new species fungus of *P. aemulans* which isolated from *C. sinensis* fruiting body using solid-state fermentation method**.** This method not only reduced the environmental pollution, but also changed the by-product to a useful material. The fermented SCR was rich in physiological active substances, low in cost and efficient in immunomodulation and anti-tumor effect, therefore the fermented SCR could be explored as ecological feed or functional food material in the further.

Author's Contributions

Yiting Li: Designed the research plan, participated in all experiments, aquisition of data, analyzed all the data and contributed to the writing of the manuscript.

Shili Meng: Participated in Chemical characterization of CPS and EAE and analyzed all the data.

Min Shi: Participated in Cell experiment and contributed to the writing of the manuscript.

Zhenya Zhang: Provided technical guidance, coordinated the writing of the manuscript, designed the research plan and organized the study.

Conflict of Interest

The author declares that they have no conflict of interest.

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