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Tyrosinase inhibitory and antioxidative effects of lactic acid bacteria, isolated from dairy cow feces

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(젖소의 분변으로부터 분리한 유산균의 Tyrosinase 억제 및 항산화 효과)

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Abstract

Overproduction and accumulation of melanin pigments cause a number of skin diseases. Reactive oxygen species (ROS), generated by metabolic intermediates in melanin biosynthesis, cause various diseases including cancer and heart diseases. Therefore, both tyrosinase inhibitors and anti-oxidants are important for the treatments of diseases, occurred by melanins accumulation and ROS. This study was aimed at the tyrosinase inhibitory and antioxidative effects of four different lactic acid bacteria (LAB) strains, isolated from dairy cow feces. We have isolated four different LAB strains designated as *Enterococcus* sp. EA3, *Enterococcus* sp. EB2, *Pediococcus* sp. PC2, and *Pediococcus* sp. PD3. To investigate optimal culture conditions of isolated LAB strains, we have performed the measurements of tyrosinase inhibitory and antioxidative activities against growth times (hour), temperatures (℃), pH, and NaCl concentrations (%) using culture filtrates of LAB. The tyrosinase inhibitory activities of LAB showed that

Enterococcus sp. EA3 was about 65% at its optimum culture condition (14 hour, 30℃, pH 8 and 0% NaCl), *Enterococcus* sp. EB2 about 65% (12 hour, 30℃, pH 9 and 0% NaCl), *Pediococcus* sp. PC2 about 80% (20 hour, 30℃, pH 6 and 0% NaCl), and *Pediococcus* sp. PD3 about 80% (20 hour, 30℃, pH 8 and 0% NaCl), respectively. In addition, anti-oxidative activities of four different LAB strains showed approximately more than 30% at optimal conditions. From the results, the culture filtrates obtained from four LAB strains can be a potential agent for developing antioxidants and inhibitors for tyrosinase. Furthermore, this study will be applied for developing skin care cosmetics.

1. Introduction

Tyrosinase is a key enzyme in mammalian melanogenesis, which is widely distributed in microorganisms, animals, and plants [1, 2]. The melanin pigment is responsible for the enzymatic browning of fresh fruits and vegetables [17]. In biosynthetic pathway for melanin formation as shown in Fig 1, tyrosinase converts tyrosine to 3,4-dihydroxyphenylalanine (Dopa or o-diphenol) and oxidizes L-dopa to form dopaquinone (o-quinones). The dopaquinone is transformed ultimately to melanin pigment in melanin biosynthesis pathway [3, 4, 5]. The melanin pigment, the end product of melanogenesis, is commonly responsible for skin color and protects the human skin cells from UV-B radiation damage, reducing the risk of cancer [14, 15, 16]. However, overproduction and accumulation of melanin pigments cause a number of skin diseases, which include hyperpigmentation, such as melasma, freckle, solar lentigo, etc [13]. Therefore, inhibitors for melanogenesis are important for the treatment of skin diseases associated with hyper-pigmentation and then will be applied in cosmetics for whitening and de-pigmentation after UV exposure [6].

The process of melanin biosynthesis pathway also produces free radicals including such as hydrogen peroxide (H_2O_2), and other reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) are generated by chemical substances and metabolic pathways in human and food. Reactive oxygen species (ROS) can cause great damage to cell membranes including membrane lipid peroxidation, resulting in membrane fluidity. Also, ROS causes DNA mutations leading to cancer, degenerative and other diseases [18, 19, 20]. Consequently, antioxidant compounds that stabilize free radicals including ROS play important roles in the prevention of diseases generated by free radicals.

Recently, lactic acid bacteria (LAB) have been isolated from various environments and fermented food [7, 8, 9]. LAB play an important role in various fermented food to prevent the growth of harmful bacteria by producing organic

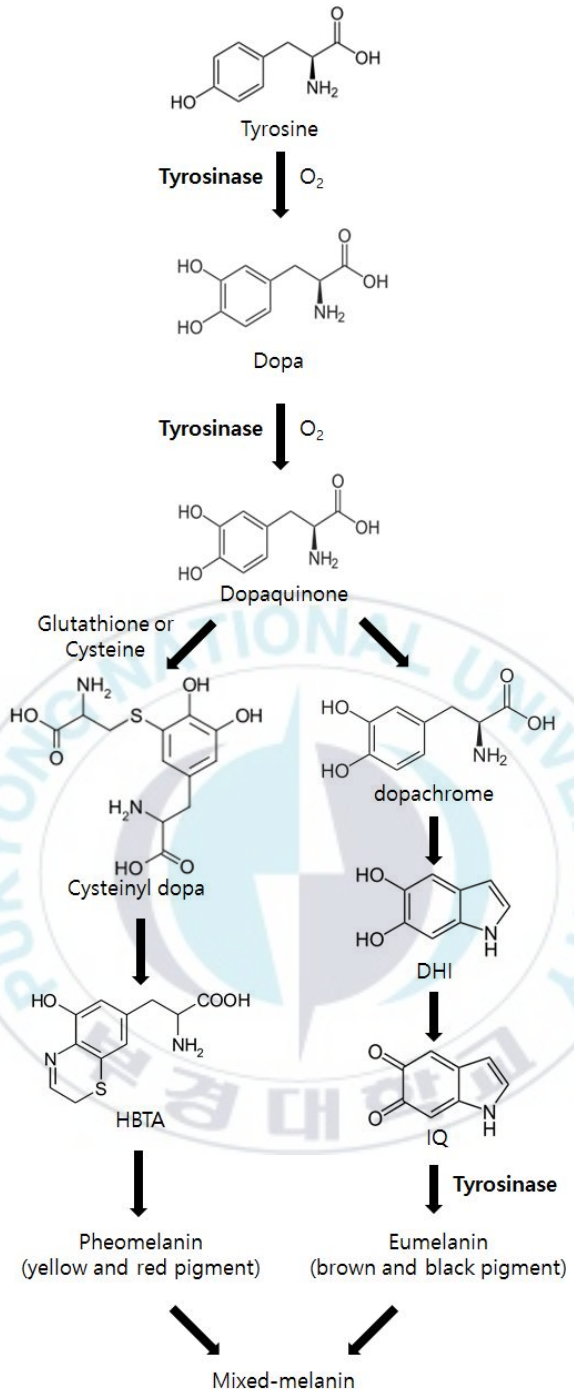

Figure 1. Melanogenesis pathway. Dopa, 3, 4-dihydroxyphenylalanine; HBTA, 5-hydroxy-1, 4-benzothiazinylalanine; DHI, 5, 6-dihydroxyindole; IQ, Indole- 5, 6-quinone.

acids and antimicrobial substances [10, 11, 12].

Previous researchers reported various tyrosinase inhibitors and antioxidants from plants or synthetic sources [1, 4]. However, there were not much data on both tyrosinase inhibition and antioxidant activities of bacterial culture filtrate. In this report, we have isolated four different LAB strains from daily cow feces and the culture filtrates showed both tyrosinase inhibitory and antioxidant effects. Optimal culture conditions for tyrosinase inhibitory and antioxidant effects were investigated through different culture conditions. In addition, we have characterized and identified isolated four LAB strains performed by 16s rRNA gene analysis and biochemical studies. Results from the present study will be provided advantages for the developing cosmetics and therapeutic agents.

2. Materials and methods

2.1 Isolation of bacterial strains.

All strains were isolated from dairy cow feces samples collected from a cow farm of Hallim-myeon, Gimhae, Korea. The samples were closed immediately in sterile 50ml corning tubes and stored in the ice box. Each sample was diluted with sterilized phosphate-buffer saline (PBS) in 10-fold serial dilution and a 100 μl aliquot of diluted samples plated on Man Rogoda Sharp (MRS) agar (Difco, Detroit, MI, USA) overnight at 30℃. After cultivation, a single colony in MRS agar medium was subcultured in a fresh medium for single-colony isolation. For sustainable usage of strains for further study, all strains were stored in MRS medium containing 40% glycerol at -80℃ deep freezer.

2.2 Identification of bacterial strains.

In order to analyze bacterial 16S rRNA gene of the strains, genomic DNA extraction was performed by Primeprep genomic DNA isolation kit (GeNet Bio, Korea). These genomic DNA preparations were used as template DNA in the 16S rDNA targeting PCR. The 16S rRNA gene was amplified in the PCR with universal primers to conserved regions of the 16S rRNA genes. The nucleotide sequences of the primers are as follow. the primer 27F (Forward primer) was 5'-AGAGTTTGATCMTGGCTCAG-3'; and the primer 1492R (Reverse primer) was 5'-TACGGYTACCTTGTTACGACTT-3'. PCR amplification was performed with GeneAmp PCR System 9700 (Applied Biosystems, USA). 10μl of PCR premix (GeNet Bio, Korea) was mixed with 2μl of purified genomic DNA for DNA template, 1μl of 10 pmol forward primer (27F), 1μl of 10 pmol reverse primer (1492R) and 6μl of sterilized water. The main PCR condition consisted of 30 cycles at 94℃ for 30 sec, 6

2℃ for 30sec, and 72℃ for 90sec. After the PCR reaction, PCR products were analyzed right away by electrophoresis in 1% (w/v) agarose gels and then by ethidium bromide (2mg/ml) staining. PCR product sizes were compared with 100bp DNA ladder (Bioneer, Korea). Appropriated DNA bands about 1600bp were purified from the gel with Primeprep gel purification kit (GeNet Bio, Korea). For cloning 16S rRNA genes of bacterial strains, the amplified 16S rRNA genes were ligated into pGEM T-easy Vector (Promega, USA). The ligation mixture was transformed into competent cells which was *Escherichia coli* strain XL1-blue. Transformed colonies were selected and cultured in LB broth containing 100 mg/ml ampicillin.

The 16S rRNA gene sequences were identified by the BLAST program of the GenBank database. A phylogenetic tree was constructed by the neighbor-joining (NJ) method with the Molecular Evolutionary Genetics Analysis version 5 (MEGA5) using the 16S rDNA sequences of related bacterial strains. The biochemical tests of all strains were performed by API 20 Strep kit (BioMerieux, France). To investigate the enzyme activities of bacterial strains, API ZYM kit (BioMerieux, France) was used.

2.3 Tyrosinase inhibitory activity assay.

Tyrosinase inhibitory activities of bacterial culture filtrates were determined by spectrophotometry. The procedure was described by Pomerantz *et al* with some modifications [21]. For breaking the cell walls, samples were treated by sonicator, and then centrifugation (10,000 rpm/ 10min) and the supernatant was passed through a cellulose acetate filter (0.45 μm). This culture filtrate was used in further assays. The reaction mixture consisted of 150 μl of 150 unit/ml mushroom tyrosinase (Sigma, Saint Louis, MO, USA), 225 μl of 2.5 mM L-tyrosine (Junsei, Japan), 225 μl of 0.4 M hepes buffer (pH 6.8), 300μl of the culture filtrate. 300μl of 0.4 M hepes buffer was used as a

negative control instead of culture filtrate. Before incubation, it was measured by spectrophotometer at 475 nm absorbance. Then the mixture was incubated at 30℃ for 15 min. After incubation, it was measured again by spectrophotometer at same conditions. It was carried out in triplicate and the calculation of tyrosinase inhibitory activity was determined as follows. In this formula, A and B was absorbance of the sample before reaction and after reaction of the mixture, respectively. C and D was absorbance of blank (0.4 M hepes buffer instead of the sample) before reaction and after reaction of the mixture, respectively.

$$Tyrosinase\ Inhibition\ (\%) = \frac{(D-C)-(B-A)}{(D-C)} \times 100$$

2.4 DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activity assay.

The anti-oxidation effect on DPPH radical was estimated according to the procedure described by Dietz *et al* with some modifications [22]. Reaction mixtures containing 80 μl of test samples (culture filtrates) or ethanol (negative control) and 200 μl of 0.4 mM 2, 2-diphenyl-1-picrylhydrazyl (Wako, Japan) in ethanol were incubated at room temperature (25℃) for 30 minutes. After incubation, absorbances at 517 nm were measured by VERSA max microplate reader (Molecular Devices, USA) in triplicate. The calculation of DPPH radical scavenging activity was calculated with this formula.

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{control\ O.D. - sample\ O.D.}{control\ O.D.} \times 100$$

2.5 ABTS (2, 2-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity assay.

The anti-oxidant activity with ABTS decolorization assay was estimated according to the procedure described by Roberta Re *et al* [23]. A test solution of ABTS decolorization assay was prepared by mixing 2.45 mM potassium persulfate (Duksan, Korea) and 7 mM ABTS (Biosesang, Korea) and incubated in the dark at room temperature for 16 hours. Before ABTS radical scavenging test, the ABTS test solution was diluted with water to the absorbance of 1.0 (±0.02) at 734 nm. 1 ml of diluted ABTS test solution was added to 10 μl of water or test sample (culture filtrate). After initial mixing, the absorbance reading at 734 nm was taken exactly 1 min and up to 6 min. These tests were carried out in triplicate. The activity was calculated by the following a formula.

$$ABTS radical scavenging activity\,(\%) = \frac{control\ O.D. - sample\ O.D.}{control\ O.D.} \times 100$$

2.6 Superoxide dismutase (SOD)-like activity assay.

Superoxide dismutase (SOD)-like activities of test samples (culture filtrates) were determined by Marklund, S., and Marklund, G. with some modifications [24]. Pyrogallol was generally used for SOD-like activity assay. Because of auto-oxidation of pyrogallol, it is a convenient method for investigating the SOD-like activity. In this experiment, pyrogallol stock solution dissolved in 10 mM HCl is stable for weeks. The mixture containing 2.6 ml of 50 mM Tris-HCl buffer (10 mM EDTA, pH 8.5), 0.2 ml of 7.2 mM pyrogallol (Junsei, Japan) diluted from stock solution and 0.2 ml of water or test sample was incubated at room temperature for 10 min. At the end of the reaction, 0.1 ml of 1 N HCl was added to the mixture for termination of the reaction. And then absorbance

reading was performed by spectrophotometer at 420 nm. Tests were carried out in triplicate. The activity was calculated by the following a formula.

$$SOD\ like\ activity\,(\%) = \frac{control\ O.D. - sample\ O.D}{control\ O.D} \times 100$$

2.7 Tyrosinase inhibitory and antioxidative activities according to culture times.

All bacterial strains were incubated in different cultivation conditions at various culture times. The tyrosinase inhibitory activities of bacterial culture filtrates in different culture times were determined with the previously-described method. Additionally, anti-oxidative activities of bacterial culture filtrates in different culture times were determined with the previously-described methods. The culture times for growth were 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hour, respectively. The temperature, pH, and NaCl concentration was 30℃, pH 7, and 0 %. All measurements were performed in triplicate.

2.8 Tyrosinase inhibitory and antioxidative activities according to temperatures.

All bacterial strains were incubated in different cultivation conditions at various temperatures. The tyrosinase inhibitory activities of bacterial culture filtrates in different temperatures were determined with the previously-described method. Additionally, anti-oxidative activities of bacterial culture filtrates in different temperatures were determined with the previously-described methods. The temperatures for growth were 4, 15, 20, 25, 30, 37, and 42 ℃, respectively. The culture time, pH, and NaCl concentration was 14 hour (EA3 and EB2) or 20 hour (PC2 and PD3), pH 7, and 0 %. All measurements were performed in triplicate.

2.9 Tyrosinase inhibitory and antioxidative activities according to pH.

All bacterial strains were incubated in different cultivation conditions at various pH. The tyrosinase inhibitory activities of bacterial culture filtrates in different pH were determined with the previously-described method. Additionally, anti-oxidative activities of bacterial culture filtrates in different pH were determined with the previously-described methods. The pH for growth were 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13, respectively. The culture time, temperature, and NaCl concentration was 14 hour (EA3 and EB2) or 20 hour (PC2 and PD3), 30 ℃, and 0 %. All measurements were performed in triplicate.

2.10 Tyrosinase inhibitory and antioxidative activities according to NaCl concentrations.

All bacterial strains were incubated in different cultivation conditions at various NaCl concentrations. The tyrosinase inhibitory activities of bacterial culture filtrates in different NaCl concentrations were determined with the previously-described method. Additionally, anti-oxidative activities of bacterial culture filtrates in different NaCl concentrations were determined with the previously-described methods. The NaCl concentrations for growth were 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 %, respectively. The culture time, temperature, and pH was 14 hour (EA3 and EB2) or 20 hour (PC2 and PD3), 30 ℃, and pH 7. All measurements were performed in triplicate.

3. Results

3.1 Isolation and identification of bacterial strains.

All strains were isolated from the dairy cow feces from the cow farm at Gimhae, Korea. Four strains showing more than 40% of tyrosinase inhibition activity were selected for the usage in further analysis among isolated 26 strains. The selected strains were designated as EA3, EB2, PC2, and PD3, respectively. All of the isolates were Gram-positive with cocci shape.

We have identified isolated four LAB strains by 16S RNA gene analysis. Phylogenetic trees based on their 16S rRNA gene sequences were constructed using neighbor-joining analysis. Both EA3 and EB2 were identified as belonging to the genus *Enterococcus* with more than 98% nucleotide homology. Both PC2 and PD3 were identified as belonging to the genus *Pediococcus* with more than 99% nucleotide homology.

The physiological characteristics of isolates were performed for the biochemiacal data by API 20 Strep kit (biomerieux, France). The results of biochemical tests are given in Table 1 and 2. With comparisons to other species in the same genus, *Enterococcus* sp. strains EA3 and EA2 were distinguished from other *Enterococcus* type strains. And *Pediococcus* sp. strain PC2 and PD3 were distinguished from other *Pediococcus* strains. In addition, enzyme activities of all strains were performed by API ZYM kit (biomerieux, France). The result of enzyme activities are given in Table 3.

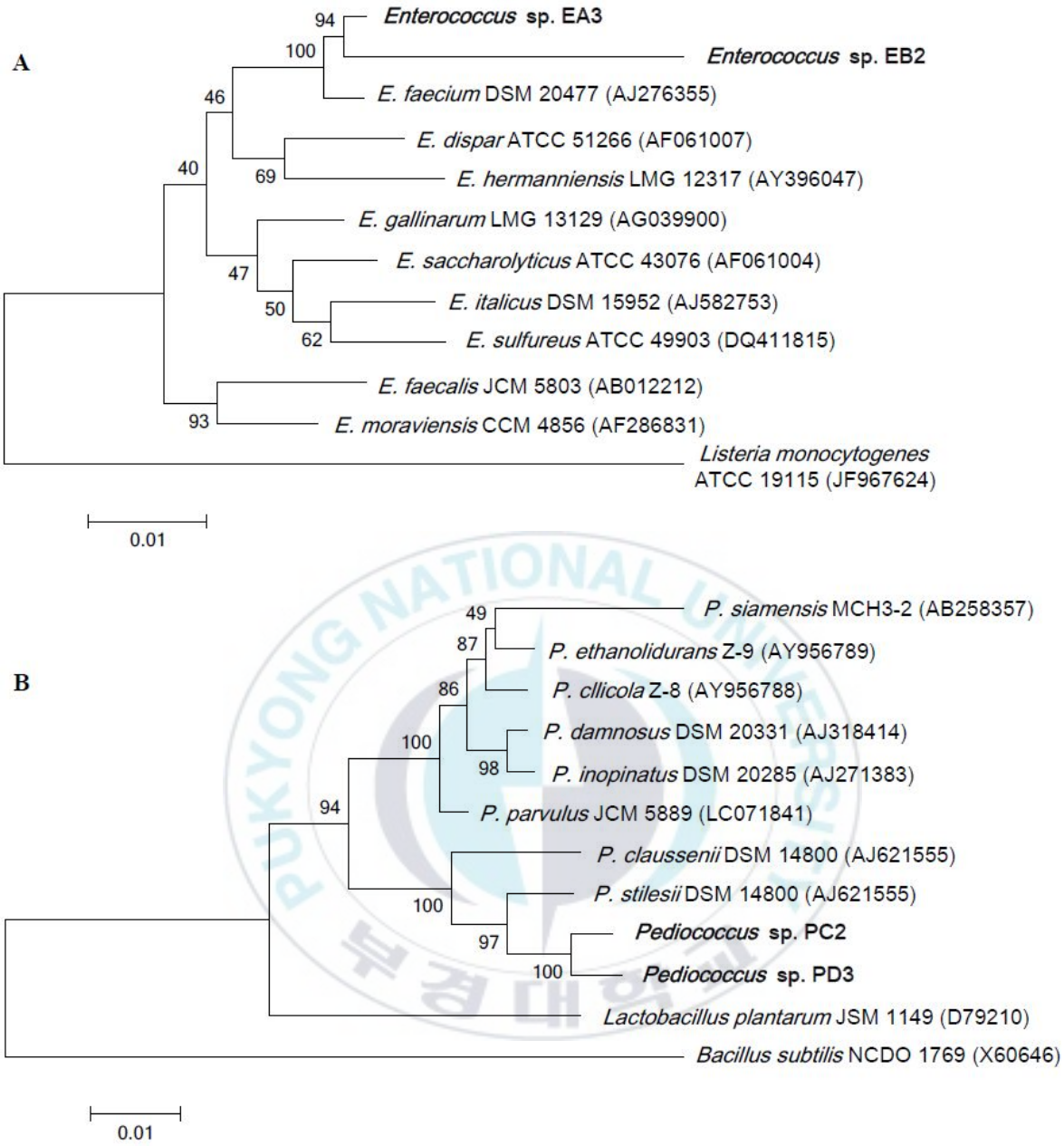

Figure 2. Neighbour-joining trees of four LAB strains. A was based on 16s rRNA gene sequences of *Enterococcus* sp. strains EA3, EB2, and all *Enterococcus* type strains. B was based on 16s rRNA gene sequences of *Pediococcus* sp. strains PC2, PD3, and related *Pediococcus* strains. Bootstrap values based upon 1000 replicates are included at the branch points. Bar, 0.01 nucleotide substitutions per site.

Table 1. Differential characteristics of *Enterococcus* sp. strains EA3, EB2, and *Enterococcus faecium*.

Characteristic		EA3	EB2	*E. faecium*
Voges-Proskauer		+	+	*NA
Hippurate hydrolysis		−	−	+
Aesculin hydrolysis		−	−	NA
Pyrrolidonylarylamidase		+	+	NA
Arginine dihydrolase		−	−	+
Hydrolysis of	Ribose	+	+	NA
	Arabinose	−	−	+
	Mannitol	−	−	+
	Sorbitol	−	−	−
	Lactose	+	+	NA
	Trehalose	+	+	NA
	Inulin	−	−	−
	Raffinose	−	−	−
	Starch	−	−	NA
	Glycogen	−	−	−

*NA: not available.

Table 2. Differential characteristics of *Pediococcus* sp. strains PC2, PD3, and *Pediococcus acidilactici*.

Characteristic		PC2	PD3	*P. acidilactici*
Voges-Proskauer		+	+	+
Hippurate hydrolysis		−	−	+
Aesculin hydrolysis		−	−	*NA
Pyrrolidonylarylamidase		+	+	−
Arginine dihydrolase		−	−	NA
Hydrolysis of	Ribose	+	+	+
	Arabinose	−	−	+
	Mannitol	−	−	−
	Sorbitol	−	−	−
	Lactose	+	+	−
	Trehalose	+	+	−
	Inulin	−	−	NA
	Raffinose	−	−	−
	Starch	−	−	NA
	Glycogen	−	−	NA

*NA: not available.

Table 3. Enzyme activities of *Enterococcus* sp. strains EA3, EB2 and *Pediococcus* sp. strains PC2, PD3.

Enzyme	EA3	EB2	PC2	PD3
Control	0	0	0	0
Alkanlin phosphatase	0	0	0	0
Esterase (C4)	5	5	1	0
Esterase lipase (C8)	4	4	2	1
Lipase (C14)	0	0	3	0
Leucine arylamidase	4	5	4	4
Crystine arylamidase	2	3	4	5
Trypsin	0	0	1	0
α-Chymotrypsin	0	2	0	1
Acid phosphatase	5	4	3	4
Naphtol-AS-BI-phosphohydrolase	4	5	4	4
α-Galactosidase	0	0	0	0
β-Galactosidase	0	0	2	3
β-Glucuronidase	0	0	0	0
α-Glucosidase	0	0	0	0
β-Glucosidase	0	0	4	1
N-Acetyl-β-glucosamidase	1	0	3	2
α-Mannosidase	0	0	0	0
α-Fucosidase	0	0	0	0

The scale of API ZYM test was used for enzyme quantification, with 1 corresponding to 5 nmol substrate metabolized, 2 to 10 nmol, 3 to 20 nmol, and 5 to 40 nmol.

3.2 Tyrosinase inhibitory activities and cell growth by various culture conditions.

3.2.1 Tyrosinase inhibitory activities and cell growth by growth times.

Tyrosinase inhibitors are chemical agents capable of reducing enzymatic reactions, such as melanin synthesis of human skin and food browning. In this study, tyrosinase inhibitory activities of four LAB strains culture filtrates were measured by triplicate. The mushroom tyrosinase inhibitory activities for 12, 14, 16 and 18 hour of *Enterococcus* sp. EA3 culture filtrates in growth times were 61.14 %, 67.11 %, 55.06 %, and 47.26 %, respectively. And cell growth of *Enterococcus* sp. EA3 showed in Figure 3A. The mushroom tyrosinase inhibitory activities for 12, 14, 16 and 18 hour of *Enterococcus* sp. EB2 culture filtrates in growth times were 64.93 %, 61.40 %, 41.95 %, and 41.44 %, respectively. And cell growth of *Enterococcus* sp. EB2 showed in Figure 3B. The mushroom tyrosinase inhibitory activities for 16, 18, 20 and 22 hour of *Pediococcus* sp. PC2 culture filtrates in growth times were 68.59 %, 75.34 %, 82.52 %, and 77.68 %, respectively. And cell growth of *Pediococcus* sp. PC2 showed in Figure 4A. The mushroom tyrosinase inhibitory activities for 16, 18, 20 and 22 hour of *Pediococcus* sp. PD3 culture filtrates in growth times were 75.26 %, 76.22 %, 82.41 %, and 65.52 %, respectively. And cell growth of *Pediococcus* sp. PD3 showed in Figure 4B. The maximum tyrosinase inhibitory activity was shown at 20 hour in PC2 strain.

3.2.2 Tyrosinase inhibitory activities and cell growth by temperatures.

The mushroom tyrosinase inhibitory activities for 25, 30 and 37℃ of *Enterococcus* sp. EA3 culture filtrates were 62.40 %, 66.74 %, and 64.96 %, respectively. And cell growth of *Enterococcus* sp. EA3 by temperatures showed in Figure 5A. The mushroom tyrosinase inhibitory activities for 25, 30, and 37℃ of *Enterococcus* sp. EB2 culture filtrates were 64.21 %, 67.61 %, and 62.08 %, respectively. And cell growth of *Enterococcus* sp. EB2 by temperatures showed in Figure 5B. The mushroom tyrosinase inhibitory activities for 25, 30, and 37℃ of *Pediococcus* sp. PC2 culture filtrates were

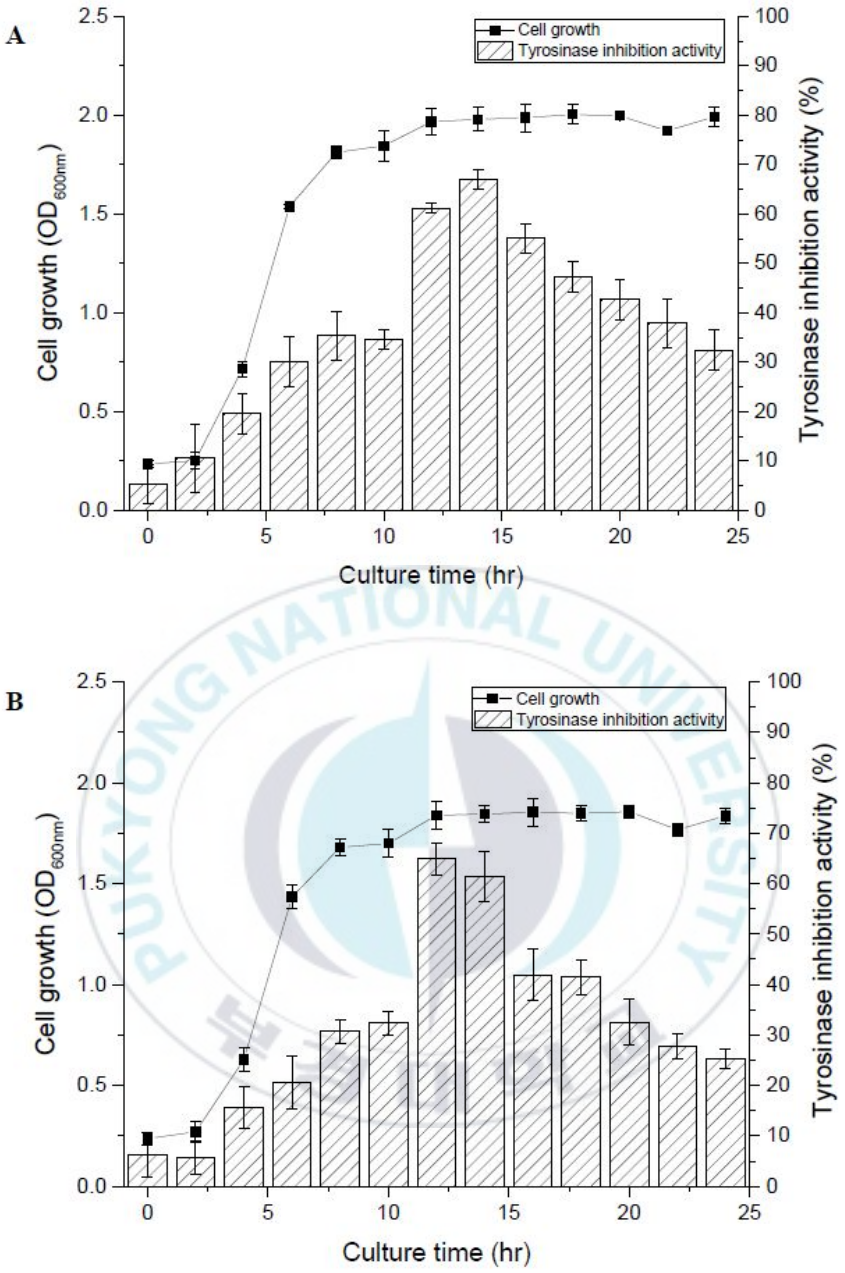

Figure 3. Tyrosinase inhibitory activities and cell growth of *Enterococcus* sp. strain EA3 (A) and EB2 (B) by growth times. The samples were collected at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hour, respectively.

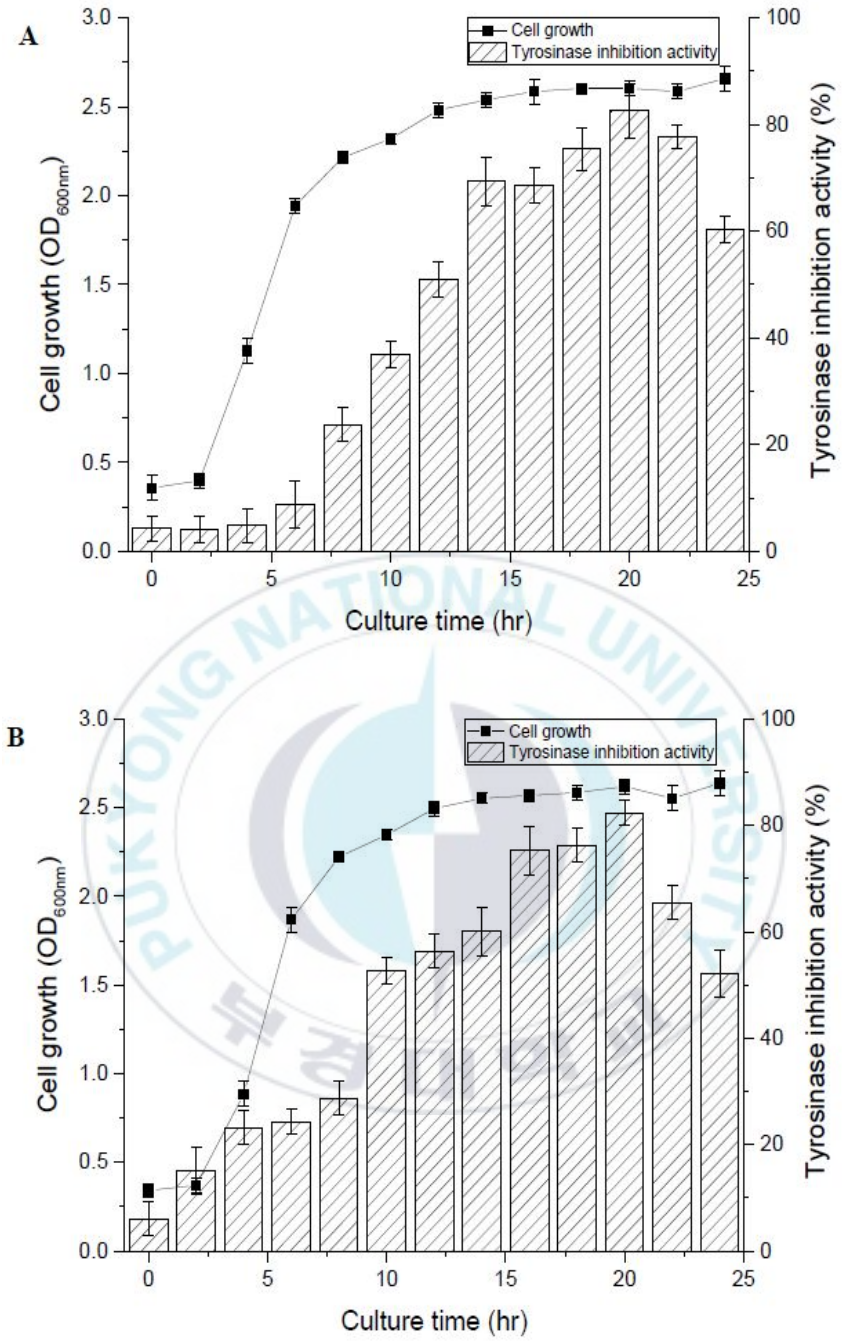

Figure 4. Tyrosinase inhibitory activities and cell growth of *Pediococcus* sp. strain PC2 (A) and PD3 (B) by growth times. The samples were collected at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hour, respectively.

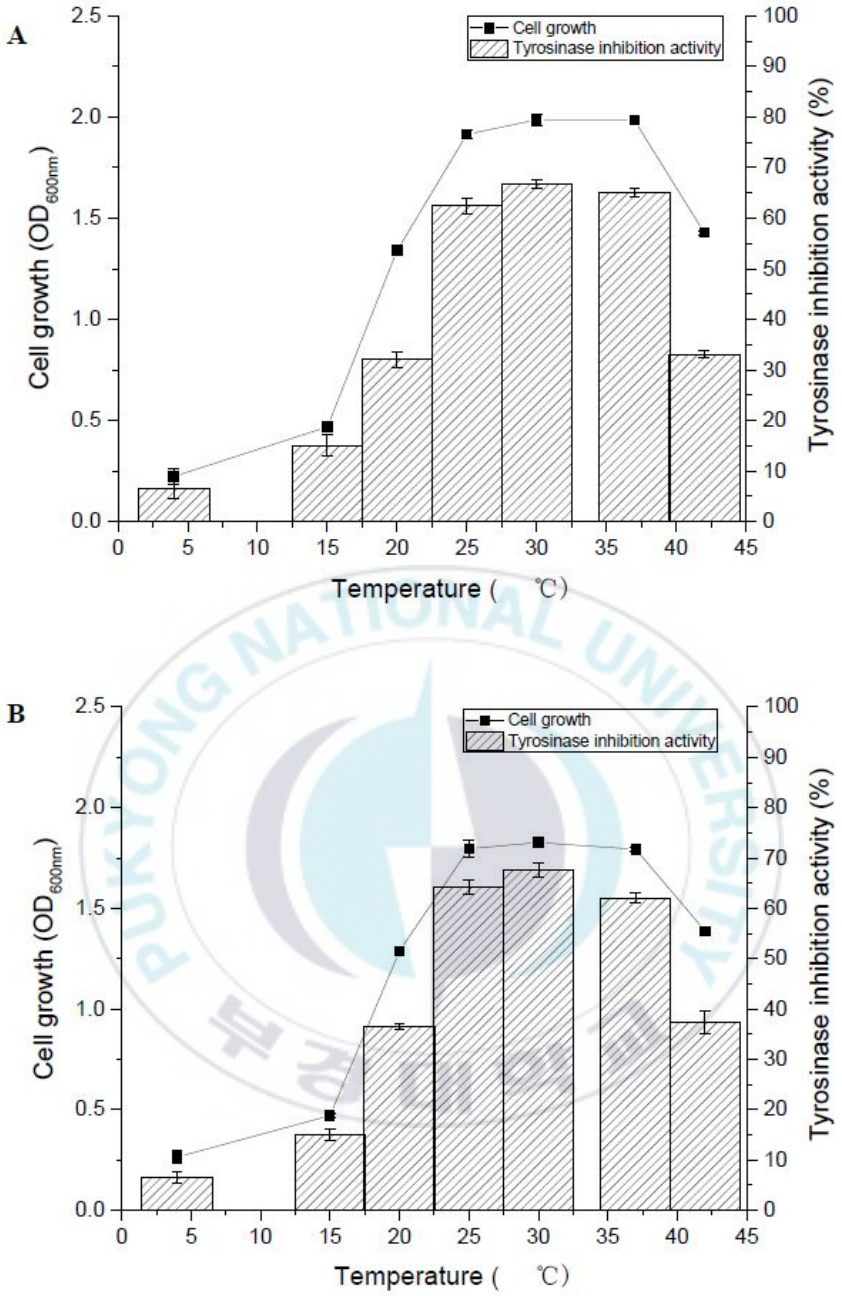

Figure 5. Tyrosinase inhibitory activities and cell growth of *Enterococcus* sp. strain EA3 (A) and EB2 (B) by temperatures (℃). The samples were collected at 4, 15, 20, 25, 30, 37, and 42 ℃, respectively.

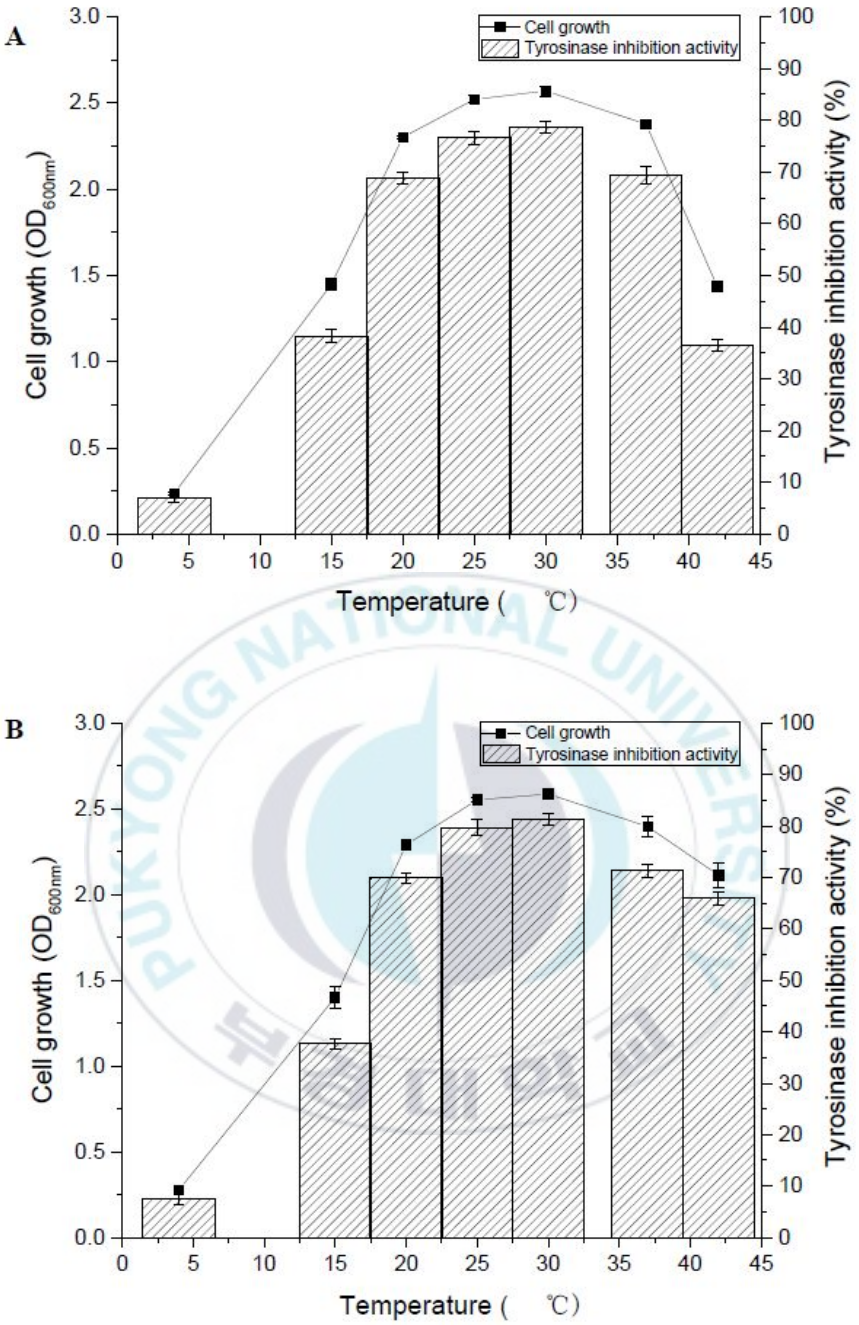

Figure 6. Tyrosinase inhibitory activities and cell growth of *Pediococcus* sp. strain PC2 (A) and PD3 (B) by temperatures (℃). The samples were collected at 4, 15, 20, 25, 30, 37, and 42 ℃, respectively.

76.59 %, 78.56 %, and 69.37 %, respectively. And cell growth of *Pediococcus* sp. PC2 by temperatures showed in Figure 6A. The mushroom tyrosinase inhibitory activities for 25, 30, and 37℃ of *Pediococcus* sp. PD3 culture filtrates were 79.69 %, 81.29 %, and 71.32 %, respectively. And cell growth of *Pediococcus* sp. PD3 by temperatures showed in Figure 6B. The maximum tyrosinase inhibitory activity was shown at 30 ℃ in PD3 strain.

3.2.3 Tyrosinase inhibitory activities and cell growth by pH.

The mushroom tyrosinase inhibitory activities for pH 6, 7, 8, and 9 of *Enterococcus* sp. EA3 culture filtrates were 55.07 %, 67.25 %, 69.78 %, and 65.37 %, respectively. And cell growth of *Enterococcus* sp. EA3 by pH showed in Figure 7A. The mushroom tyrosinase inhibitory activities for pH 9, 10, and 11 of *Enterococcus* sp. EB2 culture filtrates were 65.02 %, 64.20 %, and 57.20 %, respectively. And cell growth of *Enterococcus* sp. EB2 by pH showed in Figure 7B. The mushroom tyrosinase inhibitory activities for pH 5, 6, 7, and 8 of *Pediococcus* sp. PC2 culture filtrates were 63.32 %, 86.21 %, 83.70 %, and 63.95 %, respectively. And cell growth of *Pediococcus* sp. PC2 by pH showed in Figure 8A. The mushroom tyrosinase inhibitory activities for pH 6, 7, 8, and 9 of *Pediococcus* sp. PD3 culture filtrates were 79.05 %, 79.52 %, 82.38 %, and 53.81 %, respectively. And cell growth of *Pediococcus* sp. PD3 by pH showed in Figure 8B. The maximum tyrosinase inhibitory activity was shown at pH 7 in PC2 strain.

3.2.4 Tyrosinase inhibitory activities and cell growth by NaCl concentrations.

The mushroom tyrosinase inhibitory activities for 0, 1, 2, and 3 % NaCl concentrations of *Enterococcus* sp. EA3 culture filtrates were 61.47 %, 58.59 %, 52.37 %, and 49.98 %, respectively. And cell growth of *Enterococcus* sp. EA3 by NaCl concentrations (%) showed in Figure 9A. The mushroom tyrosinase inhibitory activities for 0, 1, and 2 % NaCl concentrations of *Enterococcus* sp. EB2 culture filtrates were 59.60

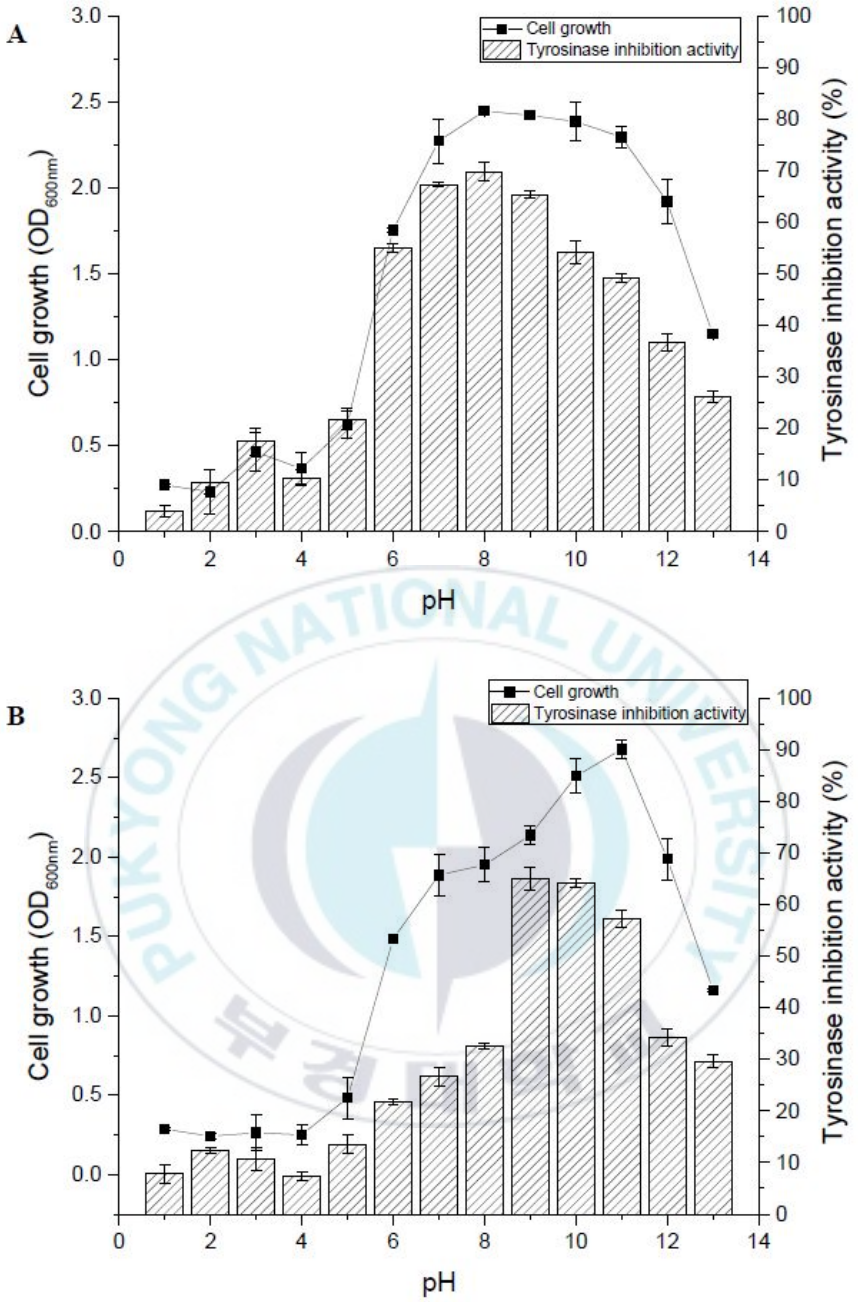

Figure 7. Tyrosinase inhibitory activities and cell growth of *Enterococcus* sp. strain EA3 (A) and EB2 (B) by pH. The samples were collected at pH 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13, respectively.

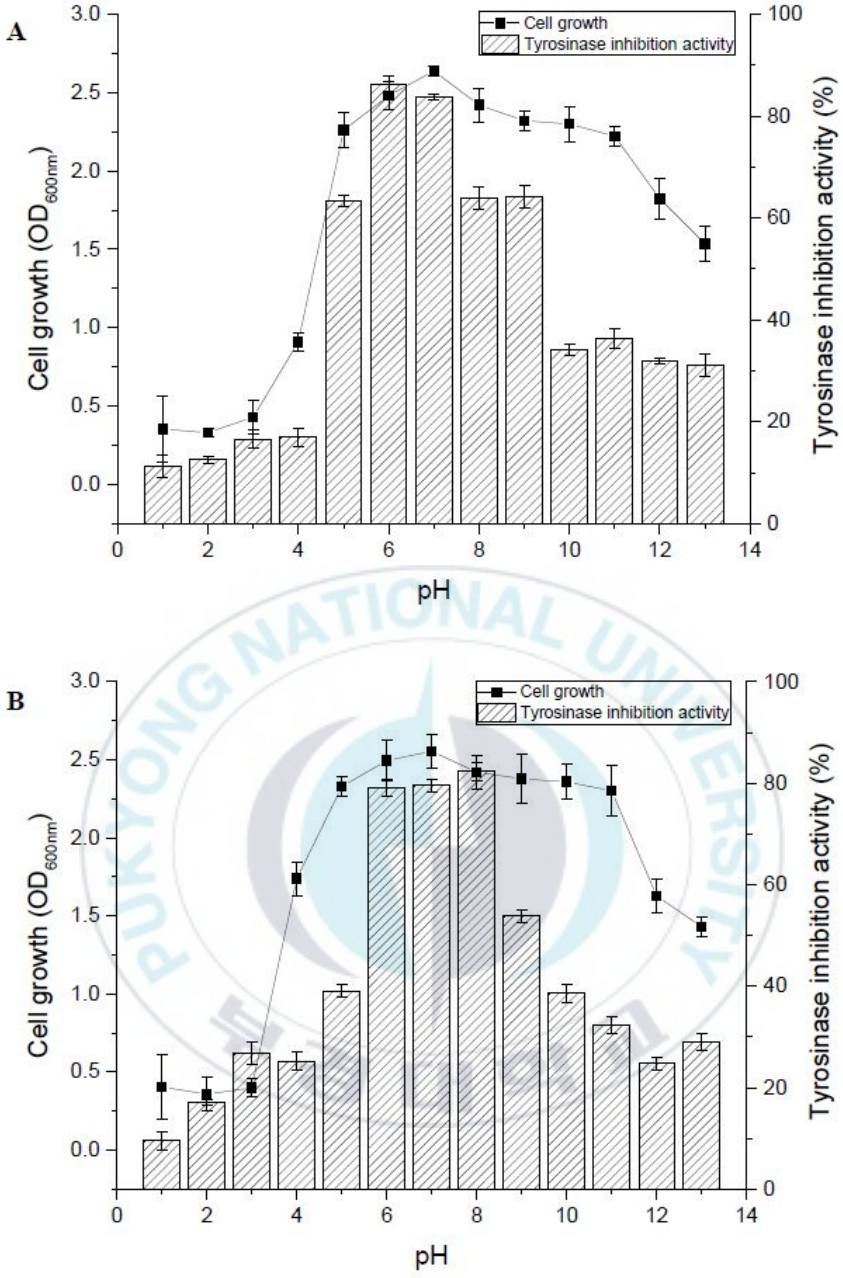

Figure 8. Tyrosinase inhibitory activities and cell growth of *Pediococcus* sp. strain PC2 (A) and PD3 (B) by pH. The samples were collected at pH 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13, respectively.

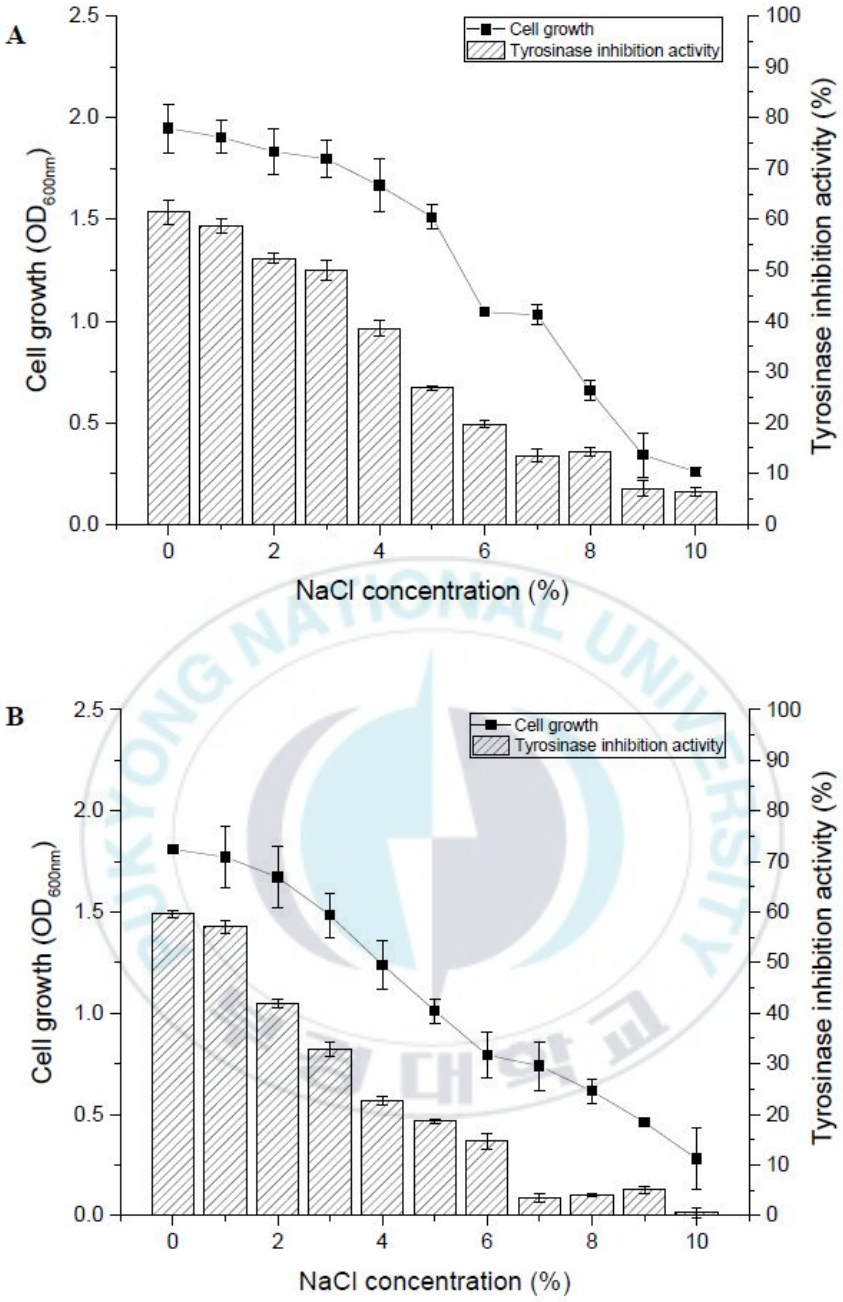

Figure 9. Tyrosinase inhibitory activities and cell growth of *Enterococcus* sp. strain EA3 (A) and EB2 (B) by NaCl concentrations (%). The samples were collected at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 % NaCl concentrations, respectively.

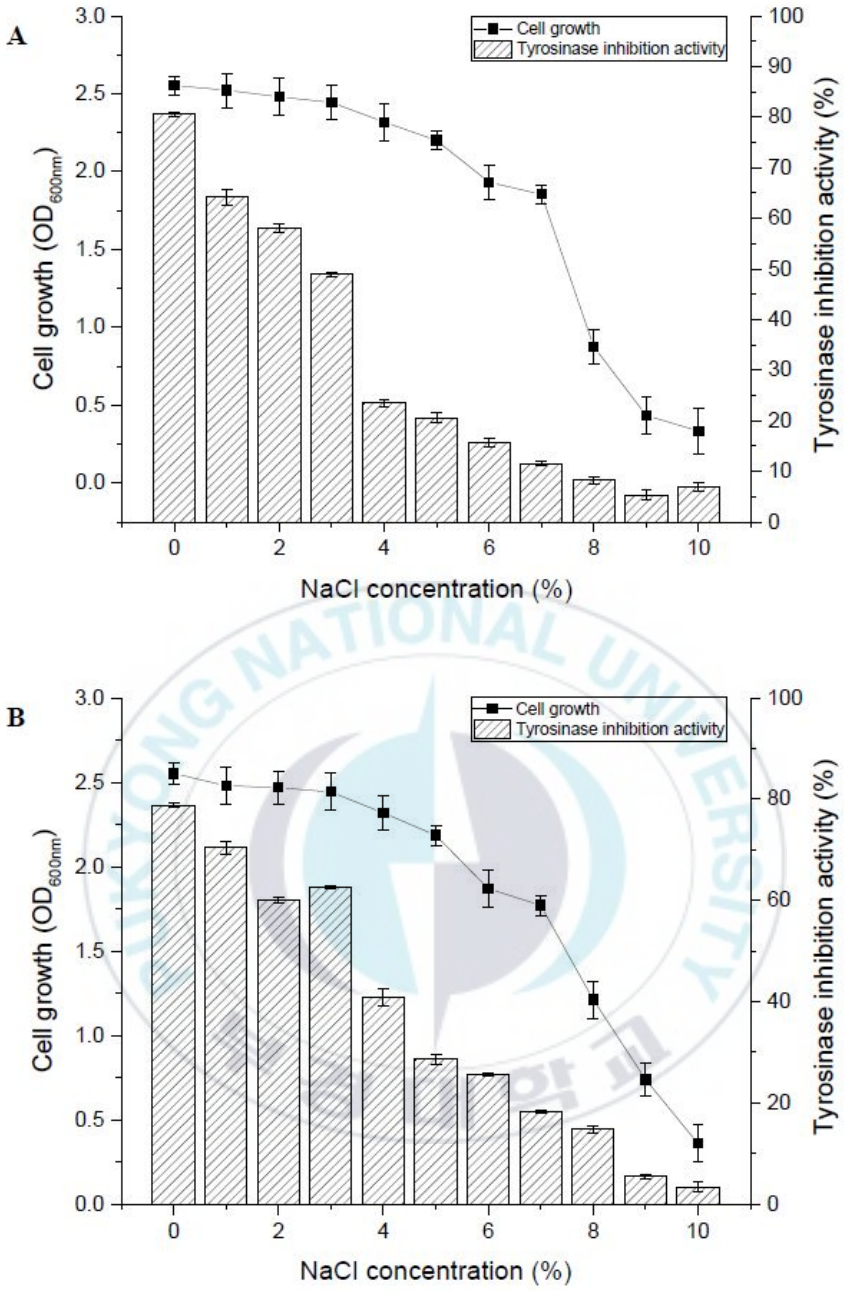

Figure 10. Tyrosinase inhibitory activities and cell growth of *Pediococcus* sp. strain PC2 (A) and PD3 (B) by NaCl concentrations (%). The samples were collected at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 % NaCl concentrations, respectively.

%, 57.07 %, and 41.92 %, respectively. And cell growth of *Enterococcus* sp. EB2 by NaCl concentrations (%) showed in Figure 9B. The mushroom tyrosinase inhibitory activities for 0, 1, 2, and 3 % NaCl concentrations of *Pediococcus* sp. PC2 culture filtrates were 80.61 %, 64.20 %, 58.02 %, and 48.97 %, respectively. And cell growth of *Pediococcus* sp. PC2 by NaCl concentrations (%) showed in Figure 10A. The mushroom tyrosinase inhibitory activities for 0, 1, 2, and 3 % NaCl concentrations of *Pediococcus* sp. PD3 culture filtrates were 78.82 %, 70.44 %, 60.10 %, and 62.56 %, respectively. And cell growth of *Pediococcus* sp. PD3 by NaCl concentrations (%) showed in Figure 10B. The maximum tyrosinase inhibitory activity was shown at 0% NaCl in PC2 strain.

3.3 Antioxidative activities by various culture conditions.

3.3.1 Antioxidative activities by growth times.

Anti-oxidative activities of four different LAB strains culture filtrates by growth times were tested for three kinds of radical scavenging activities (DPPH radical-scavenging activity, ABTS radical-scavenging activity, and SOD-like activity) (Figure 11, 12). In DPPH radical-scavenging activity assay, *Enterococcus* sp. EA3 culture filtrates showed more than 30% at 14, 16 and 18 hour of growth times, *Enterococcus* sp. EB2 culture filtrates more than 30% at 4 to 14, 20, 22 and 24 hour of growth times. *Pediococcus* sp. PC2 culture filtrates more than 30% at 10 to 14 and 18 to 24 hour of growth times, *Pediococcus* sp. PD3 culture filtrates more than 30% at 8 to 14, 22 and 24 hour of growth times, respectively. 0.5 % (w/v) of ascorbic acid was used as positive control and it showed 98.14 % of DPPH radical-scavenging activity in this assay.

ABTS radical scavenging activities were used for the procedure described by Roberta Re. Positive control of this test was 0.5 % (w/v) ascorbic acid. First, ABTS scavenging activities of *Enterococcus* sp. EA3 culture filtrates showed more than 50% at 6 hour of growth time, *Enterococcus* sp. EB2 culture filtrates more than 50% at 6 and 8

hour of growth times, *Pediococcus* sp. PC2 culture filtrates more than 55% at 6, 8, and 10 hour of growth times, *Pediococcus* sp. PD3 culture filtrates more than 55% at 6, 8, and 10 hour of growth times, respectively. In this assay, 0.5 % of ascorbic acid showed 96.31% of ABTS radical scavenging activity.

Superoxide dismutase (SOD) like activities of *Enterococcus* sp. EA3 culture filtrates showed more than 40% at 16 hour of growth time, *Enterococcus* sp. EB2 culture filtrates more than 40% at 16 hour of growth time, *Pediococcus* sp. PC2 culture filtrates more than 40% at 16 hour of growth time, *Pediococcus* sp. PD3 culture filtrates more than 40% at 14 and 16 hour of growth times, respectively. In addition, 0.5 % (w/v) of ascorbic acid was used as positive control and it showed 94.27 % of SOD-like activity.

3.3.2 Antioxidative activities by temperatures.

Antioxidative activities of four different LAB strains culture filtrates by temperatures were tested for DPPH radical-scavenging activity, ABTS radical-scavenging activity and SOD-like activity (Figure 13, 14). First, DPPH radical-scavenging activities of *Enterococcus* sp. EA3 culture filtrates showed more than 40% at 30 and 37 ℃, *Enterococcus* sp. EB2 culture filtrates more than 30% at 25, 30, and 37 ℃, *Pediococcus* sp. PC2 culture filtrates showed more than 35% at 30 ℃, *Pediococcus* sp. PD3 culture filtrates more than 35% at 25 and 30 ℃, respectively. In addition, 0.5 % (w/v) of ascorbic acid was used as positive control and it showed 97.84 % of DPPH radical-scavenging activity.

In ABTS radical scavenging activities test, 0.5 % (w/v) ascorbic acid was used as positive control. First, ABTS scavenging activities of *Enterococcus* sp. EA3 culture filtrates showed more than 40% at 25, 30, and 37 ℃, *Enterococcus* sp. EB2 culture filtrates more than 40% at 25, 30, and 37 ℃, *Pediococcus* sp. PC2 culture filtrates more than 45% at 25 and 30 ℃, *Pediococcus* sp. PD3 culture filtrates more than 50% at 25 and 30 ℃, respectively. In this test, 0.5 % of ascorbic acid showed 95.69% of ABTS

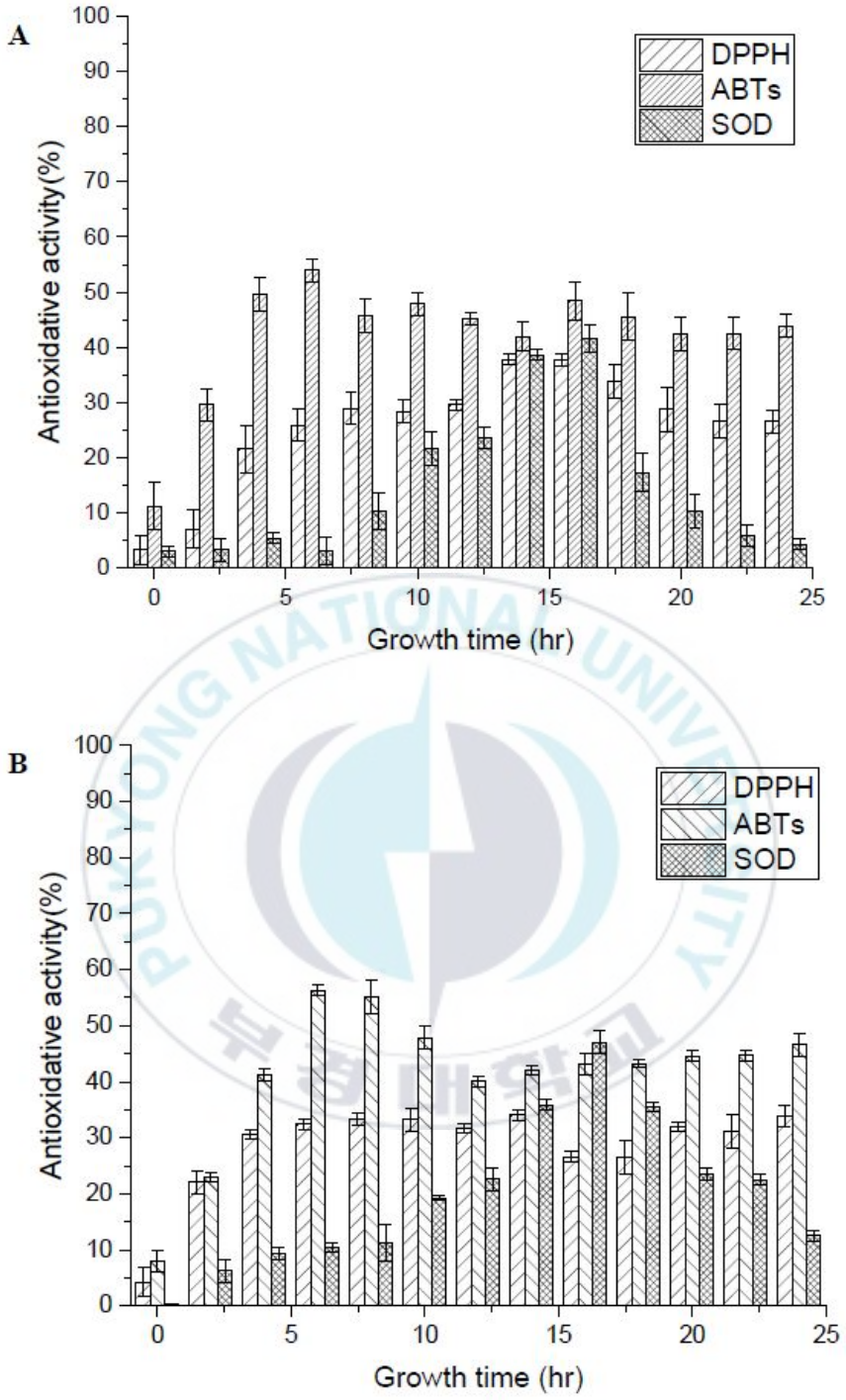

Figure 11. Antioxidative activities of *Enterococcus* sp. strains EA3 (A) and EB2 (B) by growth times. The samples were collected at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hour, respectively.

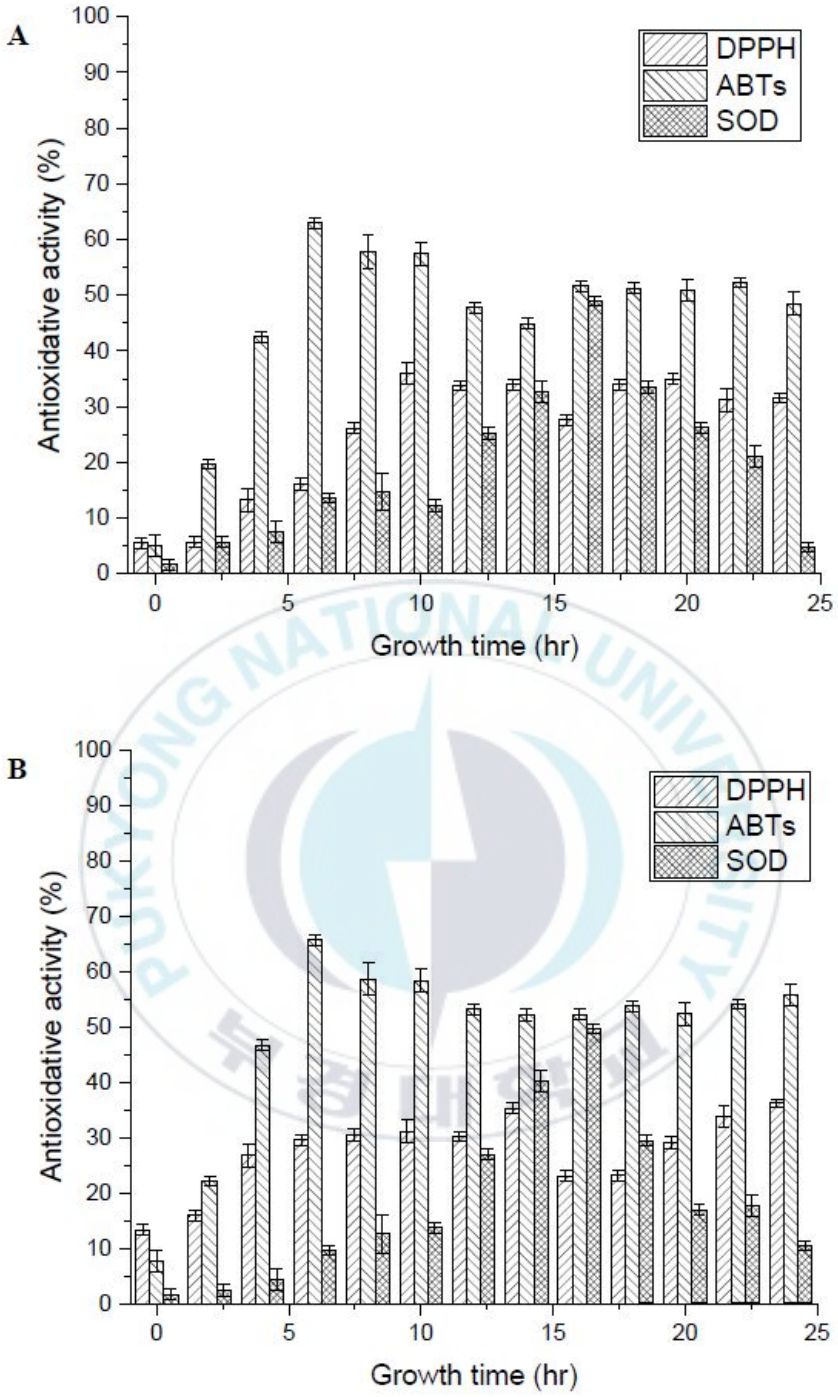

Figure 12. Antioxidative activities of *Pediococcus* sp. strain PC2 (A) and PD3 (B) by growth times. The samples were collected at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hour, respectively.

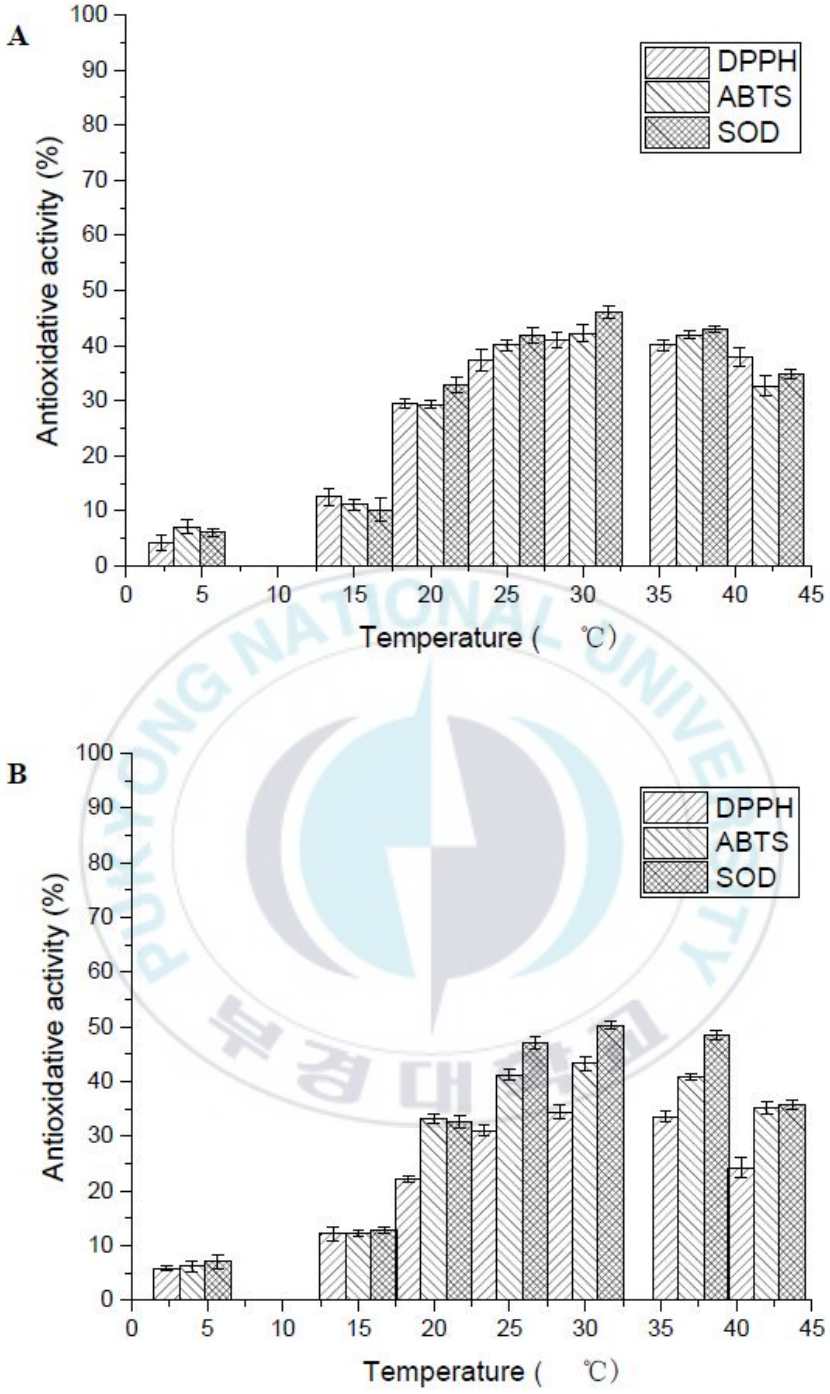

Figure 13. Antioxidative activities of *Enterococcus* sp. strain EA3 (A) and EB2 (B) by temperatures. The samples were collected at 4, 15, 20, 25, 30, 37, and 42 ℃, respectively.

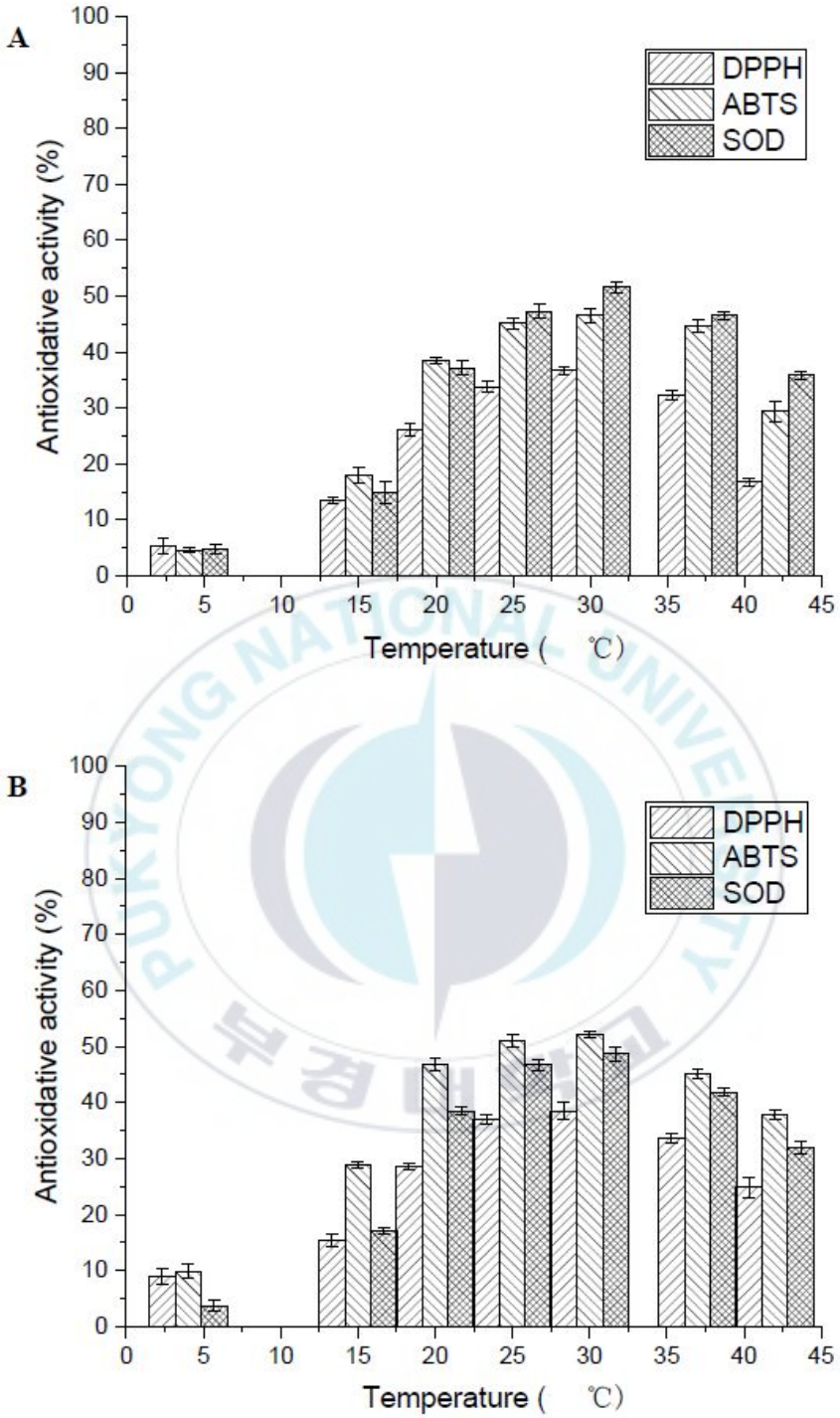

Figure 14. Antioxidative activities of *Pediococcus* sp. strain PC2 (A) and PD3 (B) by temperatures. The samples were collected at 4, 15, 20, 25, 30, 37, and 42 ℃, respectively.

radical scavenging activity.

Superoxide dismutase (SOD) like activities of *Enterococcus* sp. EA3 culture filtrates showed more than 45% at 30 ℃, *Enterococcus* sp. EB2 culture filtrates more than 50% at 30 ℃, *Pediococcus* sp. PC2 culture filtrates more than 50% at 30 ℃, *Pediococcus* sp. PD3 culture filtrates more than 45% at 25 and 30 ℃, respectively. In Addition, 0.5 % (w/v) of ascorbic acid was used as positive control and it showed 95.16 % of SOD-like activity.

3.3.3 Antioxidative activities by pH.

Anti-oxidative activities of four different LAB strains culture filtrates by pH were tested for DPPH radical-scavenging activity, ABTS radical-scavenging activity and SOD-like activity (Figure 15, 16). In DPPH radical-scavenging activity assay, *Enterococcus* sp. EA3 culture filtrates showed more than 45% at pH 8, *Enterococcus* sp. EB2 culture filtrates more than 40% at pH 7 and 8, *Pediococcus* sp. PC2 culture filtrates more than 45% at pH 8, *Pediococcus* sp. PD3 culture filtrates more than 40% at pH 5 and 8, respectively. 0.5 % (w/v) of ascorbic acid was used as positive control and it showed 98.36 % of DPPH radical-scavenging activity in this assay.

In ABTS radical scavenging activity test, 0.5 % (w/v) ascorbic acid was used as positive control. First, ABTS scavenging activities of *Enterococcus* sp. EA3 culture filtrates showed more than 40% at pH 7 and 8, *Enterococcus* sp. EB2 culture filtrates more than 45% at pH 8, 9, 10, and 11, *Pediococcus* sp. PC2 culture filtrates more than 55% at pH 6, 7, 8, and 9, *Pediococcus* sp. PD3 culture filtrates more than 55% at pH 7, 8, and 9, respectively. In this assay, 0.5 % of ascorbic acid showed 97.53% of ABTS radical scavenging activity.

Superoxide dismutase (SOD) like activities of *Enterococcus* sp. EA3 culture filtrates showed more than 40% at pH 7, *Enterococcus* sp. EB2 culture filtrates more than 35% at pH 6, *Pediococcus* sp. PC2 culture filtrates more than 40% at pH 7, *Pediococcus*

sp. PD3 culture filtrates more than 40% at pH 6, respectively. In addition, 0.5 % (w/v) of ascorbic acid was used as positive control and it showed 96.22 % of SOD-like activity.

3.3.4 Antioxidative activities by NaCl concentrations.

Antioxidative activities of four different LAB strains culture filtrates by NaCl concentrations (%) were tested for DPPH radical-scavenging activity, ABTS radical-scavenging activity, and SOD-like activity. In DPPH radical-scavenging activity test, culture filtrates of *Enterococcus* sp. EA3 showed more than 35% at 0% of NaCl concentration, culture filtrates of *Enterococcus* sp. EB2 more than 25% at 0% of NaCl concentration, culture filtrates of *Pediococcus* sp. PC2 more than 30% at 0% of NaCl concentration, culture filtrates of *Pediococcus* sp. PD3 more than 30% at 0 and 1% of NaCl concentrations, respectively. In this assay, 0.5 % (w/v) of ascorbic acid was used as positive control and it showed 98.57 % of DPPH radical-scavenging activity.

In ABTS radical scavenging activities, 0.5 % (w/v) ascorbic acid was used as positive control. First, ABTS scavenging activities of *Enterococcus* sp. EA3 culture filtrates showed more than 45% at 0 and 1% of NaCl concentrations, *Enterococcus* sp. EB2 culture filtrates more than 45% at 0% of NaCl concentration, *Pediococcus* sp. PC2 culture filtrates more than 50% at 0 and 1% of NaCl concentrations, *Pediococcus* sp. PD3 culture filtrates more than 50% at 0 and 1% of NaCl concentrations, respectively. In this assay, 0.5 % of ascorbic acid showed 95.11% of ABTS radical scavenging activity.

Superoxide dismutase (SOD) like activities of *Enterococcus* sp. EA3 culture filtrates showed more than 35% at 0 and 1% of NaCl concentrations, *Enterococcus* sp. EB2 culture filtrates more than 30% at 0 and 1% of NaCl concentrations, *Pediococcus* sp. PC2 culture filtrates more than 40% at 0% of NaCl concentration, *Pediococcus* sp. PD3 culture filtrates more than 40% at 0 and 1% of NaCl concentrations, respectively. In addition, 0.5 % (w/v) of ascorbic acid was used as positive control and it showed 96.02 % of SOD-like activity.

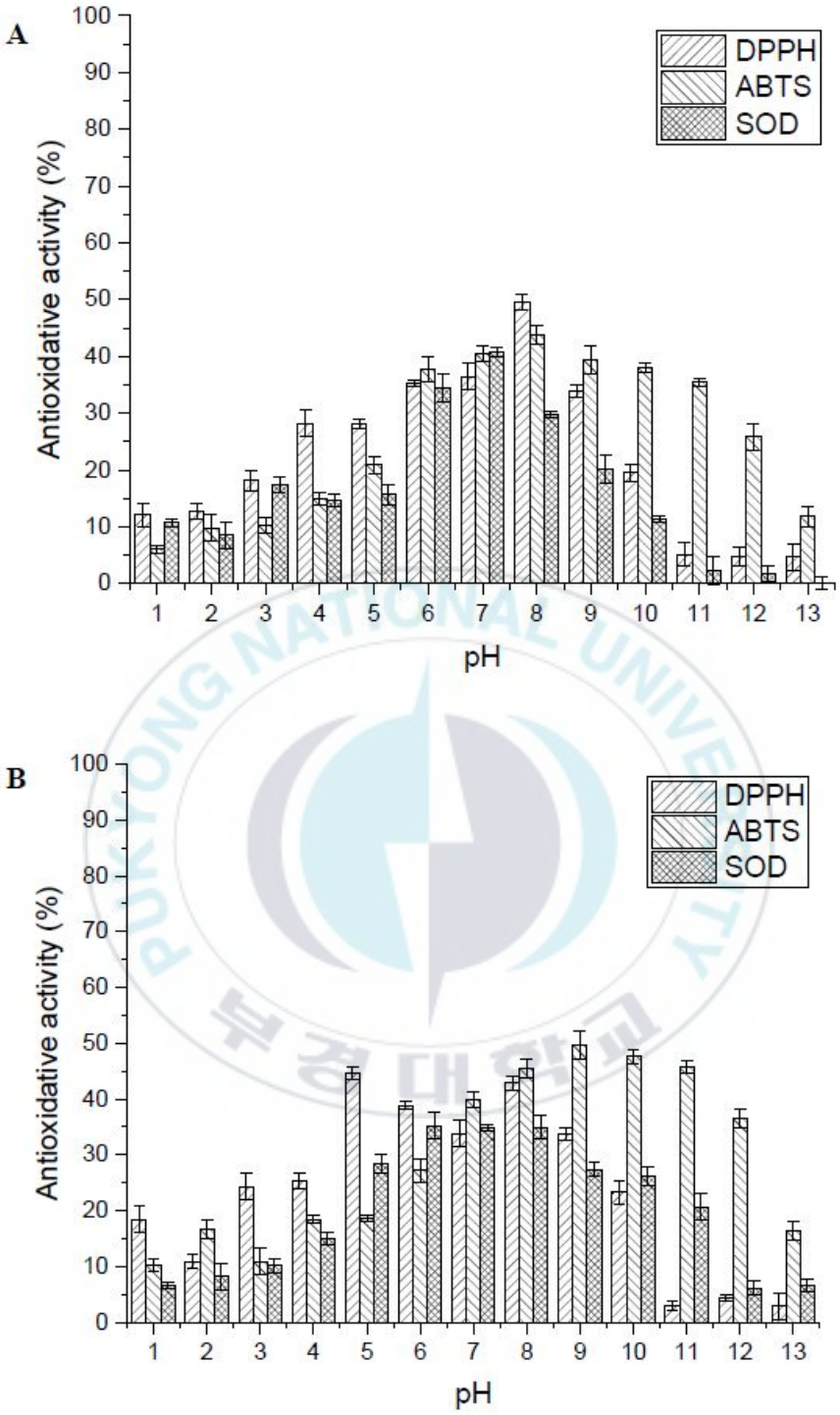

Figure 15. Antioxidative activities of *Enterococcus* sp. strain EA3 (A) and EB2 (B) by pH. The samples were collected at pH 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13, respectively.

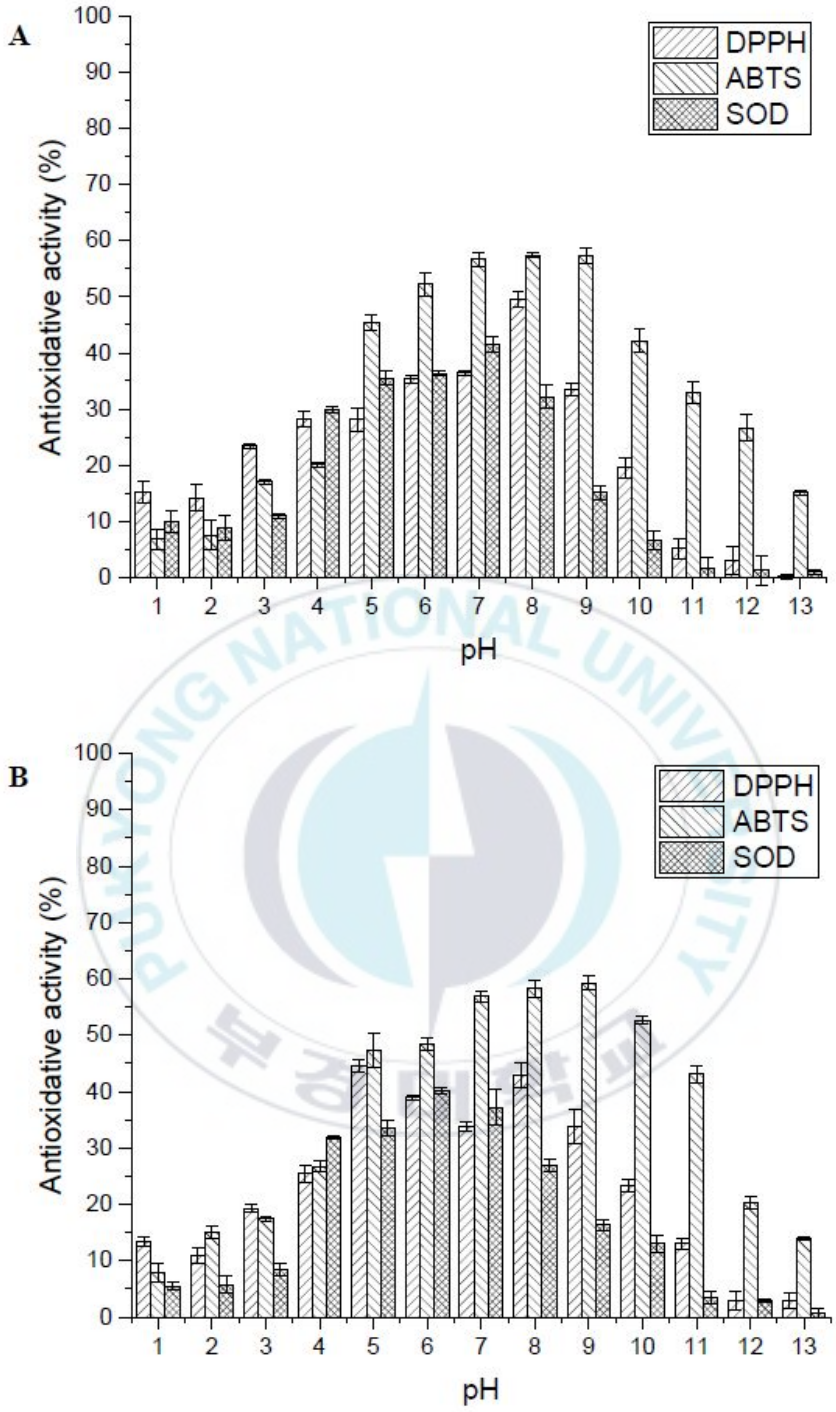

Figure 16. Antioxidative activities of *Pediococcus* sp. strain PC2 (A) and PD3 (B) by pH. The samples were collected at pH 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13, respectively.

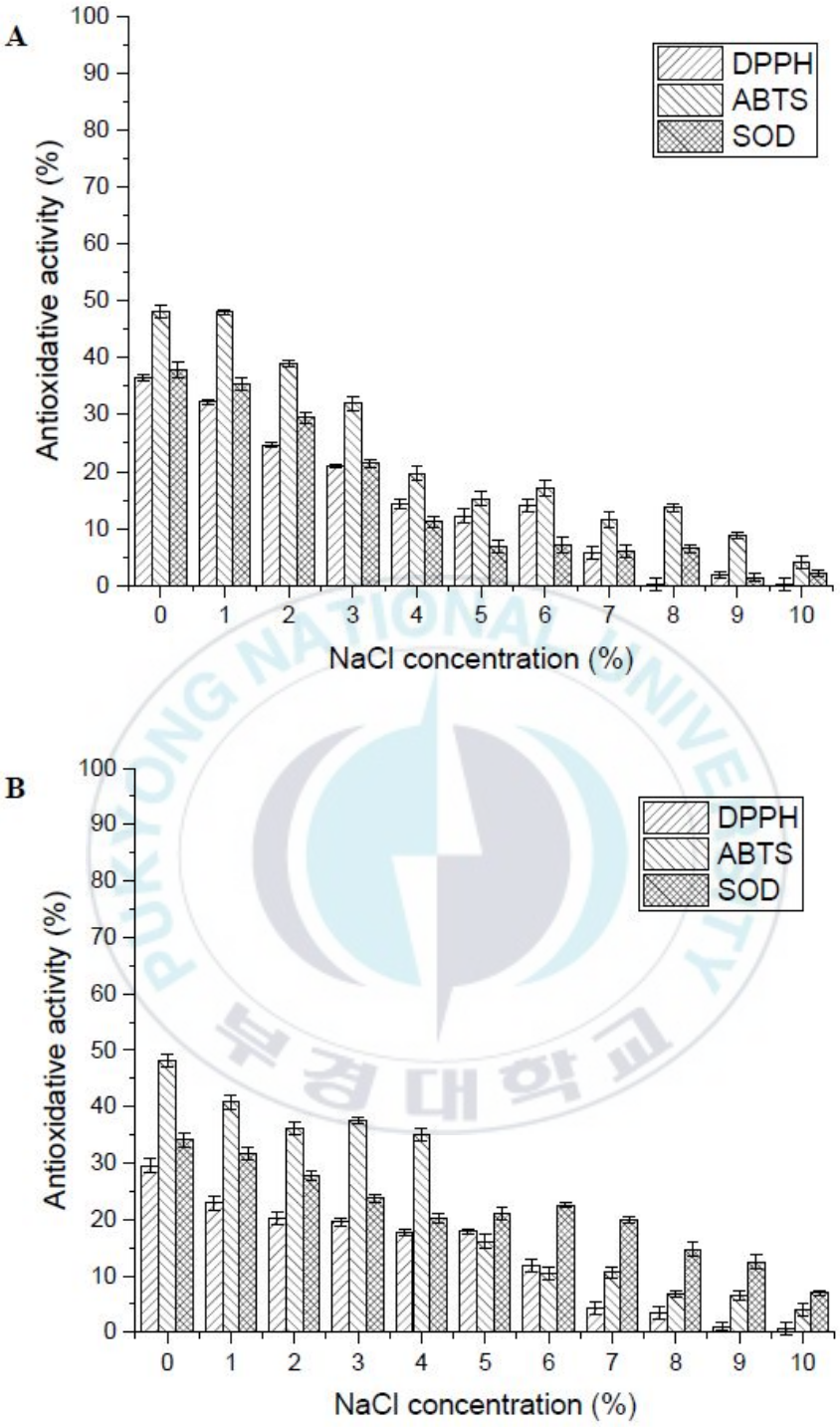

Figure 17. Antioxidative activities of *Enterococcus* sp. strain EA3 (A) and EB2 (B) by NaCl concentrations. The samples were collected at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 % NaCl concentrations, respectively.

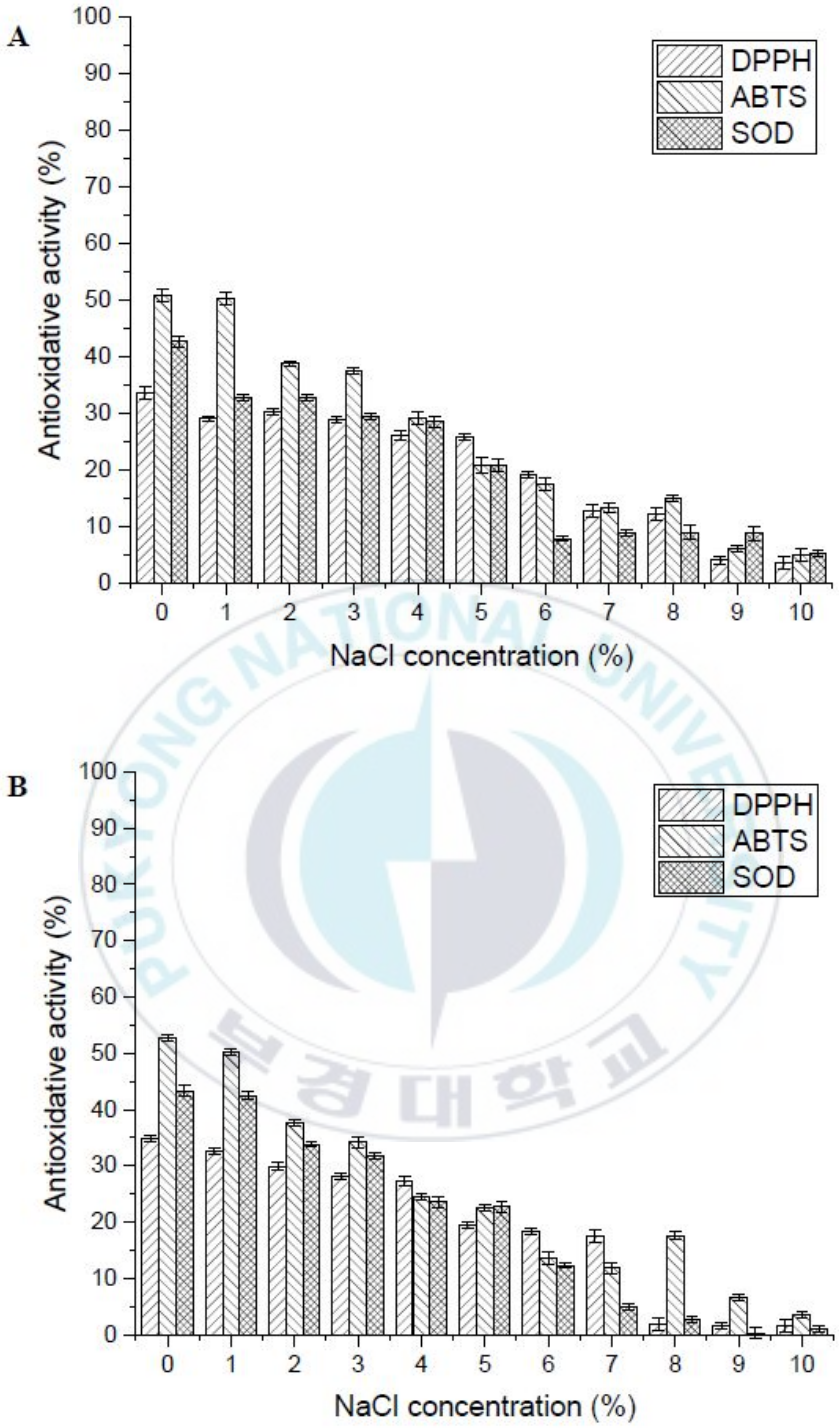

Figure 18. Antioxidative activities of *Pediococcus* sp. strain PC2 (A) and PD3 (B) by NaCl concentrations. The samples were collected at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 % NaCl concentrations, respectively.

4. Discussion

Melanin pigments are related to skin disorders [13], exogenous oxidative stress [39], and intracellular antioxidant level [40]. For a recent decade, the search for rich sources of bioactivity such as tyrosinase inhibiting and anti-oxidative property has been extended due to the increasing attention of whitening and anti-aging therapy. In many studies, plant extracts and chemical synthetic agents were tested for tyrosinase inhibitory and antioxidative activities [27, 28, 29]. For example, Sokman and other reported that the essential oils and methanol extracts of *Thymus spathulifolius* had antimicrobial and antioxidant properties [38]. However, there are a few studies about both tyrosinase inhibitory and antioxidative activities of bacteria culture filtrate.

Lactic acid bacteria (LAB) among many bacteria were widely distributed in foods their beneficial effects on human health such as increasing the immune response [30], helping to control intestinal infections [31], influencing cholesterol levels [32], antioxidant [33], whitening effects [34], and anticarcinogenic effects [35]. LAB comprise a wide range of genera including a considerable number of species. The most important genera of LAB are *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Weissella*, and *Bifidobacterium*. A number of LAB were used for food fermentation, probiotics, production of bacteriocin and other healthcare medicines. In present study, four different LAB strains were isolated from dairy cow feces. Both EA3 and EB2 were identified to *Enterococcus* sp. strain, and PC2 and PD3 were identified to *Pediococcus* sp. strain on the basis of their physiological properties and 16S rRNA gene sequences analysis. Enterococci commonly occur as inhabitants of human and animal intestinal tract, but they are also in fermented food and isolated from the environment [36]. Pediococci are lactic acid bacteria found on plants and in many fermented foods [37]. Pediococci have been added as nutritional enhancers of animal feeds and recently have been used in polymer research for production of biodegradable packaging materials.

To investigate optimal conditions for tyrosinase inhibitory activities of the culture

filtrates from four LAB strains, experiments were performed by growth times (hour), temperatures (℃), pH, and NaCl concentrations (%). In the tyrosinase inhibitory activities by growth times, culture filtrates of *Enterococcus* sp. EA3 showed approximately 65% at different culture conditions (14 hour, 30℃, pH 8 and 0% NaCl), culture filtrates of *Enterococcus* sp. strain EB2 approximately 65% (12 hour, 30℃, pH 9 and 0% NaCl), culture filtrates of *Pediococcus* sp. strain PC2 approximately 80% (20 hour, 30℃, pH 6 and 0% NaCl), culture filtrates of *Pediococcus* sp. strain PD3 approximately 80% (20 hour, 30℃, pH 8 and 0% NaCl), respectively. From this results, tyrosinase inhibitory activities of *Pediococcus* sp. PC2 and PD3 culture filtrates were approximately 15% higher than those of *Enterococcus* sp. EA3 and EB2 culture filtrates. The tyrosinase inhibitory activities in the culture filtrates from four different LAB strains were much higher than that of *Bifidobacterium bifidum* culture filtrates, reported by Huey-Chun Huang [25]. The inhibitors of tyrosinase are broadly applied in cosmetic industries. For the clinical usage, inhibitors of tyrosinase are employed to treat dermatological disorders associated with melanin hyperaccumulation, and are essential for de-pigmentation. Consequently, culture filtrates from four different LAB strains have potential use for inhibitors of tyrosinase in cosmetic industries.

The antioxidative activities of the culture filtrates isolated from four LAB were assessed by three different methods (DPPH radical scavenging, ABTS radical scavenging, and SOD-like activities assay). From the results of the DPPH radical scavenging assay, culture filtrates from all LAB strains showed approximately more than 30% at optimal conditions of tyrosinase inhibitory activities. Also, the culture filtrates from four LAB strains showed DPPH radical scavenging ability. The ABTS radical scavenging capacities of the culture filtrates from all LAB strains were approximately more than 50% at optimal conditions of tyrosinase inhibitory activities. Finally, SOD-like activities of the culture filtrates from all isolated LAB strains appeared approximately more than 40% at optimal conditions of tyrosinase inhibitory activities.

This study demonstrated the attempt to display both tyrosinase inhibitory and

antioxidative activities of the culture filtrates from four different LAB strains. The results revealed the good potentials of bacteria culture filtrates from *Enterococcus* sp. strain EA3, EB2 and *Pediococcus* sp. strain PC2, PD3 in tyrosinase inhibitory and antioxidative activities. And these suggested that the culture filtrates from all LAB strains may be attributed to its inhibitory action upon the signaling pathway regulating tyrosinase activities. In conclusion, the culture filtrates from four LAB strains have the potential as natural agents capable of applications to anti-oxidants and for inhibitors of tyrosinase. Further studies should be done to identify the active components in the culture filtrates from four different LAB strains and elucidate the mechanisms of tyrosinase inhibitory and antioxidative activities for skin care cosmetics.

5. Korean summary (국문 요약)

멜라닌의 과생산 및 축적은 많은 피부 질병들을 일으킨다. Tyrosinase 억제제는 과색소침착과 연관된 피부 질환의 치료에 중요하고 자외선 노출 후의 미백을 위한 화장품에 적용되고 있다. 그리고 과산화수소 (H_2O_2) 와 다른 활성산소종은 사람과 음식에서 화학적 물질과 대사적 경로에 의해서 생성된다. 이 때 항산화 물질들은 활성산소에 의해 발생하는 암과 심장 질환을 포함한 질병들의 감소에 있어서 중요한 역할을 한다.

이 연구는 젖소의 분변으로부터 분리한 4종의 유산균 균주 배양 여과액의 Tyrosinase 억제 및 항산화효과를 확인하는 것을 목표로 하였다. 4종의 유산균 균주 배양 여과액의 Tyrosinase 억제 및 항산화효과가 가장 높은 최적 배양조건을 확인하기 위해서 배양시간 (hour), 온도 (℃), pH, NaCl 농도 (%) 등의 조건을 달리하여 각각 균주 배양 여과액의 Tyrosinase 억제 및 항산화효과를 측정하였다. Tyrosinase 억제능 측정 결과에서 *Enterococcus* sp. EA3 균주 배양 여과액이 14시간, 30℃, pH 8, 0 % NaCl 조건에서 약 65%의 활성을 보였고, *Enterococcus* sp. EB2 균주 배양 여과액이 12시간, 30℃, pH 9, 0 % NaCl 조건에서 약 65%의 활성을 보였다. *Pediococcus* sp. PC2 균주 배양 여과액은 20시간, 30℃, pH 6, 0% NaCl 배양조건에서 약 80%의 저해활성을 보였으며 *Pediococcus* sp. PD3 균주 배양 여과액은 20시간, 30℃, pH 8, 0 % NaCl 배양조건에서 약 80%의 활성을 보였다. 추가적으로, 4종의 유산균 균주 배양 여과액의 항산화 활성은 각각 균주 배양 여과액의 tyrosinase 억제의 최적 배양 조건에서 약 30% 이상의 활성을 보였다. 그 결과 4 종의 유산균 균주들의 배양 여과액은 천연 항산화제 및 미백제로의 가능성을 갖고 있다. 이러한 연구 결과를 토대로 피부 보호 화장품의 연구 및 개발에서 네 유산균주의 배양 여과액이 후보물질로 적용될 수 있을 것이다.

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