
Production of exopolysaccharide from *Tricholoma crassum* in submerged culture and its antioxidant activities

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Abstract The effects of carbon, nitrogen sources and nutritional requirements were studied for the production of exopolysaccharide (EPS) from *Tricholoma crassum* using the statistically based experimental design in a shake flask culture, and the antioxidant activities of EPS *in vitro*. Glucose and Yeast extract was found to be the most suitable of carbon and nitrogen source. The optimization of submerged culture conditions was studied using Taguchi method; the results showed the optimized cultivation was pH 6.5, incubation temperature 30°C, cultivation time 10 days. Under these optimal conditions, the EPS content and dry cell weight were 0.727 g/L and 9.7 g/L, respectively. The EPS consisted of galactose, glucose and mannose with ratio 1:6.25:17.72, suggested that EPS from *T. crassum* could be Galacto-Gluco-Mannan. The EPS demonstrated positively antioxidant potential on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, reducing power and inhibition of lipid peroxidation assay.

Keywords: *Tricholoma crassum*, exopolysaccharide, Taguchi method, antioxidant

Introduction

Tricholoma crassum is an edible mushroom, belongs to family of Tricholomataceae (Chang *et al.*, 1978), having no toxicity, found in the Asia region mainly in Japan, Thailand and Sri Lanka. The nutritional value of *T. crassum* in 100g of fresh weight is 10.02 g carbohydrates, 0.287 g fats, 18.58 g protein, 2.71 mg calcium, and 3.35 mg iron (Teaumroong *et al.*, 2002). Ray *et al.* (2011) demonstrated the synthesis of silver nanoparticles ability of mycelium from *T. crassum*. Subrata *et al.* (2012) by disc diffusion method have found that methanolic extraction crude of fruiting body form *Tricholoma crassum* had activity of *Bacillus cereus* MTCC CODE 1306 and *Proteus vulgaris* MTCC CODE 426 growth inhibition. Recently, the structural elucidation of hetero polysaccharide and its immunological activity from alkaline extract of fruiting bodies from this mushroom were conducted by Patra

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et al. (2012). In addition, a glucan from hot aqueous extract of the fruiting bodies of *T. crassum* showed macrophage activation *in vitro* and strong splenocyte and thymocyte immunostimulation in mouse cell culture medium (Samanta *et al.*, 2013).

Compared with the polysaccharide from fruiting bodies and mycelium, the exopolysaccharide (EPS) from submerged culture is similar to physiological and biological functions. Many researchers have optimized the EPS production by many kind of mushrooms such: *Tremella fuciformis* (Cho *et al.*, 2006), *Gomphidius rutilus* (Gao *et al.*, 2012), *Grifola umbellate* (Huang and Liu, 2008), *Grifola frondosa* (Lee *et al.*, 2004), *Tricholoma matsutake* (Kim *et al.*, 2010b), *Morchella esculenta* (Meng *et al.*, 2010), *Cordyceps militaris* (Kim *et al.*, 2003), *Agrocybe cylindracea* (Kim *et al.*, 2005), *Tricholoma mongolicum* (Wu *et al.*, 2012), *Ganoderma lucidum* (Chang *et al.*, 2005). However, it has not been reported on the production of EPS by *Tricholoma crassum* in submerged culture.

Antioxidants can delay or prevent oxidation of cellular oxidative substrate. It plays an important role in preventing and curing chronic inflammation, atherosclerosis, cancer and cardiovascular disorders (Kohen and Nyska, 2002). Therefore, these compounds are widely used in food, medicine. In recently years, polysaccharide of fruiting bodies as well as in submerged culture from mushrooms has been considered as novel natural antioxidants. Meng *et al.* (2010) found that EPS of *Morchella esculenta* SO-02 had positively antioxidant potential on superoxide anion radical scavenging, reducing power and hydroxyl radical scavenging. The polysaccharide obtained by hot water extraction from *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum*, *Phelinus linteus* showed ability on DPPH scavenging and inhibition of lipid peroxidation (Kozarski *et al.*, 2011). However, knowledge is still lacking with regard to the antioxidant activities of EPS from *Tricholoma crassum*.

The evaluation of carbon, nitrogen source and optimization fermentation conditions are important for enhancing the efficiency of EPS production in submerged culture. It has been shown that the deferent carbon and nitrogen source induce the synthesis of EPS with different yields and activities (Elisashvili *et al.*, 2009; Kim *et al.*, 2010a). Cultivation parameters such pH, temperature, incubation time can affect the fermentation efficiency. In conventional optimization methods, the impact of a particular parameter is evaluated by altering it while keeping the other parameters constant. Therefore, that optimization method is time consuming and cannot provide information on interaction of the parameters. Nowadays, statistical experimental design method is widely used, such as the centre composite design, response surface

methodology and the Taguchi method. Among them, the Taguchi method is advantageous because it can optimize various factors simultaneously and substantial quantitative information can be obtained with only a few experimental trials. Taguchi methods have been successfully applied to optimize the culture medium and culture conditions for EPS production from *Gomphidius rutilus* (Gao *et al.*, 2012) and *Ganoderma lucidum* (Chang *et al.*, 2005).

The objectives of this study were to evaluate the effects of carbon, nitrogen source by single factor tests, optimize cultivation conditions by Taguchi method and to determine the antioxidant activities of EPS in vitro with the DPPH radical scavenging, reducing power and inhibition of lipid peroxidation assay.

Materials and methods

Microorganism and medium

Five strains of *Tricholoma crassum* (DOA, DOA-1, DOA-4, DOA-7, DOA-10) were kindly provided by Mrs. Achara Payapanon, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand. It was inoculated on potato dextrose agar (PDA) plates for 7 days at 25°C, maintained at 4°C and sub-cultured every 2 months.

All experiment of submerged culture was performed in 250 ml Erlenmeyer flask. The basal medium was contained 30 g/l glucose, 6 g/l yeast extract, 1 g/l KH₂PO₄, 0.5 g/l MgSO₄.7H₂O with initial at pH 6.5. *Tricholoma crassum* was first grown on PDA in a petri dish, 5mm diameter portion of agar plate was cut with sterilized cutter and then inoculated into 250 ml flask containing 100 ml with shaker at 150 rpm.

Screening of highest EPS production strain

Five strains were transferred to 100ml DMK medium in 250 ml flask by punching out 5mm diameter of PDA agar plate with sterilized cutter. The medium was grown at 25°C with shaker at 150 rpm for 10 days. After 10 days of cultivation, EPS and mycelium production were determined to find the highest EPS production strain.

Effect of carbon and nitrogen sources

The carbon source 10g/L (sucrose, glucose, maltose, lactose, fructose and yeast extract 5g/L as nitrogen source) and nitrogen source 5g/L (peptone,

tryptone, yeast extract, (NH₄)₂SO₄, NH₄NO₃ and glucose 10g/L as carbon source) were screened and selected. Variable carbon source and nitrogen source were used to respectively replace glucose and yeast extract in DMK medium.

Optimization of cultivation conditions for EPS production

Cultivation time, incubation temperature and initial pH (Table 1) were optimized by Taguchi method for EPS production. The factorial experiment was performed with 3 factors at three levels. The orthogonal L₉ (3³) was used to obtain optimal cultivation conditions for EPS production. This allowed to determine the variables affected the response. All trials were carried out in the medium which obtained from the effects of carbon and nitrogen sources section. The detailed experimental conditions for each trial were shown in Table 2.

Table 1. Value of factors in experiment

Levels	1	2	3
Factors			
Initial pH (A)	6	6.5	7
Incubation temperature (°C) (B)	20	25	30
Incubation time (day) (C)	5	10	15

Determination of dry cell weight and EPS yield

After mycelia fermentation completed, medium broth was centrifuged (3000 x g, 20 min) in a centrifuge to separate mycelium and supernatant. The mycelium of *Tricholoma crassum* was washed with distilled water for three times and dried to constant weight at 60°C for 24 hours in an oven and then quantified by subtracting the dry cell weight of the filter paper from the total weight.

For EPS yield determination, supernatant was mixed with three volume of 95% (v/v) ethanol, stirred vigorously and kept at 4°C for 18 hours. EPS precipitations were obtained by centrifugation (3000 x g, 20 min), wash three times with 95% ethanol and re-dissolved with distilled water up to initial volume. The EPS content was determined by Phenol-sulfuric acid method (Dubois *et al.*, 1956).

Determination of monosaccharide components

EPS (10 mg) was hydrolyzed with 2M hydrochloric acid (5 mL) at 100°C for 4 hours in sealed test tube. Excess sulfuric acid was neutralized with calcium carbonate and centrifuged to discard precipitation, and the supernatant was evaporated to dryness, and the solid was repeatedly re-dissolved in ethanol and evaporated to dryness. After that, the components of monosaccharide were sent to identify monosaccharide components by HPLC (High Performance Liquid Chromatography) after hydrolyzed with hydrochloric acid.

Preparation of EPS for activity testing

The crude EPS (from ethanol precipitate) was purified through the following steps: It was first deproteinized with Sevag reagent. The EPS solution was mixed with Sevag reagent (1-butanol/chloroform at 1:4 v/v) with constant stirring for 2 hours. The deproteinized EPS solution was then mixed with 1.5% (w/v) powdered activated carbon for 40 min to remove the brown pigments. After removal of the activated carbon (by filtration), the EPS solution was dialyzed (using 12-14 kDa cut off membrane) against distilled water for 24 hours. The purified EPS solution was concentrated by vacuum evaporation and to detect the antioxidant activities in vitro (Yan *et al.*, 2009).

Determination of antioxidant activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging was assayed. The reaction mixture contained 2ml of 95% ethanol, 0.1 μ M DPPH and 2 mL of the EPS solution (100 - 500 mg/L). The solution was incubated at 25°C for 15 min, and the absorbance of EPS was determined at 517 nm using BHT (Butylated hydroxyl toluene) as positive control. The antioxidant activity of EPS was evaluated according to the following formula: Scavenging activity (%) = $(1 - A_0)/A \times 100$. Where A was absorbance of EPS and A_0 was the absorbance of DPPH solution (Lin *et al.*, 2012).

The reducing power assay: of EPS was evaluated according to the method of Gao *et al.* (2012). The reaction mixtures contained 2.5 mL phosphate buffer (pH 6.6, 0.2M), 2.5 mL potassium ferricyanide (1%, w/v), and EPS solution (100 - 500 mg/L). After incubation at 50°C for 20 min, 2.5 mL trichloroacetic acid (10%, w/v) was added to the mixture to end the reaction, and then the mixture was centrifuged at 3000 rpm for 10 min. An aliquot of 1.25 mL supernatant was then collected and mixed with 1.25 mL distilled water and 0.25 mL $FeCl_3$ (0.1%, w/v). After incubation at room temperature for 15 min,

the absorbance was measured at 700 nm using BHT as the positive control. Increased absorbance value indicates higher reducing power (Gao *et al.*, 2012)

The inhibition of lipid peroxidation assay: A solution of β -carotene was prepared by dissolving 2 mg of β -carotene in 10 ml of chloroform. Two milliliters of this solution were pipetted into a 100 ml round-bottom flask. After chloroform was removed under vacuum, 40 mg of purified linoleic acid, 400 mg of Tween 40 emulsifier, and 100 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing different concentrations of EPS solution. BHT was used for positive control. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The tubes were placed at 50°C in a water bath. Measurement of absorbance was continued after 2 hours. Inhibition activity was calculated using the following equation: Inhibition activity (%) = $(A/A_0) \times 100$. Where: A was absorbance at 2 hours, A_0 was absorbance at zero time (Shon *et al.*, 2003).

Statistical analysis

Optimization of culture conditions for EPS production experiment were carried out by using Qualitek 4 software (Nutek Inc., Bloomfield Hills, MI, USA) for the automatic design of Taguchi experiments. The data were expressed in triplicate as mean \pm SD and analyzed statistically by ANOVA method.

Results

Screening of highest EPS production strain of *Tricholoma crissum*

As shown in Figure 1, the highest EPS production at 10 day (0.417 g/L) was obtained with DOA strain, whereas DOA-7 showed the highest biomass production at 10.55 g/L. The DOA-4 (0.111g/L) and DOA-1 (3.02 g/L) gave lowest EPS and biomass production, respectively. The results indicated that the EPS production was independent with cell mass production.

DOA-7 was the highest strain of converted coefficient yield of biomass (0.351) followed by DOA-10 (0.310), and DOA (0.128) as shown in Table 2. However, DOA showed the highest converted coefficient yield of EPS among five tested strains. These results revealed that DOA was potential strain for EPS production. Furthermore, this study is focused on the EPS production and its

activity therefore, DOA was a selected strain and used as a research subject of subsequent experiments.

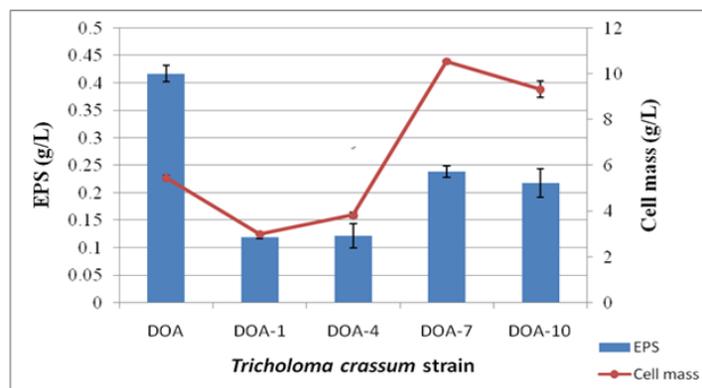


Figure 1. EPS and cell mass production form 5 strains of *Tricholoma crissum*

Table 2. Converted coefficient yield of EPS and cell mass from *Tricholoma crissum*

Strain	$Y_{p/s}$	$Y_{x/s}$
DOA	0.0139	0.182
DOA-1	0.0040	0.100
DOA-4	0.0041	0.127
DOA-7	0.0079	0.351
DOA-10	0.0072	0.310

Note: $Y_{p/s}$: Converted coefficient yield of EPS (EPS concentration/Glucose concentration);
 $Y_{x/s}$: Converted coefficient yield of biomass (Biomass concentration/Glucose concentration)

Effect of carbon and nitrogen source on EPS production of Tricholoma crissum

At different carbon sources tested, the maximum EPS production was obtained in medium supplemented by glucose (0.488 mg/L) followed by sucrose (0.391mg/L) and fructose (0.353 mg/L), this result showed that each carbon source was independently responsible in polysaccharide production.

Nitrogen may be supplied as ammonia, nitrate or in organic compounds, such as amino acids and proteins. Therefore, the omission of nitrogen in the medium greatly affects fungal growth and metabolite production. Studies on supplementation of both inorganic and organic nitrogen sources to the fermentation medium showed a mixed trend on EPS production. Among the tested nitrogen sources maximum biomass and EPS production was found when yeast extract employed, with inorganic nitrogen source $((NH_4)_2SO_4$, NH_4NO_3) microorganism was not grown (Figure 3). In comparison of inorganic

and organic nitrogen sources, it showed that the organic nitrogen source was more suitable for both EPS and biomass production.

From the above data, it was apparent that glucose and yeast extract were most beneficial to exopolysaccharide production and cell growth by submerged culture of *Tricholoma crassum*.

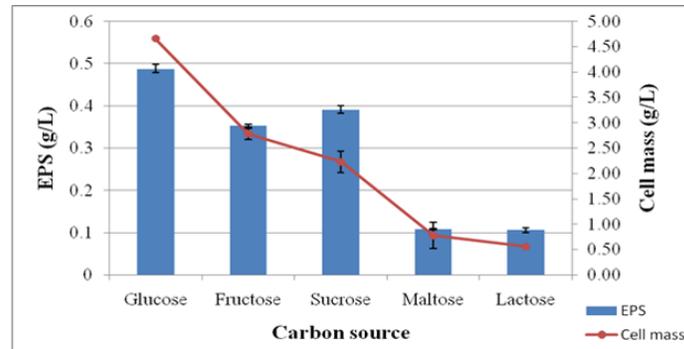


Figure 2. Effect of carbon source on EPS production of *Tricholoma crassum*

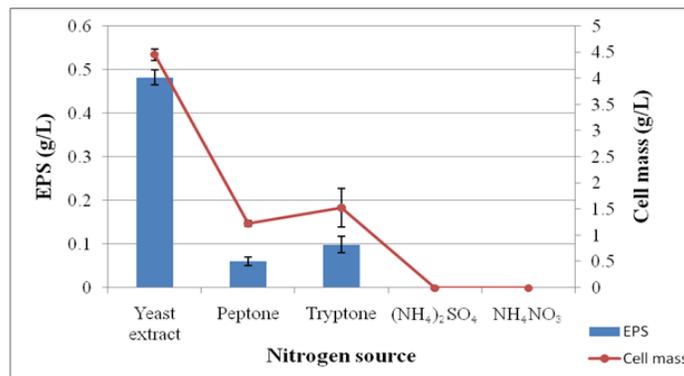


Figure 3. Effect of nitrogen source on EPS production of *Tricholoma crissum*

Optimization of culture conditions for EPS production

The Taguchi method revealed the order of effects on EPS as Temperature > pH > Incubation time (Table 3). The optimal condition for EPS production was A₂B₃C₂, namely pH 6.5, temperature 30°C, incubation time 10 days.

To confirm the results, Taguchi method used Confidence Level (C.L) which was the expected variation of the mean performance at the optimum condition. “Qualitek-4” software that used for Taguchi method is automatically using C.L at 90%. The EPS yield under optimum condition, according to the software, must be at one value that ranged from 0.673 to 0.734 g/L. After performed EPS production under optimal condition, EPS yield was obtained as

0.727 mg/L, showed good agreement between program and practical experiment. These results were used in further experiments.

Table 3. Results of Taguchi experimental design for culture medium optimisation

No.	Variables			EPS (g/L)	Cell mass (g/L)
	A	B	C		
1	6	20	5	0.058 ± 0.003	2.13 ± 0.44
2	6	25	10	0.359 ± 0.009	8.02 ± 0.12
3	6	30	15	0.721 ± 0.049	9.70 ± 0.8
4	6.5	20	10	0.260 ± 0.035	2.23 ± 0.10
5	6.5	25	15	0.305 ± 0.016	8.58 ± 0.24
6	6.5	30	5	0.586 ± 0.002	4.90 ± 0.58
7	7	20	15	0.066 ± 0.007	3.67 ± 0.13
8	7	25	5	0.123 ± 0.001	3.11 ± 0.23
9	7	25	10	0.518 ± 0.050	8.60 ± 0.12

Monosaccharide determination of EPS from Tricholoma crissum

The result showed the EPS consisting of three monosaccharides: mannose, glucose and galactose. The molar ratio between each monosaccharide was 17.72:6.25:1. Mannose occupied the highest component of total and followed by glucose and galactose. The result suggested that EPS from *Tricholoma crassum* could be Galacto-Gluco-Mannan.

Antioxidant activities

In this study, the antioxidative activities of EPS from *Tricholoma crassum in vitro* were evaluated using different methods of DPPH radical scavenging, reducing power and inhibition of lipid peroxidation assay.

DPPH is presented as a stable free radical in aqueous or methanolic solutions, and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It is usually used as a substrate to evaluate the activity of antioxidants. The scavenging activity of EPS on the inhibition of the DPPH radical was related to the concentration at the test dosage range. When the *Tricholoma crassum* EPS concentration was increased from 100 to 500 mg/L, the DPPH scavenging rate increased from 32.80 to 57.54% (Figure 6). However, the inhibition percentage of EPS was much lower than that of BHT at the same dosage range. The EC 50 value of EPS from *T. crassum* for DPPH radical scavenging activity was 338 mg/L.

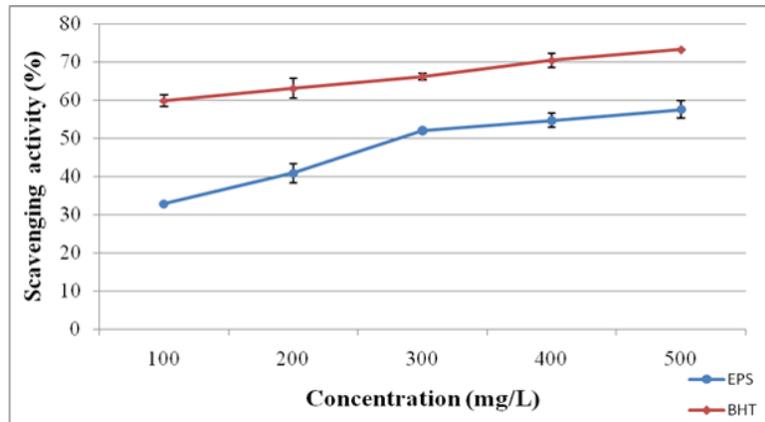


Figure 4. DPPH radical scavenging activity of EPS from *Tricholoma crassum*

The reducing power assay was determined the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. The presence of reducers in the test solution led to the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing powers of EPS from *Tricholoma crassum* increased slowly with the increase in concentration, as shown in Figure 5. It is suggested that EPS has potential antioxidant activity, however with low activity. At highest tested concentration (500 mg/L), EPS were 0.240 nm absorbance was much lower than that BHT (1.257 nm absorbance) at the same dosage.

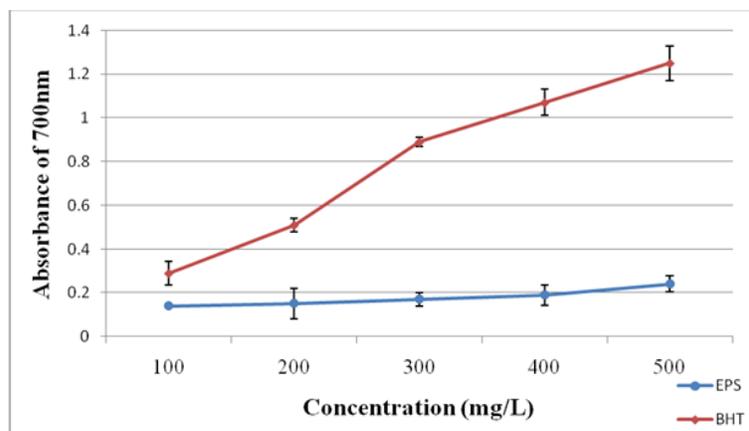


Figure 5. Reducing power activity of EPS from *Tricholoma crassum*

Lipid peroxidation is major cause of food deterioration, which affects color, flavor, texture, and nutritional value. The oxidative modification of low-

density lipoproteins may play a role in the development of atherosclerosis. Oxidative modification depends on a common initiating step in the peroxidation of polyunsaturated fatty acid components in the LDLs. As shown in Figure 6, the inhibition of lipid peroxidation activity of EPS from *Tricholoma crassum* was concentration-dependent and much lower than that of BHT at the tested dosage range. The EC50 value of EPS for inhibition of lipid peroxidation was 651 mg/L, which had significant difference from the scavenging effect of BHT.

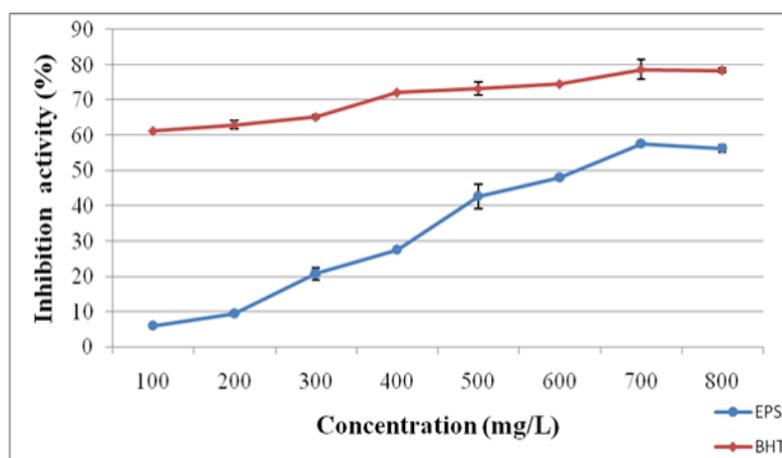


Figure 6. Inhibition lipid peroxidation activity of EPS from *Tricholoma crassum*

Discussion

In the present study, the effects of carbon and nitrogen source on EPS production and the liquid submerged culture conditions for EPS production from *Tricholoma crassum* were investigated using series of experimental design. The results revealed that DOA was a potential strain for EPS production. Furthermore, this study is focused on the EPS production and its activity therefore, DOA was a selected strain and used as a research subject of subsequent experiments. Carbohydrates are important carbon and energy sources for cultured cells. The production of EPS and cell mass was varied according to carbon sources in the medium. Therefore, even if the same fungal species needs a different carbon source for specific metabolite production, it is possible that different carbon sources might have different effects on catabolic repression on the cellular secondary metabolism. In this experiment, glucose also stimulated greatest biomass (4.66 g/L). The result was in agreement with the results reported by other investigators, who evaluated the most suitable carbon source was glucose of *Fusarium solani* (Subhadip and Banerjee, 2013).

In comparison of inorganic and organic nitrogen sources, it showed that the organic nitrogen source was more suitable for both EPS and biomass production. These findings were in accordance with required nitrogen supplements of *Furarium solani* (Subhadip and Banerjee, 2013) and *Pleurotus nebrodensis* (Le *et al.*, 2007). Glucose and yeast extract showed the most suitable carbon and nitrogen source for both EPS and cell mass production. The optimization of submerged culture conditions was studied using Taguchi method; the results showed the optimized cultivation conditions were: pH 6.5, incubation temperature 30°C, cultivation time 10 days. Under these optimal conditions, the EPS content and dry cell weight were (0.727 g/L) and (9.7 g/L), respectively. Temperature, incubation time and pH were the most effective culture conditions that effects on EPS production and mycelia growth as well. The study commonly focuses on three of these factors (Kim *et al.*, 2003) and Lai *et al.* (2014). Wu *et al.* (2012) found that temperature was the most effective on EPS of *Tricholoma mongolicum* among three factors (temperature, rotation speed, and inoculums size). The evaluation of acidic pH optimal for EPS production from fungal cultures is quite common. Formerly, Banerjee *et al.* (2009), Nehad and El-Shamy (2010), El-Dein *et al.* (2004), and Roukas and Biliaderis (1995) reported optimum medium pH 6.5, 6.0, 5.5, 5.0 for EPS production by *Stemphylium* sp., *Alternaria alternate*, *Pleurotus pulmonarius*, *Aureobasidium pullulans*, respectively. Incubation time in some species of fungi was the most effective. Fungi normally need long incubation time for maximum EPS production. Fermentation period optimal for EPS production was noticed in findings of Nehad and El-Shamy (2010) and Lee *et al.* (2004) where fermentation period of 9 and 12 days were reported as most suitable for EPS production in submerged fermentation of *Alternaria alternate* and *Ganoderma applanatum* KFRI 646, respectively. The results were quite closed to our research (10 days of incubation time). As different authors have reported different optimal values of pH, temperature and incubation time for EPS production, it seems that changes in the environmental factors lead to certain changes in the EPS yield that vary depending on the fungi species.

Monosaccharide components of exopolysaccharide from *Tricholoma crassum* DOA was determined by HPLC and composed; Galactose, Glucose and Mannose with ratio as 1:6.25:17.72. The result suggested that EPS from *T. crassum* could be Galacto-Gluco-Mannan. In comparison with another study about structure of fungal EPS, Sun *et al.* (2011) found that EPS from *Epicoccum nigrum* was composed by mannose, glucose and galactose as well but in different ratio, 5.0:2.1:1.0. This kind of EPS also named as Galacto-Gluco-Mannan. Sun *et al.* (2011) also demonstrated that EPS from *Epicoccum nigrum* had antioxidant activities.

Antioxidant activities have been attributed to various reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, binding of transition metal ion catalysts, etc. (Frankel and Meyer, 2000). The results showed the low antioxidant activity of EPS on DPPH, reducing power and inhibition of lipid peroxidation assay. In comparison with another strains, it is higher than *Fusarium solani* (570 mg/L; Subhadip and Banerjee, 2013) but much lower than *Gomphidius rutilus* (25.7 mg/L; Gao *et al.*, 2012) using DPPH.

This research provided basic data of EPS from *Tricholoma crissum*. No reports are currently available in the literature regarding the optimization of EPS production by *Tricholoma crassum* in submerged culture and its antioxidant activities *in vitro*. The results of study provided the references for large scale production of EPS and thereby open up the directions on EPS application for human health in the future.

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