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Characterization of Antioxidant-Producing *Alteromonas macleodii* HJ-14 Isolated from Seawater

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Abstract A bacterial strain HJ-14 was isolated as a producer of antioxidants from the coast of Jinhae in Korea. The isolate showed 43.4 mol% of G+C content, and contained dihydrogenated ubiquinone with Q8 as a major quinone. Chemotaxonomic analysis as well as phylogenetic analysis, based on the 16S rDNA sequence, identified the isolate as a member of *Alteromonas macleodii*. For antioxidant production, the optimum medium composition was determined to be 3% dextrin, 0.5% ammonium sulfate, and 2–6% sodium chloride. Optimum culture conditions for production of antioxidant materials with strain HJ-14 were at pH 6.0–8.0 and 25–37°C. The chloroform extract of strain HJ-14 broth showed 1.96–17.5-fold higher antioxidant activity than other organic solvents in term of electron donating ability.

Key words: *Alteromonas macleodii* HJ-14, antioxidant, 16S rDNA sequence, phylogeny

Reactive oxygen species (ROS) are a group of active oxygen molecules, which are generated as a consequence of normal metabolic events or exposure to oxidizing agents such as xenobiotics, environmental polluting agent, or ionizing radiation. All aerobic organisms are at risk of being damaged by ROS such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen. Because of their high reactivity, these ROS cause damage to biological macromolecules such as DNA, lipids, and proteins, thus influencing food quality and biological tissues [9]. The defense systems against ROS include the endogenous antioxidant of superoxide dismutase (SOD), catalase, and glutathione peroxidase as well as the exogenous (dietary) antioxidants such as tocopherol, ascorbic acid, and polyphenols. Study of the exogenous natural antioxidant materials from microorganisms has been a hot topic for many research groups [1, 4, 7, 21, 29, 32, 36]. Marine bacteria are also of considerable interest as sources of antioxidative agents [20, 23, 24, 27, 33, 34]. We identified the strain as *Alteromonas* sp. HJ-14 by phenotypic and chemotaxonomic methods. Bacteria belonging to the genus *Alteromonas* are aerobic organisms that are ubiquitous in aqueous environment. They have also been reported to be producers of anticoagulant and alginate lyase [11, 22, 26]. In order to further study this strain, 16S rDNA sequencing and phylogenetic analysis were performed in this study. To this end, we screened microorganisms from seawater samples obtained in Korea by measuring their electron donating ability, and we herein report the antioxidant characteristic of a chloroform crude extract that contained antioxidant substances. Culture conditions required for antioxidant production were optimized, and the characteristics of the antioxidant were investigated.

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Materials and Methods

Screening of Antioxidant-Producing Strains

Strains were isolated from seawater samples collected at Jinhae coast in Gyeongsangnam-do Province, Korea. The strains grown on the synthetic agar were transferred and cultured for 2 days at 25°C on medium composed of 0.25% bactopeptone, 0.01% yeast extract, 0.001% $FeSO_4 \cdot 7H_2O$, 50% seawater, and 1.5% agar (pH 7.4), as described by Tako *et al.* [34]. For the second screening, the isolated single colonies from the first screening were introduced into 10-ml test tubes of modified marine broth (2.5% dextrin and 1% ammonium sulfate added to marine broth) at 25°C for 48 h. For screening of antioxidant-producing strains, each incubated broth (200 μl) was mixed with 1 ml

of 0.041 mM DPPH (1,1-diphenyl-2-picrylhydrazyl) solution in ethanol. The mixture was kept for 10 min after shaking by vortex, and then centrifuged at 12,000 rpm for 3 min at 4°C. Finally, the absorbance of the mixture was measured at 525 nm using a spectrophotometer (Shimazu UV-1601, Japan). The antioxidant activity was expressed as electron donating ability (EDA). The strain HJ-14 showed the most intensive activities, and was used throughout this study as the producer of antioxidant.

Cultivation Conditions

Cultivation was carried out with marine medium [Difco 2216 (pH 7.6)]. For the preculture preparation, a suspension (10^2–10^4) was inoculated into 20 ml of the marine medium in a 100-ml Erlenmyer flask and cultivated at 25°C for 24 h on a reciprocating shaker (120 strokes/min). The main cultivation was performed with 70 ml of the same medium in a 500-ml Erlenmyer flask by inoculating 2.1 ml of the preculture, followed by incubation at 25°C for 2 days on a rotating shaker at 150 rpm. The culture was filtered by suction filtration (Advance Co., No. 20), and the filtrate was used as the source of crude antioxidant.

Morphological and Physiological Characteristics

For morphological characterization, strain HJ-14 was cultivated for 24 h at 30°C on marine medium. The morphology of strain HJ-14 was examined by light microscopy as well as by scanning electron microscopy (Hitachi S-4200, Japan). For SEM observations, cells from early growth and stationary phases were prepared by the method described by Bozzola and Russell [3]. The cultural and physiological characteristics were determined at 30°C as described by Shirling and Gottlieb [30] and Locci [19]. The hydrolysis of starch and tyrosine was tested by the method of Cowan and Steel [5]. The hydrolysis of esculin was determined according to the method of Kurup and Fink [15], catalase activity was determined by bubble formation in a 3% hydrogen peroxide solution, oxidase activity was determined by the oxidation of 1% *p*-aminodimethylaniline oxalate, and nitrate reduction was examined as described by Lanyi [16]. The utilization of various substrates as sole carbon sources was determined as described by Shirling and Gottlieb [30], at the concentration of 1.0% (w/v). Assays for lipase, amylase, and gelatinase activities were performed as described by Skerman [31]. Color was determined by comparison with color chips from the ISCC-NBS color charts Standard sample No. 2106 [12].

Chemotaxonomic Characterization

The strain HJ-14 for the analyses of ubiquinone and fatty acid were obtained after 24 h of growth at 30°C on marine agar (Difco). A whole cell sample was prepared and analyzed as described by Becker *et al.* [2] and Kim *et al.* [13]. Isomers of diaminopimelic acid (DAP) in the cell wall peptidoglycan was determined by TLC (Merck; No. 5716) after hydrolysis of cell wall amino acids with 6 N HCl at 100°C for 18 h [14]. Ubiquinone was analyzed by the procedures described by Yamada and Kondo [38], using reverse-phase HPLC. Whole-cell fatty acids were extracted [17], converted to methyl esters by anhydrous methanolic HCl, and analyzed according to the instructions of the Microbial Identification System (MIDI; Microbial ID).

Isolation of DNA and Determination of the G+C Content

Chromosomal DNA of strain HJ-14 was extracted after growth for 24 h at 30°C in liquid marine medium on a reciprocating shaker at 150 rpm, and DNA was purified according to the method previously described [39]. The concentration and quality of the DNA preparation were determined by spectroscopic measurement and agarose gel electrophoresis, respectively. After enzymatic hydrolysis and dephosphorylation, the G+C content of the genomic DNA was determined by reverse-phase HPLC according to the method of Tamaoka and Komagata [35], using an equimolar mixture of nucleosides (Yamasa Shoyu Co., Ltd., Chiba, Japan) as the standard.

16S rDNA Sequencing

16S rDNA was sequenced, as described previously [18], with reverse primer 704R (5'-TCTRCGNATTTCACCNCTAC-3'; positions 704 to 685 in *E. coli* 16S rRNA numbering). In some cases, the sequencing reactions were performed with dITP from a DNA sequencing kit (Amersham, U.S.A.), or with the SequiTherm EX-CEL II DNA sequencing kit (Epicentre Technologies, U.S.A.), in order to relieve compression artifacts.

Phylogenetic Analysis and Nucleotide Sequence Accession Numbers

The 16S rDNA sequence of strain HJ-14 was aligned with the 16S rRNA/16S rDNA sequences of *Alteromonas* species and other related reference strains by using CLUSTAL W (ver 1.7) software [37]. The 16S rDNA similarity values were calculated from the alignment. Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. The evolutionary distances matrices were calculated using the algorithm of Jukes and Cantor [11] with the DNADIST program within the PHYLIP package [8]. A phylogenetic tree was constructed using the neighbor-joining method [28] from a distance matrix calculated by CLUSTAL W. The stability of the relationship was assessed by a bootstrap analysis of 1,000 data sets by using the programs SEQBOOT, DNADIST, NEIGHBOR, and DONSENSE of the PHYLIP package. The reference sequences were obtained from the GenBank, EMBL, and DDBJ databases: *C. psychrotropica*

ACAM 179^T (T=Type strain, U85846), *C. rossensis* ACAM 608^T (U14581), *C. maris* JCM 10085^T (AB002630), *C. demingiae* ACAM 459^T (U85845), *C. psychrerythraea* ATCC 27364^T (AF001375), *C. hornerae* ACAM 607^T (U85847), *A. macleodii* IAM 12920^T (X82145), *P. haloplanktis* ATCC 14393^T (X67024), *P. antarctica* NF3^T (X98336), *P. atlantica* IAM 12927^T (X82134), *P. aurantia* ATCC 33046^T (X82135), *P. rubra* ATCC 29570^T (X82147), *P. denitrificans* ATCC 43337^T (X82138), *P. bacteriolytica* IAM 14595^T (D89929), *P. antarctica* DSM 10704^T (Y14697), *F. balearica* PAT^T (X93021), *S. putrefaciens* LMG 26268^T (X81623), and *V. cholerae* CECT 514T (X76337).

Extraction of Antioxidant Substances

The culture broth was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant (250 ml) was extracted three times with an equal volume of chloroform, ethyl acetate, butanol, or hexane for 20 min. Each organic solvent layer (750 ml) collected was evaporated to 10 ml in vacuum and used for further analyses.

Scavenging Effect on DPPH Radicals

The scavenging effect of isolated compounds on the DPPH radical was estimated by slightly modifying the method of Rhee *et al.* [25]. Each sample solution (200 μl) was mixed with 1 ml of 0.041 mM DPPH solution. The mixture was shaken by vortex and kept for 10 min at room temperature. After centrifuging at 12,000 rpm and 4°C for 3 min, the absorbance of the mixture was measured at 525 nm. EDA was calculated as follows: EDA=[1−(absorbance of sample at 532 nm)/(absorbance of control at 532 nm)]×100.

Thiocyanate Method for Antioxidant Activity

The antioxidant activity of inhibiting linoleic acid model was assayed using the thiocyanate method [9]. Each sample solution (200 μl in ethanol) was placed into 800 μl of mixtures that consisted of 200 μl (25 mg/ml in EtOH) of linoleic acid, 100 μl (0.3 g/ml in dH_2O) of NH_4SCN, 100 μl (2.45 mg/ml in 3.5% hydrochloric acid) of ferrous chloride, and 400 μl of 50 mM sodium phosphate buffer (pH 7.0). After shaking, the mixture was kept at 50°C in the dark. Each solution (100 μl) was placed into test tubes together with 3 ml of 70% ethanol, 100 μl of NH_4SCN, and 100 μl of ferrous chloride. The absorbance was measured at 500 nm. For the control, ethanol solution (100 μl) was used instead of sample solution.

Optimum pH and Temperature

For the optimal temperature, antioxidant activity was assayed at temperatures of 20, 25, 30, and 37°C. The pH dependence of the antioxidant activity was measured using 0.1 M Tris-acetate buffer for the pH range of 5.0–10.0. The pH of the reaction mixture was then adjusted to 6.0, and the remaining activity was measured at 30°C for 10 min.

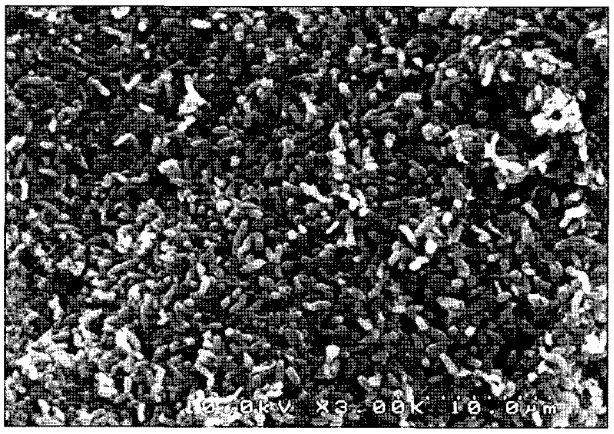
Results and Discussion

Isolation of Strain HJ-14 as a Producer of Antioxidants

In order to obtain a strain that might serve as a new source of antioxidant, more than 60 strains of bacteria were isolated from seawater samples obtained in Korea, and were screened for antioxidant production. Five strains (strains HJ-6, HJ-14, HJ-29, HJ-43, and HJ-57) showed antioxidant activity (data not shown), and the strain HJ-14 was selected, because it exhibited the strongest antioxidant-producing activity among the strains tested, as determined by EDA assay.

Morphological Characteristics of Strain HJ-14

Colonies of the strain HJ-14 were creamy, smooth, and convex on marine agar at 30°C. The strain HJ-14 was found to be Gram-negative, non-spore-forming, and motile. Scanning electron microscopy indicated cells with a smooth surface, straight rod-shaped (Fig. 1), and 0.5–0.6×0.9–1.9 μm in size (Table 1). Morphological properties of the



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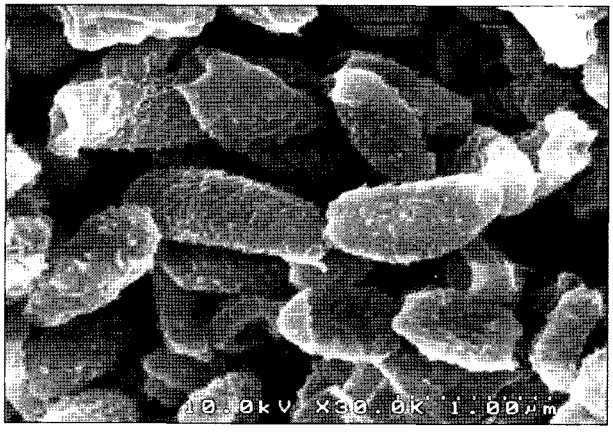

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Fig. 1. Scanning electron micrograph of strain HJ-14 grown on marine agar for 2 days at 30°C.
Bars; 10 μm (**A**) and 1 μm (**B**).

Table 1. Comparison of various characteristics for strain HJ-14 and *A. macleodii* IAM 12920^T.

Characteristics	HJ-14	*A. macleodii* IAM 12920^T	Characteristics	HJ-14	*A. macleodii* IAM 12920^T
Cell			L-Alanine	+	+
Size	0.5–0.19 μm	0.7–3.0 μm	L-Arabinose	–	–
Surface	Spiny, irregular	Spiny, irregular	L-Arginine	–	d
Shape	Straight	Straight	L-Lysine	–	–
Gram reaction	–	–	L-Ornithine	–	–
Mobility	+	+	L-Tartarate	–	–
Pigmentation	–	–	L-Tyrosine	+	+
Na^+ requirement	+	+	Maltose	+	+
Hydrolysis of			*m*-Hydroxybenzoate	–	–
Casein	–	+	*N*-Acetylglucosamine	–	d
Esculin	–	–	Propionate	–	+
Gelatin	+	+	Raffinose	+	+
Lipase	+	+	Salicin	+	+
Starch	+	+	Starch	+	+
			Succinate	–	–
Utilization of			Sucrose	+	+
Acetate	–	+	Nitrate reduced	–	–
Butanol	–	–	to nitrite		
Cellobiose	+	+	Catalase	+	+
Citrate	–	–	Oxidase	+	+
D-Fructose	–	+	Urease	–	–
D-Galactose	+	+	ONPG	+	+
D-Glucose	+	+	KCN	–	–
DL-Malate	–	–	O/F test	Oxidation	Oxidation
D-Mannitol	–	d	Hydrogen sulfide	–	–
D-Mannose	–	–	Growth at		
D-Sorbitol	–	–	4°C	–	–
D-Xylose	–	d	35°C	+	+
Ethanol	+	d	40°C	+	d
Glycerol	+	+	DAP	LL-A_2Pm	LL-A_2Pm
Glycine	–	d	Cell wall	Peptidoglycan	Peptidoglycan
Melibiose	+	+	Ubiquinone	Q8	Q8
			GC content (mol%)	43.4	44–47

All results are from current study. Symbols: +, positive reaction; –, negative reaction; d, 11–89% of strains are positive; T, type strain.

isolated strain HJ-14 were determined according to *Bergey's Manual of Systematic Bacteriology* [6].

Cultural, Physiological, and Chemotaxonomic Characteristics

Strain HJ-14 exhibited poor growth on TCBS (Thiosulfate Citrate Bile Sucrose, Difco Co., U.S.A.) and *Pseudomonas* agar base medium (Oxoid Ltd., U.K.). This strain did not produce any diffusible pigment on any of the media tested. Strain HJ-14 grew optimally at 25–40°C, but did not grow at 4°C and 50°C. In addition, the strain HJ-14 required Na^+ and tolerated about 12% NaCl levels. The phenotypic and chemotaxonomic properties of the strain HJ-14 are shown in Table 1. Production of amylase, gelatinase, and lipase were positive. Of the organic substrates tested, D-galactose, L-tyrosine, sucrose, maltose, cellobiose, melibiose, galactose, salicin, and glycerol were utilized by the strain HJ-14. The strain was found to have a G+C content of 43.4 mol%, *LL-A_2Pm* (*LL-meso*-diaminopimelic acid) as the diamino acid in the cell wall peptidoglycan contained Q-8 as the major ubiquinone (Table 1). The strain HJ-14 contained Sum in feature 3 ($C_{16:1\,\omega}$-7_c/$C_{15:0}$ *iso*-2OH), $C_{16:0}$ and $C_{18:1\,\omega}$-7_c as the major cellular fatty acids, with small amounts of $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:1\,\omega}$-7_c alcohol, Sum in feature 2 ($C_{14:0}$ 3OH/$C_{16:1}$ *iso*-I), $C_{16:0}$ N alcohol, $C_{17:1\,w}8_c$, and $C_{17:0}$ (Table 2). All of these characteristics suggested that the strain HJ-14 was very similar to those of *Alteromonas macleodii* IAM 12920^T (Table 1, Table 2).

Phylogenetic Analysis Based on 16S rDNA Sequences

The 16S rDNA of the strain HJ-14 was amplified by PCR, and 1,488 bp of 16S rDNA (corresponding to nucleotide

Table 2. Cellular fatty acid composition of strain HJ-14 and *A. macleodii* IAM 12920^T.

Fatty acids	Composition (%)	
	Strain HJ-14	*A. macleodii* IAM12920^T
$C_{12:0}$	3.28	2.50
$C_{14:0}$	2.29	2.48
Sum in feature 2	4.11	3.26
$C_{15:0}$	2.99	2.48
Sum in feature 3	22.92	24.64
$C_{16:0}$ N alcohol	2.69	6.64
$C_{16:0}$	19.86	23.82
$C_{16:1}$ ω7c alcohol	3.35	4.33
$C_{17:1}$ ω8c	5.25	4.33
$C_{17:0}$	3.14	2.60
$C_{18:1}$ ω7c	14.33	9.86

Summed feature represents groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Sum in feature 2, $C_{14:0}$ 3OH/$C_{16:1}$ *iso*-I; Sum in feature 3, $C_{16:1}$ ω7c/$C_{15:0}$ *iso*-2OH.

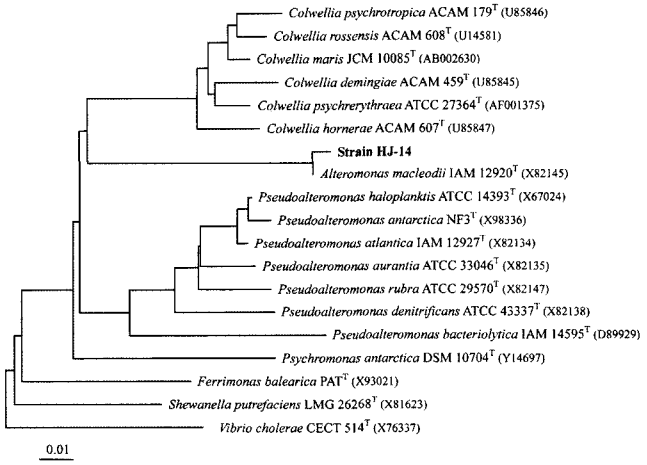

Fig. 2. Phylogenetic tree based on 16S rDNA sequences showing the position of strain HJ-14 and representatives of some other related taxa.
Scale bar represents 0.01 substitution per nucleotide position.

28 and 1,524 of the *E. coli* 16S rRNA gene) were determined. The unrooted phylogenetic tree (Fig. 2) obtained by the neighbor-joining method [28] indicated that the strain HJ-14 is similar to the type strains of the *Alteromonas* species, indicating that strain HJ-14 is a member of the genus *Alteromonas* (Table 3). The strain HJ-14 was found to form a coherent cluster with the type strain of *A. macleodii*, a finding supported by a high bootstrap resampling value of 99.7%. Therefore, the strain HJ-14 was determined to be *Alteromonas macleodii* HJ-14, a taxon that is physiologically, chemotaxonomically, and phylogenetically distinct from the related genera (*Colwellia*, *Pseudoalteromonas*, *Psychromonas*, *Ferrimonas*, *Shewanella*, and *Vibrio*).

Culture Conditions for Antioxidant Production

To investigate the effects of carbon sources on the EDA of the strain HJ-14, various carbon sources (1%, w/v) were added into basal medium (Marine broth). As shown in Table 4, the EDA and cell growth of the strain HJ-14 did not show any significant difference in the presence of mannitol, fructose, xylose, or sorbitol, whereas high levels of antioxidant were produced in the presence of dextrin. Therefore, the optimum dextrin concentration was further investigated.

Table 3. Percentage 16S rDNA/16S rRNA similarity between strain HJ-14 and reference strains used in the phylogenetic analysis.

Species (Taxon)	(%) Sequence similarity																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. Strain HJ-14																		
2. *Alteromonas macleodii* IAM 12920^T	99.7																	
3. *Colwellia psychrotropica* ACAM 179^T	88.4	89.1																
4. *Colwellia rossensis* ACAM 608^T	88.2	88.9	97.7															
5. *Colwellia maris* JCM 10085^T	88.9	89.3	97.7	98.2														
6. *Colwellia demingiae* ACAM 459^T	88.9	89.3	95.5	96.4	96.9													
7. *Colwellia psychrerythraea* ATCC 27364^T	88.9	89.6	96.1	97.7	97.3	97.0												
8. *Colwellia hornerae* ACAM 607^T	88.6	89.2	95.6	96.3	96.9	95.6	96.4											
9. *Pseudoalteromonas haloplanktis* ATCC 14393^T	88.9	89.7	90.0	89.2	90.2	90.5	90.3	90.0										
10. *Pseudoalteromonas antarctica* NF3^T	88.3	88.8	89.2	88.5	89.6	89.5	89.4	89.5	99.0									
11. *Pseudoalteromonas atlantica* IAM 12927^T	89.1	89.7	90.2	89.4	90.2	90.4	90.3	90.3	99.3	98.6								
12. *Pseudoalteromonas aurantia* ATCC 33046^T	88.7	89.4	89.8	88.5	89.8	90.0	90.1	89.7	96.9	96.5	96.8							
13. *Pseudoalteromonas rubra* ATCC 29570^T	89.9	90.1	89.2	88.3	89.2	89.6	89.5	89.4	96.2	95.4	96.7	96.2						
14. *Pseudoalteromonas denitrificans* ATCC 43337^T	87.3	88.1	89.4	89.5	90.0	90.0	90.9	89.9	94.7	94.4	95.1	95.2	94.3					
15. *Pseudoalteromonas bacteriolytica* IAM 14595^T	87.1	87.3	87.9	88.3	87.8	88.9	88.8	88.2	91.1	90.6	91.3	90.0	90.4	90.3				
16. *Psychromonas antarctica* DSM 10704^T	87.5	87.8	89.0	88.3	89.2	88.7	88.7	88.8	89.6	89.8	89.5	89.4	89.2	88.8	88.2			
17. *Ferrimonas balearica* PAT^T	87.8	87.9	89.3	88.2	89.2	88.9	88.9	88.8	88.8	88.9	89.4	88.9	89.2	88.2	86.5	89.0		
18. *Shewanella putrefaciens* LMG 26268^T	87.7	88.0	90.3	89.3	89.1	90.2	89.2	89.2	89.3	89.4	89.7	89.7	88.8	88.5	87.8	88.2	91.7	
19. *Vibrio cholerae* CECT 514^T	87.0	87.2	88.2	87.7	87.8	88.0	88.1	88.1	88.5	88.2	88.5	88.6	87.9	87.0	87.5	87.9	87.0	87.5

Table 4. Effects of carbon and nitrogen sources and sodium chloride concentrations on the electron donating activity (EDA) of strain HJ-14.

Sources	Components	Cell growth ($OD_{660\,nm}$)			EDA (%)[a]		
		12 h	24 h	48 h	12 h	24 h	48 h
Carbon[b]	Control	3.62	4.46	2.80	47.6	28.2	22.2
(1%, w/v)	Mannitol	2.83	4.52	2.48	48.0	29.9	21.1
	Sucrose	3.71	7.40	5.47	53.0	52.1	42.4
	Lactose	3.94	6.63	4.40	51.2	35.2	43.5
	Fructose	2.73	4.63	3.78	43.0	19.9	19.5
	Glucose	3.23	6.06	4.63	48.9	44.9	43.7
	Xylose	2.42	4.40	1.92	48.5	34.6	32.4
	Soluble starch	3.39	5.40	4.04	51.7	41.4	43.0
	Sorbitol	2.32	4.46	2.09	46.7	33.1	27.3
	Dextrin	4.19	8.72	4.61	56.1	55.0	45.5
Inorganic nitrogen[c]	Control	6.05	10.1	12.1	76.0	79.0	80.0
(0.5%, w/v)	NH_4Cl	4.68	9.65	10.0	89.3	90.2	90.4
	NH_4NO_3	5.02	8.45	12.4	84.4	83.2	84.2
	$(NH_4)_2SO_4$	5.12	9.45	13.2	91.1	91.4	92.1
	KNO_3	4.68	8.12	13.0	81.1	80.7	82.5
	$(NH_4)_2HPO_4$	7.09	7.95	13.3	89.0	82.2	82.0
	$NaNO_3$	7.02	10.4	12.3	76.1	75.1	79.2
Sodium chloride	2	5.08	8.75	12.4	88.3	87.6	90.0
concentrations[d]	4	3.65	7.27	9.28	85.3	86.3	86.3
(%, w/v)	6	3.42	6.14	7.99	83.7	82.1	80.0
	8	2.60	5.28	7.95	74.4	82.2	80.8
	10	1.26	4.16	7.78	44.3	72.4	74.2

[a]EDA (%)=[1−(absorbance of sample at 525 nm)/(absorbance of control at 525 nm)]×100. Each basal medium is [b]Marine broth (Difco Co., U.S.A.), [c]Marine broth/2.5% dextrin, and [d]Marine broth/2.5% dextrin/$(NH_4)_2SO_4$. All cultures were carried out at 25°C, pH 7.0.

The EDA of more than 2.5% dextrin concentration was higher than that of dextrin concentration below 2% and was continuously maintained during 48 h. Based on the above results, the optimum carbon source for obtaining antioxidant production by the strain HJ-14 was determined to be 3% dextrin (Table 5). To investigate the effects of nitrogen sources on the EDA, various nitrogen sources (0.5%, w/v) were added respectively into basal medium containing 3% dextrin. The result showed that only ammonium chloride, ammonium sulfate, and ammonium phosphate produced the highest levels of antioxidant (EDA), whereas the other sources gave poor yields (Table 4). In addition, the effects of sodium chloride concentrations were examined at 2, 4, 6, 8, and 10%. Among these concentrations, 2% showed the highest growth and EDA. There was no growth and EDA at 10% sodium chloride during 12 h of culture, but cell growth and EDA increased after 12 h (Table 4). Therefore, the strain HJ-14 appears to be resistant to high sodium chloride concentration.

Table 5. Effects of dextrin concentration on the electron donating activity (EDA) of strain HJ-14.

Conc. (%)	Cell growth ($OD_{660\,nm}$)			EDA (%)		
	12 h	24 h	48 h	12 h	24 h	48 h
0	3.62	4.46	2.80	47.6	28.2	22.2
0.5	4.51	6.48	4.02	50.0	48.0	42.1
1.0	4.19	8.72	4.61	56.1	55.0	45.5
1.5	6.24	8.98	8.24	62.1	63.8	60.1
2.0	6.48	8.56	10.0	70.8	72.3	68.5
2.5	6.08	8.37	9.79	73.1	77.0	72.7
3.0	5.89	9.24	9.57	70.6	79.5	73.0
4.0	6.12	7.87	8.64	72.5	72.1	71.9
5.0	5.79	7.95	8.46	71.2	73.1	74.2

Optimal pH and Temperature

The optimal pH for cell growth and EDA at 25°C was determined to be at pH 5.0–10.0 (Table 6). Although each

Table 6. Effects of optimal pH on the electron donating activity (EDA) of strain HJ-14.

Various pH	Cell growth ($OD_{660\,nm}$)			EDA (%)		
	12 h	24 h	48 h	12 h	24 h	48 h
5	0.34	0.26	5.69	0.84	0.68	90.0
6	6.62	8.65	11.7	90.1	89.7	90.3
7	6.15	8.45	11.9	90.8	90.1	91.0
8	8.12	11.4	12.0	90.3	88.8	90.0
9	5.12	8.71	11.8	8.05	85.0	89.3
10	3.22	5.21	5.04	0.08	0.04	0.16

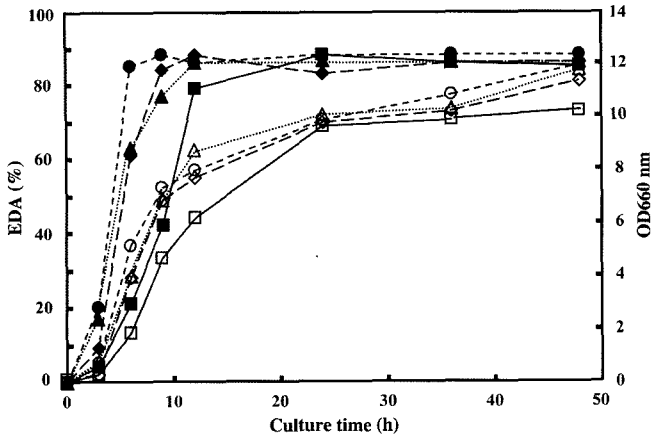

Fig. 3. Effects of culture temperature at antioxidant production.
■: EDA at 20°C; ◆: EDA at 25°C; ▲: EDA at 30°C; ●: EDA at 37°C; □: cell growth at 20°C; ◇: cell growth at 25°C; △: cell growth at 30°C; and ○: cell growth at 37°C.

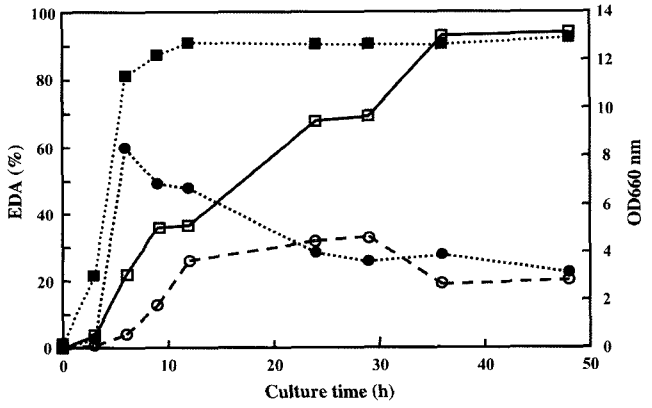

Fig. 4. Comparison of cell growth and EDA (%) on marine and optimized broth.
■: EDA in optimized broth; ●: EDA in marine broth; □: cell growth in optimized broth; and ○: cell growth in marine broth.

EDA of pH 5.0 and 9.0 was inhibited during the early culture period (12 h), the EDA increased with the increase of culture period. However, the EDA and cell growth at pH 10.0 were very poor, compared with those at pH 6.0–8.0.

The optimum temperature of the EDA and cell growth was similar at all temperatures except 20°C (Fig. 3). As shown in Fig. 4, the optimized medium showed 1.36-fold increase of EDA, compared with basal medium (marine broth).

Table 7. Electron donating ability (EDA) of various organic solvent fractions of broth of strain HJ-14.

Fractions	Total volume (ml)	EDA (%)
Butanol	10	38.4±0.18
Chloroform	10	75.1±2.14
Ethyl acetate	10	22.3±1.41
Hexane	10	4.3±1.30

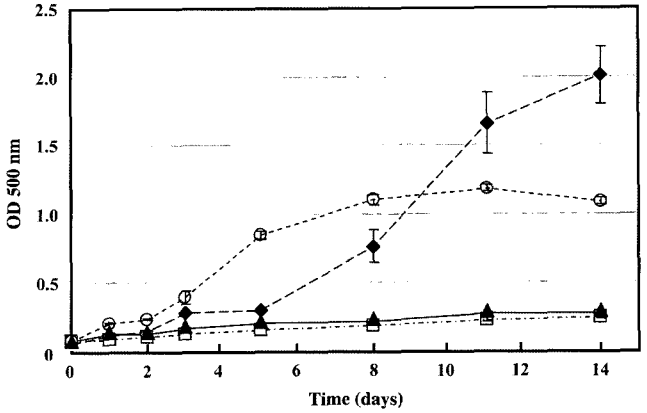

Fig. 5. Antioxidative activities measured by the thiocyanate method (linoleic acid model).
◆: Control (Ethanol); ○: chloroform extract (1%, w/v); ▲: butylated hydroxyanisole (1%, w/v); and □: α-tocopherol (1%, w/v).

Electron Donating Ability of Organic Solvent Extracts

To separate the antioxidant materials from the strain HJ-14, chloroform, ethyl acetate, butanol, and hexane were used. Each organic solvent extract was obtained by differences in polarity. The chloroform extract showed the highest EDA among the organic solvent extracts (Table 7).

Inhibition of Linoleic Acid Peroxidation

The antioxidant activities of $CHCl_3$ extract, BHA, and α-tocopherol were determined using the thiocyanate method (Fig. 5). The antioxidant activity of chloroform (1%, w/v) extract was always slightly higher than that of BHA (1%, w/v) during the incubation period. In addition, the antioxidant activity of α-tocopherol (1%, w/v) was inferior to the chloroform extract. The remarkable antioxidant activity on linoleic acid shown above indicates that the chloroform extract has potential as a natural antioxidant of unsaturated fatty acid.

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