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RESEARCH ARTICLE

Isolation and Identification of Antioxidant Producing Marine-Source Actinomycetes and Optimal Medium Conditions

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Abstract Actinomycetes strain S-1 containing antioxidant components was isolated from the coast of Jeju Island in Korea and identified to the genus level using 16S ribosomal DNA sequencing and cellular fatty acids analysis as *Nocardiopsis* sp. (S-1). S-1 cultured supernatants showed high DPPH radical scavenging activities and hydroxyl radical scavenging abilities of up to 53%. Nutritional and culture conditions for antioxidant production were optimized. Galactose and yeast extracts were the best carbon and nitrogen sources for growth and antioxidant production. An initial medium acidity of pH 7.6, an incubation temperature of 25°C, a sodium chloride concentration of 2.5%, and an incubation time of 8 days were optimal. The DPPH scavenging activity of culture supernatants was 88% under optimum conditions. The S-1 strain can be useful for the functional fermented food and medicine industries.

Keywords: antioxidant, actinomycetes, *Nocardiopsis*, optimum conditions

Introduction

Antioxidants are usually used as additives in the food industry to prevent lipid peroxidation. Although synthetic antioxidants have been widely applied in food processing, they have been reassessed for possible toxic and carcinogenic components formed during their degradation (1,2). Due to these health concerns, natural antioxidants have been extensively used instead of synthetic ones in recent years (3). Oxidative stress has been implicated both in the physiological process of aging and in many pathological progressions in the central nervous system (4,5). Free radicals are known to take part in lipid peroxidation, which causes food deterioration, aging in organisms, and cancer promotion. Free radicals also exert detrimental effects, including lipid peroxidation of cell membranes, alteration of lipid-protein interactions, enzyme inactivation (6), and DNA breakage (7). Cellular defense mechanisms against oxidative damage include enzymatic conversion of reactive oxygen species (ROS; H_2O_2, O_2^-•, and •OH^-) into less reactive species, chelation of transition metal catalysts, and detoxification of ROS by antioxidants. Thus, application of antioxidants might be an effective therapeutic strategy to cure both physiological and pathological disorders that are initiated by ROS (8). Many synthetic chemicals are found to be strong radical scavengers, but they usually have some severe adverse effects (9). Mainly due to the suspected health risk that can be associated with synthetic antioxidative compounds, recent research has shown that microbial sources can be used for production of natural antioxidants (10,11).

Microorganisms can live in environments of extreme temperature, pH, salinity, and hydropressure. These microorganisms have acquired the ability to survive under such environmental conditions through long-term evolutionary processes and they may possess specific defense mechanisms for survival against oxidative stress in such extreme environments (12,13).

Marine microorganisms, the subject of growing natural product research interest, are now considered to be efficient

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producers of biologically active and/or chemically novel compounds (9). Natural antioxidants are usually more expensive and inferior in effect to natural products. Finding safer, more effective, and low-cost natural antioxidants is highly desirable. A few reports dealing with isolation and function of antioxidative materials of marine microbial origin have been published. Secondary metabolites of microbes from the ocean have been investigated to identify natural products of biological and chemical interest (14).

In this study, bacteria from sea water and marine animals were isolated and identified. Culture supernatant of the selected bacterial exhibited antioxidative effects, providing potential candidates for a natural antioxidant source.

Materials and Methods

Isolation and maintenance Samples for isolation of antioxidant-producing actinomycetes were collected along the coast of Jeju Island of South Korea in June of 2012. Marine algae and marine animals were homogenized using blender (Model 985-370; Biospec, Bartlesville, OK, USA) in sterilized seawater (20 mL) to produce a suspension that was diluted appropriately using sterilized seawater. The diluted suspensions (0.2 mL) were spread on Marine Agar (MA; Difco, Franklin Lakes, NJ, USA) plates and cultured at 25°C for 7 days. The stock cultures were made in MA broth containing 20% (v/v) glycerol and at -20°C, and propagated twice before experimental use.

Isolation of antioxidant-producing actinomycetes All strains were grown on 50 mL of MB broth (Difco) for 10 days at 25°C, then harvested by centrifugation (Micro 17TR; Hanil, Incheon, Korea) at 4°C, 5,200×*g* for 1 min. An amount of 1 mL of supernatant and 2 mL of a freshly prepared 150 μM DPPH solution (in methanol) were mixed. The mixture was incubated at room temperature for 30 min. After standing for 30 min, the absorbance at 525 nm was measured using a UV-VIS spectrophotometer (Opron 3000; Hanson Tech. Co., Seoul, Korea). A control contained MB medium (Difco) instead of the culture supernatant.

Physiological, biochemical, and morphological characteristics Media used were recommended by Shirling and Gottlieb (15) in the International Streptomyces Project (ISP) and by Waksman (16). Mycelia were observed after incubation at 25°C for 7 days. Colors were determined according to Prauser (17). Carbohydrate used was determined by growth on a carbon utilization medium (ISP medium No. 9) (18) supplemented with a 1% carbon source at 25°C. The NaCl concentrationand the pH range for growth were determined on an inorganic salt starch agar medium (ISP medium No. 4) using a temperature gradient incubator (VS-123P3V; Vision, Daejeon, Korea). All culture characteristics were recorded after 2 weeks. Knowledge of the morphological characteristics of mycelia and spores is required to determine the genus of actinomycetes, based on observation of mycelial grown on a tryptone yeast glucose agar medium (ISP medium No. 1), an oatmeal agar medium (ISP medium No. 3), a starch inorganic salt agar medium (ISP medium No. 4), a glycerin asparagine agar medium (ISP medium No. 5), a peptone yeast iron agar medium (ISP medium No. 6), or a tyrosine agar medium (ISP medium No. 7).

16S rDNA analysis In order to identify strains, 16S rDNA analysis was carried out. Strains were incubated in MB medium (Difco) for 8 days and the cultured broth was centrifuged at 10,000×*g* for 15 min (Micro 17TR; Hanil) for extraction of DNA carried out using a Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). DNA amplification was performed using a PCR. The forward and reverse primers were 5'-GGC AGC AGT GGG GAA TAT TG-3' and 5'-TCG TCA GCT CGT GTC GTG AG-3', respectively. The thermal cycle profile commenced with an initial denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 2 min. In the last cycle, the reaction mixture was kept at 72°C for 10 min and cooled to 4°C. The PCR products were purified using a PCR purification Kit (Bioneer).

Cellular fatty acid analysis (CFA) Fatty acid methyl esters were prepared using the method described by Miller and Berger (19). Briefly, bacteria were saponified for 30 min at 100°C in 1 mL of 15% NaOH in 50% aqueous methanol. After cooling, 2 mL of a methylation reagent (325 mL of 6.0 N HCl plus 275 mL of methanol) was added, and the samples were heated at 80°C for 10 min. After cooling, 1.25 mL of a 1:1 (v/v) hexane:ether mixture was added and the samples were mixed using end-to-end rotation for 10 min. The phases were allowed to separate by standing for several minutes, and the aqueous (lower) phase was carefully removed using a pasteur pipette and discarded. Next, 3 mL of base wash (10.8 g of NaOH in 900 mL of distilled water) was added to each sample and mixed using end-to-end rotation for 5 min. The phases were allowed to separate, and the organic (top) phase was removed and placed in autosampler vial for gas chromatograph analysis. Fatty acid methyl ester (FAME) mixtures were analyzed by capillary GC using a Hewlett Packard model 5898A GC (Hewlett Packard, Palo Alto, CA, USA) run using Microbial Identification software (Microbial ID; MIDI, Newark, DE, USA).

Measurement of the growth rate and antioxidant activity The growth rate of strain S-1 was measured

based on the dry weight of mycelia. The antioxidant activity was determined based on the DPPH free radical and hydroxyl radical scavenging activities (20). The absorbance of the supernatant was measured at 525 nm using a UV-VIS spectrophotometer (Opron 3000). The percentage of inhibition was defined as the absorbance at 525 nm in the absence of the supernatant relative to the absorbance measured with a sample. A control sample contained MB medium instead of a culture supernatant. The DPPH radical scavenging ability (%) was defined as:

$$\text{EDA (Electron donating ability)} = [1-(A_{525}(\text{sample})/A_{525}(\text{control}))]\times 100$$

The hydroxyl radical scavenging activity was determined according to a modification of the 2-deoxyribose oxidation method (21). The hydroxyl radical was generated using a Fenton reaction in the presence of $FeSO_4 \cdot 7H_2O$. A reaction mixture containing 0.2 mL of 10 mM $FeSO_4 \cdot 7H_2O$, 10 mM EDTA, and 10 mM 2-deoxyribose was mixed with 0.2 mL of a culture supernatant solution, and a 1 M phosphate buffer (pH 7.4) was added to the reaction mixture until the total volume reached 1.8 mL. Then 0.2 mL of 10 mM H_2O_2 was added to the reaction mixture and incubated at 37°C for 4 h. After incubation, 1 mL of 2.8% trichloroacetic acid (TCA) and 1.0% thiobarbituric acid (TBA) were added. Then, the mixture was placed in a boiling water bath for 10 min. The absorbance was measured at 532 nm and the hydroxyl radical scavenging activity (%) was defined as:

$$\text{HSA (hydroxyl radical scavenging activity)} = [1-(A_{532}(\text{sample})/A_{532}(\text{control}))]\times 100$$

Optimization of nutrient sources and culture conditions To determine the optimal carbon and nitrogen sources for growth and antioxidant production, MB medium (Difco) was used as a base supplemented with different carbon and nitrogen sources to study the effects on growth and antioxidant production. MB medium (Difco) (50 mL in 250 mL Erlenmeyer flask) was inoculated with a 2% (v/v) bacterial suspension grown in MB at 25°C for 24 h and further incubated on a rotary shaker (110 rpm, VS-8480SF; Vision) for 10 days. To determine the nutritional requirements for production of antioxidants, different carbon and nitrogen sources and NaCl were tested. To investigate the effect of carbon sources on production of antioxidants, carbon sources were provided at a concentration of 1% (w/v) instead of carbon sources in the MB medium. The carbon sources tested were mannose, starch, sucrose, xylose, glycerin, fructose, lactose, dextrose, mannitol, maltose, and galactose. Peptone, yeast extract, tryptone, malt extract, $NaNO_3$, KNO_3, NH_4NO_3, NH_4SO_4, and $(NH_4)_2PO_4$ were used as nitrogen sources. NaCl was provided at concentrations of 2 to 7% (v/v). The effects of culture conditions, such as different incubation temperatures (4, 16, 20, 25, 30, 35, and 40°C), initial pH (6, 6.8, 7.6, 8, 8.4, and 9.2), and incubation time (1 to 13 days) on growth and antioxidant production were also studied.

Statistical analysis Data for antioxidative activities are presented as a mean±standard deviation (SD). Data were analyzed using Duncan's multiple range test with SAS software (SAS, Cary, NC, USA). Differences were considered statistically significant at $p<0.05$.

Results and Discussion

Screening and identification of antioxidant-producing actinomycetes Strain S-1 was collected from the sand of Hamdeok Beach in Jeju Island and the bacteriological characteristics of isolated strain S-1 were investigated (Table 1). After incubation for 7 days on MA medium, dirty-white aerial mycelia were observed and identified as a Gram-positive, aerobic bacterium. The optimum growth temperature was 25°C, and the range of NaCl concentration for growth was 0 to 10% (w/v) with an optimum at 2.5%. The optimum growth pH was 7.6. The strain produced acid from xylose, cellobiose, rhamnose, sucrose, galactose, and maltose, so S-1 was considered to be genus *Nocardiopsis* or a closely related genus. S-1 showed moderate or good growth in different media. Vegetative mycelia showed a dark-yellow color and aerial mycelia showed a white color. A dark-yellow diffusible pigment was produced in most of the tested media (data not shown).

The 16S rDNA sequence was determined using BLAST (National Center for Biotechnology Information, Bethesda, MD, USA) to construct a phylogenetic tree (data not shown). An almost complete 16S rDNA sequence of S-1, consisting of 1,446 nucleotides, was compared with all other known 16S rDNA sequences, and a phylogenetic tree was constructed using the neighbor-joining method. The phylogenetic tree indicated that S-1 belongs to the genus *Nocardiopsis*. The 16S rDNA sequence similarities of S-1 to *N. dassonvillei, N. Antarctica,* and *N. synnemataformans* were 99, 99, and 98% homology, respectively. Therefore, S-1 was a presumed member of the genus *Nocardiopsis,* according to 16S rDNA sequence analysis.

CFA composition as an adjunct to identification dependent on species or taxonomic groups is important. The CFAs of S-1 were extracted and analyzed according to the Microbial Identification System (Microbial ID; MIDI). The CFA composition of S-1 is shown in Table 2 The fatty acid pattern of this strain contained iso/anteiso-branched fatty acids. Smaller amounts of 10-methyl-branched and unbranched fatty acids were also found. The high amount of anteiso fatty acids in combination with 10-methyl-

Table 1. Comparison of the physiological and biochemical characteristics of isolated S-1

Character	Strain S-1	Type strains[1)]			
		N. dassonvillei	*N. alborubida*	*N. antarcticus*	*N. listeri*
Growth under anaerobic conditions					
Gram reaction	+	+	+	+	+
Motile spores					
Color of aerial mycelium	White to grayish	White to grayish	Gray	Gray	White on HT agar
Color of substrate mycelium	Dark brown	Yellowish to brownish	Yellow to orange	Dark brown	Yellow
Optimum pH for growth	7-8	7	10	8	8
Optimum NaCl for growth(w/v)	2.5%	ND	ND	ND	ND
Growth on:					
D-Xylose Melezitose	+	+	+	+	+
Cellobiose	ND		+		
Rhamnose	+	+	+		+
Sucrose	+	+	+	+	+
Adonitol	ND		+		
Inositol	ND		+		
D-Galactose	+	V	ND	ND	
Maltose	+		ND	ND	

[1)]Data complied from references 10, 15, 17, and 26. Carbohydrate use was determined by growth on carbon utilization medium (ISP medium No. 9) supplemented with 1% carbon sources at 25ºC. NaCl and pH ranges for growth were determined on an inorganic salt starch agar medium (ISP medium No. 4); +, positive; , negative; ND, no data; V, variable testing results

Table 2. Cellular fatty acid profile of S-1

Fatty acid	Composition (%)
14:0 *ISO*	0.48
15:0 *ISO*	0.57
15:0 *ANTEISO*	3.56
16:0 *ISO*	26.82
16:1 *CIS* 9	1.81
16:0	1.14
17:0 *ISO*	2.07
17:0 *ANTEISO*	23.42
17:1 *CIS*9	5.26
17:0	2.00
17:0 10*METHYL*	1.29
18:0 *ISO*	2.35
18:1 *CIS*9	21.01
18:0	6.69
TBSA 18:0 10 *METHYL*	1.53

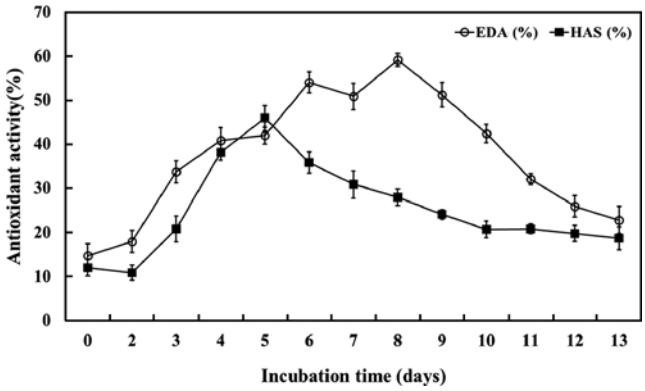

Fig. 1. A time course of fermentation production of antioxidants by strain S-1 cultured in MB medium (pH 7.6) for 13 days at 25ºC, 110 rpm.

branched fatty acids is diagnostic for species of the genus *Nocardiopsis* (22). Based on the phenotype, phylogenetic characterization, and cellular fatty acid analysis, strain S-1 was identified as a member of the genus *Nocardiopsis*.

Antioxidant activity Free radicals are highly reactive molecules or chemical species capable of an independent existence. Generation of highly ROS is an integral feature of normal cellular functions, including the mitochondrial respiratory chain, phagocytosis, arachidonic acid metabolism, evolution, and fertilization (23). ROS production; however, multiplies several times under certain pathological conditions. Release of oxygen free radicals has been also reported during the recovery phase of cerebral tissues from the effects of many pathological noxious stimuli (24). The antioxidant activity of the S-1 culture supernatant was estimated by measuring EDA and HSA levels using the 2-deoxyribose oxidation method (21). The free radical scavenging ability during an incubation period was determined based on changes in absorbance due to reduction of DPPH. The percentage scavenging activity of each supernatant against DPPH is shown in Fig. 1. The DPPH radical scavenging activity (EDA) at day 8 showed the highest antioxidant activity and the maximum HSA level was observed at day 5. The DPPH radical and hydroxyl radical scavenging

activities of the S-1 strain supernatant were 60 and 45%, respectively. These results indicate that S-1 produces antioxidant components that are effective for removing ROS via a non-enzymatic pathway.

Effect of temperature and pH on cell growth and antioxidant production The optimum temperature for growth and production of antioxidants from *Nocardiopsis* S-1 are shown in Fig. 2A. S-1 showed a broad growth temperature range for relatively good growth and antioxidant production. *Nocardiopsis* S-1 was able to grow between 4 to 40°C, and temperatures for reasonable growth were 25 and 30°C. Above 35°C, production of antioxidant material was reduced. The optimum temperature for antioxidant production of *Nocardiopsis* S-1 was 25°C. The organism appeared to be mesophilic in nature.

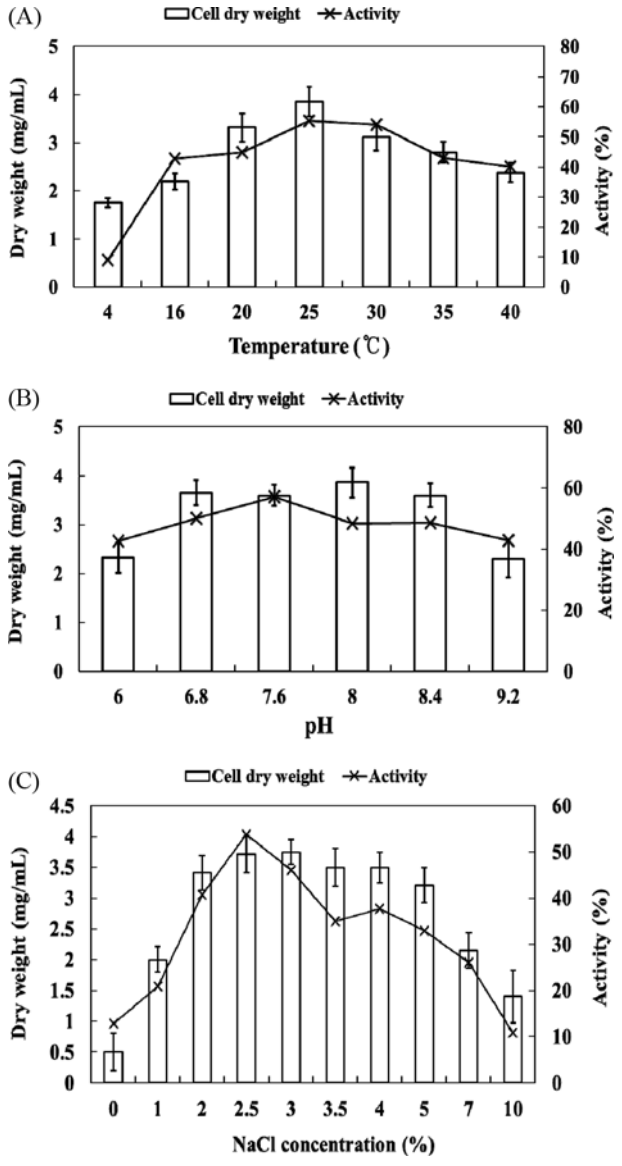

Fig. 2. The effect of (A) temperature, (B) pH, and (C) the NaCl concentration on the antioxidative activity of *Nocardiopsis* S-1. The antioxidative activity was tested using the DPPH method. Each value represents a mean value of 3 independent experiments.

The pH of a medium is important but is often neglected as an environmental factor. Many investigators have claimed that the different morphologies of fungi and bacteria under different initial pH values were the critical factor in biomass accumulation and metabolite formation. The medium pH may affect cell membrane function, cell morphology and structure, uptake of nutrients, and production of biosynthetic components (25). In the present study, *Nocardiopsis* S-1 was able to grow at pH 6 to 9.2 with a high antioxidant activity. The optimum pH for growth and antioxidant production of *Nocardiopsis* S-1 was 7.6 (Fig. 2B).

Effect of the NaCl concentration on cell growth and antioxidant production The effect of the NaCl concentration on growth and antioxidant production of *Nocardiopsis* S-1 is shown in Fig. 2C. *Nocardiopsis* S-1 was able to grow at NaCl concentrations of 0 to 11% (w/v) (data not shown). Antioxidants were produced at all tested NaCl concentrations. The optimum NaCl concentration for *Nocardiopsis* S-1 growth and antioxidant production was 2.5% (w/v). Apparently, this strain originated from a marine environment or adapted to a high salt concentration.

Effect of carbon and nitrogen sources on cell growth and antioxidant production Optimization of antioxidant production was carried out in a flask culture. Mannose, starch, sucrose, xylose, glycerin, fructose, lactose, dextrose, mannitol, maltose, and galactose were used to determine the effect of carbon sources on production of antioxidants. S-1 was able to grow using all the tested carbon sources (Table 3). Among 11 variables tested, a medium supplemented with galactose as the sole carbon source showed maximum growth and antioxidant activity, followed by glycerin and mannitol. Elimination of nitrogen from the growth medium greatly affected bacterial growth and antioxidant production. Results of nitrogen source use are shown in Table 3. *Nocardiopsis* S-1 was able to grow well in a medium containing yeast extract and peptone, but not with KNO_3 and $(NH_4)_2SO_4$. The highest radical scavenging activity was obtained in a medium containing yeast extract as a nitrogen source, followed by cultures containing tryptone, peptone, and malt extract. However, according to another report using *Cordyceps militaris*, a medium containing tryptone provided the highest productivity of antioxidant materials (26). Therefore, it appears that carbon and nitrogen sources for antioxidant production depend on the microorganism used.

Effect of an optimized medium on antioxidant production Antioxidant production was tested using an

Table 3. Effects of different carbon and nitrogen sources on the growth and radical scavenging activities of a *Nocardiopsis* S-1 supernatant

Source[1)]	Compounds	Dry weights (mg/mL)	EDA (%)
Carbon[a] (1%, w/v)	D-Mannose	2.543	53
	Starch	2.684	64
	Sucrose	2.880	35
	Xylose	1.568	64
	Glycerin	3.789	71
	D-fructose	1.952	37
	Lactose	2.639	38
	Dextrose	1.316	49
	Mannitol	3.576	70
	Maltose	3.468	44
	Galactose	4.782	76
Nitrogen[b] (0.5%, w/v)	None	4.452	74
	Peptone	4.832	81
	Yeast extract	5.431	88
	Tryptone	4.994	83
	Malt extract	4.788	79
	$NaNO_3$	4.654	74
	$(NH_4)_2SO_4$	4.453	72
	$(NH_4)HPO_4$	4.887	78
	NH_4NO_3	4.993	77
	KNO_3	4.326	66

[1)]Each basal medium was [a]Marine broth, [b]Marine broth/1.0% galactose. All cultures were carried out at 25, pH 7.6. Each value represents the mean value of 3 independent experiments.

optimized medium and culture conditions. The DPPH radical scavenging activity was the highest (88%) under optimized conditions (Fig. 3). Results indicated a dependence of antioxidant synthesis on medium constituents. In fact, the characteristics of carbon and nitrogen sources strongly affect antibiotic and antioxidant activities in different organisms (11).

Novel antioxidants can be produced and the antioxidative potential of marine actinomycetes has been shown. However, further research is required for identification and purification of antioxidative compounds from cultured bacterial supernatants.

Strain S-1 was identified as *Nocardiopsis* sp. using 16S ribosomal DNA sequence and cellular fatty acids analysis. The S-1 culture supernatant showed high DPPH radical scavenging and hydroxyl radical scavenging activities. Galactose and yeast extracts were found to be the best carbon and nitrogen sources for growth and antioxidant production. An initial medium acidity of pH 7.6, an incubation temperature of 25ºC, a sodium chloride concentration of 2.5%, and an incubation time of 8 days were found to be optimal. The DPPH scavenging activity of culture supernatants was 88% under optimum conditions.

Nocardiopsis sp. can produce a few types of nocardiopsin

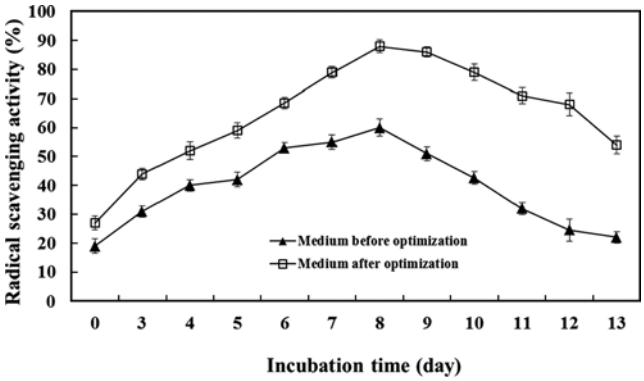

Fig. 3. The effect of optimized media on production of antioxidants by *Nocardiopsis* S-1. The antioxidative activity was tested using the DPPH method. Each value represents a mean value of 3 independent experiments.

with hydroxypipecolic acid in the residue, which is known to have an antioxidative effect (27,28). Therefore, the antioxidative activity of strain S-1 may result from hydroxypipecolic acid. This bacterial strain could be applicable for use in functional fermented food, medicine, and probiotics fields.

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Disclosure The authors declare no conflict of interest.

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