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Antioxidant activity and cultural characterization of metabolites from marine bacteria and fungus

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국문초록

해양은 지구상에 남아있는 마지막 자원의 보고로서, 해양에 서식하고 있는 동식물 등은 식량자원 뿐만 아니라 근래에는 의약산업, 정밀 화학소재, 신 기능물질 등의 고부가가치 소재로서 이용되고 있다. 특히 해양은 지구 생물종의 약 80%가 존재한다는 것을 고려할 때, 잠재적 유전자원으로서의 그 중요성은 매우 크다고 볼 수 있다. 지금까지 천연물로부터의 생리활성물질에 관한 연구는 주로 육상 자원을 대상으로 하여 왔지만, 육상생물로부터 새로운 활성도를 가진 물질을 개발하는 것에 투입된 연구비에 비해 경제성이 점차 떨어지므로 선진국 등에서는 이미 탐색 대상을 육상생태계가 아닌 해양이나 극한 생태계 같은 새로운 환경에 눈을 돌려 연구를 시작하고 있다. 해양은 특이한 생태계를 이루는 환경 때문에 해양 생물이 만드는 대사산물은 육상생물에 비하여 특이한 골격의 화학구조를 가지게 되며 적자 생존의 경쟁속에서 살아남기 위하여 이들 생물들은 다양한 생리활성 물질들을 함유하고 있다. 해양미생물에서 발견된 주목받는 생리활성물질로는 marinostatin, isatin, aplasmomycin, marinactam, istamycin 등이 있는데 이것들은 항생, 항암, 항바이러스 효과가 뛰어나다고 알려져 있으며, 열대의 산호에서 분리된 방선균 *Streptomyces* sp.가 생산하는 octalactin 또한 항암효과가 우수한 물질로 밝혀졌다. 예로부터, 항산화 물질에 대한 연구는 육상식물들을 위주로 활발히 진행되어 왔다. 그러나 이러한 육상식물에서의 연구에 비해 해양 유래의 식물 및 미생물에 대해서도 연구가 미약한 실정이지만, 해조류, 곰팡이, 효모 및 세균등의 일부에서 항산화 물질에 대한 연구가 보고된 바 있다.

따라서 본 연구에서는 천연 생리활성 물질을 개발하기 위한 목적으로 해양 유래의 미생물을 이용하여 *in vitro* 항산화 실험계를 이용하여 항산화능을 가진 균주를 선별 및 동정하였고, 이러한 균주로부터 항산화물질 생성을 위한 최적 조건을 확립하여 (균주배양액에서의) 항산화 활성을 조사함으로써, 해양 미생물 유래 천연 항산화제의 이용 가능성을 조사 하였

다. 그 결과 제주연안의 해수 및 생물시료로부터 분리된 116개의 해양 유래 균주들 중에 항산화 활성이 가장 뛰어난 Bacillus 계열의 그람양성 균주(genus *Exiguobacterium*)와 해양유래 방선균(genus *Nocardiopsis*), 총 2균주를 분리하였으며, 분리균주의 생화학적특성, 배양학적특성, 생리학적특성, 16S rDNA 염기서열 분석 및 세포벽 지방산 분석방법을 이용하여 분석한 결과, 최종적으로 각각 *Exiguobacterium* sp. SC2-1, *Nocardiopsis* sp. S-1으로 동정되었다. 또한 이들 균주에 적합한 최적조건의 배양조건을 맞추어 준 후, 균주의 배양액을 이용하여 세포외로 방출하는 항산화 활성을 측정해 본 결과 일반 합성항산화제와 거의 유사한 활성을 나타내는 것을 확인 할 수가 있었다.

또한, 부가적인 내용으로 총 9종류의 버섯균주 배양 추출액으로부터 생리활성 물질을 탐색 및 천연 배지의 개발 가능성을 확인 할 목적으로 항균활성 및 항산화 실험을 실시하였다. 그 결과 버섯균사체 추출물들이 농도에 의존하여 항균 및 항산화 활성이 높게 나타났으며, 감귤농축액을 천연배지로 이용하였을 경우에 일반 합성배지에서와 유사한 결과를 얻을 수가 있어서 차후 값비싼 합성배지를 대체할 수 있는 천연물로서의 가치를 확인할 수가 있었다. 차후 항균 및 항산화 활성을 나타내는 물질이 어떠한 것인지에 대한 연구와 *in vivo*상에서 독성실험을 거쳐 동물에 어떠한 영향을 주는지에 대한 연구는 계속해 보아야 할 것으로 사료된다.

이러한 결과를 종합해 볼 때, 해양 미생물의 2차 대사산물은 각종 질병을 일으키고 있는 활성 산소종을 효과적으로 억제 시킬 수 있을 뿐만 아니라 대량 배양이 용이 하기 때문에 산업적 용도가 매우 유용할 것으로 판단되며, 미생물 유래의 대사산물의 독성평가가 이루어진다면 기존의 합성 항산화제의 단점인 안전성문제를 해결할 수 있을 것으로 사료된다.

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GENERAL 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 their possible toxic and carcinogenic components formed during their degradation (Maeura *et al.*, 1984; Ito *et al.*, 1985). Due to these health concerns, natural antioxidants have been extensively employed instead of synthetic ones in recent years (Yen *et al.*, 2003). Oxidative stress has been implicated both in the physiological process of aging and in many pathological progression in the central nervous system (CNS) leading usually to some neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Benzi and Moretti, 1995; Finkel and Holbrook, 2000). Free radicals are known to take part in lipid peroxidation, which causes food deterioration, aging in organisms, and cancer promotion. Free radicals are ascertained to exert some detrimental effects, including lipid peroxidation of cell membranes, alteration of lipid-protein interact-ions, enzymes inactivation (Kim *et al.*, 1985) and DNA breakage (Imlay and Linn, 1988). Cellular defense mechanisms against oxidative damage include enzymatic conversion of reactive oxygen species (ROS; e.g., 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 neurodegenerative disorders initiated by ROS (Sun *et al.*, 2004). Though, many synthetic chemicals such as phenolic compounds are found to be strong radical scavengers, they usually have some severe adverse effects (Zhou and Zheng, 1991). Recent research show that microbial sources could be a potential means of producing natural antioxidants (Ishikawa, 1992).

There are microorganisms living in environments of extreme temperature,

pH, salinity, and hydropressure. These microorganisms have apparently acquired the ability to survive under such environmental conditions through long-term evolutionary processes and they possess specific mechanisms for survival in such extreme environments. Screening for natural antioxidants has been mainly done among secondary metabolites of terrestrial plants (Larson, 1988; Pratt and Hudson, 1990; Nakatani, 1990). Our attention has been focused on marine microorganisms, which are known to contain much polyunsaturated fatty acids.

Marine microorganisms, the subject of a growing number of natural product researches, are now considered as efficient producers of biologically active and/or chemically novel compounds (Zhou and Zheng, 1991). Natural antioxidants are usually more expensive and inferior in effect, however finding safer, more effective and low-cost natural antioxidants are highly desirable. A few reports dealing with the isolation and function of antioxidative materials of marine microbial origins could be found to date although secondary metabolites of microbes from ocean have been well investigated to discover a lot of natural products of fascinating biological and chemical interest.

In this paper, we wish to describe a simple screening procedure for antioxidant-producing microbes and isolation and identification of a few antioxidants from marine bacteria isolated from sea water and marine animal.

Part Ⅰ.

Isolation, Identification and Cultural Characterization of Antioxidant-Producing Marine Bacteria Isolated from the Ocean

Part I .

Isolation, identification and cultural characterization of antioxidant-producing marine bacteria isolated from the ocean

1. ABSTRACT

An antioxidant-producing bacterium was isolated from sea water in Jeju island. The isolated strain, SC2-1 was Gram-positive, catalase positive, facultatively anaerobic, oxidase positive, motile and small rods. The strain utilized sucrose, dextrose, fructose, mannitol and maltose as a sole carbon and energy source and sodium chloride required for the bacteria growth. The bacterium was identified based on phenotype characterizations, cellular fatty acids analysis and 16S rDNA sequencing then named *Exiguobacterium* sp. SC2-1. The radical scavenging activity of the culture supernatants was determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) method. It might be explained by stable radical (DPPH) scavenging effect and by weak hydroxyl radical scavenging effect. Additionally, culture supernatant appeared to weak activity of the inhibit superoxide radical scavenging ability. The optimum culture conditions for production of antioxidant were 25℃, pH 7.8 and NaCl concentration were 4%. The modified optimal medium compositions were maltose 2.5% (w/v), yeast extract 1.5% (w/v) and KH_2PO_4 0.05% (w/v). Free radical scavenging activity of under optimal culture conditions were 93%.

2. MATERIALS AND METHODS

2.1 *Strains and culture conditions*

Antioxidant producing bacteria were collected along Jeju Island coast of Korea during a period from August 2003 to October 2003. All strains were grown in MA (Marine Agar, Difco. Co. USA) and MB (Marine Broth, Difco. Co. USA) at 25℃ for 24 h with reciprocal shaking. Bacterial stocks were kept frozen at -20℃ in a solution of 1:4 v/v of glycerol to adequate broth and propagated twice before experimentation.

2.2 *Screening method for antioxidant-producing strains*

Sea water, marine algae and marine animal were homogenized in sterilized sea water (20 ml). Each suspension was diluted with sterilized from 10^{-1} to 10^{-4}. The suspensions from 10^{-4} to 10^{-6} (0.2 ml) were spread on agar plates made from the medium and cultured at 25℃ for a few day. A sterilized filter paper was placed on the agar plate so that colonies and their metabolites were replaced on the agar plate so that was further continued at 25℃ for a few days. Then the filter paper was taken out and sprayed with a 1×10^{-4} DPPH solution (dissolved in EtOH) after drying. Strains showing a white-on-purple spot were regarded as antioxidant-producing strains (Takao *et al.*, 1994).

2.3 Physiological characterization of strain SC2-1

The isolate was identified for its physiological and biochemical properties according to Bergey's Manual of Systematic Bacteriology (Bauman *et al.*, 1984), Manual of identification of Medical Bacteria, and the procedure previously described.

2.4 *16S rDNA analysis*

The nearly complete nucleotide sequence of 16S rDNA was determined as described previously. The primer using Universal primer 27F 5'-AGA TGA TCC TGG CTC AG-3' and 1522R 5'-AAG GAA GTG ATC CAG CCG CA-3' (Bioneer, Korea). The resultant sequence of strain SC2-1 was manually aligned with representatives of the genus *Exiguobacterium* and related taxa using known 16S rDNA secondary structure information. Phylogenetic trees were inferred by using the neighbour-joining method (Fengrong *et al.*, 2003). The resultant unrooted tree topology was evaluated in bootstrap analyses of the neighbour-joining method based on 1000 resamplings.

2.5 Cellular Fatty acid analysis (CFA)

After incubation, sufficient quantities of bacterial growth to produce minimum total-area counts were harvested into a tube (13 by 100 mm) fitted with a Teflon-lined cap; this open amounted to one loopful of bacteria by using a calibrated disposable loop. The fatty acid methyl esters were prepared by using the method described by Miller and Berger (1985). Briefly, the bacteria were saponified for 30 min at 100℃ in 1 ml of 15% sodium hydroxide in 50% aqueous methanol. After cooling, 2 ml of the methylation reagent (325 ml of 6.0 N hydrochloric acid plus 275 ml of methanol) was added, and the samples were heated at 80℃ for 10 min. After cooling, 1.25 ml of a 1:1 (v/v) hexane-ether mixture was added and the samples were mixed by 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 with 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 by end-to-end rotation for 5 min. The phases were allowed to separate, and the organic (top) phase was removed to a Teflon-lined, septum-cap autosampler vial for analysis on the gas chromatograph. Fatty acid

methyl esters (FAME) mixtures were analysed by capillary gas chromatography (GC) using a Hewlett Packard model 5898A GC run by Microbial Identification software (Microbial ID).

2.6 Scavenging effect on DPPH free radical of isolated strain SC2-1

Free radical scavenging activity (Electron donating ability, EDA) of the supernatant of bacterial culture broth was determined by using stable free radical, DPPH, according to the modified method of Blosis (1958). DPPH solution was prepared at the concentration of 150 uM in methanol. During the assay, the cultural broth supernatant of 1 ml was mixed 3.0 ml DPPH solution. The mixture was incubated in the room temperature for 30 min. After standing for 30 min, absorbance at 525 nm, and the percentage of inhibition was defined by the absorbance at 525 nm in the absence of supernatant to that measured with sample. The control sample contained a MB medium instead of sample. The absorbance of the mixture was measured at 525 nm, and DPPH radical scavenging ability (%) was defined as follows : EDA(Electron donating ability) = $[1-(A_{525}(\text{sample})/A_{525}(\text{control}))] \times 100\%$.

2.7 *Hydroxyl radicals scavenging activity*

Hydroxyl radical scavenging activity was determined according to the modified method of the 2-deoxyribose oxidation method (Kawagan, 1996 and Kogukuchi, 1999). Hydroxyl radical was generated by Fenton reaction in the presence of $FeSO_4 \cdot 7H_2O$. A reaction mixture containing each 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 cultural supernatant solution and 1.0 M phosphate buffer (pH 7.4) was added into the reaction mixture until the total volume reached to 1.8 ml. then 0.2 ml of 10 mM H2O2 was finally added to the reaction mixture

and incubated at 37℃ for 4 h. After incubation, each 1 ml of 2.8% TCA (trichloroacetic acid) and 1.0% TBA (thiobarbituric acid) were added. Then, the mixture was placed in a boiling water bath for 10 min. Absorbance was measured at 532 nm.

2.8 Superoxide radical scavenging ability

Superoxide radical scavenging ability was by measuring the inhibition of the auto-oxidation of pyrogallol using a modified method of Marklund and Marklund (Eugene, 1983 and Marklund, 1974). A sample solution (0.1 ml) and 2.61 ml of 50 mM phosphate buffer (pH 8.24) were added into freshly prepared 90 ㎕ of 3 mM pyrogallol (dissolved in 10 mM HCl). The inhibition rate of pyrogallol auto-oxidation was measured at 325 nm. Absorbance of each sample was recorded at every 20 sec interval for 3 min and the increment of absorbance was calculated by the difference (the absorbance at 3 min - the absorbance at the starting time).

2.9 Optimization of nutritional and cultural conditions

To determine the optimal nutritional and cultural conditions for growth and antioxidant production, MB medium was used as the base. It was supplemented with different carbon and nitrogen sources to study their effect on growth and antioxidant production. The medium (50 ml in 250 ml Erlenmeyer flask) was inoculated with 2% (v/v) bacteria suspension and incubated with at 25℃ on a rotary shaker (120 rpm) for 2 days. To investigate the effect of carbon and nitrogen sources on the production of antioxidant, carbon and nitrogen sources were provided at the concentration of 1 and 0.5%, respectively, instead of carbon and nitrogen sources in MB medium. Carbon sources tested were sucrose, xylose, glycerin, fructose,

lactose, dextrose, mannitol and maltose. Peptone, yeast extract, tryptone, malt extract, $NaNO_3$, KNO_3, NH_4NO_3, NH_4SO_4 and $(NH_4)_2PO_4$ were used as the nitrogen sources. NaCl was provided at the concentration of between 0 to 11% (v/v). The effect of cultural conditions like different incubation temperatures (0, 4, 18, 25, 30 and 35℃) and initial pH (4, 5, 6, 7, 8, 9 and 10) on growth and antioxidant production was studied. Antioxidant activity of the strain was determined by using DPPH method.

3. RESULTS AND DISCUSSION

3.1 Screening and identification of the antioxidant-producing bacterium

Guided by the screening method with DPPH, we obtained a hundred sixteen antioxidants producing bacterial isolated from the sea water. The bacteriological characteristics of isolated strain SC2-1 were investigated (Table 1). After incubating for one days on the MA medium under orange, colonies were circular, convex. Strain SC2-1 was gram-positive, rod shaped and aerobic bacterium. Strain was positive for oxidase and catalase. Cell grow at temperatures between 4℃ and 40℃, but not at 50℃. Its optimum growth temperature was 25-30℃. The NaCl concentration for growth was 0 to 11% (w/v) with an optimal growth at 1.5~2% (w/v). The strain was produced acid from fructose, maltose and mannitol. From these results, the strain SC2-1 was considered to being to the *Exigubacterium* genus or related one.

In recent years, the nucleotide sequence comparison of the 16S rDNA sequence has been used as a powerful tool for identifying bacterial species and for determining exact phylogenetic and taxonomic positions similar genera and species, since 16S rDNA is highly conserved in the evolutionary aspects

(Nubel *et al.*, 1996; Yoon *et al.*, 1998). The 16S rDNA sequence is shown in Fig. 1. It was analyzed by using BLAST (National Center for Biotechnology Information). The almost-complete 16S rDNA sequence of SC2-1, which, consists of 1,515 nucleotides, was compared with all other known 16S rDNA sequences, and a phylogenetic tree was constructed using the neighbor-joining method. As a result of its homology search with GenBank databases, the phylogenetic tree constructed from the sequence data showed that the strain SC2-1 appeared within the evolutionary radiation area encompassing the genus *Exiguobacterium* species. The phylogenetic tree indicated that SC2-1 falls into the genus *Exiguobacterium* (Fig. 1.). The 16S rDNA sequence similarities of SC2-1 to *E. undae, E. acetylicum* and *E. oxidotolenum* were 98, 97 and 98% , homology respectively. The phylogenetic analysis clearly established that the strain SC2-1 was a member of the *Exiguobacterium* species and it was therefore named as *Exiguobacterium* sp. SC2-1.

The importance of CFA profiles of as an adjunct to identification was dependent on which species or taxon group was under consideration. CFA was especially useful in discriminating between group which otherwise are nearly identical biochemically by the Hollis and Weaver (1981). The cellular fatty acids (CFA) of the strain SC2-1 were extracted and analyzed according to the instructions of Microbial Identification System (MIDI; Microbial ID). Table 2 shows the cellular fatty acid composition of strain. The strain SC2-1 is consisted of $isoC_{12:0}$ (1.25%), $isoC_{13:0}$ (12.84%), $anteisoC_{13:0}$ (12.22%), $C_{14:0}$ (1.31%), $isoC_{15:0}$ (15.22%), $anteisoC_{15:0}$ (3.21%), $isoC_{16:0}$ (1.23%), $C_{16:1w11c}$ (5.7%), $C_{16:0}$ (12.93%), $isoC_{17:0\ w10c}$, $isoC_{17:0}$ (8.83%), $anteisoC_{17:0}$ (2.36%), $C_{18:3\ w6c}$ (3.52%), $C_{18:1\ w7c}$ (1.29%), $C_{18:0}$ (2.66%). Based on our phenotype, phylogenetic characterization and cellular fatty acid analysis, strain SC2-1 was identified as a member of the genus *Exiguobacterium.*

Table 1-1. Differential phenotypic feature of isolated SC2-1 and *Exiguobacterium oxidotolerans* T-2-2^T

Characteristics	Strain SC2-1	E. *Oxidotolerans* T-2-2^T
Gram reaction	+	+
Cell shape	Rod	Rod
Growth at:		
4℃	+	+
35℃	+	+
40℃	+	+
Mobility	+	+
Pigmentation	Orange	Orange
Maximum NaCl concn tolerated	0~11%	ND
Na+ requirement for growth	+	+
Catalase	+	+
Oxidase	+	-
Methyl red	+	+
Acid produced from:		
D-fructose	+	+
D-maltose	+	+
Mannitol	+	+
L-arabinose	-	-
D-xylose	+/-	-
Sorbitol	-	-
D-galactose	+/-	-

Symbols: +, Positive; -,Negative; +/-, Weak reaction; ND, no data

Fig. 1-1. Phylogenetic tree based on 16S rDNA sequences comparing isolated strain with members of *Exiguobacterium* and related genera. DNA distance were established by using the neighbor-joining method. The scale bar indicates 0.01 substitution per nucleotide position. The numbers at the branch nodes are bootstrap values from 1000 bootstrap trails.

Table 1-2. Cellular fatty acid profile of the strain SC2-1.

Fatty acid	Composition(%)
iso$C_{11:0}$	0.35
iso$C_{12:0}$	1.25
$C_{12:0}$	0.52
iso$C_{13:0}$	12.84
antei so$C_{13:0}$	12.22
iso$C_{14:0}$	0.86
$C_{14:0}$	1.31
Sum in feature 1	1.38
iso$C_{15:0}$	15.22
antei so$C_{15:0}$	3.21
$C_{15:0}$	0.21
$C_{16:1}$ w7c alcohol	0.59
iso$C_{16:1}$ H	0.45
iso$C_{16:0}$	1.23
$C_{16:1}$ w11c	5.70
Sum in feature 3	3.56
$C_{16:1}$ w5c	0.25
$C_{16:0}$	12.93
iso$C_{17:1}$ w10c	5.16
Sum in feature 4	1.45
iso$C_{17:0}$	8.83
antei so$C_{17:0}$	2.36
$C_{17:0}$	0.65
$C_{18:3}$ w6c	3.52
$C_{18:1}$ w7c	1.29
$C_{18:0}$	2.66
Total	**99.99**

Fatty acid methyl esters (FAME) mixtures were analysed by capillary GC using a Hewlett Packard model 5898A GC run by Microbial Identification software (Microbial ID).

Optimum cultural and nutritional conditions for growth and production of antioxidant

1. Effect of temperature

The optimum temperature for cell growth and production of antioxidant from the *Exiguobacterium* sp. SC2-1 were shown in Fig. 1-4. SC2-1 showed a narrow range of incubation temperature for relatively good cell growth rate and antioxidant production. The *Exiguobacterium* sp. SC2-1 was cultivated at various temperatures ranging from 10 to 40℃. Both maximum cell growth and antioxidant were observed at 25℃. The optimum temperature for production of antioxidant of *Exiguobacterium* sp. SC2-1 cultural supernatant was 25℃. The organism appeared to be mesophilic in nature.

2. Effect of pH

The pH of medium is a very important but is often a neglected environmental factor. Many investigators claimed that the different morphology of fungi and bacteria under a different initial pH value was the critical factor in biomass accumulation and metabolite formation (Shu and Lung, 2004; Wang and McNeil, 1995). The medium pH may affect cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis (Gerlach *et al.*, 1998; Shu and Lung, 2004). In the present study, *Exiguobacterium* sp. SC2-1 was able to grow at pH 4 to 10 and also showed the antioxidant activity at the same pH range. The optimum pH for growth and production of antioxidant of *Exiguobacterium* sp. SC2-1 cultural supernatant was 7~8 (Fig. 1-5).

3. Effect of NaCl concentration

The effect of Nacl concentration on growth and production of antioxidant

of *Exiguobacterium* sp. SC2-1 cultural supernatant were shown in Fig. 1-6. *Exiguobacterium* sp. SC2-1 was able to grow at 0 to 11% (w/v) of NaCl concentration. The production of antioxidant was little different by NaCl concentration. The optimum NaCl concentration for growth and production of antioxidant fo *Exiguobacterium* sp. SC2-1 was 4% (w/v). These results suggest that this strain was originated from marine environment or adapted itself to marine environment.

4. Effect of various carbon sources on production of antioxidant

It is generally understood that cell of many bacteria grow to some extend over a wide range of carbon source (Yang *et al.*, 2003). To find out the suitable carbon source for the antioxidant production and cell grown in *Exiguobacterium* sp. SC2-1, eight carbon sources were separately provided at 10 g/l for 3 days in basal medium (Fig. 1-7.). *Exiguobacterium* sp. SC2-1 was able to very well in medium containing mannitol, xylose, sucrose, lactose, maltose and glycerin, but not fructose and dextrose. Radical scavenging activity of cultural supernatant was measured by DPPH method. Antioxidant was produced well in medium containing maltose, but not sucrose and xylose. The results showed that, as to antioxidant production, maltose was the optimal carbon sources. Fructose had similar positive effects, while sucrose and xylose played badly. Similarly, Kim et al. (2005) reported that the production of exopolysaccharides by *Agrocybe cylindracea* was increased by maltose. The data of some authors indicated that the effect of carbon sources significantly depends on its concentration in the nutrition medium (Galhaup *et al.* 2002). In this study, increased the extracellular antioxidative activity of Exigubacterium sp. SC2-1 more than 1.4-fold (Table 1-3) presumably by increasing the content of fermentable carbohydrates. It is possible that different carbon sources might have different effects of catabolic repression on the

cellular secondary metabolism. Such as phenomenon was also claimed in submerged cultivation of many kinds of mushrooms (Hwang *et al.*, 2003; Kim *et al.*, 2003).

5. *Effect of various nitrogen sources on production of antioxidant*

Many previous studies have proved that both the nature and concentration of nitrogen sources are powerful nutrition factors regulating antioxidant production by microorganism fermentation process. Nitrogen may be supplied as ammonia, nitrate or in organic compounds, such as amino acids and proteins. Therefore, the omission of nitrogen in the medium greatly affects bacteria growth and metabolite production. To investigate the effect of nitrogen sources on cell growth and antioxidant production, cells were cultivated in the medium containing various nitrogen sources, where each nitrogen source was added to the basal medium at a concentration level of 0.5 g/l. The results of nitrogen source utilization were shown in Table 1-4. *Exiguobacterium* sp. SC2-1 was able to grow very well in medium containing yeast extract and tryptone, but not $NaNO_3$ and $(NH_4)_2SO_4$. The highest antioxidant production was obtained in culture of *Exiguobacterium*. sp. SC2-1 containing yeast extract as a nitrogen source, followed by cultures containing tryptone and peptone. When inorganic nitrogen source were used, very poor antioxidative activities were achieved. And much higher activities were obtained as to the organic nitrogen sources. Tryptone worked worse than the other three organic nitrogen sources. It reported that reduced nitrogen (as found in ammonium ions, amino groups of amide groups) is the form utilized in biosynthesis (Fisher, 1991), which may be the reason for the bad performance of ammonium sulfate.

The result also showed that the concentration of yeast extract (Fig. 1-8) greatly influenced the production of the antioxidant with maximum

antioxidant yield being obtained in cultures supplemented with 1.5% (w/v) of yeast extract. At this yeast extract concentration, the antioxidant activity was The antioxidant was about 1.2 fold increased. The results showed that antioxidant activity was higher in yeast extract-grown cell. Similarly, Shin *et al.* (2000) reported that the production of antibiotic by *Streptomyces* sp. NS 13239 was increased by yeast extract.

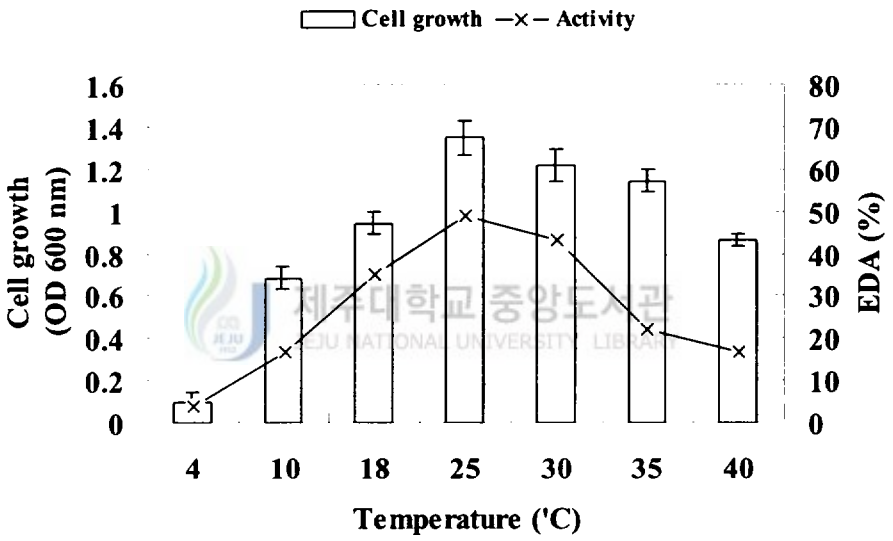

Fig. 1-2. Effect of temperature on the producing antioxidative activity by strain SC2-1. The antioxidative activity was tested DPPH method.

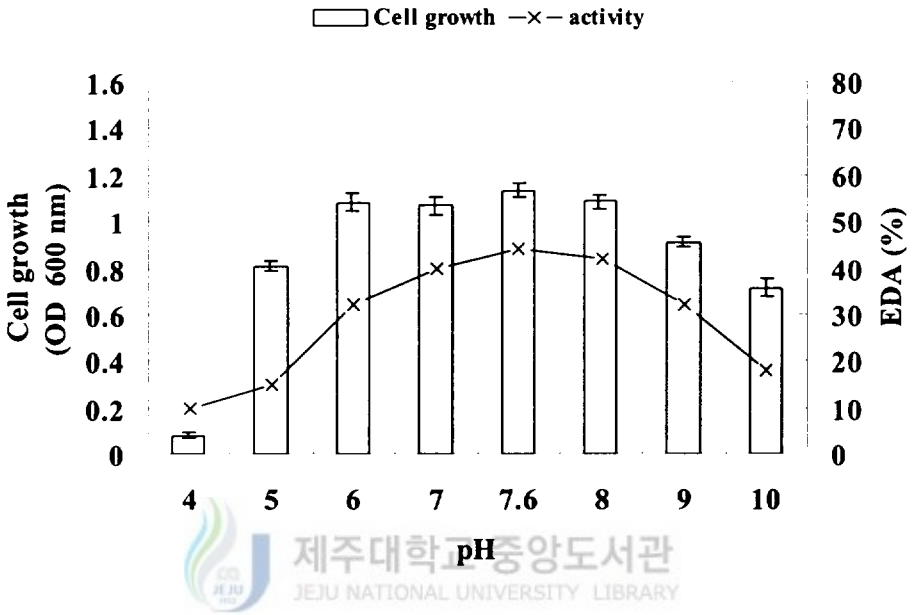

Fig. 1-3. Effect of pH on the producing antioxidative activity by strain SC2-1. The antioxidative activity was tested DPPH method. Each value represents the average of three independent experiments.

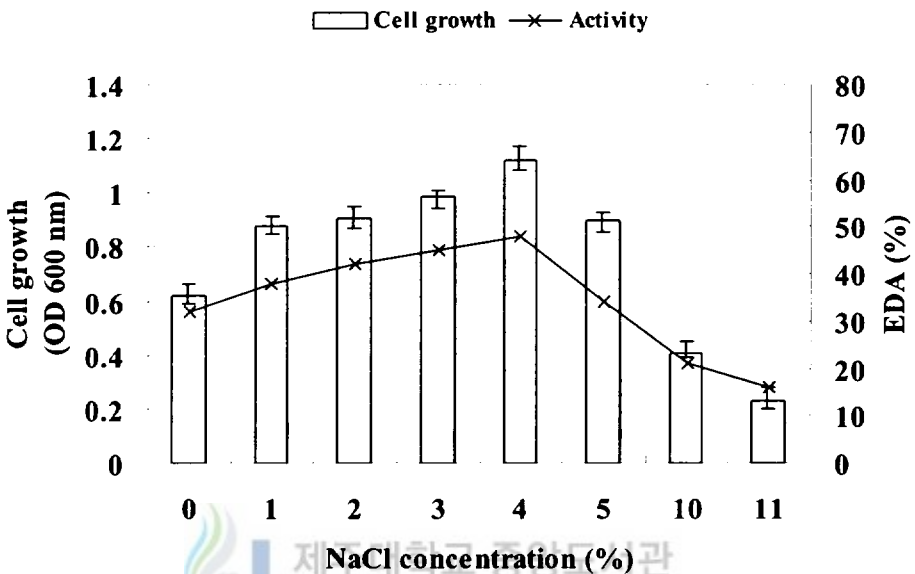

Fig. 1-4. Effect of NaCl concentration on the producing antioxidative activity by *Exiguobacterium* sp. SC2-1. The antioxidative activity was tested DPPH method. Each value represents the average of three independent experiments.

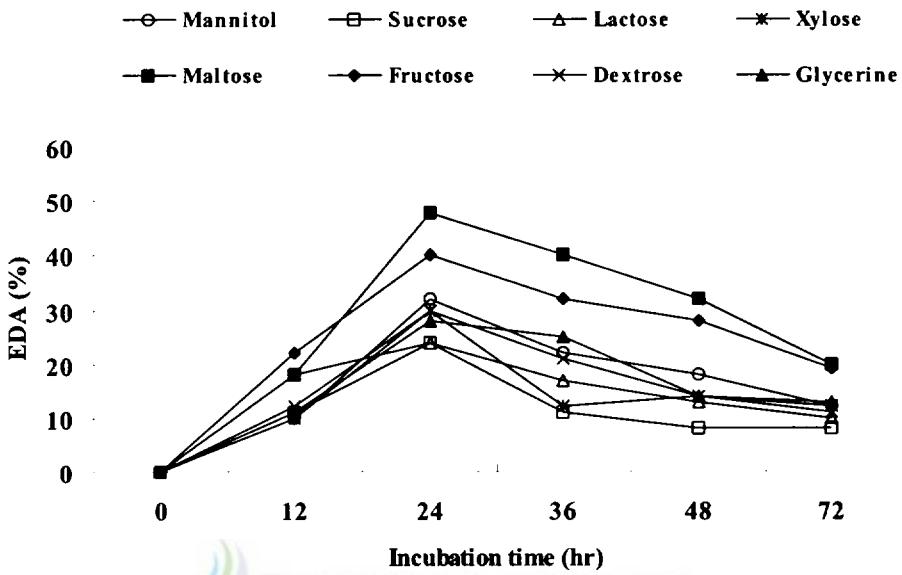

Fig. 1-5. Effect of various carbon sources on the DPPH free radical scavenging activity of *Exiguobacterium* sp. SC2-1 supernatant. The carbon sources were provided at the concentration of 1% in MB medium. Each value represents the average of three independent experiments.

Table 1-3. Effect of maltose concentration on the production of antioxidant materials.

Concentration	Cell growth (OD_{600nm})			EDA(%)		
	24hrs	30hrs	48hrs	24hrs	30hrs	48hrs
None	0.830	0.820	0.840	30	22	14
0.5	0.897	0.912	0.912	38	30	20
1.0	0.970	0.865	0.865	48	40	35
1.5	1.254	1.223	1.322	48	50	52
2.0	1.093	1.134	0.877	50	52	54
2.5	1.127	1.176	0.988	60	62	56
3.0	1.092	1.107	0.885	60	51	52
4.0	1.052	1.076	0.819	53	54	54

Each basal medium is Marine broth (Difco Co., USA). Each value represents the average of three independent experiments.

EDA(%)=[1-(absorbance of sample at 525nm)/(absorbance of control at 525nm)]100

Table 1-4. Effect of nitrogen source on the production of antioxidant materials.

Sourse	Compounds	Cell growth ($OD_{600\ nm}$)			EDA (%)[a]		
		24hrs	30hrs	48hrs	24hrs	30hrs	48hrs
Nitrogen [b] (0.5% w/v)	None	1.067	1.180	1.174	60	62	56
	Peptone	1.191	1.283	1.253	62.3	58.2	61
	Yeast extract	1.328	1.394	1.371	79.2	74	77
	Tryptone	1.398	1.439	1.519	76.6	67.6	51.3
	$NaNO_3$	1.022	1.113	1.023	49	53.1	44.9
	$(NH_4)_2SO_4$	1.036	1.133	1.156	44.5	53	42.1
	$(NH_4)HPO_4$	1.242	1.277	1.212	59.8	58.5	72
	Malt extract	1.058	1.174	1.253	61.3	59.5	59.4
	NH_4NO_3	1.080	1.161	1.156	45.8	53	51.3
	KNO_3	0.976	1.096	1.093	52.2	52.3	49.7

[a] EDA (Electron donating ability)

[b] Each basal medium is Marine broth (Difco Co., USA) and added nitrogen sources of 0.5% (w/v), respectively. Each value represents the average of three independent experiments.

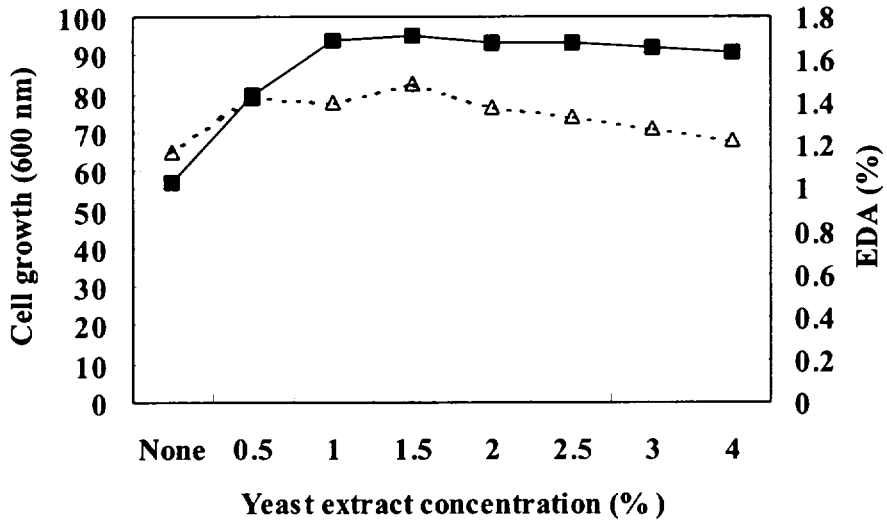

Fig. 1-6. Effect of yeast extract concentration on the cell growth and DPPH free radical scavenging activity of *Exiguobacterium* sp. SC2-1. supernatant. -△-, cell growth in broth: -■-, EDA.

6. Effect of various mineral sources

The effect of mineral sources on cell growth and antioxidant production was examined by employing various mineral sources at a concentration of 0.05% (w/v) in the basal medium. The results of mineral source utilization were shown in Table 1-5. *Exiguobacterium* sp. SC2-1 was able to grow very well in medium containing KH_2PO_4 but not $MgSO_4 \cdot 7H_2O$, $MgCl_2$ and Na_2HPO_4. The highest antioxidant production was obtained in culture of *Exiguobacterium.* sp. SC2-1 containing KH_2PO_4 as a mineral source, followed by cultures containing K_2HPO_4.

Table 1-5. Effect of various mineral sources on the growth and radical scavenging activity of *Exiguobacterium* sp. SC2-1 supernatant.

Mineral sources (0.05%)	Cell growth	EDA (%)
None	1.322	93
K_2HPO_4	1.340	94
$MgSO_4 \cdot 7H_2O$	1.221	93.6
$MgCl_2$	1.252	93.8
Na_2HPO_4	1.359	93
KH_2PO_4	1.351	95

Each basal medium is Marine broth (Difco Co., USA) and added maltose and yeast extract of 2.5, 0.5% (w/v), respectively. Each value represents the average of three independent experiments.

Effect of optimized media on the producing of antioxidant

Medium optimization by the one-fact-at-a-time method involves changing on independent variable (i. e. nutrient, temperature, pH, NaCl) while fixing the others at certain levels. DPPH is a free radical donor which has been widely used to test the free radical scavenging effect of natural antioxidants. DPPH free radical scavenging activity of *Exiguobacterium sp.* SC2-1 in optimum condition for production of antioxidant by the DPPH method was Fig. 1-9. The antioxidant from *Exiguobacterium* sp. SC2-1 showed strong activity against DPPH solution. The supernatant of bacterial cultured from synthesis medium having 90% scavenging activities were inferior to the commercial antioxidants such as α-tocopherol, BHA and little to BHT. These result support ability to invent some novel natural antioxidant.

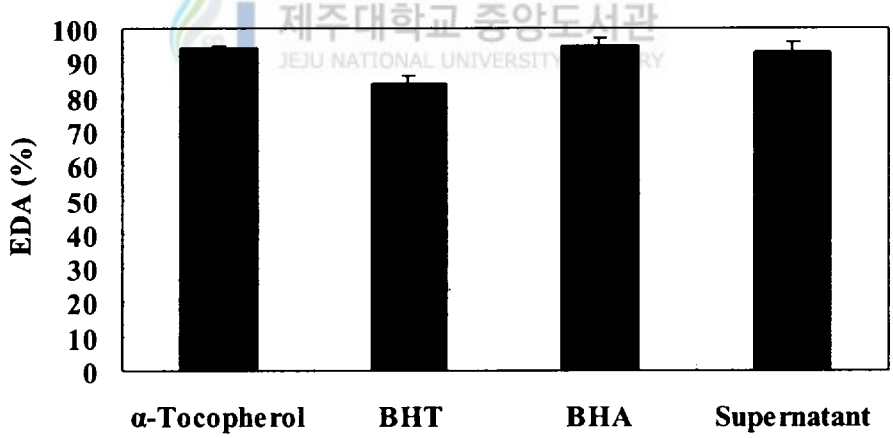

Fig. 1-7. Antioxidant activity of culture supernatant of strain *Exiguobacterium* sp. SC2-1. The concentration of α-tocopherol, BHT and BHA added in reaction mixture were 0.05 mg/ml, respectively.
BHT: butylated hydroxytoluene, BHA: butylated hydroxyanisole.

Hydroxyl radical scavenging ability

Reactive oxygen species (ROS) such as hydroxyl radical produced by sunlight, ultraviolet, chemical reaction, and metabolic processes have a wide variety of pathological effects on cellular processes. The hydroxyl free radicals are very reactive species and rapidly attack the macromolcules in cell (Boveris *et al.*, 1972). Hydroxyl radical scavenging activity of the bacterial cultural supernatant were measured as the percentage of inhibition of hydroxyl radicals generated in the using Fenton reaction mixture (Fe^{2+} + $H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$). and these results were expressed as inhibition rates. In general, bioactivity accumulation was observed during the exponential growth phase, and the maximum titre was resistered when the culture reached the stationary phase. Usually, at 18 h most of the runs showed a considerable level of activity in all experiments. However, some treatments resulted in continued production of antioxidant after this period, reaching their highest levels at 24-36 h. Then production stopped and decreased slightly with time. The cultural supernatant from *Exiguobacterium* sp. SC2-1 showed a slight scavenging ability on hydroxyl radical. However, the scavenging ability of BHT was 44% at 0.5 mg/ml (Fig. 1-2). The inhibitory rate was inferior to those of the commercial antioxidants such as BHA and similar to that of BHT.

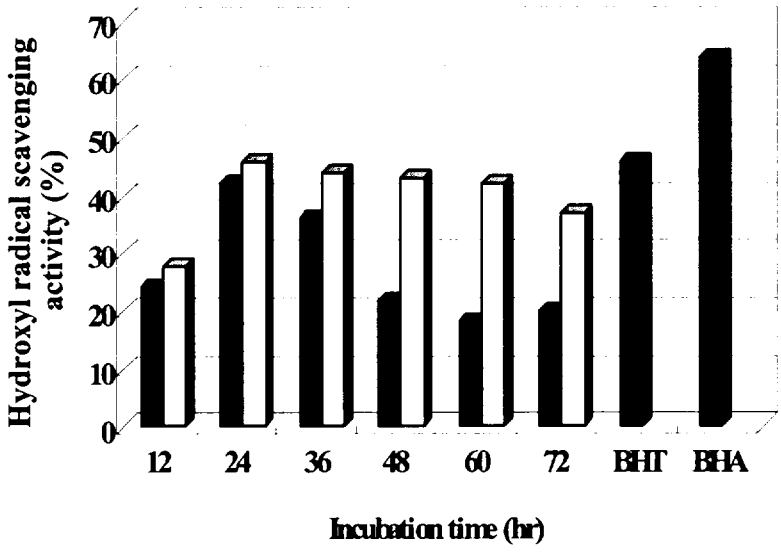

Fig. 1-8. Hydroxyl racical scavenging activities of culture supernatant of *Exiguobacterium* sp. SC2-1 by 2-deoxyribose oxidation method and strain were cultured from optimum condition for 72 h at 25℃.

Superoxide radical scavenging ability

Superoxide radical is one of the strongest free radicals in cellular oxidation reactions because, once it forms, it further produces various kinds of cell-damaging free radicals and oxidizing agents. Most of the superoxide radicals are formed in the mitochondrial and microsomal electron transport chain. Except for cytochrome oxidase, which retains the partially reduced oxygen intermediated bound to its active site, all other elements in the mitochondrial respiratory chain, e.g., ubiquinone, etc., transfer the electron directly to oxygen and do not retain

the partially reduced oxygen intermediates in their active sites. On the internal mitochondrial membrane, the superoxide anion may also be generated by auto-oxidation of semiquinones (Hemnani and Parihar, 1998). Superoxide radical scavenging activity of the cultural supernatant from *Exiguobacterium* sp. SC2-1 was measured using the pyrogallol auto-oxidation system and these results are indicated as inhibitory rate the superoxide productivity. After 24 h incubation, the supernatant having around 38% scavenging activities were inferior to the commercial antioxidants such as BHA and little superior to BHT.

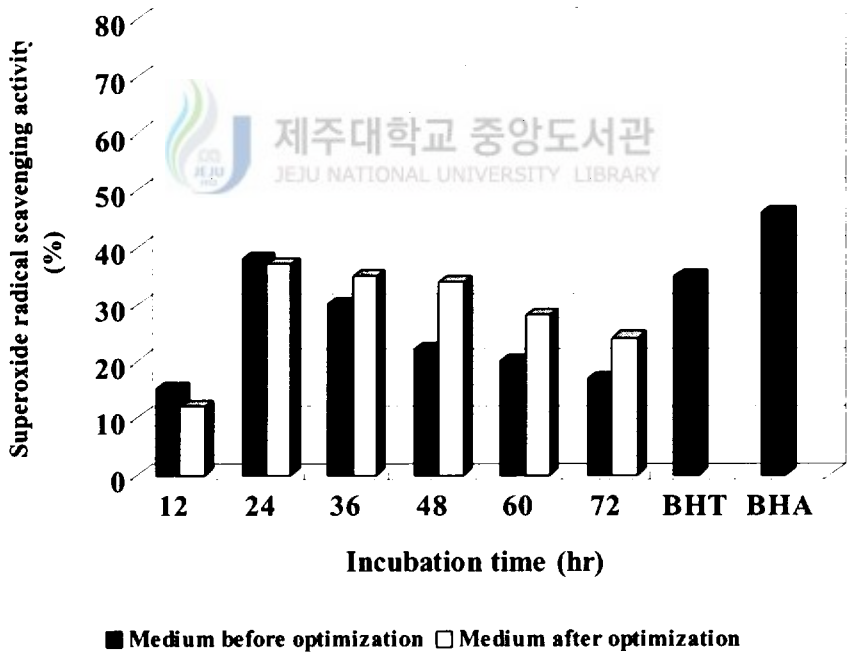

Fig. 1-9. Superoxide radical scavenging activity of *Exiguobacterium* sp. SC2-1 supernatant by pyrogallol auto-oxidation colormetric method and strain were cultured from optimum condition for 72 h at 25℃.

4. CONCLUSION

Antioxidant activities are closely linked with various diseases, aging and food processing and storage. Natural antioxidants may also have correlations with pharmacological actions of plant and microbial sources. An antioxidant-producing bacterium was isolated from sea water in Jeju island. The isolated strain, SC2-1 was gram-positive, catalase positive, facultatively anaerobic, oxidase positive, motile and small rods. The strain utilized sucrose, dextrose, fructose, mannitol and maltose as a sole carbon and energy source and sodium chloride required for the bacteria growth. Based on our phenotypic, phylogenetic, and fatty acid characterization strain SC2-1 was identified as a member of the genus *Exiguobacterium*. The optimum culture conditions for production of antioxidant were 25℃, pH 7.8 and NaCl concentration were 4%. The modified optimal medium compositions were maltose 2.5% (w/v), yeast extract 1.5% (w/v) and KH_2PO_4 0.05% (w/v).

In this study, the potential antioxidant activity of *Exiguobacterium* sp. SC2-1 has been assessed based on three different assay methods and compared with BHA and BHT. It might be explained by stable radical (DPPH) scavenging effect and by weak hydroxyl radical scavenging effect. Additionally, culture supernatant appeared to weak activity of the inhibit superoxide radical scavenging ability. The inhibitory rate was inferior to those of the commercial antioxidants such as BHA and similar to that of BHT. The DPPH method was proved to be a useful optimization technique for determining submerged culture condition of *Exiguobacterium* sp. SC2-1. Further study was required for identification of antioxidant active compounds from culture supernatant of bacteria.

Part Ⅱ.

Isolation and Identification of Antioxidant Producing Marine Sources *Actinomycetes* and Optimal Medium Condition

Part Ⅱ.

Isolation and Identification of Antioxidant Producing Marine Sources Actinomycetes and Optimal Medium Condition

1. ABSTRACT

For the research of the natural marine antioxidant, several bacteria were isolated from the coast of Jeju in Korea. An *actinomycetes* strains, S-1, containing antioxidant component was isolated from sea sand and was identified to a genus level 16S ribosomal DNA sequence and cellular fatty acids analysis. From these results and other characteristics described in the Bergey's Manual, this strain was identificated as a *Nocardiopsis* sp. Strain S-1 showed high activity of 1,1-diphenyl-2-picrylhydrazyl radical scavenging. The hydroxyl radical scavenging ability of *Nocardiopsis* sp. S-1 broth was 53%. Nutritional and cultural conditions for the production of antioxidant by this organism under shake-flask conditions have optimized. Similary initial medium pH 7.6, incubation temperature of 25℃, sodium chloride concentration 2.5% and incubation time of 8 day were found to be optimal. The optimum conditions for the production of antioxidant for carbon, organic and inorganic nitrogen sources were galactose and yeast extract. The DPPH free radical scavenging ability of *Nocardiopsis* sp. S-1 cultural supernatant was 88% from optimum culture condition.

2. MATERIALS AND METHODS

2.1 Isolation and maintenance

Antioxidant producing actinomyces were collected along Jeju Island coast of Korea during a period from August 2003 to October 2003. The strain S-1 has been derived from the sediments sand of Hamdeok beach. Sea water, marine algae and marine animal were homogenized in sterilized sea water (20 ml). Each suspension was diluted with sterilized from 10^{-1} to 10^{-4}. The suspensions from 10^{-4} to 10^{-6} (0.2 ml) were spread on agar plates made from the medium and cultured at 25℃ for a few day. It was isolated on MA (Marine Agar. Difco. Co. USA) while incubating at 25℃. Plates containing the culture were stored at 4℃. For long storage, it was grown in MB (Marine Broth. Difco. Co. USA) for 7 days. Bacterial stocks were kept frozen at -20℃ in a solution of 1:4 v/v of glycerol to adequate broth and propagated twice before experimentation.

2.2 Antioxidant producing actinomyces isolation

All strains were grown on 50 ml Marine broth for 10 days at 25℃ and then harvested by centrifugation at 4℃ (8,000 rpm) for 1 min, A 1 ml of supernatant and 2 ml of freshly prepared 150 uM DPPH solution (in methanol) were mixed. The mixture was incubated in the room temperature for 30 min. After standing for 30 min, absorbance at 525 nm. The control sample contained a MB medium instead of sample. A mixtures showing a white-on-purple spot were regarded as antioxidant producing strains.

2.3 Physiological, biochemical and morphological characteristics

Media used were those recommended by Shirling and Gottlieb (1966) in the international Streptomyces Project (ISP) and by Waksman (1961).

Mycelium was observed after incubation at 25℃ for 2 weeks. Colors were determined according to Prauser (1964). Carbohydrate utilization was determined by growth on carbon utilization medium (ISP medium No. 9) (Pridham and Gottlieb, 1948) supplemented with 1% carbon sources at 25℃. NaCl and pH range for growth was determined on inorganic salts starch agar medium (ISP medium No. 4) using a temperature gradient incubator. All cultural characteristics were recorded after 2 weeks. It is required to describe morphological characters of mycelia, spore, etc. which differentiate the taxonomic genus of species of the actinomycetes, based on observation of the species grown on tryptone yeast glucose agar medium (ISP medium No. 1), oatmeal agar medium (ISP medium No. 3), starch inorganic salt agar medium (ISP medium No. 4), glycerin asparagine agar medium (ISP medium No. 5), peptone yeast iron agar medium (ISP medium No. 6) or tyrosine agar medium (ISP medium No. 7).

2.4 16S rDNA analysis

In order to identify the strain that produced the test sample, 16S rDNA analysis was carried out. The strain was fermented in Marine Broth (Difco. Co. USA) medium for eight days and the fermented broth was centrifiged for the extraction of DNA. It was carried out by using the Genomic DNA Extraction Kit (Bioneer. Co. Korea). An amplification of DNA was performed by using PCR Kit. The primers were p364f 5'-GGC AGC AGT GGG GAA TAT TG-3' and p1037r 5'-TCG TCA GCT CGT GTC GTG AG-3'. The thermal cycle profile commenced with an initial denaturation at 94℃ for 45 sec, annealing at 50℃ for 45 sec, and extension at 72℃ for 2 min. In the last cycle, the reaction mixture was kept at 72℃ for 1 min and cooled to 4℃. The PCR products were purificated by using PCR purification Kit (Bioneer. Co. Korea)

2.5 Cellular fatty acid analysis (CFA)

The fatty acid methyl esters were prepared by using the method described by Miller and Berger (1985). Briefly, the bacteria were saponified for 30 min at 100℃ in 1 ml of 15% sodium hydroxide in 50% aqueous methanol. After cooling, 2 ml of the methylation reagent (325 ml of 6.0 N hydrochloric acid plus 275 ml of methanol) was added, and the samples were heated at 80℃ for 10 min. After cooling, 1.25 ml of a 1:1 (v/v) hexane-ether mixture was added and the samples were mixed by 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 with 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 by end-to-end rotation for 5 min. The phases were allowed to separate, and the organic (top) phase was removed to a Teflon-lined, septum-cap autosampler vial for analysis on the gas chromatograph. Fatty acid methyl esters (FAME) mixtures were analysed by capillary GC using a Hewlett Packard model 5898A GC run by Microbial Identification software (Microbial ID).

2.6 Measurement of growth rate and antioxidant activity

Growth rate of the strain S-1 was measured as dry weight of the mycelium. Antioxidant activity was performed by DPPH free radical and hydroxyl radical scavenging activity. DPPH solutrion was prepared at the concentration of 150 uM in methanol. During the assay, the supernatant of 1 ml was mixed 3.0 ml DPPH solution. The mixture was incubated in the room temperature for 30 min. Absorbance of the supernatant was measured at 525 nm using UV-VIS spectrophotometer (Opron 3000 Hanson Tech. Co. Korea). The percentage of inhibition was defined by the absorbance at 525

nm in the absence of supernatant to that measured with sample. The control sample contained a MB medium instead of sample. DPPH radical scavenging ability (%) was defined as follows :

EDA(Electron donating ability) = [1-(A_{525}(sample)/A_{525}(control))] × 100%

Hydroxyl radical scavenging activity was determined according to the modified method of the 2-deoxyribose oxidation method. Hydroxyl radical was generated by Fenton reaction in the presence of $FeSO_4 \cdot 7H_2O$. A reaction mixture containing each 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 cultural supernatant solution and 1.0 M phosphate buffer (pH 7.4) was added into the reaction mixture until the total volume reached to 1.8 ml. then 0.2 ml of 10 mM H_2O_2 was finally added to the reaction mixture and incubated at 37℃ for 4 h. After incubation, each 1 ml of 2.8% TCA (trichloroacetic acid) and 1.0% TBA (thiobarbituric acid) were added. Then, the mixture was placed in a boiling water bath for 10 min. Absorbance was measured at 532 nm.

HSA (Hydroxyl radical Scavenging Activity)=[1-(A_{532}(sample)/A_{532}(control))]×100%

2.7 Optimization of carbon sources and cultural conditions

To determine the optimal nutrient sources and cultural conditions for growth and antioxidant production, MB medium was used as the base. It was supplemented with different carbon and nitrogen sources to study their effect on growth and antioxidant production. The medium (50 ml in 250 ml Erlenmeyer flask) was inoculated with 2% (v/v) bacteria suspension and incubated with at 25℃ on a rotary shaker (110 rpm) for 10 days.

To determine nutritional requirement for the production of antioxidant, several nutrients as carbon, nitrogen sources and NaCl were tested. The strain S-1 was cultured in 50 ml of MB medium as the basal medium for 10 days. The cultivation was carried out on a reciprocal shaker at 25℃ and at 110

rpm. To investigate the effect of carbon sources on the production of antioxidant, carbon sources were provided at the concentration of 1%(w/v) instead of carbon sources in MB medium. 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 the nitrogen sources. NaCl was provided at the concentration of between 2 to 7% (v/v). The effect of cultural conditions like different incubation temperatures (4, 16, 20, 25, 30, 35 and 40℃), initial pH (6, 6.8, 7.6, 8, 8.4 and 9.2) and incubation time (1~13 days) on growth and antioxidant production was studied.

3. RESULT AND DISCUSSION

3.1 Screening and identification of the antioxidant-producing actinomyces

We obtained a five antioxidants producing actinomyces isolated from the coastal sand. The bacteriological characteristics of isolated strain S-1 were investigated (Table 2-1). After incubating for seven days on the MA medium under white. Strain S-1 was gram-positive and aerobic bacterium. Its optimum growth temperature was 25℃. The NaCl concentration for growth was 0 to 10% (w/v) with an optimal growth at 2.5% (w/v). Its optimum growth pH was 7.6. The strain was produced acid from xylose, cellobiose, rhamnose, sucrose, D-galactose and maltose. From these results, the strain S-1 was considered to being to the *Nocardiopsis* genus or related one. The cultural characteristics of the isolated S-1 are shown in Table 2-2. The isolated showed moderated to good growth in different media. Vegetative mycelium showed dark-yellow color and aerial mycelium showed white color. It produced dark-yellow diffusible pigment in most of the media.

The 16S rDNA sequence is shown in Fig. 2-1. It was analyzed by using BLAST (National Center for Biotechnology Information). The almost-complete 16S rDNA sequence of S-1, which, consists 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 falls into the genus *Nocardiopsis* (Fig. 2-1.). The 16S rDNA sequence similarities of S-1 to *N. dassonvillei, N. antarctica* and *N. synnemataformans* were 99, 99 and 98% , homology respectively. According to 16S rDNA sequence analysis, S-1 is a member of the genus *Nocardiopsis.*

The importance of CFA composition as an adjunct to identification was dependent on which species or taxon group was under consideration. The cellular fatty acids of the actinomycetes S-1 were extracted and analyzed according to the instructions of Microbial Identification System (MIDI; Microbial ID). Table 2-3 shows the cellular fatty acid composition of strain. The strain S-1 is consisted of $isoC_{14:0}$ (0.48%), $isoC_{15:0}$ (0.57%), $anteisoC_{15:0}$ (3.56%), $isoC_{16:0}$ (26.82%), $cis9C_{16:1}$ (1.81%), $C_{16:0}$ (1.14%), $isoC_{17:0}$ (2.07%), $anteisoC_{17:0}$ (23.42%), $cis9C_{17:1}$ (5.26%), $C_{17:0}$ (2.00%), 10 $methylC_{17:0}$ (1.29%), $isoC_{18:0}$ (2.35%), $cis9C_{18:1}$ (21.01%), $C_{18:0}$ (6.69%). Based on our phenotype, phylogenetic characterization and cellular fatty acid analysis, strain S-1 was identified as a member of the genus *Nocardiopsis.*

Table 2-1. Comparision physiological and biochemical characteristics of the isolated S-1

Character	Strain S-1	Type strains[a]			
		N dassonvillei	*N alboniida*	*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
Opimum 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	ND	−	+	−	−
Cellobiose	+	+	−	ND	+
Rhamnose	+	+	+	−	+
Sucrose	+	+	+	+	+
Adonitole	ND	−	+	−	−
Inositole	ND	−	+	−	−
D-galactose	+	V	ND	ND	−
Maltose	+	−	ND	ND	−

+, positive; -, negative; ND, no data; V, variable results of test. [a]Data complied from references 10, 15, 17, and 26. Carbohydrate utilization was determined by growth on carbon utilization medium (ISP medium No. 9) supplemented with 1% carbon sources at 25℃. NaCl and pH range for growth was determined on inorganic salts starch agar medium (ISP medium No. 4)

Table 2-2. Cultural characteristics of the isolated S-1 cultured from International Streptomyces Project medium.

Medium	Characteristics				
	Growth	Vegetative mycelia	Aerial mycelia	Spore	Soluble pigment
Glycerol tyrosine agar	G	G, yellow	Abundant, white	Poor	Brown
Peptone yeast extract	M	M, dark white yellow	M, white yellow	Poor	Dark brown
Oatmeal agar	M	M, yellow white	Abundant, white	M	Yellow brown
Glycerol asparagine agar	M	M, yellow white	M, light gray	Poor	Yellow brown
Starch agar	M	M, dark white	M, dark white	M	Yellow brown
Tryptone yeast glucose agar	G	G, Dark white brown	M, gray	M	Dark
Nutrient agar	G	M, Dark white	M, white	M	Dark brown
Marine agar	G	G, Dark white	Abundant, white	M	Yellow brown

M, moderate; G, good

Table 2-3. Cellular fatty acid profile of the strain 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

Fatty acid methyl esters (FAME) mixtures were analysed by capillary GC using a Hewlett Packard model 5898A GC run by Microbial Identification software (Microbial ID).

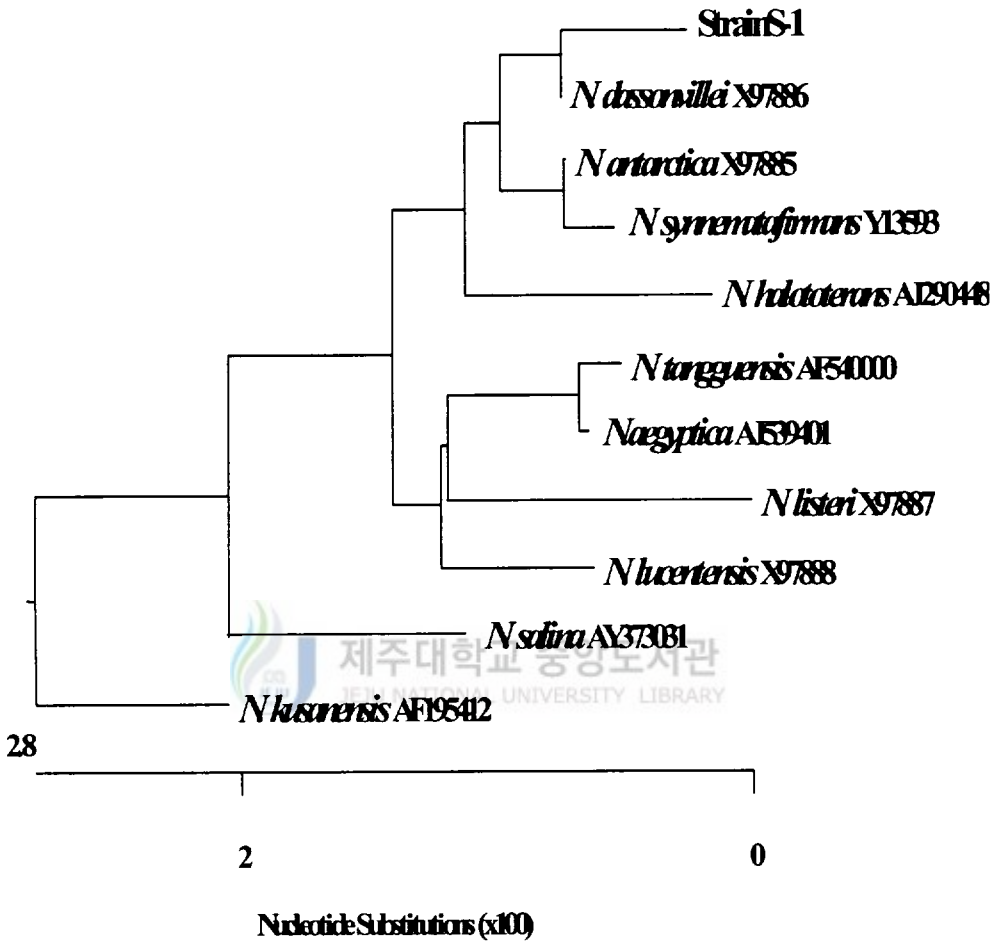

Fig. 2-1. Rooted neighbor-joining tree based on nearly complete 16S rDNA sequences showing relationships between strain S-1 and members of the genus *Nocardiopsis*. The numbers at the branch nodes are bootstrap values from 1000 bootstrap trails.

3.2 Antioxidant activity

Free radicals are highly reactive molecules or chemical species capable of independent existence. Generation of highly Reactive Oxygen Species (ROS) is an integral feature of normal cellular function like mitochondrial respiratory chain, phagocytosis, arachidonic acid metabolism, evolution, and fertilisation. Their production however, multiplies several folds during pathological conditions. The release of oxygen free radicals has also been reported during the recovery phases from many pathological noxious stimuli to the cerebral tissues (Halliwell and Gutteridge, 1989). Antioxidant activity of strain S-1 cultural supernatant were estimated by measuring EDA and HSA with 2-deoxyribose oxidation method. Free radical scavenging ability of various incubation time was evaluated with the change of absorbance caused by the reduction of DPPH. The percentage scavenging activity of each supernatant against DPPH was shown in Fig. 2-2. The radical scavenging activity cultivation 8th showed a most high antioxidant activity and HSA were observed cultivation 5th most high activity. Radical and hydroxyl radical scavenging activity of strain S-1 supernatant measured about 60% and 45%.

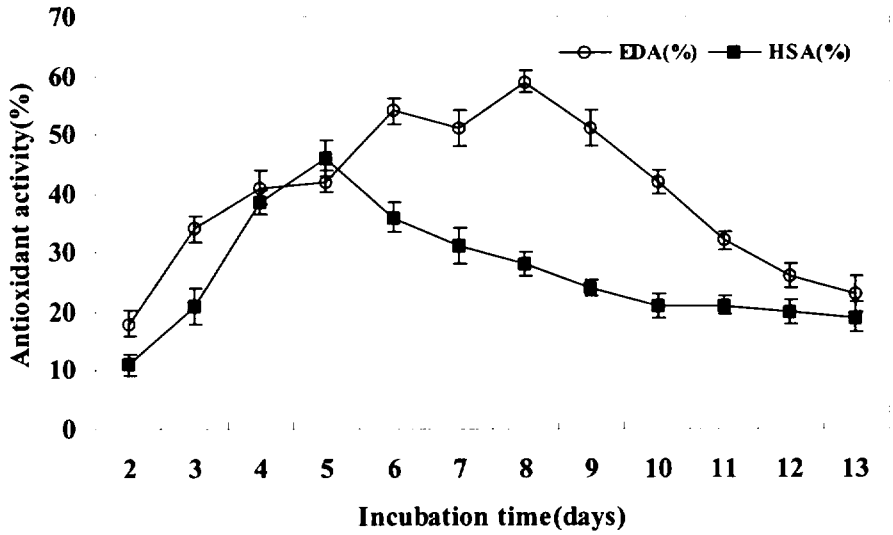

Fig. 2-2. A time course of fermentative production of antioxidant by the strain S-1. The strain was cultured in MB medium (pH 7.6) for 13 day at 25℃, 110 rpm.

3.3 Optimum cultural and nutritional conditions for growth and production of antioxidant

Effect of temperature and pH

The optimum temperature for growth and production of antioxidant from the *Nocardiopsis dassonvillei* S-1 were shown in Fig. 2-3. The strain S-1 showed a narrow range of incubation temperature for relatively good growth and antioxidant production. The *Nocardiopsis dassonvillei* S-1 was able to grow at 4 to 40℃ and the reasonable temperatures for growth were 25℃ and 30℃. At above 35℃, the productivity of antioxidant material was reduced, contrast to an reduced in the productivity of dark brown pigments (data not

shown). From these results, we considered that the production of antioxidant material is related with the production of pigments. The optimum temperature for production of antioxidant of *Nocardiopsis dassonvillei* S-1 cultural supernatant was 25℃. The organism appeared to be mesophilic in nature.

The pH of medium is a very important but is often a neglected environmental factor. Many investigators claimed that the different morphology of fungi and bacteria under a different initial pH value was the critical factor in biomass accumulation and metabolite formation. The medium pH may affect cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis. In the present study, *Nocardiopsis dassonvillei* S-1 was able to grow at pH 6 to 9.2 and also showed the antioxidant activity at the same pH range. The optimum pH for growth and production of antioxidant of *Nocardiopsis dassonvillei* S-1 cultural supernatant was 7.6 (Fig. 2-4).

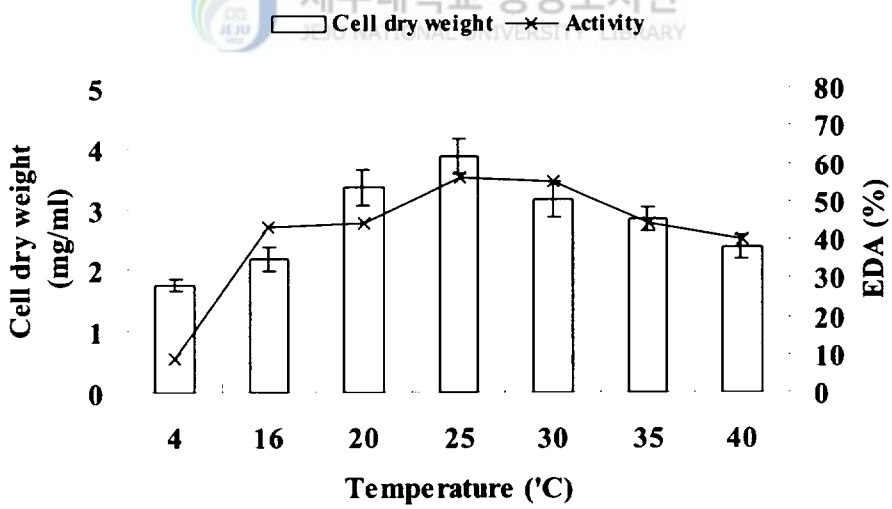

Fig. 2-3. Effect of temperature on the growth and producing antioxidative activity by *Nocardiopsis dassonvillei* S-1. The antioxidative activity was teasted DPPH method.

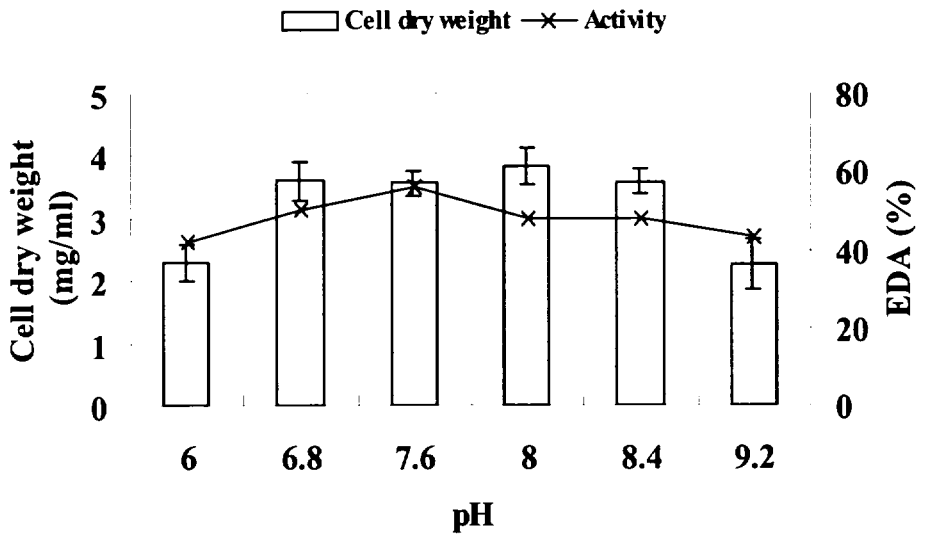

Fig. 2-4. Effect of pH on the producing antioxidative activity by *Nocardiopsis dassonvillei* S-1. The antioxidative activity was tested DPPH method. Each value represents the average of three independent experiments.

Effect of NaCl concentration

The effect of Nacl concentration on growth and production of antioxidant of *Nocardiopsis dassonvillei* S-1 cultural supernatant were shown in Fig. 2-5. *Nocardiopsis dassonvillei* S-1 was able to grow at 0 to 11% (w/v) of NaCl concentration (not shown data). The production of antioxidant was little different by NaCl concentration. The optimum NaCl concentration for growth and production of antioxidant fo *Nocardiopsis dassonvillei* S-1 was 2.5% (w/v). These results suggest that this strain was originated from marine environment or adapted itself to marine environment.

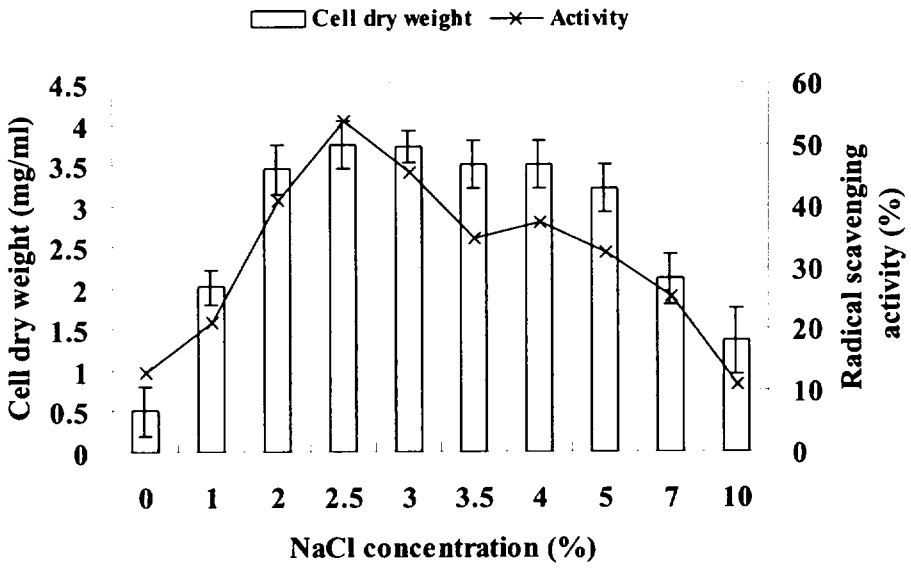

Fig. 2-5. Effect of NaCl concentration on the producing antioxidative activity by *Nocardiopsis dassonvillei* S-1. The antioxidative activity was tested DPPH method. Each value represents the average of three independent experiments.

Effect of carbon and nitrogen sources on the production of antioxidant

Optimization of antioxidant production was carried out in flask culture. Mannose, starch, sucrose, xylose, glycerin, fructose, lactose, dextrose, mannitol, maltose and galactose were used to determine the effect of carbon sources on the production of antioxidant. This strain was able to grow in all the tested carbon sources (Table 2-4). However, maximum growth and antioxidant activity was obtained in medium supplemented with galactose as a sole carbon sources followed by glycerin and mannitol. *Nocardiopsis dassonvillei* S-1 was able to very well in medium containing mannitol, glycerin and

galactose, but not mannose, sucrose, fructose, lactose, dextrose and maltose.

Nitrogen may be supplied as ammonia, nitrate, or in organic compounds such as ammonia acids and proteins. Therefore, the omission of nitrogen in the medium greatly affects bacterial growth and metabolite production. The results of nitrogen sources utilization were shown in Table 2-3. In case of nitrogen sources, *Nocardiopsis dassonvillei* S-1 was able to grow very well in medium containing yeast extract, and peptone, but not KNO_3 and $(NH_4)_2SO_4$. The highest radical scavenging activity was obtained in culture of isolated S-1 containing yeast extract as a nitrogen source, followed by cultures containing peptone, tryptone and malt extract, but not KNO_3 and $(NH_4)_2SO_4$. However, in case of *Sphingomonas pancimobilis*, tryptone provided the highest productivity of antioxidant materials. Therefore, it appears that carbon and nitrogen source for antioxidant production would depend on microbial and sorts of antioxidant.

Table 2-4. Effect of various carbon and nitrogen sources on the growth and radical scavenging activity of *Nocardiopsis dassonvillei* S-1 supernatant.

Sourse	Compounds	Dry weight (mg/ml)	EDA (%)[a]
Carbon [b] (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 [c] (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

[a]EDA(%)=[1-(absorbance of sample at 525nm)/(absorbance of control at 525nm)]100. Each basal medium is [b]Marine broth (Difco Co. USA), [c]Marine broth/1.0% galactose. All cultures were carried out at 25, pH 7.6. Each value represents the average of three independent experiments.

Effect of optimized media on the production of antioxidant

Antioxidant production was tested employing the modified medium and optimized cultral conditions. As results indicated (Fig. 2-6), the radical scavenging activity very high (88%) under optimized condition. The results indicated the dependence of the antioxidant synthesis on medium constituents. in fact, it has been shown that the nature of carbon and nitrogen sources, strongly affect antibiotic production in different organisms. These results support ability to invent some noble antioxidant.

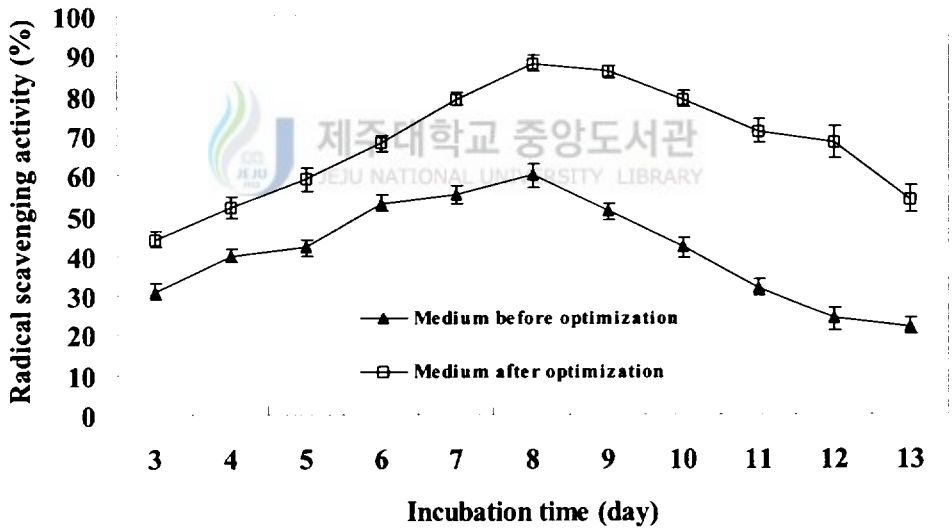

Fig. 2-6. Effect of optimized media on the production of antioxidant by *Nocardiopsis dassonvillei* S-1. Each value represents the average of three independent experiments.

4. CONCLUSION

The genus *Nocardiopsis* include aerobic, spore-forming actinomycetes that produce a branched, vegetative mycelium and aerial hyphae. We isolated antioxidant-producing marine actinomyces from the coastal regions of Jeju island in Korea. After incubating for seven days on the MA medium under white. The NaCl concentration for growth (mycelial) was 0 to 10% (w/v) with an optimal growth at 2.5% (w/v). Its optimum growth pH was 7.6. The strain was produced acid from xylose, cellobiose, rhamnose, sucrose, D-galactose and maltose. From these results, the strain S-1 was considered to being to the *Nocardiopsis* genus or related one. Strain S-1 was gram-positive and aerobic bacterium. The screened strains having high antioxidative activities ability strain Based on our phenotypic, phylogenetic, and cellular fatty acid characterization strain S-1 was identified as a *Nocardiopsis dassonvillei*. The antioxidative activity of isolated *Nocardiopsis dassonvillei* S-1 ranged from about 18% to 60% scavenging activity on the DPPH radical and from about 10% to 44% hydroxyl radical scavenging activity. The optimum culture conditions for production of aqntioxidant materials with *Nocardiopsis dassonvillei* S-1 were at 25℃, pH 7.6. and NaCl concentration 2.5% (w/v). The optimum conditions for the production of antioxidant for carbon, organic and inorganic nitrogen sources were galactose and yeast extract. The radical scavenging ability of *Nocardiopsis dassonvillei* S-1 cultural broh was 88% from optimum culture condition.

Further work is required for identification and purification of antioxidative compounds from bacterial cultural supernatant. The results of this study demonstrate the antioxidative potential of marine actinomycetes, and suggest these strain are useful for functional fermented food and probiotics.

Part Ⅲ.

Antibacterial and Antioxidant Activities of Fungus mycelium Culture Extracts

Part Ⅲ.

Antibacterial and antioxidant activities of fungus mycelium culture extracts

1. INTRODUCTION

Free radicals are defined as any molecules or atoms with one or more unpaired electrons (Yashikawa, Naito and Kondo, 1997). With the possession of the unpaired electrons, free radical is a key step in lipid peroxidation and is an important cause of cell membrane destruction and thus tissue damage (Halliwell, 1995). Antioxidants can scavenge free radicals and inhibit lipid peroxidation. During the past decades there has been an increasing interest in the production of antioxidnat from mushrooms due to their various physiological activities (Chihara *et al.*, 1970; Lee *et al.*, 1996; Maziero *et al.*, 1999; Rosado *et al.*, 2003; Song *et al.*, 1998). Vegetables and fruits are rich sources of antioxidants such as vitamin C, vitamin E and beta-carotene, which are suggested to be antiatherogenic in epidemiological studies (Enstrom, Kanim, and Klein, 1992; Rimm *et al.*, 1993; Stampfer et al., 1993). Some common mushrooms, which are widely consumed in Asian culture, have currently been found to posses antioxidant activity which is well correlated with their total phenolic content (Cheung, Cheung, and Ooi, 2003). There are many reports regarding the culture condition of mushroom, characterization of antioxidants and their biological activities produced from the extract of mushroom.

However, most investigators have made efforts to cultivate this mushroom on solid artificial media rather than submerged culture. Due to the fact that fruiting body formation most of mushroom has not developed successfully in solid-state

fermentation, the production of mycelial biomass by submerged culture deserves investigating to obtain bioactive materials (Kim *et al.*, 2005). In this paper, we describe antioxidant and antimicrobial activity of mushroom mycelia culture liquid extract (MMCE). Also, it confirmed the use possibility as the natural media of citrus extract.

2. ABSTRACT

This study was carried out to investigate the antimicrobial and antioxidative effects of mycelium cultural extract from mushroom. Mushroom mycelium was grown in a defined synthetic liquid medium and citrus extracts, and the culture extracts were examined for antioxidant activity and antibacterial. Myceliums of *Phellinus linteus*, *Cordyceps militaris*, *Coriolus versicolor*, *Sparassic crispa*, *Agaricus blazei*, *Inonotus obliquus*, *Lentinus edodes*, *Hericium erinacium*, *Gonoderma lucidium* in 10% citrus extract supplemented medium and synthesis medium were incubated in a shaking incubator (120rpm, 24~30℃) for 7~15days. The antimicrobial activities of the culture fluid of mushroom mycelium grown in submerged liquid culture was tested against 12 microorganisms which were fish pathogens and common bacterial species. The culture extracts showed high activity against *Vibrio* sp and had poor effect on *Streptococcus* sp, *S. parauberis*, *S. iniae*. The culture extracts obtained from the synthetic medium showed 30~93% of the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenger activity, the culture extracts obtained from the citrus extracts medium exhibited antioxidant activity up to 55%.

2. MATERIAL AND METHOD

2.1 Microorganism and media

Mushroom mycelia (*Phellinus linteus*, *Cordyceps militaris*, *Coriolus versicolor*, *Sparassic crispa*, *Agaricus blazei*, *Inonotus obliquus*, *Lentinus edodes*, *Hericium erinacium*, *Gonoderma lucidium*) (Table 3-1) was obtained from the Dae Woo Environment (Co. Korea) and maintained in a potato dextrose agar (PDA; Difco. Co. USA) and YM (Dextrose 1%, Peptone 0.5%, Yeast extract 0.3%, Agar 1.8%) slant at 4℃. The seed culture of mushroom mycelium was transferred to Petri dishes containing PDA and YM media and incubated at 25℃ for two weeks. Mycelial agar discs (5 mm) were obtained by Cork borer and were used as inocula for subsequent experiments. Additional, natural medium used added 10% (v/v) citrus extract in water. *Vibrio harvey* (KCTC 2717), *Vibrio mimicus* (KCTC 2732), *Vibrio alginolyticus* (KCTC 2472), *Vibrio vulnificus* (KCTC 2959), *Vibrio parahaemolyticus* (KCTC 2471), *Vibrio anguillarum* (KCTC 2711), *Edwardsiella tarda* (KCTC 12267), *Vibrio pelagius* (KCTC 2732) obtained from the Korean Collection for Type Cultures (KCTC), and wild type strain *Streptococcus* sp, *Streptococcus iniae* and *Streptococcus parauberis* were mainly used in the assay of the antibacterial activity of MMCE (Mushroom Mycelia Cultural Extract). Media for growth of bacteria used MA (Marine agar) and NA (Nutrient Agar, Difco. Co. USA) and media for antibacterial activity measuring used Mueller Hinton Agar (Difco. Co. USA).

Table 3-1. The mushroom mycelium used for the tests of antibacterial and antioxidant activities

Name of mushroom		Part used
Korean name	Scientific name	
Sanghwang	*Phellinus linteus*	Mycelium
Eunji	*Coriolus versicolor*	Mycelium
Dongchunghacho	*Cordyceps militaris*	Mycelium
Sinlyung	*Agaricus blazei Murill*	Mycelium
Chaga	*Inonotus obliquus*	Mycelium
Norugungdengi	*Hericicum erinacium*	Mycelium
Youngji	*Ganoderma lucidum*	Mycelium
Pyogo	*Lentinus edodes*	Mycelium
Kkotsong i	*Sparassic crispa*	Mycelium

2.2. *Cultivation Mushroom mycelium from dilution citrus extracts and synthesis medium*

All mushroom mycelium was grown at 25℃ in submerged liquid cultures. The synthesis medium used Potato Dextrose Broth and YM. One hundred millitres of the medium in a 300 ml Erlenmeyer flask was inoculated with agar plugs covered by the mycelium. This study, using of Carboy culture method. An inoculum of 100 ml of the 7-10 days-old shaken culture (120 rpm) started the fermentation in a sterilized bottle (1000 ml medium inoculated in a 2000 ml bottle). The culture fluid was harvested after 2 weeks of fermentation (25℃, with stirring at 120 rpm). The natural citrus medium contained citrus extract (Jeju Provincial Develpoment Corp. Korea. 62 brix) in distilled water (2 L), final concentration of citrus extract in medium were 10%.

2.3 Extraction of mushroom mycelium culture liquid

The culture fluid was treated at 121℃ for 60 min from autoclave and after centrifugation (4000 Xg, 10min), the culture fluid was filtered by filter paper (Whatman No. 2) and mixed with three times volume of absolute ethanol, stirred vigorously and left overnight at 4℃. The extraction solvents were removed using a rotary evaporator and freeze dried. residues were removed and extracts was stored at 4℃ (Fig. 3-1).

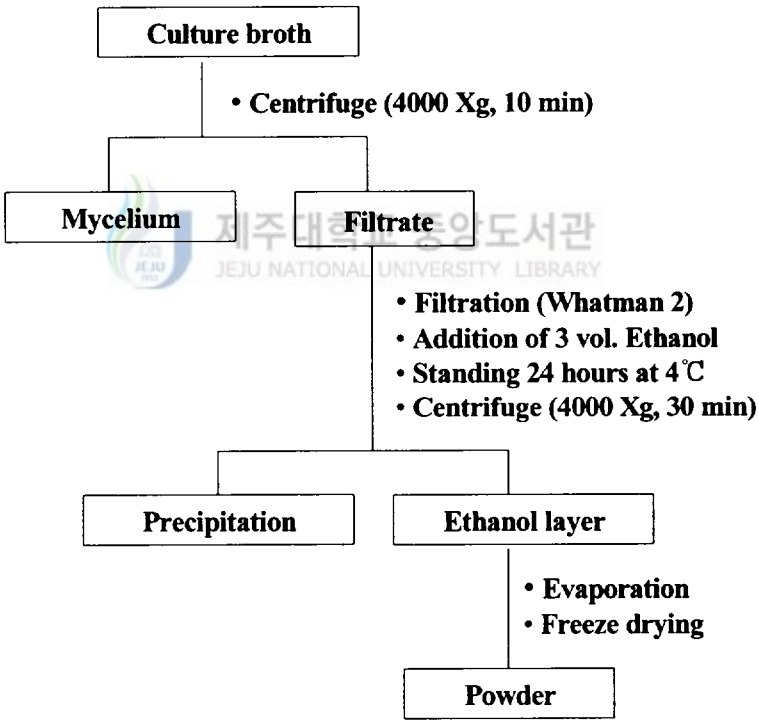

Fig. 3-1. Extraction procedure from culture broth of mushroom mycelium

2.4 Assay for antibacterial activity

The agar disc diffusion method was employed for the determination of antibacterial activity of the crude extract. Briefly, Petri dishes were prepared with a base layer of Muller Hinton agar (Difco. Co. USA) (15ml). Sterile filter discs (ADVANTEC F0424695, 6 mm in diameter) were impregnated with 100 ㎕ of dilutions of known extracts concentrations (1 and 2 mg/disc), placed on the inoculated plates, and after staying at 4℃ for 30 min, they were incubated moderation temperature for 24 h. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the discs. The growth inhibition diameter was average of four measurements, taken at four different directions. All tests were performed in triplicate. In addition, MIC values for Penicillin, Erythromycin, Neomycin, Kanamycin, Chloramphenicol, Nalidixic acid and Tetracycline were determined.

2.5 Scavenging activity of DPPH method of extracts

This assay was based on the scavenging of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by the radical scavenging components in mushroom mycelium culture extracts (MMCE). Modified method of Brand-Williams (1995) was used to investigate the free radical scavenging activity. Various concentrations of the stock solutions (diluted to final concentrations of 10, 5, and 1 mg/ml) were mixed with 150 uM DPPH in methanol. The mixture was vigorously shaken and left to stand for 30 min in the room temperature, and its absorbance was measured at 525 nm. BHA (butylated hydroxyanisole) was used as the control.

2.6 Superoxide radical scavenging ability of extracts

The scavenging potential of the MMCE for superoxide radicals was

analyzed using a inhibition of the auto-oxidation of pyrogallol using a modified method of Marklund and Marklund (1974). A sample solution (0.1 ml) and 2.61 ml of 50 mM phosphate buffer (pH 8.24 at 25℃) was mixed in a cuvette. Freshly prepared 0.09 ml of 3 mM pyrogallol in 10 mM HCl was added and the inhibition of pyrogallol autoxidation was measured at 325 nm using UV-VIS spectrophotometer. Absorbance of each extract was recorded at every 20 sec interval for 3min and the increment of absorbance was calculated by the difference (the absorbance at 3 min - the absorbance at the starting time).

2.7 Hydroxyl radical scavenging activity of extracts

Hydroxyl radical was generated by Fenton reaction in the presence of $FeSO_4 \cdot 7H_2O$. A reaction mixture containing each 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 cultural supernatant solution and 1.0 M phosphate buffer (pH 7.4) was added into the reaction mixture until the total volume reached to 1.8 ml. then 0.2 ml of 10 mM H2O2 was finally added to the reaction mixture and incubated at 37℃ for 4 h. After incubation, each 1 ml of 2.8% TCA (trichloroacetic acid) and 1.0% TBA (thiobarbituric acid) were added. Then, the mixture was placed in a boiling water bath for 10 min. Absorbance was measured at 532 nm.

3. RESULT AND DISCUSSION

3.1 Antibacterial activity of the MMCE

Treatment with mushroom mycelium culture fluid was effective against twelve of the bacterial strains tested. The antibacterial activity results are shown in Table 3-2, 3-3. Table 3-2 showed antibacterial activities of the liquid culture extracts of mushroom mycelium from the synthetic liquid media. Table 3-3 showed Antibacterial activities of the liquid culture extracts of mushroom mycelium from the citrus extract media. Our findings showed that the ethanol extract from mycelium culture fluid had interesting activity against fish pathogenic bacteria and human pathogenic bacteria. From our results, the antioxidant activity of the mushroom mycelium cultural extract (MMCE) was concentration dependent, with stronger antibacterial ability occurring as higher concentrations of the extracts in synthesis and citrus medium. Almost MMCE obtained high activity against Gram negative bacteria, but the MMCE showed poor activity against Gram positive bacteria (*S. iniae* and *S. parauberis*). *Sparassic crispa* mycelium culture extract showed very high antibacterial activity against all tested Gram-negative bacteria. The diameters of growth inhibition area were in the range of 8-25 mm.

Table 3-2. Antibacterial activities of the liquid culture extracts of mushroom mycelium from the synthetic liquid media

Strain	*PL*		*CV*		*CM*		*AB*		*IO*		*HE*		*GL*		*LE*		*SC*	
	Inhibition zone (mm)																	
	Concentration (mg/ml)																	
	10	20	10	20	10	20	10	20	10	20	10	20	10	20	10	20	10	20
V. harvey	+	+	++	+++	+	++	++	+++	-	+	+	++	+	+	+	+	++	+++
V. mimicus	+	+	+	++	-	+	+	++	+	+	+	+	+	+	+	+	+++	+++
V. alginolyticus	+	++	+	+	-	+	+	+	-	-	+	++	+	+	+	+	++	+++
V. vulnificus	+	++	++	++	+	+	+	++	+	+	+	++	-	+	+	+	++	+++
V. parahaemolyticus	++	++	++	+++	+	+	+	++	+	+	+	+	+	++	-	+	++	+++
V. campbelli	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+	+++	+++
V. anguillarum	+	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+++	+++
V. pelagius	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+	+++	+++
E. tarda	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	++	++
Streptococcus sp.	+	++	+	++	+	+	+	+	+	+	+	+	-	-	+	+	++	++
S. iniae	-	+	-	+	+	+	-	-	+	+	-	-	-	+	+	+	+	++
S. parauberis	-	+	+	+	+	+	-	-	+	++	-	-	-	+	-	-	+	++

Cell were grown on MHA plate for 24h at 26, 37℃ after 10 mg, 20 mg each of mushroom liquid cultures was absorbed into paper disc (6 mm in diameter) and then the diameter (mm) of the growth inhibition zone was measured. Each value represents the average of three independent experiments. PL, *Phellinus linteus*; CV, *Coriolus versicolor*; CM, *Cordyceps militaris*; AB, *Agaricus blazei*; IO, *Inonotus obliquus*; HE, *Hericium erinacium*; GL, *Ganoderma lucidum*; LE, *Lentinus edodes;* SC, *Sparassic crispa.* (-, bellow 6 mm; +, 6~12 mm; ++, 13~19 mm; +++, above 20 mm).

Table 3-3. Antibacterial activities of the liquid culture extracts of mushroom mycelium from the citrus extract media

Strain	*PL*		*CV*		*CM*		*AB*		*IO*		*HE*		*GL*		*LE*		*SC*	
	Inhibition zone (mm)																	
	Cocentration(mg/ml)																	
	10	20	10	20	10	20	10	20	10	20	10	20	10	20	10	20	10	20
V. harvey	+	++	+	++	+	++	+	++	+	++	+	++	+	++	+	+	++	+++
V. mimicus	+	++	+	++	+	+	+	+	+	+	+	++	+	+	+	+	+	+++
V. alginolyticus	+	++	+	++	+	+	+	+	+	+	+	++	+	+	+	++	++	++
V. vulnificus	+	++	+	++	-	+	+	+	+	++	+	++	+	++	+	++	+	++
V. parahaemolyticus	++	++	++	++	-	-	+	+	+	+	+	++	+	+	+	++	++	+++
V. campbelli	+	+	+	+	+	+	-	-	-	+	+	++	+	+	+	++	+++	+++
V. anguillarum	++	++	+	++	+	+	+	++	+	++	+	++	+	++	+	++	++	+++
V. pelagius	-	+	+	+	-	-	-	-	-	-	+	+	-	-	+	+	+	++
E. tarda	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	++
Streptococcus sp.	++	++	+	+	+	+	+	+	+	+	+	++	-	-	+	+	++	+++
S. iniae	-	+	+	++	+	+	-	-	+	+	-	-	-	+	-	+	++	++
S. parauberis	-	+	+	+	+	+	-	-	+	++	-	-	+	-	-	-	+	-

Cell were grown on MHA plate for 24h at 26, 37℃ after 10 mg, 20 mg each of mushroom liquid cultures was absorbed into paper disc (6 mm in diameter) and then the diameter (mm) of the growth inhibition zone was measured. Each value represents the average of three independent experiments. PL, *Phellinus linteus*; CV, *Coriolus versicolor*; CM, *Cordyceps militaris*; AB, *Agaricus blazei*; IO, *Inonotus obliquus*; HE, *Hericicum erinacium*; GL, *Ganoderma lucidum*; LE, *Lentinus edodes;* SC, *Sparassic crispa.* (-, bellow 6 mm; +, 6~12 mm; ++, 13~19 mm; +++, above 20 mm).

Table 3-4. Antibiotics resistance of *Vibrio* sp. and fishes disease bacteria

	P	E	N	K	C	NA	T
Streptococcus sp.	25	23	25	21	27	30	22
E. tarda	18	30	23	18	22	30	20
V.pelagius	20	30	24	19	20	30	35
V. campbelli	25	25	25	22	25	28	35
V. alginolyticus	25	21	20	19	24	30	20
V. parahaemolyticus	30	20	19	19	25	28	18
V. mimicus	32	22	24	20	25	31	20
V. anguillarum	30	22	20	21	28	30	15
V. harvey	32	24	21	20	30	27	20
V. vulnificus	24	20	20	16	26	26	18

P, penicillin; E, erythromycin; N, neomycin; K, kanamycin; C, chloramphenicol; NA, nalidixic acid; T, tetracycline.

Each value represents the average of three independent experiments.

3-2 Antioxidant activity

3-2-1 DPPH radical scavenging activity

Free radicals are produced continuously in cells either as by-products of metabolism or deliberately as in phagocytosis (Cheesman and Slater, 1993). DPPH is a free radical donor which has been widely used to test the free radical scavenging effect of natural antioxidants. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation.

The method of scavenging DPPH free radicals can be used to evaluate the antioxidant activity of specific compounds or extract in a short time. The use of the stable DPPH radicals has the advantage of being unaffected by side reactions, such as enzyme inhibition and metal chelation (Wettasinghe and Shahidi, 1999).

The MMCE cultured from synthesis media was shown to scavenge directly the stable DPPH radical over a concentration range of 1 mg/ml (4~75% inhibition, synthesis media) to 10 mg/ml (30~90% inhibition, synthesis media) (Fig. 3-2). MMCE cultured from citrus media was shown to scavenge directly the stable DPPH radical over a concentration range of 1 mg/ml (5~30% inhibition, citrus media) to 10 mg/ml (57~94% inhibition, citrus media) (Fig. 3-3). It seems that the scavenging abilities of the ethanol extracts concentration (1 and 5 mg/ml) from mycelium culture broth by synthesis media were relatively comparable, and more effective than those of the ethanol extracts concentration (1 and 5 mg/ml) from mycelium culture broth by citrus media. We was found that the best scavenging ability of the ethanol extract from *Sparassic crispa* mycelium culture broth was 94%. Scavenging abilities of ethanol extracts from other medicinal mushrooms in synthesis media were measured at up 5 mg/ml and were 90%, 40%, 60%, 55%, 44%, 62%, 18%, and 82% for *P. linteus*, *C. versicolor*, *C. militaris*, *A. blazei*, *I. obliquus*, *H. erinacium*, *G. lucicum*, and *L. edodes*, respectively. Scavenging abilities of ethanol extracts from other medicinal mushrooms in citrus media were measured at up 5 mg/ml and were 40%, 34%, 62%, 73%, 37%, 52%, 35%, and 52% for *P. linteus*, *C. versicolor*, *C. militaris*, *A. blazei*, *I. obliquus*, *H. erinacium*, *G. lucicum*, and *L. edodes*, respectively.

At 0.5 mg/ml, BHA and α-tocopherol showed excellent scavenging abilities of 92% and 90%, respectively (not shown data). Many reseachers have been reported positive correlation between free radical scavenging activities and total phenolics. Oki *et al.* (2002) observed that the radical scavenging activity increased with the increase of phenolic compound content. In this study, the MMCE from synthesis media showed higher free radical scavenging ability over the MMCE from citrus media (Fig. 3-2, 3-3).

From the earlier results, it was found that the ethanol extract of mushroom mycelium culture broth contained active substances, including phenolic compounds, that had a high hydrogen-donating capacity to scavenge DPPH radicals as a possible mechanism for their antioxidative activities. These activities of mushroom mycelium would provide one of pharmacological backgrounds for its use in folk medicine.

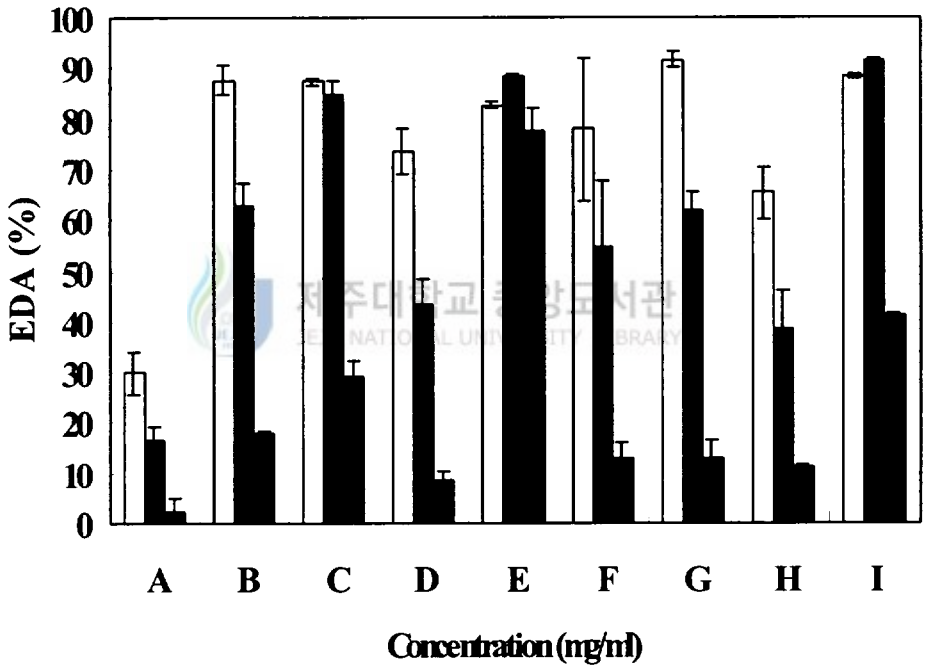

Fig. 3-2. Electron donating activities of extracts from mushroom mycelia cultured from synthesis media. A: *Ganoderma lucidum*, B: *Hericicum erinacium*, C: *Lentinus edodes* D: *Inonotus obliquus*, E: *Sparassic crispa*, F: *Agaricus blazei*, G: *Cordyceps militaris*, H: *Coriolus versicolor*, *I: Phellinus linteus*. □: 10mg/ml, ▦: 5mg/ml, ▥: 1mg/ml. Each value represents the average of three independent experiments.

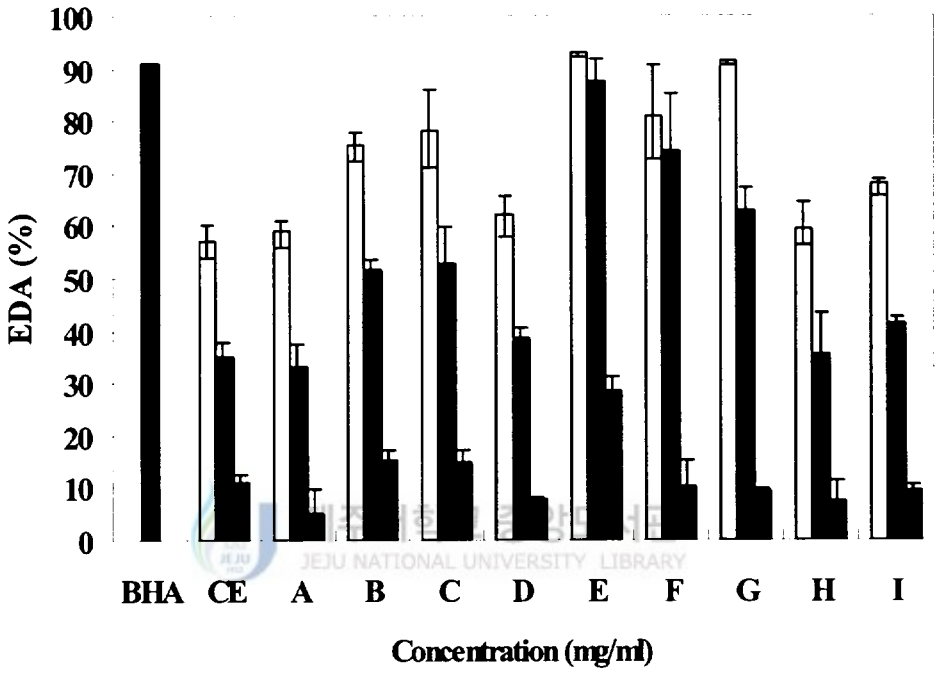

Fig. 3-3. Electron donating activities of extracts from mushroom mycelia cultured with citrus (10%) extracts. A: *Ganoderma lucidum,* B: *Hericicum erinacium,* C: *Lentinus edodes* D: *Inonotus obliquus,* E: *Sparassic crispa,* F: *Agaricus blazei,* G: *Cordyceps militaris,* H: *Coriolus versicolor,* I: *Phellinus linteus.* □: 10mg/ml, ▦: 5mg/ml, ▥: 1mg/ml. ■: BHA(butylated hydroxyanisole), CE: Citrus extract (Negative control). Each value represents the average of three independent experiments.

3-2-2 Superoxide radical scavenging ability

Superoxide and hydroxyl radicals are the two most effective representative free radicals. Superoxide anion (O_2^-) are formed in living cells during several biochemical reaction (Fridovich, 1974) and its effects can be magnified because it produces other kinds of free radicals and oxidizing agent inducing cell damage (Lui and Ng., 1999). The superoxide radical scavenging activity of the extracts was investigated (Fig. 3-4). In this study, the MMCE showed higher superoxide radical scavenging activity over the commercial antioxidants, such as BHA and BHT. All of the extracts showed dose dependent superoxide radical scavenging activity (not shown data). In particullar, the *Sparassic crispa* mycelium culture extract had the highest scavenging activity, which was higher than that of BHA. The superoxide radical scavenging activity of the MMCE was also generally quite high. This was especially the case for the E (*Sparassic crispa*) extract, whose activity was higher than that of BHA. It is well known that polyphenolic compounds are able to efficiently scavenge superoxide radicals (Valentao *et al* ., 2002). These phenolic compounds may react with the superoxide radical via a one-electron transfer mechanism or by a hydrogen abstration mechanism to form the corresponding semiquinone (Wang *et al.*, 1996). Futhermore, polyphenolic crude extracts are known to have a certain inhibitory activity towards xanthine oxidase.

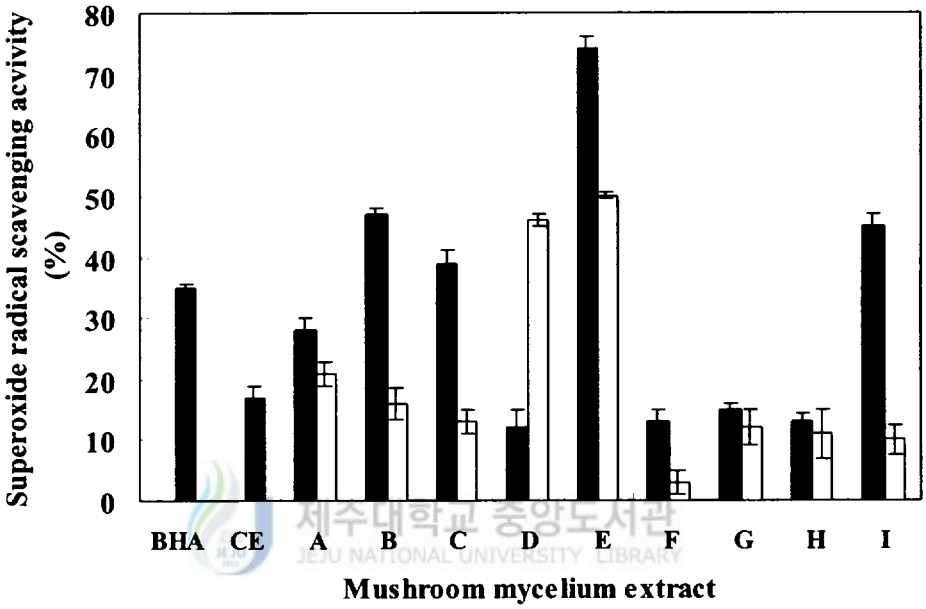

Fig. 3-4. SOD-like activity of the liquid culture extracts of mushroom mycelium from the synthetic liquid and the citrus extract media. A: *Ganoderma lucidum*, B: *Hericicum erinacium*, C: *Lentinus edodes* D: *Inonotus obliquus*, E: *Sparassic crispa*, F: *Agaricus blazei*, G: *Cordyceps militaris*, H: *Coriolus versicolor*, I: *Phellinus linteus*. ▪: liquid culture extracts of the synthetic media, □: liquid culture extracts of the citrus media. BHA(butylated hydroxyanisole), CE: Citrus extract (Negative control). Each value represents the average of three independent experiments.

3-2-3 Scavenging effect on hydroxyl free radical

The cell-damaging action of hydroxyl radical is the strongest among free radicals. Superoxide and hydroxyl radicals actively participate in the initiation of lipid oxidation (Lui and Ng, 2000). Hydroxyl radical scavenging activity of the MMCE was measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. All the ethanol extracts from mushroom mycelium culture broth showed a slight scavenging ability on hydroxyl radicals and this ability was concentration-dependent (Fig. 3-5, 3-6). The hydroxyl radical scavenging activities of some extractions were higher than all the commercial antioxidants (not shown data). However, the scavenging ability of BHA was 42% at 0.5 mg/ml. At 2 mg/ml, the MMCE from synthesis media scavenged hydroxyl radicals by 38~76% (Fig. 3-5) and the MMCE from citrus media scavenged hydroxyl radicals by 44~73% (Fig. 3-6). Hydroxyl radicals are known to be capable of abstracting hydrogen atoms from membranes and they bring about peroxidic reactions of lipids (Kitada *et al.*, 1979). Therefore, the strong hydroxyl radical scavenging activity of MMCE from synthesis and citrus media is an advantage due to the fact that the hydroxyl radical is an extremely reactive oxidant radical that will react with most biomolecules at diffusion controlled rates (Cheeseman and Slater, 1993).

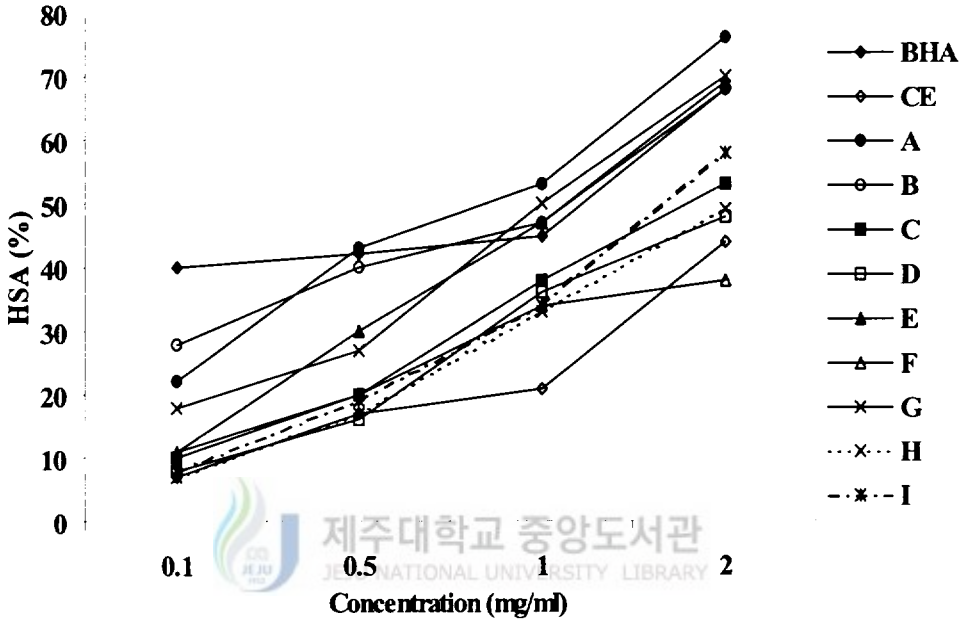

Fig. 3-5. Hydroxyl radical scavenging activity of the liquid culture extracts of mushroom mycelium from the synthetic liquid. A: *Ganoderma lucidum*, B: *Hericicum erinacium*, C: *Lentinus edodes* D: *Inonotus obliquus*, E: *Sparassic crispa*, F: *Agaricus blazei*, G: *Cordyceps militaris*, H: *Coriolus versicolor*, I: *Phellinus linteus*, CE: Citrus extract (Negative control). Each value represents the average of three independent experiments.

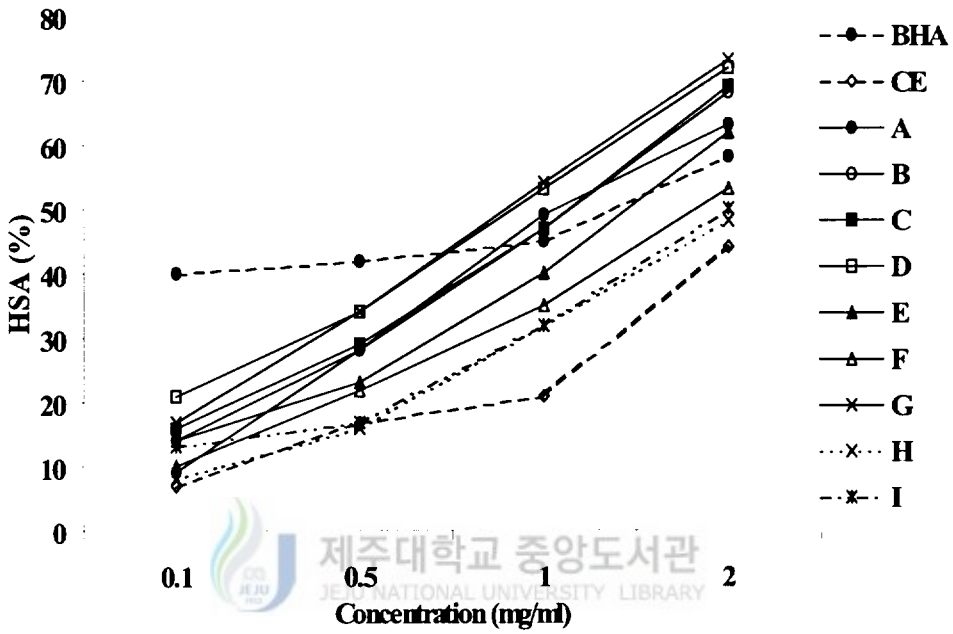

Fig. 3-6. Hydroxyl radical scavenging activity of the liquid culture extracts of mushroom mycelium from the citrus extract media. A: *Ganoderma lucidum*, B: *Hericicum erinacium*, C: *Lentinus edodes* D: *Inonotus obliquus*, E: *Sparassic crispa*, F: *Agaricus blazei*, G: *Cordyceps militaris*, H: *Coriolus versicolor*, I: *Phellinus linteus*, CE: Citrus extract (Negative control). Each value represents the average of three independent experiments.

CONCLUSION

The results of our experiments showed that the sterile, mycelium-free culture fluid fermented by the mycelial biomass in a submerged culture had a similar effect. The culture fluid was less toxic to human tissue culture cells than to microbes. From our results, the antibacterial and antioxidant activity of the mushroom mycelium cultural extract (MMCE) was concentration dependent, with stronger inhibition of radical scavenging ability occurring ay higher concentrations of the extracts in most cases. The culture extracts (from the synthetic and citrus extracts medium) showed high activity against *Vibrio* sp. and had poor effect on *Streptococcus* sp., *S. parauberis*, *S. iniae*. Whereas the culture extracts (5 mg/ml) obtained from the synthetic medium and the un-inoculated citrus extracts showed about 18~92 and 34% of the DPPH radical scavenger activity, the culture extracts obtained from the citrus extracts medium exhibited antioxidant activity about 33~89%. The Superoxide radical scavenging activity of the culture extracts (1mg/ml) obtained from the citrus extracts, synthetic liquid and citrus extract medium were about 18, 12~75, and 4~50%, respectively. The hydroxyl radical scavenging activity of the culture extracts (1mg/ml) obtained from the citrus extracts, synthetic liquid and citrus extract medium were about 21, 33~47, and 32~53%, respectively. The results clearly showed that the fermentation of mushroom mycelium in the citrus and in the defined synthetic medium similarly the antibacterial and antioxidant activity. However, there is no study so far concerning the characterization of the active antioxidative components in mushroom mycelium culture liquid. Future work on the isolation and structural characterization of the active components is needed. Also, the antioxidant activity of these components, in regard to the mechanisms for radical, hydroxyl, and superoxide radical scavenging activity, will be the primary objective of further investigation.

SUMMARY

It is well known that the O_2 molecule has low reactivity and its toxicity stems mostly from its excited state (singlet oxygen) or from its semi-reduced radical forms, which can cause deleterious or lethal oxidative damage to the cells. The four electron reduction of O_2 to H_2O_2 gives rise successively to the formation of the reactive oxygen intermediates superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^·$). Several models have been proposed to explain the mechanism of O_2 sensitivity in micro-organisms. O_2^- and H_2O_2 are moderately reactive in aqueous solutions but they both contribute to the formation of the highly reactive oxidant $HO^·$ via the Fenton and Haber-Weiss reactions. In bacteria, generation of the $HO^·$ radicals is facilitated by the presence of O_2^-, H2O2 and free iron. Cellular components such as hemoproteins, lipids and DNA are targets for $HO^·$ formed by a site-specific Fenton-driven mechanism. Therefore, its removal is required to ensure cellular homeostasis. Many microorganisms posses enzymatic and non-enzymatic antioxidative mechanisms and minimize generation of reactive oxygen species (ROS) to levels that are harmful to the cells. These antioxidative mechanisms of bacteria are well studied in *Escherichia coli* and *Salmonella typhimurium*.

Special geographical conditions in Jeju coastal provide optimum environmental conditions for a variety of marine species. Different compositions of bioactive materials can be expected in marine sources, comparing to the land plants, because marine bacteria are growing under quite different environmental conditions over the land sources.

The objective of this study was to develop bio-active, antibacterial and beneficial natural antioxidants from the marine bacteria and mushroom mycelium (fungus) culture liquid. Guided by the screening method with DPPH, we obtained a hundred sixteen antioxidants producing bacterial isolated from the sea water and animal sources. Based on our phenotypic, phylogenetic,

and fatty acid characterization strain SC2-1 and S-1 (actinomyces) was identified as a member of the species *Exiguobacterium* sp. SC2-1 and *Nocardiopsis dassonvillei* S-1 respectively. The potential antioxidant activity of isolated two strains (SC2-1, S-1) has been assessed based on two different assay methods and compared with BHA and BHT. It might be explained by stable radical (DPPH) scavenging effect and by weak hydroxyl radical scavenging effect. Additionally, culture supernatant appeared to weak activity of the inhibit superoxide radical scavenging ability. Also, the antioxidant activity from bacterial culture broth showed strong activity against DPPH solution in nutritional optimum condition.

The antibacterial and antioxidant activity of the fungus (mushroom mycelium) cultural extract (MMCE) was concentration dependent, with stronger inhibition of DPPH free radical, hydroxyl radical, superoxide radical scavenging ability occurring ay higher concentrations of the extracts in most cases.

The results of this study demonstrate the antioxidative potential of marine bacterial, actinomycetes and fungus. Further work is required for identification and purification of antioxidative compounds from bacterial cultural supernatant. So suggest these strain are useful for functional fermented food, medicine industry and probiotics.

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대학에 들어와서 학문이란 분야를 이렇게 까지 열심히 할 줄은 꿈에도 몰랐었던 나에게 지금까지의 길을 걸을 수 있게 많은 격려와 조언을 아끼지 않으시고 지도하여 주신 허문수 교수님에게 고개 숙여 깊은 감사를 드립니다. 또한 늘 부족했던 저에게 용기와 힘을 주셨던 부산대학교 박근태 교수님에게 깊은 감사의 말씀을 드립니다. 그리고 늘상 바쁘신 일정에도 아랑곳 하지 않고 시간을 할애하여 저의 논문의 심사와 수정을 도와주셨고 항상 조언을 아끼지 않으신 전유진 교수님과 여인규 교수님에게 다시금 감사드립니다. 그리고 학부생부터 지금까지 많은 가르침을 주신 송춘복 교수님, 이재희 교수님에게도 깊은 감사의 말씀을 드립니다.

대학원생 생활을 하면서 항상 옆에서 버팀목이 되어주시고, 관심을 아끼지 않았던 제주지방 해양청에 진창남 지도관님, 국립수산물검역소의 고대희 계장님, 해양수산자원연구소의 양병규 연구사님 그리고 인생선배로서 저에게 형 노릇을 해주었던 영환이형에게 진심으로 감사의 말씀을 드리며, 실험실 생활을 하는 동안 나의 모든 불만을 너그럽게 받아주셨던 용욱이형, 철영이형과 친구이자 동반자 같은 영건이형, 아무런 내색 없이 친 동생 처럼 잘 따라주었던 태원, 주상, 수미, 현식, 상혁, 선경, 봉근, 승현에게 이 글을 빌어 진심으로 감사의 마음을 전합니다. 또한 유전 육종실험실원, 분자유전학실험실원, 해양자원이용공학 실험실원, 어류분자생리학 실험실원, 해양 생물공학과 외국인 유학생들에게도 감사 드립니다.

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정말로 많은 분들의 도움으로 제가 이 자리에 올라올 수 있었던 것 같습니다. 알게 모르게 많은 도움을 주신 분들에 이 글을 빌어 감사의 말씀을 드립니다.

끝으로 오늘날에 제가 있기까지 보살펴주시고, 걱정해주시고, 믿어주신 사랑하는 어머니, 아버지 그리고 사랑하는 나의 동생들과 믿음으로 저를 성원해준 친구들에게 깊은 감사에 마음을 전합니다.