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Medium Optimization for the Production of Antioxidants from *Aspergillus candidus*

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ABSTRACT

The objective of this study was to optimize the factors for the production of antioxidant from *Aspergillus candidus* CCRC 31543. Extracts of broth filtrate had higher antioxidant activity (inhibition of peroxidation [IP] >98%) when sucrose or lactose was used as a carbon source. Sucrose in the medium also resulted in a higher yield of extracts. Ethyl acetate extracts had the highest yield and antioxidant activity compared with the other two solvents. For the production of antioxidant, inorganic nitrogen sources were found to be more suitable than organic nitrogen sources, and ammonium sulfate was better than sodium nitrate. Yeast extract had a strong influence on the yield of antioxidant extracts. Both mycelium and broth filtrate of *A. candidus* CCRC 31543 showed similar antioxidant activity (IP = 95%), and they also had similar extraction yields.

In food processing, storage, and transportation, oxidation of lipid causes the nutritive value and quality of food to deteriorate. It has been reported that certain diseases are related to toxic substances produced by lipid oxidation *(2, 22).* Antioxidants are used to protect food quality by preventing oxidation of its lipids. The most widely used synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene, may be a safety concern *(17).* α-Tocopherol, a natural antioxidant, is an effective antioxidant for lipid-containing foods but has limited use *(14).* Therefore, both the development and utilization of more effective antioxidants of natural origin are desired.

It has been suggested that microorganisms can produce antioxidant metabolites. For example, 2,3-dihydroxy benzoic acid is derived from the broth of *Penicillium roquefortti* IFO 5956 *(4),* and *N*-(4,6-dihydroxy-2,3,5-trimethylbenzoyl)-glycine is derived from *Mortierella* sp. USF-406 *(5).* Extracts from *Aspergillus* spp. (*A. oryzae, A. sojae,* and *A. niger*) culture broth also exhibited various antioxidant activities *(3, 15).* Esaki et al. *(3)* reported an antioxidant, hydroxy benzoic acid, isolated from the extracts of *A. saitoi* cultured on soybean medium. An antioxidant produced by *Rhizopus oligosporus* in fermented soybean (tempeh) was identified by Hoppe et al. *(6).*

Ishigawa *(7)* demonstrated that *Pencillium* and *Aspergillus* could produce antioxidants, and this was confirmed by Yen and Lee *(23).* In a study by Yen and Lee, *A. candidus* CCRC 31543 was found to have the strongest antioxidant activity from 10 molds. The antioxidant activity of ethyl acetate extracts from *A. candidus* (EAEAC) broth filtrate has been investigated. However, the optimum cultural conditions, especially the carbon and nitrogen sources of the medium, for production of antioxidant from *A. candidus* CCRC 31543 have not yet been studied. This article reports the effects of carbon sources and nitrogen sources in cultural medium on the antioxidant activity of *A. candidus* CCRC 31543. Furthermore, extracts from both mycelium and broth filtrate were compared to verify the origin of antioxidant activity.

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MATERIALS AND METHODS

Microorganism. Lyophilized culture of *A. candidus* CCRC 31543 was obtained from the Culture Collection and Research Center (CCRC) (Food Industry and Development Institute, Hsinchu, Taiwan, Republic of China). The stock culture was grown on potato dextrose agar and maintained at 25°C.

Medium and chemicals. The standard medium used for production of antioxidants in this study consisted of 3% sucrose, 0.1% yeast extract, 0.1% polypeptone, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl and 0.001% $FeSO_4 \cdot 7H_2O$ at pH 7.0 *(23).* Different carbon sources and nitrogen sources were used in the medium to assess their effects on the production of antioxidants from the culture. The carbon sources assessed included sucrose, maltose, glucose, and lactose. The nitrogen sources used included soybean protein isolate, casein, polypeptone, hydrolyzed soybean protein, sodium nitrate ($NaNO_3$), and ammonium sulfate [$(NH_4)_2SO_4$]. The experimental design using different nitrogen sources in the culture medium is shown in Table 1. All the reagents used in the medium were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Difco Laboratories (Detroit, Mich.).

Culture conditions. The culture was performed according to the method of Yen and Lee *(23).* From the slant culture, *A. candidus* CCRC 31543 was inoculated into a 500-ml Hilton flask containing 100 ml of medium. The flask was incubated at 25°C with shaking at 130 rpm for 12 days on a orbital shaker (Model S302A, Firstek orbital shaker, Taiwan) .

Preparation of test samples. The culture filtrate was separated from the mycelium by filtration through Whatman no. 1 filter paper under vacuum and mixed with equal volumes of different solvents. The solvents ethyl acetate, *n*-hexane, and ethyl ether

TABLE 1. *Experimental design for the culture media of different nitrogen sources*[a]

Nitrogen sources	No. of culture medium (%)								
	N1	N2	N3	N4	N5	N6	N7	N8	N9
Yeast extract	0.1		0.1	0.1	0.1	0.1	0.1		0.1
Polypeptone	0.1	0.1		0.1			0.1		
Ammonium sulfate					0.3		0.3		
Sodium nitrate	0.3	0.3	0.3			0.3		0.3	0.3
Soybean protein hydrolyzate						0.1			
Casein									0.1
Soybean protein isolate								0.1	

[a] In each experiment, sucrose (3%) was used as a carbon source.

were used individually for extraction. The mixtures were shaken in a separator funnel for 15 min, and the phase-separated organic solvent layer was collected. The solvent extracts were passed through Whatman no. 1 filter paper and dehydrated using anhydrous sodium sulfate (1%, wt/vol). The solvent was removed in vacuo using a rotary evaporator below 30°C. The yield of each prepared extract was calculated and expressed as a percentage (wt/vol) based on the original broth volume.

Antioxidant activity determination. The antioxidant activities of different solvent extracts from broth filtrate of *A. candidus* CCRC 31543 were determined using the thiocyanate method *(10).* Briefly, each sample (1 mg) redissolved in the original extractant (0.5 ml) of an extract was mixed with linoleic acid emulsion (2.5 ml, 0.02 M, pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0) in a test tube and incubated at 37°C. At regular intervals, the extent of oxidation was determined using the thiocyanate method. A 0.1-ml aliquot of this solution was mixed with 4.7 ml of ethanol (75%), 0.1 ml of ammonium thiocyanate (30%) , and 0.1 ml of ferrous chloride (20 mM in 3.5% HCl) added sequentially. After stirring for 3 min, the absorbance of the mixture was measured at 500 nm with a spectrophotometer (Hitachi U-2000, Japan). The peroxide value was expressed as the absorbance at 500 nm. A high absorbance at 500 nm indicated low antioxidant activity.

The antioxidant activity was also expressed as a percentage of the IP = [1 − (absorbance of the sample at 500 nm)/(absorbance of the control at 500 nm)] × 100. The antioxidant activities of the samples were compared with those of controls composed of BHA and α-tocopherol at the same concentrations.

Comparison of antioxidant activity of broth filtrate and mycelium. The antioxidant activity of broth filtrate and mycelium from *A. candidus* CCRC 31543 was compared. *A. candidus* CCRC 31543 was cultured in forty 500-ml Hilton flasks, each flask containing 100 ml of broth with sucrose and ammonium sulfate as carbon and nitrogen sources, at 25°C with shaking at 130 rpm for 12 days. The culture broth and mycelium were suspended by means of filtration through Whatman filter no. 1 paper under vacuum. Both broth filtrate and mycelium were extracted with ethyl acetate. The antioxidant activity of ethyl acetate extracts prepared from the broth filtrate and mycelium was determined using the thiocyanate method described above.

Statistical analysis. Statistical analysis were performed using SAS software *(16).* Analyses of variance were performed by ANOVA procedures. Significant differences ($P < 0.05$) between means were determined by Duncan's multiple range tests. All experiments were run in three replicates and averaged.

TABLE 2. *Antioxidative activity of various solvent extracts from broth filtrate of* Aspergillus candidus *CCRC 31543 cultured with different carbon sources*

Sample	Absorbance at 500 mm	Inhibition of peroxidation (%)[a]
Control		
Ethyl acetate	2.52 ± 1.06[b]	—
n-Hexane	2.14 ± 0.72	—
Ether	2.21 ± 0.08	—
Sucrose		
Ethyl acetate	0.13 ± 0.01	94.2 ± 1.9 A
n-Hexane	0.18 ± 0.01	91.0 ± 3.3 A
Ether	0.16 ± 0.02	93.3 ± 1.3 A
Lactose		
Ethyl acetate	0.14 ± 0.01	94.1 ± 3.3 A
n-Hexane	0.19 ± 0.01	90.5 ± 3.6 A
Ether	0.15 ± 0.01	93.7 ± 1.0 A
BHA	0.14 ± 0.02	90.8 ± 2.3 A
α-Tocopherol	0.61 ± 0.02	74.9 ± 9.1 B

[a] Inhibition of peroxidation values were calculated as follows: [1 − (absorbance of sample at 500 nm)/(absorbance of control at 500 nm)] × 100. A high inhibition of peroxidation value indicates high antioxidative activity.

[b] All values are the mean and standard deviation of three replicate analyses. Data bearing different letters in the same column are significantly different ($P < 0.05$).

RESULTS AND DISCUSSION

Antioxidant from different solvent extractions. Broth filtrates from sucrose and lactose media were extracted using the solvents *n*-hexane, ethyl acetate, and ethyl ether to optimize the extraction of antioxidant substances. The antioxidant activity (IP) and yields of extracts with different solvents are shown in Table 2 and Figure 1, respectively. All six samples had an IP value of above 90% at a concentration of 200 ppm (Table 2). No significant differences ($P > 0.05$) on antioxidative activity were found among these samples. As shown in Figure 1, culture medium with sucrose produced a higher yield than lactose, which was in agreement with the results of the above experiment. The yields of EAEAC CCRC 31543 broth filtrate from both sucrose and lactose in the medium were in the order of ethyl acetate > ethyl ether > *n*-hexane. It was found that ethyl acetate was the most suitable extractant.

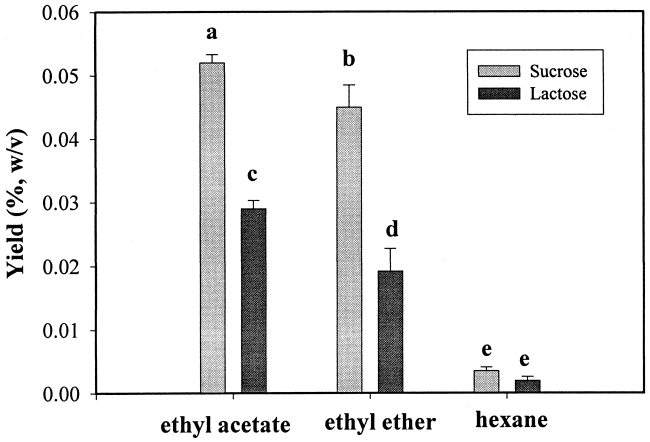

FIGURE 1. *Yields of various solvent extracts from broth filtrate of* A. candidus *CCRC 31543 cultured with different carbon sources. (Yields are percentage of extracts from 500-ml broth). Values in each column with different letters are significantly different (*P *< 0.05).*

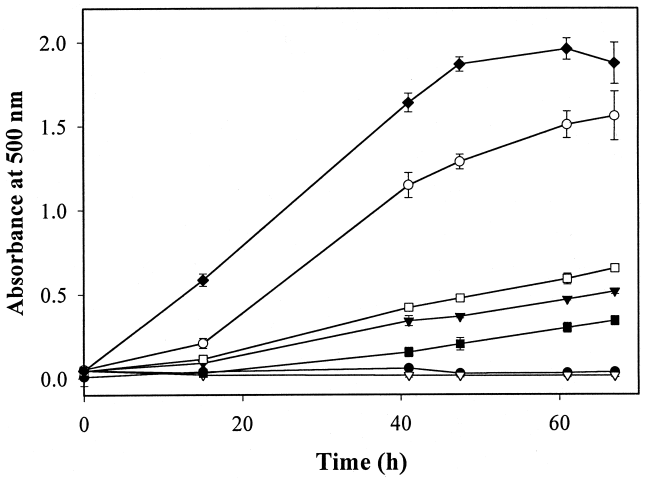

FIGURE 2. *Effects of carbon sources in culture medium on the antioxidative activity of EAEAC CCRC 31543 broth filtrate as determined by thiocyanate method. A high absorbance at 500 nm indicates a low antioxidant activity. Sucrose (●), maltose (○), glucose (▼), lactose (▽), BHA (■), α-tocopherol (□), and control (◆).*

Ethyl acetate has been widely used as solvent for extraction of antioxidant from various sources, including 2,3-dihydroxy benzoic acid from *P. roquefortii (4),* indophenol-reducing phenol from *Moretierella* sp. *(5),* and antioxidant substances from *A. candidus (23).*

Effect of carbon sources on the production of antioxidant substances. Figure 2 shows the the antioxidant activity of EAEAC CCRC 31543 broth filtrate with different carbon sources after extraction. The results indicated that EAEAC broth filtrate from cultured medium with maltose exhibited the weakest antioxidant activity, whereas EAEAC broth filtrate from sucrose and lactose in the medium exhibited marked antioxidant activity. Moreover, the antioxidant activity of EAEAC broth filtrate from both sucrose and lactose in the medium was equal to that of BHA at the same concentration (200 ppm).

As shown in Table 3, the IP of EAEAC broth filtrate from medium with sucrose and lactose as carbon sources was significantly ($P < 0.05$) higher than with other carbon sources such as glucose and maltose. The IP of EAEAC broth filtrate from both sucrose and lactose in the medium was close to that of BHA and higher than that of α-tocopherol. Among the four carbon sources, the EAEAC broth filtrate from medium with maltose exhibited the lowest IP, indicating that maltose was not a suitable carbon source for the production of antioxidant.

The yields of EAEAC broth filtrate from different carbon sources are also shown in Table 3. Three carbon sources, sucrose, maltose and glucose, produced almost the same yields of EAEAC broth filtrate, which were higher than that for lactose. Sucrose, with an IP of 98.4% and the highest yield of 0.079%, was the most suitable carbon source for antioxidant production. Nihei et al. *(12)* reported that target products could be obtained from a culture with different carbon sources for various applications. Sucrose has been used as a carbon source for microbial antioxidant production *(4, 23).* The carbon sources may influence the type of glycolipid formed by a microorganism *(19).* Makkar and

TABLE 3. *Antioxidative activity and yields of the ethyl acetate extracts from broth filtrate of* Aspergillus candidus *CCRC 31543 cultured with different carbon sources*

Sample	Absorbance at 500 nm	Inhibition of peroxidation (%)[a]	Yield percentage (w/v)[b]
Sucrose	0.029 ± 0.010[c]	98.4 ± 0.6 A	0.079 ± 0.010 A
Maltose	1.508 ± 0.080	18.6 ± 4.5 D	0.007 ± 0.002 B
Glucose	0.467 ± 0.007	74.7 ± 2.6 C	0.012 ± 0.004 B
Lactose	0.013 ± 0.009	99.3 ± 0.4 A	0.010 ± 0.003 B
Control	0.298 ± 0.030	—	—
BHA	0.589 ± 0.032	95.2 ± 1.5 A	—
α-Tocopherol	1.859 ± 0.187	79.3 ± 2.6 B	—

[a] Inhibition of peroxidation values were calculated as follows: [1 − (absorbance of sample at 500 nm)/(absorbance of control at 500 nm)] × 100. A high inhibition of peroxidation value indicates high antioxidative activity.

[b] Yields are the percentage of extract from 500 ml of culture broth extracted with the same volume solvent.

[c] All values are the mean and standard deviation of three replicate analyses. Data bearing different letters in the same column are significantly different ($P < 0.05$).

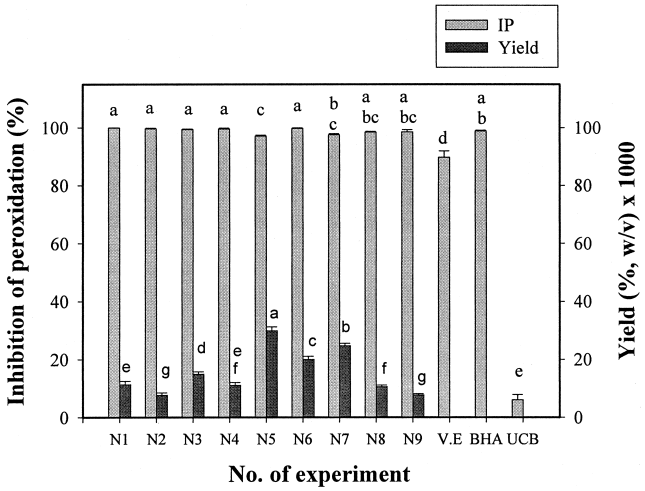

FIGURE 3. *The yield and antioxidative activity of the EAEAC CCRC 31543 broth filtrate cultured with various nitrogen sources. (N1 through N9 are the same as in Table 1; V.E. is α-tocopherol; UCB is uncultured broth). Each value is the mean ± SD of three replicates. Values in each column with different letters are significantly different (*P < 0.05*).*

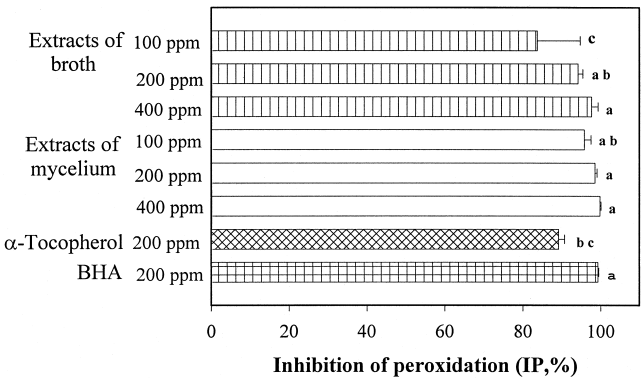

FIGURE 4. *Comparison of the antioxidative activity of the EAEAC CCRC 31543 broth filtrate and mycelia. Each value is the mean ± SD of three replicates. Values in each column with different letters are significantly different (*P < 0.05*).*

Cameotra *(9)* obtained the maximal yield of biosurfactant from medium with sucrose as a carbon source.

Effect of nitrogen sources on the production of antioxidant substances. Nitrogen sources were assigned to nine sets of experiments, and 3% of sucrose was used as carbon source as shown in Table 1. The extract yields and antioxidant activities of EAEAC broth filtrate from each group are shown in Figure 3. The yield of EAEAC broth filtrate from each group ranged from 0.06 to 0.3 mg/ml. The yield from N5 was the highest, and the yields from N2 and N9 were lower. No significant differences ($P < 0.05$) were found in IP for all EAEAC broth filtrate from each group. The IP of each EAEAC broth filtrate was above 95% at a concentration of 200 ppm, which was close to that of BHA but higher than that of α-tocopherol. Therefore, the variation among the groups was determined based on the yields of the extracts. The lowest yield was obtained from the broth filtrate of N2, the medium which lacked yeast extract. Thomas and Ingledew *(21)* reported that yeast extract containing amino acid mixture could stimulate microbial growth and shorten the time of fermentation. Very low antioxidant activity (IP < 10%) was observed in the uncultured medium that contained yeast extract; thus, yeast extract might play an important role in production of antioxidants. The yield of N3 was higher than that of N1, showing that the yield could be increased by adding sodium nitrate instead of polypeptone. The yields of N5 and N7 were higher than those of N1 and N3, indicating that ammonium sulfate was much more beneficial than sodium nitrate. Compared with N1 and N7, the lower yields of N3 and N5 showed that the addition of polypeptone to inorganic nitrogen broth decreased the yield. The highest yield in cultured medium with ammonium sulfate might have resulted because it could produce more glycerol than could other nitrogen sources *(1)*. In summary, inorganic nitrogen sources were better than organic sources, and ammonium sulfate was more suitable than sodium nitrate. In addition, yeast extract was an essential medium component for production of antioxidant substances by *A. candidus.*

Comparison of ethyl acetate extracts from broth filtrate and mycelium. The yield of extracts from both mycelia and broth filtrate expressed as percentage (wt/vol) was compared. The amount of original broth medium was 4000 ml. The yield of extracts (170.3 mg) from wet mycelia was 0.0043%, whereas the yield of extracts (186.9 mg) from broth filtrate was 0.0046%. There was no significant difference ($P > 0.05$) in the yields between broth filtrate and mycelium. The yields are comparable to previous studies. Oeda et al. *(13)* reported that the yield of antioxidant substances from *A. niger* mycelia were 0.00023%. Extracts from 30-liter broth cultured with *Streptomyces* sp. had a yield of 0.011% *(11)*. Moreover, the antioxidant substances of indole and 3,4-dimethoxyphenol from 4-liter cultured broth of *Tapes philippinarum* were only 1.0 to 2.0 mg *(20)*.

The IP of all the samples in various concentrations were above 95% (Figure 4), indicating that both extracts exhibited high antioxidant activity with no significant difference ($P > 0.05$) in their IP. In addition, both extracts' IPs were equal to that of BHA (99.3%) and higher than that of α-tocopherol (89.8%) at 200 ppm. Few studies have been focused on a comparison of mycelium and broth filtrate antioxidants from the same culture.

Other antioxidant substances from either mycelium or broth filtrate have been reported. For example, naphterpin, a free radical scavenger, was extracted from mycelium of *Streptomyces aeriouvifer (18)*. Carazostatin, which exhibits scavengering activity, is also a mycelium extract and has been obtained from *S. chromofucus (8)*, and ansamycin from the broth filtrate of *Streptomyces* sp. USF-319 has been found to show scavenging activity *(11)*. Few studies were concerned with the comparison of yield of antioxidants from mycelium and broth filtrate. In the preliminary study of chemical properties of the antioxidants, broth filtrate extract was separated into four fractions by thin-layer chromatography. Both extracts from broth filtrate and mycelium contained the same four major components according to the high-pressure liquid chromotography analysis.

Thus, the antioxidant activity might be due to a combination of substances. The extract is a brown solid and was observed to be soluble in methanol, acetone, and ethyl acetate but not in hexane. In addition, the extracts redissolved in ethyl acetate exhibited a maximal absorbance at 275 to 280 nm, indicating that antioxidants in extracts contained aromatic compounds. Further studies on the isolation and identification of major antioxidant components are necessary and are currently under way.

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