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Optimisation of exopolysaccharide production by *Gomphidius rutilus* and its antioxidant activities *in vitro*

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ABSTRACT

The production conditions of the *Gomphidius rutilus* exopolysaccharides (GREP) in submerged culture were optimised, and the antioxidant activities of GREP *in vitro* were evaluated. The optimal culture medium constituents were determined as follows: 30 g/L sucrose, 3.0 g/L soybean meal, 0.25 g/L $MgSO_4$, 1.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.03 g/L $ZnSO_4$, and 0.01 g/L $FeSO_4$. The optimum parameters for the liquid fermentation were as follows: temperature, 25 °C; cultivation time, 6 d; initial pH, 8.0; volume of medium, 150 mL; and rotary speed, 180 rpm. GREP content and dry cell weight in optimised conditions were 540.1 ± 15.9 mg/L and 8.2 ± 0.3 g/L, respectively. GREP content under the optimised conditions was 2.5 times than that under the basic culture medium and initial conditions. GREP demonstrated positive antioxidant potential on superoxide anion radical, 1,1-diphenyl-2-picrylhydrazyl, and hydroxyl radical scavenging, and reducing power.

1. Introduction

In Asian countries, such as China, Korea, and Japan, mushrooms have long been used as traditional food and medicines. Along with the development of cytobiology, immunology, and molecular biology, mushroom polysaccharides and their composites have recently attracted the attention of biochemists and pharmacologists all over the world for their various biological activities (Borchers, Keen, & Gershwin, 2004; Li, Zhou, & Han, 2006; Zhang, Cui, Cheung, & Wang, 2007). Many reports have provided evidence that these compounds have biological functions, such as anti-tumour (Li et al., 2008), anti-oxidant (Yan et al., 2011), anti-cancer (Chen et al., 2010), and immunobiological activities (Li et al., 2008; Sun et al., 2008), among others.

Gomphidius rutilus, a traditional Chinese medicinal and edible fungus in China, belong to *Basidiomycotina* and is often found beneath pine trees. This mushroom is widely distributed in China including Hebei, Shanxi, Liaoning, Jilin, Heilongjiang, Hunan, Sichuan, and Tibet. The extraction and anti-oxidant activity of polysaccharides from the fruiting bodies of *G. rutilus* have been previously studied (Sun & Kennedy, 2010). The agrestal *G. rutilus* has become scarce in recent years, and the fruiting bodies of this fungus cannot be harvested by artificial cultivation. Thus, the use of *G. rutilus* polysaccharides has been restricted in large-scale and industrial processes. Even if this fungus can be cultivated artificially, the traditional method of fruiting body production still has several disadvantages including long fruiting body cultivation period, difficulty of controlling the product quality, and vulnerability to environmental changes., The submerged fermentation technology for exopolysaccharides and mycelium of many mushroom species (Chen, Zhang, Qu, & Xie, 2008; Xiao et al., 2004; Meng et al., 2010; Tang & Zhong, 2002) has been developed. This technology has gained considerable attention as a promising alternative because it has none of the aforementioned weaknesses. Using submerged fermentation technology in harvesting exopolysaccharides and mycelium is economic and cost-efficient.

The design of fermentation conditions and medium composition is vital for enhancing the efficiency of production in submerged culture. Many variables such as medium composition, initial pH, temperature, and rotary speed can affect the fermentation efficiency. Statistical experimental design methods provide a systematic and efficient means of reaching particular goals and simultaneously studying several control factors. Hence, these methods can be used to examine and optimise the operational variables. Orthogonal design is one of the important statistical methods that use the Taguchi parameter design methodology. This method is advantageous because it can optimise various factors simultaneously and substantial quantitative information can be extracted with only a few experimental trials. These methods have been successfully applied to improve the culture medium or culture

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conditions used in the cultivation of exopolysaccharide and mycelium (Chen, Zhang, et al., 2008; Xiao et al., 2004; Meng et al., 2010; Tang & Zhong, 2002).

Antioxidants can delay or prevent oxidation of cellular oxidative substrates. Thus, these compounds are widely used in the fields of food, medicine, and personal care, among others. Although synthetic antioxidants are effective and cheaper, their safety and toxicity have been of great concern (Imaida et al., 1983). As a result, the development and effective use of natural, instead of synthetic, antioxidants is essential. In recent years, polysaccharides have been explored as novel natural antioxidants because they play an important role as free radical scavengers *in vitro* and *in vivo* (Chen, Zhao, Chen, & Li, 2008; Deng et al., 2011; Ke et al., 2009; Li, Liu, Fan, Ai, & Shan, 2011; Xiong, Li, Huang, Lu, & Hou, 2011).

The present work attempts to establish suitable culture conditions for GREP production in submerged culture using the orthogonal matrix method and evaluate the antioxidant activities of GREP for use in food and pharmaceutical industries.

2. Materials and methods

2.1. Chemicals

Butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), methionine (MET), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and riboflavin (RF) were purchased from Sigma Chemicals Co (St. Louis, USA). Unless otherwise stated, all chemicals used were of analytical grade.

2.2. Microorganism

The fresh fruiting bodies of *G. rutilus* were purchased from a local market (Fushun, Liaoning Province, PR China). The strain was isolated from the fruiting body in the laboratory. The strain was maintained on potato dextrose agar (PDA) slant at 4 °C and activated every 2 months.

2.3. Inoculum preparation and liquid culture

G. rutilus was initially grown on PDA medium in a Petri dish for 6–7 d. A 10 mm^2 portion of the agar plate culture was cut with a sterilised cutter and then inoculated into a 500 mL flask containing 100 mL basal medium. The basal liquid medium with pH 6.8 was composed of glucose (20 g/L), peptone (2.0 g/L), K_2HPO_4 (1.0 g/L), KH_2PO_4 (0.5 g/L), and $MgSO_4$ (0.5 g/L).

2.4. Optimisation of culture medium for GREP production

The carbon sources (glucose, maltose, lactose, sucrose, corn flour, and starch) and nitrogen sources [NH_4Cl, $(NH_4)_2SO_4$, soybean meal, beef extract, yeast extract, and bran] were selected and screened. Variable carbon and nitrogen sources were used to replace glucose and peptone in the basic medium, respectively.

A seven factor-two level orthogonal test was applied to optimise the medium composition of inorganic salts for GREP production with the optimal carbon and nitrogen sources. The orthogonal design of inorganic salts with variable sources and levels is presented in Table 1. A seven factor-four level orthogonal test was subsequently applied to optimise the medium composition for GREP production after the determination of the optimal carbon and nitrogen sources as well as inorganic salts. The orthogonal design of the culture medium with variable sources and levels is presented in Table 2. *G. rutilus* was cultured in a 500 mL flask containing 100 mL culture medium in a rotary shaker (200 rpm) at 28 °C for 6 d in all aforementioned experiments.

Table 1
Orthogonal design of inorganic salts optimisation.

Variable (g/L)	Levels	
	1	2
(A) K_2HPO_4	0	1.0
(B) KH_2PO_4	0	0.5
(C) $MgSO_4$	0	0.5
(D) $ZnSO_4$	0	0.01
(E) $MnSO_4$	0	0.01
(F) $FeSO_4$	0	0.01
(G) $CaCl_2$	0	0.01

Table 2
Orthogonal design of culture medium optimisation.

Variable (g/L)	Levels		
	1	2	3
(A) Sucrose	10.0	20.0	30.0
(B) Soybean meal	1.0	2.0	3.0
(C) K_2HPO_4	0.5	1.0	1.5
(D) KH_2PO_4	0.25	0.50	0.75
(E) $MgSO_4$	0.25	0.50	0.75
(F) $ZnSO_4$	0.01	0.02	0.03
(G) $FeSO_4$	0	0.01	0.02

2.5. Cultivation condition optimisation for GREP production

Cultivation time (1, 2, 3, 4, 5, 6, 7, and 8 d), culture medium initial pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0), culture medium loading volume (50, 100, 150, 200, 250, and 300 mL/500 mL) and revolution (140, 160, 180, and 200 rpm) were investigated for GREP production. All trials were conducted in the optimum liquid medium obtained from Section 2.4.

2.6. Measurement of GREP content and dry cell weight (DCW)

The original block of inoculation in the liquid medium was discarded, and the mycelia of *G. rutilus* were obtained by centrifugation (3000 × *g*, 20 min) after cultivation according to the experimental design. DCW was measured after the mycelia precipitate was rinsed with distilled water thrice and dried to constant weight at 60 °C.

The supernate (5 mL) was precipitated by the addition of ethanol to a final concentration of 75% (v/v). The precipitates collected by centrifugation (3000 × *g*, 20 min) were solubilised in deionised water and lyophilised to obtain GREP. GREP content was measured by the phenol–sulphuric acid method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.7. Preparation of GREP for antioxidant activity assay

The filtrates of *G. rutilus* were collected after they were cultured under optimal conditions. The culture filtrate was concentrated five-fold under vacuum and was precipitated by the addition of ethanol to a final concentration of 75% (v/v). The precipitate (GREP) was obtained after centrifugation (3000 × *g*, 20 min) and was applied to detect antioxidant activities *in vitro*.

2.8. Antioxidant activity assay of GREP

2.8.1. Superoxide radical (O_2^-) scavenging assay

Superoxide anion radical scavenging activity was determined according to the method of Stewar and Beewley (1980). The reaction mixture with 3 mL final volume contained the following reagents at final concentrations: 13 mM MET, 10 mM RF, 75 μM NBT, 100 mM EDTA, 50 mM phosphate buffer (pH 7.8), and 5–250 mg/L GREP. After irradiating the reaction mixture with a

fluorescent lamp at 25 °C for 30 min, the absorbance was measured at 560 nm, using BHT as positive control. The inhibition capability of scavenging the superoxide radical was calculated using the following formula:

$$\text{Scavenging rate}\,(\%) = \left[\frac{A_0 - A_1}{A_0}\right] \times 100\%$$

where A_0 is the absorbance of the blank, and A_1 is the absorbance of GREP/BHT.

2.8.2. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was measured according to the method of Winterbourn and Sutton (1984). The reaction mixture contained 1 mL 0.15 M phosphate buffer (pH 7.4), 1 mL 40 μg/mL safranin, 1 mL 0.945 mM EDTA–Fe(II), 1 mL 3% (v/v) H_2O_2, and 0.5 mL 5–250 mg/L GREP. After incubation at 37 °C for 30 min, the absorbance was measured at 560 nm using BHT as a positive control. The EC_{50} value (mg/L) of GREP or BHT is the effective concentration at which the hydroxyl radicals were scavenged by 50%. The hydroxyl radical scavenging activity was expressed as:

$$\text{Scavenging rate}\,(\%) = \left[\frac{A_0 - A_1}{A_0}\right] \times 100\%$$

where A_0 is the absorbance of the blank, and A_1 is the absorbance of GREP/BHT.

2.8.3. DPPH scavenging assay

The scavenging of DPPH radical was performed according to the method of Shimada, Fujikawa, and Yahara (1992) with slight modification. The reaction mixture contained 2 mL DPPH (0.1 μM in 95% ethanol) and 2 mL 5–250 mg/L GREP. The mixture was incubated at 25 °C for 15 min, and the absorbance of the mixture was determined at 517 nm using BHT as the positive control. The EC_{50} value (mg/L) of GREP is the effective concentration at which the DPPH radicals were scavenged by 50%. The antioxidant activity of GREP was evaluated according to the following formula:

$$\text{Scavenging rate}\,(\%) = \left[\frac{1 - A}{A_0}\right] \times 100\%$$

where A is the absorbance of GREP/BHT, and A_0 is the absorbance of the DPPH solution.

2.8.4. Determination of the reducing power of GREP

The reducing power of GREP was evaluated according to the method of Deng et al. (2011). The reaction mixtures contained 2.5 mL phosphate buffer (pH 6.6, 0.2 M), 2.5 mL potassium ferricyanide (1%, w/v), and GREP (5–250 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 1200 × *g* for 10 min. An aliquot of 2.5 mL supernate was collected and mixed with 2.5 mL deionised water and 0.5 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.

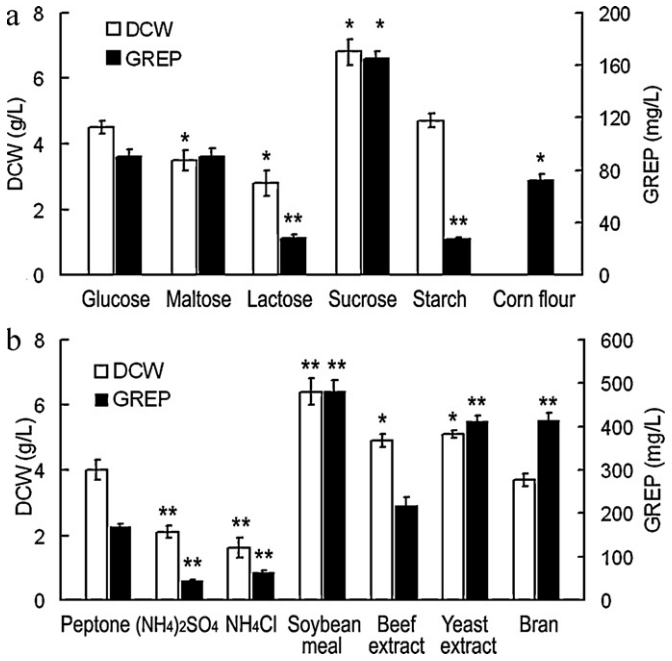

Fig. 1. Effects of carbon sources (a) and nitrogen sources (b) on GREP content and DCW (*P < 0.05; **P < 0.01).

3. Results and discussion

3.1. Medium optimisation for GREP production

As shown in Fig. 1a, the maximal GREP production (164.6 ± 5.4 mg/L) was obtained in the presence of sucrose as the carbon source in the medium, whereas maximal DCW was 6.8 ± 0.4 g/L. The GREP content obtained from the medium containing maltose had no significant difference with that from the basic medium containing glucose. However, the DCW was significantly lower. The GREP content and DCW from the medium containing lactose and starch were remarkably lower than those from the other media. The medium containing corn flour was not able to

Table 3
Results of orthogonal experiments for inorganic salts optimisation.

No.	(A) K_2HPO_4	(B) KH_2PO_4	(C) $MgSO_4$	(D) $CaCl_2$	(E) $ZnSO_4$	(F) $FeSO_4$	(G) $MnSO_4$	GREP (mg/L)	DCW (g/L)
1	1	1	1	1	1	1	1	172.7	2.4
2	1	1	1	2	2	2	2	169.9	2.7
3	1	2	2	1	1	2	2	115.2	4.4
4	1	2	2	2	2	1	1	319.8	5.7
5	2	1	2	1	2	1	2	362.5	6.8
6	2	1	2	2	1	2	1	304.8	3.6
7	2	2	1	1	2	2	1	510.4	2.9
8	2	2	1	2	1	1	2	173.8	3.8
k_1	194.4	252.5	256.7	290.2	191.6	257.2	326.9		
k_2	337.9	279.8	275.6	242.0	340.6	275.1	205.4		
R^a	143.5	27.3	18.9	−48.2	149.0	17.9	−121.5		
k'_1	3.8	3.9	2.9	4.1	3.5	4.7	3.6		
k'_2	4.3	4.2	5.1	3.9	4.5	3.4	4.4		
R'^b	0.5	0.3	2.2	−0.2	1.0	−1.3	0.8		

[a] GREP.
[b] DCW.

harvest mycelium because the corn flour was suspended in the fermenting liquor, which influenced the harvest of mycelium.

The Fig. 1b showed the GREP content (482.2 ± 23.4 mg/L) and DCW (6.4 ± 0.4 g/L) were highest in the medium containing soybean meal as the nitrogen source. The DCW and GREP content from the medium containing $(NH_4)_2SO_4$ and NH_4Cl were remarkably lower than those from the other media. Organic nitrogen sources are generally better than inorganic nitrogen sources for GREP production in submerged culture of mushrooms (Confortin et al., 2008; De Baets, Du Laing, Francois, & Vandamme, 2002).

Considering that sucrose and soybean meal are the best carbon and nitrogen sources, they were selected and applied to optimise the medium composition. Considering that metal ions also have a great effect on the GREP production, a seven-factor-two-level orthogonal test for metal ions was designed and performed. The results are described in Table 3. According to Table 3, the negative action of Ca^{2+} and Mn^{2+} on GREP production was obvious in the present culture medium, and the mycelium growth was inhibited by Ca^{2+} and Fe^{2+}. The aim of the present study is to optimise the liquid culture conditions of GREP, such that Ca^{2+} and Mn^{2+} are abnegated. Although Fe^{2+} has negative effects on the mycelium growth, this ion also has positive effect on GREP production. Aside from K_2HPO_4, KH_2PO_4, $MgSO_4$, and $ZnSO_4$, $FeSO_4$ was chosen for the next optimisation step.

The carbon sources, nitrogen sources, and metal ions were chosen for the optimisation of culture medium composition. An orthogonal design was used for further investigation (Table 2). The results and analysis are shown in Table 4. The *R*-value in Table 4 shows that the effect of these variables was reduced in the order of B > G > D > C > E > A > F. Accordingly, soybean meal is a remarkable factor and should be controlled in a high level. On the other hand, the *R*-value of soybean meal is also a remarkable factor for DCW production. The optimised culture medium was chosen as

Table 5
Effects of culture conditions on DMW and GREP.

Cultivation condition	DMW (g/L)	GREP (mg/L)
Culture time (*d*)		
1	1.7 ± 0.2**	126.5 ± 6.0**
2	2.7 ± 0.3**	147.8 ± 7.4**
3	4.1 ± 0.2**	184.3 ± 9.2**
4	4.4 ± 0.2**	212.0 ± 11.7**
5	5.0 ± 0.3*	377.7 ± 13.3**
6	5.9 ± 0.2	515.5 ± 16.9
7	5.9 ± 0.2	491.0 ± 11.2
8	5.8 ± 0.3	471.5 ± 12.0
Initial pH		
5.5	4.0 ± 0.4**	91.3 ± 7.9**
6.0	4.4 ± 0.4**	133.6 ± 9.8**
6.5	5.3 ± 0.2**	154.1 ± 13.3**
7.0	5.7 ± 0.1**	261.4 ± 16.9**
7.5	6.0 ± 0.4	378.0 ± 21.3*
8.0	6.2 ± 0.2	515.5 ± 27.0
8.5	6.1 ± 0.2	378.2 ± 21.2*
9.0	5.7 ± 0.1**	298.1 ± 16.9**
Temperature (°*C*)		
25	5.7 ± 0.2	512.3 ± 23.6
28	6.1 ± 0.3	504.4 ± 32.1
30	6.2 ± 0.2	517.3 ± 24.9
Volume of medium (*mL/500 mL*)		
50	5.5 ± 0.3**	445.6 ± 34.2
100	6.0 ± 0.4	499.8 ± 43.4
150	6.1 ± 0.3	485.4 ± 34.3
200	5.3 ± 0.4**	375.5 ± 27.5*
250	5.5 ± 0.4**	362.8 ± 36.1**
300	5.3 ± 0.2**	287.4 ± 15.0**
Rotary speed (*rpm*)		
140	5.4 ± 0.2**	359.9 ± 21.8*
160	6.0 ± 0.1**	373.9 ± 17.1*
180	6.6 ± 0.1	496.3 ± 21.8
200	6.3 ± 0.1*	460.8 ± 14.3

* $P < 0.05$.
** $P < 0.01$.

Table 4
Results of orthogonal experiments for culture medium optimisation.

No.	Variables							GREP (mg/L)	DCW (g/L)
	A	B	C	D	E	F	G		
1	1	1	1	1	1	1	1	150.2	1.8
2	1	2	2	2	2	2	2	143.3	5.0
3	1	3	3	3	3	3	3	184.7	5.4
4	2	1	1	2	2	3	3	97.3	1.7
5	2	2	2	3	3	1	1	167.3	3.6
6	2	3	3	1	1	2	2	433.2	7.9
7	3	1	2	1	3	2	3	98.3	2.3
8	3	2	3	2	1	3	1	276.7	5.3
9	3	3	1	3	2	1	2	387.3	6.1
10	1	1	3	3	2	2	1	134.4	1.6
11	1	2	1	1	3	3	2	276.4	5.2
12	1	3	2	2	1	1	3	154.9	5.1
13	2	1	2	3	1	3	2	104.2	1.8
14	2	2	3	1	2	1	3	235.7	4.1
15	2	3	1	2	3	2	1	145.6	7.9
16	3	1	3	2	3	1	2	97.3	2.4
17	3	2	1	3	1	2	3	105.3	4.4
18	3	3	2	1	2	3	1	278.9	5.1
k_1	174.0	113.6	193.7	245.5	204.1	198.8	192.2	$\sum$ = 3471	$\sum'$ = 76.7
k_2	197.2	200.8	157.8	152.5	212.8	176.7	240.3		
k_3	207.3	264.1	227.0	180.6	161.6	203.0	146.0		
R[a]	33.3	150.5	69.2	93.0	51.2	26.3	94.3		
k'_1	4.0	1.9	4.5	4.4	4.4	3.8	4.2		
k'_2	4.5	4.6	3.8	4.6	3.9	4.8	4.7		
k'_3	4.3	6.2	4.4	3.8	4.4	4.1	3.8		
R'[b]	0.5	4.3	0.7	0.8	0.5	1.0	0.9		

[a] GREP.
[b] DCW.

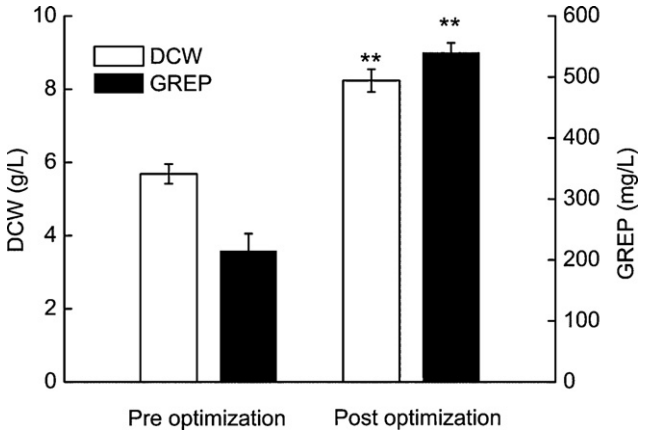

Fig. 2. Comparison of pre and post optimisation of GREP content and DCW.

$A_3B_3C_3D_1E_1F_3G_2$. The optimal medium composition was obtained as follows: 30 g/L sucrose, 3.0 g/L soybean meal, 1.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.25 g/L $MgSO_4$, 0.03 g/L $ZnSO_4$, and 0.01 g/L $FeSO_4$.

3.2. Cultivation condition optimisation for GREP production

The effects of cultivation conditions on GREP production and DCW are shown in Table 5. Results show that with prolonged culture time, GREP content remarkably increased, reaching its maximum (515.5 ± 16.9 mg/L) in 6 d. A higher DCW was simultaneously obtained. When the cultivation time was extended after 6 d, both the DCW and GREP did not increase remarkably. *G. rutilus* grew at initial pH 5–9, and the GREP content reached its maximum at initial pH 8.0, whereas more DCW was harvested at initial pH 7.5–8.5 (Table 5). The initial pH had a significant influence on the mycelial biomass and GREP content because the medium pH affects cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis (Shu & Lung, 2004). The effect of culture temperature on the DCW and GREP content had no statistical significance ($P > 0.05$). The culture temperature was chosen as 25 °C because the lower the temperature, the more energy would be saved. The volume of liquid medium had a significant effect on the DCW and GREP content. Higher DCW and GREP production were obtained in the 500 mL Erlenmeyer flask containing 100–150 mL liquid medium. The DCW increased gradually with the rotary speed at 100–180 rpm, and GREP content increased at 180–200 rpm. The cultivation conditions were determined as follows: temperature, 25 °C; cultivation time, 6 d; initial pH, 8.0; volume of medium, 150 mL; and rotary speed, 180 rpm.

3.3. Comparison of culture medium and conditions

G. rutilus was cultured under initial and optimal conditions. The DCW and GREP content are shown in Fig. 2. The GREP content and DCW in initial conditions were 214.9 ± 28.4 mg/L and 5.7 ± 0.3 g/mL, respectively, compared with 540.1 ± 15.9 mg/L and 8.2 ± 0.3 g/L, respectively, under optimised conditions. GREP content under optimised conditions was 2.5 times than that under the basic culture medium and initial conditions.

3.4. Antioxidant activity

Antioxidant activities have been attributed to various reactions and mechanisms, such as radical scavenging, reductive capacity, prevention of chain initiation, and binding of transition metal ion catalysts, among others (Frankel & Meyer, 2000). In the present experiment, the antioxidant activities of GREP *in vitro* were evaluated using different biochemical methods of superoxide anion,

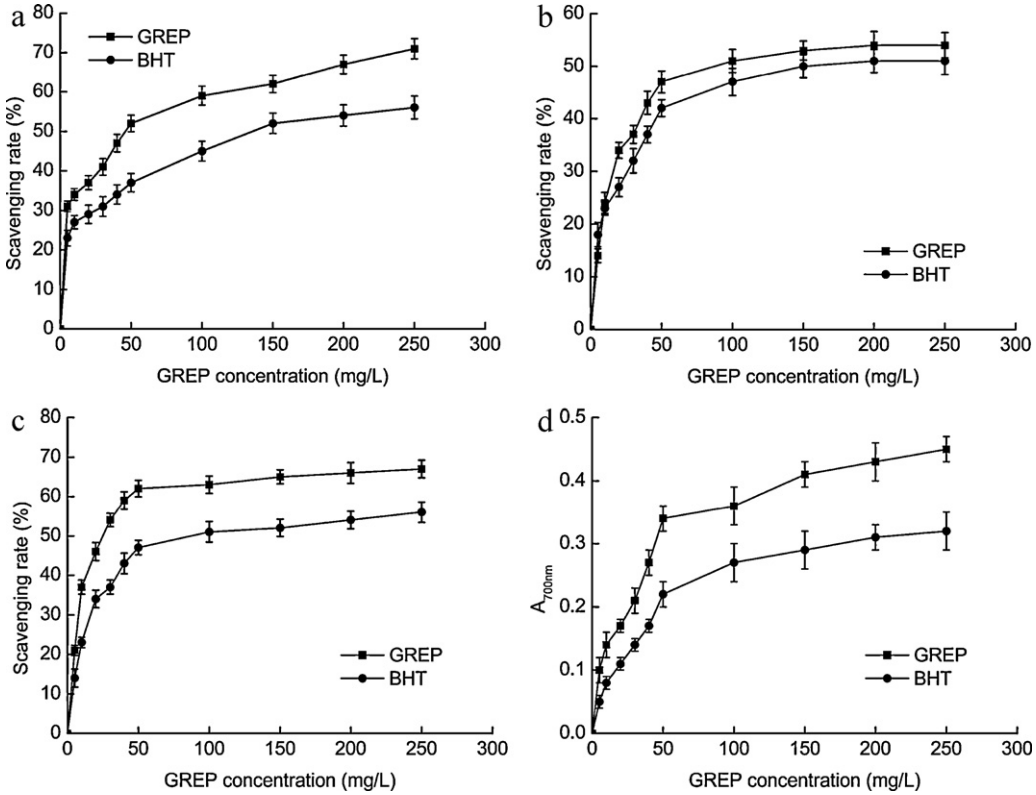

Fig. 3. Antioxidant activity assay of GREP and BHT. (a) Scavenging effects on superoxide anion radicals. (b) Scavenging effects on hydroxyl radicals. (c) Scavenging effects on DPPH. (d) Reducing power of GREP and BHT.

reducing power analysis, and hydroxyl, as well as DPPH, radical scavenging assay.

Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals. Therefore, superoxide anion indirectly initiates lipid peroxidation. The presence of a superoxide anion can magnify cellular damage because it produces other free radicals and oxidizing agents (Athukorala, Kim, Jeon, 2006). The results of superoxide radical scavenging assay are described in Fig. 3a. The rates of GREP and BHT to scavenge superoxide radical are directly proportional to their concentrations. The inhibition percentage of GREP was remarkably higher than that of BHT at the test dosage range. The EC_{50} value of GREP was 47.6 ± 1.6 mg/L, approximately 34% that of BHT (142.2 ± 3.1 mg/L). The EC_{50} value of GREP obtained indicates that GREP significantly affects the scavenging of superoxide anion radical.

Hydroxyl radicals can easily cross cell membranes, readily react with most biomolecules (including carbohydrates, proteins, lipids, and DNA in cells), and cause tissue damage or cell death. Thus, removing hydroxyl radicals is important for the protection of living systems (Cheng, Ren, Li, Chang, & Chen, 2002). As shown in Fig. 3b, the hydroxyl radical scavenging activity of GREP was concentration-dependent and slightly higher than that of BHT at the test dosage range. The EC_{50} value of GREP for hydroxyl radical scavenging activity was 88.2 ± 3.8 mg/L, which had significant difference from the scavenging effect of BHT (149.4 ± 3.5 mg/L).

The DPPH free radical is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant. Thus, the DPPH free radical has been widely accepted as a tool for evaluating the free radical scavenging activities of natural compounds (Leong & Shui, 2002). The scavenging effects of GREP on the DPPH radical were measured and are shown in Fig. 3c. The scavenging activity of GREP on the inhibition of the DPPH radical was related to the concentration at the test dosage range. Furthermore, the DPPH scavenging activities of GREP significantly increased with increasing concentration and were higher than that of BHT at every concentration point. The EC_{50} value of GREP was 25.7 ± 1.9 mg/L, significantly higher than 96.7 ± 2.2 mg/L.

The reducing capacity of a compound serves as a significant indicator of its potential antioxidant activity (Kallithraka, Bakker, & Clifford, 2001). The reducing power of GREP and BHT were investigated. Fig. 3d shows that the reducing capacity of GREP was remarkably higher than that of BHT. The results indicate that GREP has potential antioxidant activities.

4. Conclusion

In the present study, the liquid submerged fermentation conditions for GREP production was optimised utilising a series of experimental designs. No reports are currently available in the literature regarding the optimisation of exopolysaccharide production by *G. rutilus* in submerged culture and its antioxidant activities *in vitro*. The results of the present study provide references for large-scale production of GREP as potentially functional food or antioxidant. However, the biological activities and antioxidant mechanism of GREP are areas for future studies.

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