Advance Journal of Food Science and Technology 6(9): 1067-1075, 2014
DOI:10.19026/ajfst.6.161
ISSN: 2042-4868; e-ISSN: 2042-4876
© 2014 Maxwell Scientific Publication Corp.
Submitted: April 29, 2014 Accepted: May 25, 2014 Published: September 10, 2014

Research Article

Antioxidative Activity of Exopolysaccharide Extract from Fermented Wheat Distillers'dried Grains Using UV-Irradiation Degradation Pretreatment by *Preussia aemulans*

[1]Yiting Li, [2]Shili Meng, [2]Linbo Wang, [2]Yuepeng Wang, [3]Xiaoyan Zu, [2]Yingnan Yang and [2]Zhenya Zhang
[1]Key Laboratory of Food Nutrition and Safety, Tianjin University of Science and Technology, Ministry of Education, Tianjin 300457, China
[2]Graduate School of Life and Environmental Science, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
[3]Institute for Farm Products Processing and Nuclear-Agricultural Technology, Hubei Academy of Agricultural Sciences, Wuhan, Hubei 430064, P.R. China

Abstract: A novel approach for utilizing wheat Dried Distillers' Grains with Solubles (DDGS) to produce exopolysaccharide by *Preussia aemulans* was explored. For degrading the insoluble polymer of wheat DDGS, the UV-irradiation method was used. After irradiation of 24 h, the soluble saccharide content of wheat DDGS medium was increased by 67%. The optimum fermentation conditions were: 5% (w/v) of fructose, 0.5% (w/v) of yeast extract, 7.5 of pH value, 5% (v/v) of the inoculum size, 60 rpm of shaking condition and 5 days of the fermentation time. The maximum exopolysaccharides yield of fermented broth under the optimum fermentation conditions was 3.51±0.24 g/L. Comparing with the unfermented wheat DDGS water extract, the exopolysaccharide yield of fermented broth was increased by 36%. The crude exopolysaccharide (EPS) indicated remarkable antioxidative activities though four antioxidant assays. Therefore, the crude EPS could be utilized as antioxidative food additives and functional feed in the future.

Keywords: Antioxidative activity, *Cordyceps sinensis*, Fermentation, *Preussia aemulans*, UV-irradiation, Wheat dried distillers' grains with solubles

INTRODUCTION

In recent years, distilled spirit is becoming popular not only in the eastern countries but also all over the world. Dried Distillers' Grains with solubles (DDGS) are the principal by-products of distilled spirit production from the fermentation of dry milled whole grains (Rasco *et al*., 1987) and wheat DDGS produced by the distilled spirit industry is discharged as an agro-industrial waste. Approximately 0.81 million tons and 0.26 million tons of wheat DDGS are disposed in Japan and Canada every year. Traditionally, a part of wheat DDGS has been primarily used as ruminant feeds (Greter *et al*., 2008; Penner *et al*., 2009) and produce biogas by compost method account to the high polymer contents. But most of wheat DDGS is incinerated and buried, which has caused severe environmental pollution. However, there are lots of physiological active substances contained in wheat DDGS, such as phenolic compounds, vitamin B, vitamin E and essential amino-acid etc. Thus, we considered wheat DDGS could be potentially utilized as a high quality media for the microbial fermentation.

Cordyceps sinensis (*C. sinensis*) (Berk) Succ., is a parasitic fungus and it is often used as a fungal traditional Chinese medicine to treat various kinds of diseases. According to Chinese tradition and the Chinese Pharmacopoeia, *C. sinensis* can tonify the lung, replenish the kidneys, arrest bleeding, dissolve phlegm, treat chronic coughs, treat spontaneous sweating and restore strength after an illness (Zhu *et al*., 1998). Recent studies have demonstrated its multiple pharmacological actions in potentiating the immune system (Xu *et al*., 1992), the anti-tumor activity (Chen *et al*., 1997) and the anti-oxidation activity (Yu and He, 1998). Furthermore, according to previous research, 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 pharmacological activities as *C. sinensis* (Zhang *et al*., 2010).

Exopolysaccharides (EPS) produced by fungi, are secreted into the growth media or remain tightly attached to the cell surface (Seviour *et al*., 1992). Some

Corresponding Author: Zhenya Zhang, Graduate School of Life and Environmental Science, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan, Tel.: +81-29-853-4712; Fax: +81-29-853-4712
This work is licensed under a Creative Commons Attribution 4.0 International License (URL: http://creativecommons.org/licenses/by/4.0/).

of these EPS have shown useful biological activities (Wang and Luo, 2007; Miranda *et al*., 2008; Kanekiyo *et al*., 2005) including antioxidative activities (Sun *et al*., 2009; Guo *et al*., 2010). Polysaccharides extracted from cultivated *C. sinensis* indicated anti-tumor activity and its mechanisms were considered as its modulation in immunity (Zhang *et al*., 2008; Yang *et al*., 2005). In previous research, solid-state fermentation was used for producing polysaccharides, however in order to produce EPS, bioactive compounds and other biomass, process of submerged fermentation is more efficient than that of solid-state fermentation (Leung *et al*., 2009).

Antioxidants play an important role in defending the body against free radicals damage (Hajar *et al*., 2010). And antioxidants were considered as a group of compounds that are able to delay or inhibit the oxidation of tissues, prevent or repair the damage of the body (Tachakittirungrod *et al*., 2007). There is a considerable amount of epidemiological evidence revealing natural antioxidants, including ascorbic acid, polyphenol, β-carotene and flavonoids which could function as free radical interrupters (Yen *et al*., 2002; Kelly *et al*., 2002).

Up to now, wheat DDGS was generally use as animal feed or agricultural fertilizer, but it has not been used as a microbial medium for fungus, thus the approach of utilizing wheat DDGS in this study was extremely novel. The *Preussia aemulans* (*P. aemulans*), a new species fungus isolated from *C. sinensis* fruiting body, the fermentation condition and bioactivity of *P. aemulans* have not been reported in previous researches. Therefore, the wheat DDGS water extract was utilized as a growth medium for *P. aemulans*, in order to establish the optimum conditions with orthogonal experiment for producing exopolysaccharide by submerged fermentation. Due to the high polymer contents of wheat DDGS are difficult to be decomposed, the photodegradation method was used in this research. Then, the crude exopolysaccharide and Ethanol-Supernatant (ES) fraction were subsequently separated from the fermented broth by ethanol-precipitation method to investigate the antioxidative activity.

MATERIALS AND METHODS

Chemicals and reagents: Peptone, beef extract, potato extract, yeast extract, agar, ethanol, Protein Quantification Kit-Rapid, rutin, ascorbic acid, hydrogen peroxide and ethylenediamine tetraacetic acid (EDTA) were obtained from Wako Pure Chemical Industries, Ltd, Osaka, Japan. SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Gallic acid, 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 2-diphenyl-1-picry-hydrazyl radical (DPPH) were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). All other chemical reagents were of analytical grade.

Isolation and cultivation of *preussia aemulans*: The fruiting body of *C. sinensis* was purchased from Qinhai, China and the isolated *P. aemulans* mycelium (SIID11759-01) was identified by TechnoSuruga laboratory co., ltd, Japan. The stroma of *C. sinensis* fruiting body was sterilized with ethanol three times, air-dried, cut into small segment and transferred to slant tube fermentor to incubate for 7 days, at room temperature.

The white mycelium appeared on the surface during slant fermentation. Then, mycelium was transferred to ager medium, which contained (per liter): 20 g of surcose, 10 g of peptone, 20 g of ager powder, 1.5 g of $MgSO_4$, 3 g of KH_2PO_4. After 7 days of culture, when white mycelium appeared on the surface of the medium, the mycelium was transferred into liquid medium, which was containing (per liter): 20 g of surcose, 10 g of peptone, 4 g of potato powder, 1.5 g of $MgSO_4$, 3 g of KH_2PO_4. The *P. aemulans* 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.

Extraction of wheat DDGS and photodegradation: The wheat DDGS was obtained from Kyushu, Japan. The carbon nitrogen ratio and pH value of wheat DDGS were 11.79 and 4.03±0.02, respectively. According to the wheat DDGS contains a mass of insoluble polymers which difficult to be decomposed by *P. aemulans* mycelium, therefore the UV-irradiation was considered to be used. Wheat DDGS was extracted with 95% moisture content, in a shaking incubator at room temperature and irradiated by two of $\lambda = 254$ nm UV-lamp (TUV 5W, NEC Japan) for 2, 4, 6, 8, 12, 24, 48 and 72 h. Incident light intensity, measured by radiometer IL 1400A (International Light, USA), was 1μW/cm^2. Then the supernatant was centrifuged and filtrated to remove the sediment. The soluble saccharide contents of collected supernatant was detected and used as basal medium for culture experiment.

Orthogonal experiment for yield of exopolysaccharide: Media and fermentation conditions were assessed for the optimum conditions for the yield of exopolysaccharide by submerged fermentation. The carbon sources, the nitrogen sources, adding dosage of the carbon sources and the fermentation time, were regarded as correlated factors of the culture conditions. Due to the preliminary test results (data not shown), the pH value of the wheat DDGS water extract was adjusted to 7.5 and the shaking condition was 60 rpm. The inoculum size of *P. aemulans* was 5% (v/v). The optimum fermentation conditions were obtained by an orthogonal layout L_9 (3^4) in shake flask culture. The

Table 1: $L_9(3^4)$ orthogonal design of wheat DDGS by *P. aemulans*

Factor /Level	Carbon source (CS)	Nitrogen source (NS) (0.5% w/v)	Adding dosage of carbon source (ADCS) (% w/v)	Fermentation time (FT) (day)
1	Glucose	Peptone	3%	5
2	Sucrose	Beef extract	5%	10
3	Fructose	Yeast extract	7%	15

levels of the factors were shown in Table 1. After the fermentation, the fermented wheat DDGS and mycelium mixture was centrifuged at 8000 rpm for 10min and then filtered by glass microfibre filters (47 Ø circles, whatman) to remove the *P. aemulans* mycelium. The fermented broth was collected for the determination of exopolysaccharide content.

Separation of the fermented broth: After evaporation and concentration, the fermented broth of the optimum fermentation conditions for exopolysaccharide yield was precipitated by four volumes of 99.5% ethanol and store at 4°C overnight. Then, the solution was collected by centrifuging at 7,000 rpm for 15 min. The supernatant was concentrated and lyophilized to obtain the Ethanol-Supernatant (ES) fraction of fermented broth. The precipitate was washed twice by 99.5% of ethanol and dried at room temperature to remove residual ethanol and then dissolved in distilled water. The water solution was concentrated and lyophilized to obtain the crude exopolysaccharide (EPS). In contrast to the crude EPS of fermented broth (EPS-1), the crude EPS of unfermented wheat DDGS (EPS-2) was also separated by the same method.

Determination of physiological active substances:
Determination of exopolysaccharide content: Total exopolysaccharide content was measured by modified phenol-sulfuric acid method (Masuko *et al*., 2005). The sample was precipitate by 87.5% of ethanol at 4°C for 12 h. Then, the supernatant was centrifuged at 9500 rpm for 10 min. The precipitate was washed twice by 99.5% of ethanol and dried at room temperature to remove residual ethanol. Then, the precipitate was dissolved in distilled water and used for exopolysaccharide analysis. The color reaction was initiated by mixing 1 mL of crude polysaccharide solution with 0.5 mL of phenol solution and 2.5 mL of concentrated sulfuric acid and the reaction mixture was kept in a 100°C water bath for 15 min. After cooling its temperature to room temperature, the Optical Density (OD) of the mixture was determined at 490 nm and the crude exopolysaccharide content was calculated with D-glucose as the standard.

Determination of phenolic compounds content: The total phenolic compounds content of fermented powder was estimated according to Folin-Ciocalteu, colorimetric method with some modifications (Mau *et al*., 2002). 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 OD of the mixture was measured at 725 nm. The phenolic compounds content was calculated with gallic acid as the standard and expressed as milligram gallic acid equivalent (mg GAE/g extract).

Determination of flavonoids content: Flavonoids determination was based on a modified colorimetric method and rutin was used as a standard (Jia *et al*., 1999). Samples (2 mL) were placed in test tube, with 0.25 mL of $NaNO_2$ solution (5%). After reaction for 10 min, the mixture was combined with 0.25 mL of $AlCl_3$ solution (10%). 10 min later, 1.25 mL of NaOH (1 M) was added into the mixture and another 10 min later, the mixture was diluted to 6.25 mL with distilled water. Then the OD of the mixture was measured at 510 nm. The results were expressed as milligram rutin equivalent (mg RE/g extract).

Determination of protein content: The protein content was determined by the Protein Quantification Kit-Rapid (Shi *et al*., 2011). 6 μL of samples (10 mg/mL) and 300 μL of Coomassie Brilliant Blue (CBB) were added into a 96-well plate separately, then the OD of the mixture was measured at 595 nm and the protein content was calculated by a Bovine serum albumin (BSA) solution as the standard.

Determination of antioxidative activity:
DPPH radical-scavenging activity: DPPH radical-scavenging activities were assayed following the method with some modifications (Blois, 2002). Aliquots (0.5 mL) of various concentrations of samples were mixed with 2 mL (25 μg/mL) of a MeOH 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. A decrease of the solution absorbance indicated an increase of the DPPH radical-scavenging activity. Ascorbic acid was used as the positive control. DPPH free radical-scavenging activity was calculated according to the following equation:

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

where,
A_0 = The absorbance without samples
A_1 = The absorbance in the presence of the samples, respectively:

$ABTS^{\cdot+}$ radical-scavenging activity: The determination of $ABTS^{\cdot+}$ radical-scavenging activities was used a colorimetric method with some modifications (Re *et al*., 1999). $ABTS^{\cdot+}$ was mixed with a potassium persulphate solution at a final concentration of 2.45 mM. The reaction mixture was left to settle at room temperature for 12-16 h in the dark before use. $ABTS^{\cdot+}$ solution was diluted with methanol to adjust its absorbance to within 0.70±0.02 at 734 nm wavelength. Then 2.85 mL of $ABTS^{\cdot+}$ solution was mixed with 0.15 mL of various concentrations of samples. Finally, the absorbances were measured at 734 nm after reaction at room temperature for 10 min. The $ABTS^{\cdot+}$ free radical scavenging activity was calculated by the following equation:

$$ABTS^{\cdot+} \text{ scavenging activity } (\%) = \{(C–D) – (A–B)/(C–D)\} \times 100$$

where,

A = Absorbance of ABTS solution+sample/standard
B = Absorbance of potassium persulphate+sample /standard
C = Absorbance of ABTS solution+distilled water/methanol
D = Potassium persulphate+distilled water/methanol, respectively

Scavenging activity on hydroxyl radicals: The determination of hydroxyl radicals scavenging activity was measured with a few modifications (Smirnoff and Cumbes, 1989). Hydroxyl radicals were generated from $FeSO_4$ and H_2O_2 and detected by their ability to hydroxylate salicylate. 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:

$$\% \text{ HO} \bullet \text{ scavenged} = [1 - \frac{A_1 - A_2}{A_0}] \times 100\%$$

where,

A_1 = The absorbance of the sample or ascorbic acid
A_0 = The absorbance of the solvent control
A_2 = The absorbance of the reagent blank without sodium salicylate, respectively

Ferrous metal ions chelating activity: Ferrous metal ions chelating activities were measured with a few modifications (Decker and Welch, 1990). Sample or Ethylene Diamine Tetra Acetic acid (EDTA) solution (50 μL) were mixed with 180 μL of methanol, 50 μL of ferrous chloride (2 mM) and 10 μL of ferrozine (5 mM), then the mixture was shaked carefully, kept to stay for 10 min at room temperature and the absorbance of the mixture was determined at 560 nm, by a microplate spectrophotometer (BIO-RAD Model 550, USA). EDTA was included as the positive control. The chelating activity was calculated as:

$$\text{Chelating rate} = [1 - \frac{A_1 - A_2}{A_0}] \times 100 \%$$

where,

A_0 = The absorbance of the control (without sample)
A_1 = The absorbance in the presence of the sample
A_2 = The absorbance without ferrozine, respectively

Statistical analysis: The obtained data were analyzed by student's t-test and results were expressed as mean±SD. Statistic difference was considered to be significant at $p<0.01$ (**).

RESULTS AND DISCUSSION

Soluble saccharide contents of wheat DDGS by UV-irradiation: After UV-irradiation (Data shown in Fig. 1), the soluble saccharide contents were increased at time-depended manner and the soluble saccharide contents were 36.58±1.03, 37.32±0.98 and 37.36±1.25 mg/g, at 24, 48 and 72 h of irradiation time. For Energy conservation, the suitable irradiation time of wheat DDGS was considered as 24 h. Contrast to unirradiated wheat DDGS, the *P. aemulans* mycelium weight of 24h irradiation time was increased by 16% (shown in Fig. 2).

Orthogonal experiment for the yield of exopolysaccharide: The exopolysaccharide yield was shown in Table 2. The highest mean yield of exopolysaccharide from *P. aemulans* was 3.25 g/L according to the orthogonal experiment. The levels of corresponding factors were involved respectively. CS 3, NS 3, ADCS 2 and CT 1 were named fructose, yeast extract, 5% adding dosage of the carbon source and the 5 days of the fermentation time, respectively. The *R* value of various factors indicated that the fermentation time (5 days) was the highest among these factors. And the significant levels were shown in Table 3, the results were suggested that wheat DDGS water extract culture medium of *P. aemulans* had significantly related with the fermentation time and varieties of the nitrogen source. The carbon source and adding dosage of the carbon source indicated little influence in the yield of exopolysaccharide. Further, the CS 3, NS 3, ADCS 2, CT 1, conditions were demonstrated, the mean exopolysaccharide content of the fermented broth was reached to 3.51±0.24 g/L and the *P. aemulans* mycelium yield was 9.64±0.22 g/L (data not shown). In

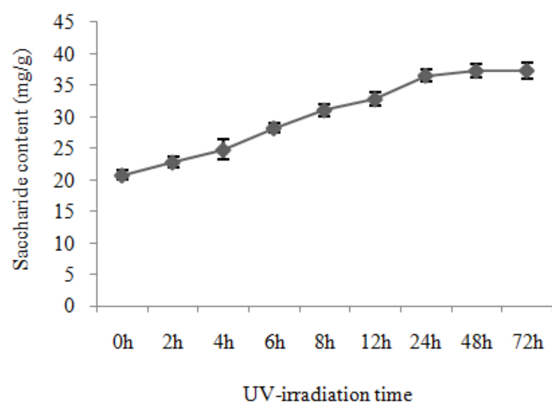

Fig. 1: The saccharide contents of wheat DDGS by UV-irradiation degradation method. Data were expressed as means±S.D. of three independent experiments

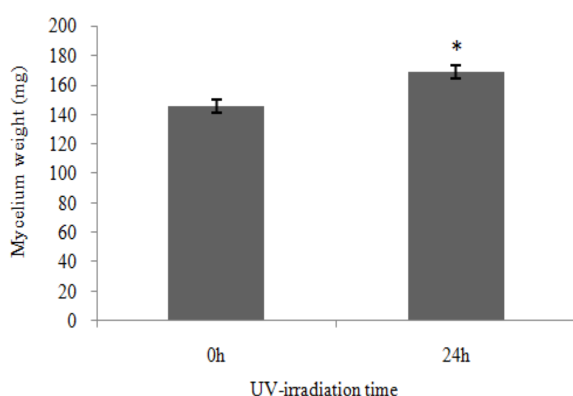

Fig. 2: The *Preussia aemulans* mycelium weight of fermented wheat DDGS with non-irradiation and 24 h UV-irradiation time. Data were expressed as means±S.D. (n = 3). (*p<0.05 in comparison with 0h)

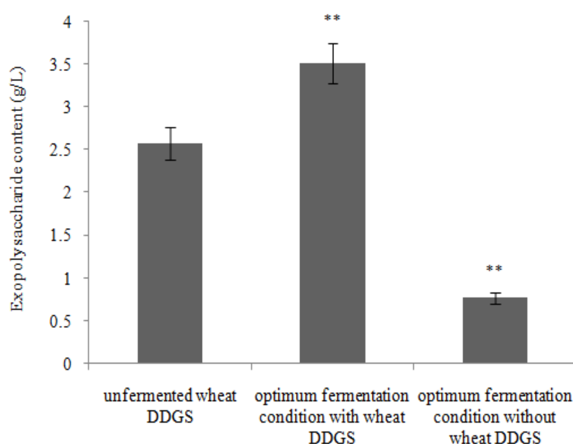

Fig. 3: Exopolysaccharide contents of unfermented and optimum fermentation condition by phenol-sulfuric acid method. Unfermented wheat DDGS was control. Data were expressed as means±S.D. (n = 3); (**p<0.01 in comparison with unfermented Wheat DDGS)

Table 2: Orthogonal test according to orthogonal design L_9 (3^4) of wheat DDGS by *P. aemulans*

Experimental group	CS	NS	ADCS	FT	Exopolysaccharide content (g/L)
	Level				
1	1	1	1	1	2.50±0.17
2	1	2	2	2	2.88±0.11
3	1	3	3	3	2.50±0.18
4	2	1	2	3	1.96±0.19
5	2	2	3	1	2.86±0.24
6	2	3	1	2	2.86±0.11
7	3	1	3	2	2.62±0.25
8	3	2	1	3	2.30±0.18
9	3	3	2	1	3.25±0.08
I_j	23.64	21.24	22.98	25.86	
II_j	23.04	24.11	24.26	25.06	
III_j	24.51	25.84	23.95	20.26	
R	1.47	4.60	1.28	5.60	
Optimum Level	3	3	2	1	

* Ij, IIj, IIIj, are the crude exopolysaccharide contents from liquid culture of level 1, level 2 and level 3, respectively; *R* means the maximum of Ij, IIj and IIIj minus the minimum of Ij, IIj and IIIj, respectively; CS: carbon source; NS: nitrogen source; ADCS: adding dosage of carbon source; FT: fermentation time, respectively

Table 3: The variance analysis of L_9 (3^4) orthogonal test of wheat DDGS by *P. aemulans*

Factor	Sum of square deviation (SS)	Degree of freedom (υ)	Mean square (MS)	*F* ratio	Significance level
CS	0.12	2	0.06	1.92	
NS	1.20	2	0.60	18.92	***
ADCS	0.10	2	0.05	1.55	
FT	2.04	2	1.02	32.27	***
e	0.57	18	0.03		

* $F_{0.10}$ (2, 18) = 2.78; $F_{0.05}$ (2, 18) = 3.55; $F_{0.01}$ (2, 18) = 6.01; * *F* ratio > $F_{0.1}$; ** $F_{0.01}$ > *F* ratio > $F_{0.05}$; *** *F* ratio > $F_{0.01}$; CS: carbon Source; NS: nitrogen source; ADCS: adding dosage of carbon source; FT: fermentation time; e, error

contrast to the fermented broth with wheat DDGS, the exopolysaccharide content of the fermented broth without wheat DDGS under optimum condition and the unfermented wheat DDGS were 0.77±0.07 g/L and 2.58±0.19 g/L, respectively (Fig. 3). It was demonstrated that the produced exopolysaccharide was mainly originated from fermentation of wheat DDGS. In accordance with the fermentation, exopolysaccharide content of the broth could be enhanced during the submerged fermentation by *P. aemulans*. In addition, the obtained *P. aemulans* mycelium could be used as functional material, such as functional food, food additive and ecological feed.

Antioxidative activities:

DPPH radical-scavenging activity: DPPH has been widely used for free radical scavenging activity due to its ease and convenience (Mohsen *et al*., 2009). The DPPH radical solution possesses a distinct purple color which disappears as antioxidants react with DPPH and it breaks the radical chain reaction by donating a hydrogen atom (Krizkova *et al*., 2006). In this study, the results of DPPH radical-scavenging activities were shown in Fig. 4. It was indicated that ES, EPS-1, EPS-2 and ascorbic acid (positive control) were found to be effective scavenger against DPPH radical. Moreover, EPS-1, EPS-2 and ES exhibited highest DPPH radical-scavenging activities at the dosage

Table 4: EC_{50} of various antioxidative activities

	EC_{50} mg/mL		
Antioxidative activity method	EPS-1	EPS-2	ES
DPPH radical-scavenging activities	1.07	1.45	3.30
$ABTS^{+}$ radical-scavenging activities	0.88	2.13	3.05
Scavenging activities of hydroxyl radical	0.12	0.25	0.58
Ferrous metal ions chelating activities	1.31	6.88	>100

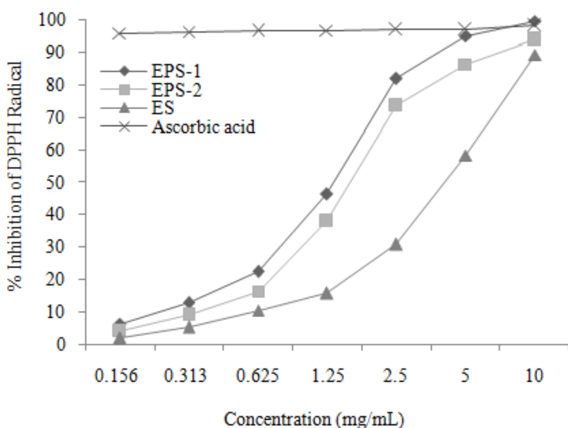

Fig. 4: Inhibition of DPPH radical capacities of EPS-1, EPS-2 and ES. Ascorbic acid was the positive control. Data were expressed as means±S.D. of three independent experiments

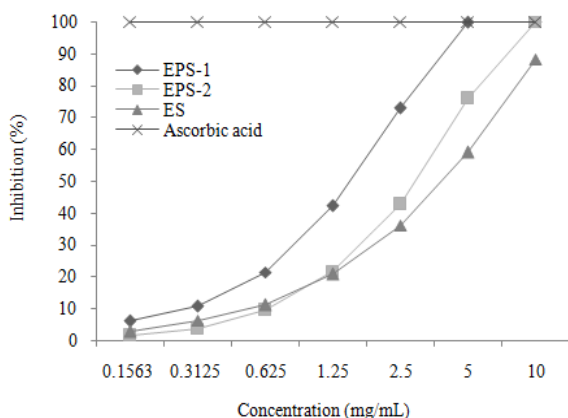

Fig. 5: $ABTS^{\cdot+}$ radical-scavenging activities of EPS-1, EPS-2 and ES. Ascorbic acid was the positive control. Data were expressed as means±S.D. of three independent experiments

of 10 mg/mL with the inhibition of 99.73, 94.07 and 89.16%, respectively. Furthermore, as the concentration of 5 mg/mL, EPS-1 was showed no significant difference with ascorbic acid. And the EC_{50} (Table 4) of EPS-1, EPS-2, ES and ascorbic acid were 1.07, 1.45, 3.30 and 0.04 mg/mL, respectively.

$ABTS^{\cdot+}$ radical-scavenging activity: $ABTS^{\cdot+}$ radical cation decolourisation assay, which is applicable for both lipophilic and hydrophilic antioxidants, exhibited

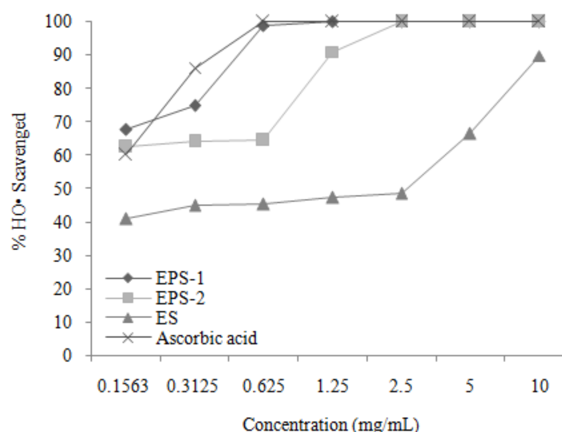

Fig. 6: Scavenging activities of hydroxyl radical in EPS-1, EPS-2 and ES. Ascorbic acid was the positive control. Data were expressed as means±S.D. of three independent experiments

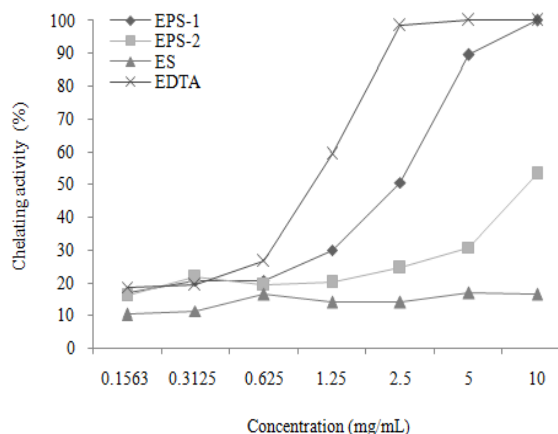

Fig. 7: Ferrous metal ions chelating activities of EPS-1, EPS-2 and ES. EDTA was the positive control. Data were expressed as means±S.D. of three independent experiments

quite similar tendency to DPPH reaction. The inhibitory activity of $ABTS^{\cdot+}$ radical-scavenging activities was shown in Fig. 5. EPS-1, EPS-2, ES scavenged $ABTS^{\cdot+}$ radicals in a dose-dependent-manner. Compared with the two fractions, the scavenging effect of EPS-1 was stronger than that of EPS-2 and ES at each concentration. And at 10 mg/mL, $ABTS^{\cdot+}$ radical-scavenging activity of EPS-1, EPS-2 and ES were the highest, which were 100, 100 and 88.21%, respectively. Furthermore, as the concentration of 5 mg/mL, EPS-1 was showed no significant difference with ascorbic acid. And the EC_{50} of EPS-1, EPS-2 and ES were shown in Table 4, they were 0.88, 2.13 and 3.05 mg/mL, respectively. The ascorbic acid (positive control) showed the strongest $ABTS^{\cdot+}$ radical-scavenging activity, which exhibited 100% inhibitory activity at low concentration (EC_{50}, 0.027 mg/mL).

Scavenging activity of hydroxyl radical: The hydroxyl radical scavenging activities of EPS-1, EPS-2 and ES in various concentrations were shown in Fig. 6. EPS-1 exhibited the same scavenging activity of hydroxyl radical, compared with ascorbic acid. Both ascorbic acid and EPS-1 exhibited 100% inhibitory activity at 0.625 mg/mL. However the scavenging effect of ES exhibited the low inhibitory activity and the inhibition of scavenging activity of hydroxyl radical was 100% at 10 mg/mL. As shown in Table 4, the EC_{50} of EPS-1, EPS-2, ES and ascorbic acid were 0.12, 0.25, 0.58 and 0.05 mg/mL, respectively.

Ferrous metal ions chelating activity: The results of ferrous metal ions chelating activities were shown in Fig. 7. The chelating activity of EPS-1, EPS-2 and EDTA were in a dose-dependent-manner. The chelating activity of EPS-1 was much higher than that of EPS-2 and ES. Furthermore, EPS-1 showed the significant chelating activity at those dosage (0.16-10.00 mg/mL) and at 10 mg/mL, chelated 100% ferrous metal ions. EDTA (the positive control) showed a high chelating ability of 92.40% at 2.5 mg/mL. The EC_{50} (Table 4) of EPS-1, EPS-2 and EDTA were 1.31, 6.88 and 0.63 mg/mL, respectively.

As above, the antioxidative activities of EPS-1 were much higher than these of EPS-2 and ES. Thus, it is necessary to determine the physiological active substances of EPS-1, EPS-2 and ES.

Physiological active substances contents of EPS-1, EPS-2 and ES: Exopolysaccharides content: Polysaccharides have been demonstrated to play an important role as dietary free radical scavenger for the prevention of oxidative damage (Rice *et al*., 1995). In recent years, a few of evidences have suggested that some polysaccharides isolated from cultivable microbial sources exhibited antioxidant capabilities and low cytotoxicity (Kondo *et al*., 1996; Mazur *et al*., 1999). As shown in Table 5, the exopolysaccharides content of EPS-1 (423.61±3.31 mg/g extract), EPS-2 (398.59±1.66 mg/g extract) was higher than that of ES (262.42±2.23 mg/g extract).

Phenolic compounds content: Phenolic compounds have been established to exhibit a scavenging effect on free radicals and the antioxidative activity of phenolics compounds are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Bi *et al*., 2011; Chatchai *et al*., 2011). Data were shown in Table 5, the phenolic compounds content of EPS-1 was 13.09±0.52 mg GAE/g extract, much higher than that of EPS-2 and ES.

Flavonoids content: Flavonoids are a class of secondary plant phenolics with significant antioxidative

Table 5: Physiological active substances contents of EPS-1, EPS-2 and ES

	EPS-1	EPS-2	ES
Exopolysaccharide content (mg/g extract)	423.61±3.31	398.59±1.66	262.42±2.23
Polyphenol compounds content (mg GAE/g extract)	13.09±0.52	11.12±0.34	7.41±0.40
Flavonoid content (mg RE/g extract)	12.28±0.59	10.67±0.56	3.44±0.42
Protein content (mg/g extract)	81.65±2.32	86.75±4.33	13.23±0.89

and chelating properties (Kelly *et al*., 2002). By virtue of their capacity to inhibit LDL oxidation, flavonoids have demonstrated unique cardioprotective effects (Kondo *et al*., 1996; Mazur *et al*., 1999).

Proteins content: The proteins contents of EPS-1, EPS-2 and ES were also determined. As shown in Table 5, the protein of EPS-1, EPS-2 and ES were 81.65±2.32 mg/g extract, 86.75±4.33 mg/g extract and 13.23±0.89 mg/g extract, respectively. Compared with another research, the proteins content was similar to this study of the crude polysaccharides, which were extracted from L. *polychrous Lév* mycelium, fresh fruiting bodies and dry fruiting bodies by ethanol-precipitate method (Shi *et al*., 2011).

As above, the exopolysaccharides contents were much higher than those of phenolic compounds, flavonoids and proteins. Therefore, the excellent antioxidative activities of EPS-1 could be mainly related to the exopolysaccharides contents.

CONCLUSION

In conclusion, with the popularity of distilled spirit, the generation of the by-products, wheat DDGS, has increased massively. Most of wheat DDGS is incinerated and buried, which has caused severe environmental pollution. Therefore, ecological treatment of wheat DDGS is indispensable. Traditionally, wheat DDGS was used as animal feed or agricultural fertilizer. Furthermore, in this study, the crude exopolysaccharide which isolated from fermented wheat DDGS broth exhibited remarkable antioxidative activity, thus the isolated exopolysaccharide could be explored as natural antioxidative food or feed additive. In brief, this biological treatment of wheat DDGS is extremely novel and it not only could reduce the influence on environment, but also could convert agro-industrial waste to functional material.

For further study, the bioactivity of other physiological active substances in fermented wheat DDGS by *P. aemulans* should be explored and demonstrated in the future.

REFERENCES

Bi, J., Q.L. Yang, J. Sun, J. Chen and J. Zhang, 2011. Study on ultrasonic extraction technology and oxidation resistance of total flavonoids from peanut hull. Food Sci. Technol. Res., 17: 187-198.

Blois, M., 2002. Antioxidant determinations by the use of a stable free radical. Nature, 26: 1199-2000.

Chatchai, T., K. Saranyu, C. Khajeelak, S. Chantragan and S. Rakrudee, 2011. Antioxidant properties and cytotoxicity of crude polysaccharides from *Lentinus polychrous* Lév. Food Chem., 128: 634-639.

Chen, Y.J., M.S. Shiao, S.S. Lee and S.Y. Wang, 1997. Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. Life Sci., 60: 2349-2359.

Decker, E. and B. Welch, 1990. Role of ferritin as a lipid oxidation catalyst in muscle food. J. Agr. Food Chem., 38: 674-677.

Greter, A.M., G.B. Penner, E.C. Davis and M. Oba, 2008. Effect of replacing corn dry distillers grains' with triticale dry distillers' grains on lactation performance and plasma metabolites. Can. J. Anim. Sci., 88: 129-132.

Guo, S.D., W.J. Mao, Y. Han, X.H. Zhang, C.L. Yang, Y. Chen, Y.L. Chen, J. Xu, H.Y. Li, X.H. Qi and J.C. Xu, 2010. Structural characteristics and antioxidant activities of the extracellular polysaccharides produced by marine bacterium *Edwardsiella tarda*. Bioresource Technol., 101: 4729-4732.

Hajar, I.I., W.C. Kim, A.M. Abdalbasit and I. Maznah, 2010. Phenolic content and antioxidant activity of cantaloupe (*Cucumis melo*) methanolic extracts. Food Chem., 119: 643-647.

Jia, Z.S., M.C. Tang and J.M. Wu, 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem., 64: 555-559.

Kanekiyo, K., J.B. Lee, K. Hayashi, H. Takenaka, Y. Hayakawa, S. Endo and T. Hayashi, 2005. Isolation of an antiviral polysaccharide, nostoflan, from a *terrestrial cyanobacterium*, *Nostoc flagelliforme*. J. Nat. Prod., 68: 1037-1041.

Kelly, E.H., R.T. Anthony and J.B. Dennis, 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem., 13: 572-584.

Kondo, K., R. Hirano, A. Matsumoto, O. Igarashi and H. Itakura, 1996. Inhibition of LDL oxidation by cocoa. Lancet, 348: 1514-1518.

Krizkova, L., I. Zitnanova, D. Mislovicova, J. Masarova, V. Sasinkova and Z. Durackova, 2006. Antioxidant and antimutagenic activity of mannan neoglycoconjugates: Mannan-human serum albumin and mannan-penicillin Gacylase. Mutat. Res-Gen. Tox. En., 606: 72-79.

Leung, P.H., S. Zhao, K.P. Ho and J.Y. Wu, 2009. Chemical properties and antioxidant activity of exopolysaccharides from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1. Food Chem., 114: 1251-1256.

Masuko, T., A. Minami, N. Iwasaki, T. Majima, S. Nishimaru and Y.C. Lee, 2005. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal. Biochem., 339: 69-72.

Mau, J.L., H.C. Lin and S.F. Song, 2002. Antioxidant properties of several specialty mushrooms. Food Res. Int., 35: 519-526.

Mazur, A., D. Bayle, C. Lab, E. Rock and Y. Rayssiguier, 1999. Inhibitory effect of procyanidin-rich extracts on LDL oxidation in vitro. Atherosclerosis, 145: 421- 422.

Miranda, C.C., R.F. Dekker, J.M. Serpeloni, E.A. Fonseca, I.M. Cólus and A.M. Barbosa, 2008. Anticlastogenic activity exhibited by botryosphaeran, a new exopolysaccharide produced by *Botryosphaeria rhodina* MAMB-05. Int. J. Biol. Macromol., 42: 172-177.

Mohsen, M.S.A., M.A. Youssri and F.R. Mohamed, 2009. Chemical characteristics and antioxidant activity of exopolysaccharide fractions from *Microbacterium terregens*. Carbohyd. Polym., 77: 563-567.

Penner, G.B., P. Yu and D.A. Christensen, 2009. Effect of replacing forage or concentrate with wet or dry distillers'grains on the productivity and chewing activity of dairy cattle. Anim. Feed Sci. Tech., 153: 1-10.

Rasco, B.A., F.M. Dong, A.E. Hashisaka, S.S. Gazzaz, S.E. Downey and M.L. San Buenaventura, 1987. Chemical composition of Distillers Dried Grains with Solubles (DDGS) from soft white wheat, hard red wheat and corn. J. Food Sci., 52: 236-237.

Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, E.C. Rice, 1999. Antioxidant activity applying an improved ABTS radical cation decolorization. Free Radical. Bio. Med., 26: 231-237.

Rice, E.C., N.J. Miller, G.P. Bolwell, P.M. Bramley and J.B. Pridham, 1995. The relative antioxidants activities of plant-derived poly-phenolic flavonoids. Free Radical Res., 22: 375-383.

Seviour, R.J., S.J. Stasinopoulos, D.P.F. Auer and P.A. Gibbs, 1992. Production of pullulan and other exopolysaccharides by *filamentous* fungi. Crit. Rev. Biotechnol., 12: 279-298.

Shi, M., Y.N. Yang, Y.T. Li, Y.P. Wang and Z.Y. Zhang, 2011. Optimum condition of ecologic feed fermentation by *Pleurotus ostreatus* using soybean curd re sidue as raw materials. Int. J. Biol., 3: 2-12.

Smirnoff, N. and Q. Cumbes, 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry, 28: 1057-1060.

Sun, H.H., W.J. Mao, Y. Chen, S.D. Guo, H.Y. Li, X.H. Qi, Y.L. Chen and J. Xu, 2009. Isolation, chemical characteristics and antioxidant properties of the polysaccharides from marine fungus *Penicillium sp*. F23-2. Carbohyd. Polym., 78: 117-124.

Tachakittirungrod, S., S. Okonogi and S. Chowwanapoonpohn, 2007. Study on antioxidant activity of certain plants in Thailand: Mechanism of antioxidant action of guava leaf extract. Food Chem., 103: 381-388.

Wang, Z.J. and D.H. Luo, 2007. Antioxidant activities of different fractions of polysaccharide purified from *Gynostemma pentaphyllum* Makino. Carbohyd. Polym., 68: 54-58.

Xu, R.H., X.E. Peng, G.Z. Chen and G.L. Chen, 1992. Effects of *Cordyceps sinensis* on natural killer activity and colony formation of B16 melanoma. Chinese Med. J-Peking, 105: 97-101.

Yang, J.Y., W.Y. Zhang, P.H. Shi, J.P. Chen, X.D. Han and Y. Wang, 2005. Effects of exopolysaccharide fraction (EPSF) from a cultivated *Cordyceps sinensis* fungui on c-Myc, c-Fos and VEGF expression in B16 melanoma-bearing mice. Pathol. Res. Pract., 201: 745-750.

Yen, G.C., P.D. Duh and H.L. Tsai, 2002. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. Food Chem., 79: 307-313.

Yu, Z. and J.X. He, 1998. Study of water extract of *Cordyceps sinensis* on antiperoxidation. Res. Tradit. Chinese Med., 14: 51-52.

Zhang, W.Y., J. Li, S.Q. Qiu, J.P. Chen and Y. Zheng, 2008. Effects of the exopolysaccharide fraction (EPSF) from a cultivated *Cordyceps sinensis* on immunocytes of H22 tumor bearing mice. Fitoterapia, 79: 168-173.

Zhang, Y.J., B.D. Sun, S. Zhang, M. Wang, X.Z. Liu and W.F. Gong, 2010. Mycobiotal investigation of natural *Ophiocordyceps sinensis* based on culture-dependent investigation. Mycosystema, 29: 518-527.

Zhu, J.S., G.M. Halpern and K. Jones, 1998. The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis*: Part I. J. Altern. Complem. Med., 4: 289-303.