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Isolation and Characterization of Lactic Acid Bacteria Strains with Ornithine Producing Capacity from Natural Sea Salt

Jin-Ju Yu and Suk-Heung Oh*

Department of Food and Biotechnology, Woosuk University, Jeonju 565-701, Republic of Korea

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Two lactic acid bacteria (LAB) having ornithine-producing capacity were isolated from Korean natural sea salt. They were Gram-positive, short rod-type bacteria, and able to grow anaerobically with CO_2 production. The isolates grew well on MRS broth at 30-37°C and a pH of 6.5-8.0. The optimum temperature and pH for growth are 37°C and pH 7.0. The isolates fermented D-ribose, D-galactose, D-lactose, D-maltose, D-cellobiose, D-tagatose, D-trehalose, sucrose, D-melezitose, gentiobiose, D-glucose but not D-melibiose, inositol, and L-sorbose. The 16S rDNA sequences of the two isolates showed 99.5% and 99.6% homology with the *Weissella koreensis* S5623 16S rDNA (Access no. AY035891). They were accordingly identified and named as *Weissella koreensis* MS1-3 and *Weissella koreensis* MS1-14, and produced intracellular ornithine at levels of 72 mg/100 g cell F.W. and 105 mg/100 g cell F.W. and extracellular ornithine at levels of 4.5 mg/100 ml and 4.6 mg/100 ml medium, respectively, by culturing in MRS broth supplemented with 1% arginine. High cell growth was maintained in MRS broth with a NaCl concentration of 0-6%. These results show for the first time that Korean natural sea salts contain lactic acid bacteria *Weissella koreensis* strains having ornithine producing capacity.

***Keywords*:** natural sea salt, ornithine, lactic acid bacteria

Natural sea salt is derived from the crystallization of salt by vaporizing seawater in the saltern by solar heat and wind (Han *et al*., 2009). In Korea, traditionally, it has been widely used for the production of fermented foods, such as Kimchi, soybean paste, soy sauce, and salted fish. The Korean natural sea salt is reported to have lower NaCl concentration and richer mineral contents including Ca, Mg, K, etc as compared to imported salt (Ha and Park, 1998; Park *et al*., 2000; Han *et al*., 2009). Also, some recent studies reported that the Kimchi and soybean paste that used natural sea salt, especially natural sea salt without bittern showed more excellent performance in terms of the growth promotion of effective microorganisms and anti-cancer effects as compared to those that used refined salt (Ham *et al*., 2008; Chang, 2009; Han *et al*., 2009). Some reported that halophilic microorganism including *Haloarcula* sp. existed in natural sea salt and these halophilic microorganisms and enzymes are used in industry-applied research for salt waste treatment and heavy metal retrieval (Norberg and Hofsten, 1969; Park and Jeong, 1996). In this context, the natural Korean sea salt is expected to have lactic acid bacteria that are possibly originating from mud flats and seas containing the living effective microorganism; however, there has been no report concerning it.

Ornithine is a type of medicinal amino acid that is frequently used in the U.S. as a food supplement as well as anti-obesity medicinal agent since it increases muscle synthesis by promoting growth hormone secretion and accelerates basal metabolism (Evain-Brion *et al*., 1982; Elam, 1988). Recently, it is known to have a new function including skin aesthetic effect and wrinkle improvement (Shi *et al*., 2002). Moreover, L-ornithine is widely used as an anabolic material for muscle development (Wernerman and Hammarqvist, 1987) and immunity improvement (Kawai *et al*., 1999, 2000; Robinson *et al*., 1999) in the form of L-ornithine-α-Ketoglutaric Acid (OKG) that contains L-ornithine and α-ketoglutaric acid in the ratio of 2 to 1. It is being used medicinally for improving liver disorders in the form of L-ornithine-L-aspartate in Europe (Muting and Kalk, 1992). Ornithine is known to be abundant in freshwater shells and in the brackish water bivalve, *Corbicula japonica* (160 mg/100 g) (Uchisawa *et al*., 2004). It has also been known that fermented foods such as wine and cheese contain ornithine which is produced by the transformation of arginine via the arginine deaninase (ADI) pathway or the arginase-urease pathway (Arena *et al*., 1999; Liu *et al*., 2003).

Recently, this research team reported the characteristics of Kimchi lactic acid bacteria *Weissella koreensis* OK1-4 and *Weissella koreensis* OK1-6 exhibiting an excellent capacity for the ornithine production (Yu *et al*., 2009). Kimchi is a fermented food that is rich in lactic acid bacteria as it is made by mixing various seasonings with salted cabbages or radishes. For this reason, it is expected to introduce functional lactic acid bacteria into various ingredients. Since salted fish and cabbage are also fermented foods, our research team designed this study to isolate and characterize effective lactic acid bacteria strains from natural sea salt. This approach is to elucidate the route of lactic acid bacteria introduction into Kimchi and to find new characteristics of Korean natural sea salts. Thus, in the study, the results show that Korean natural sea salts contain lactic acid bacteria having ornithine

* For correspondence. E-mail: shoh@woosuk.ac.kr; Tel: +82-63-290-1433; Fax: +82-63-290-1429

producing capacity.

Materials and Methods

Screening of ornithine-producing lactic acid bacteria *Weissella* strains

Natural sea salts (Chunil Yum) were purchased from Shinan-Gun (www.shinan.go.kr) through internet and used for isolation of lactic acid bacteria (LAB) with high ornithine producing ability. The salts were dissolved in sterilized water and diluted with 1% (v/v) peptone solution. Subsequently, 1 ml of diluted solution was spread on DE MAN, ROGOSA, and SHARPE (MRS) (Difco, USA) agar with 0.002% bromophenol blue (Sigma Chemical Co., USA), grown at 30°C for 48 h. Among the colonies, those with no ring, but with dark yellow color were classified as *Weissella* strains (Yu *et al*., 2009). The classified colonies were inoculated with an MRS medium containing 1% of arginine, cultivated at 30°C for 48 h, and then verified for the existence or non-existence of ornithine productivity using Thin Layer Chromatography (TLC, silica gel 60 F_{254}, Merck, Germany) (Yu *et al*., 2009).

Mesurement of intracellular and extracellular ornithine content

Ornithine content produced by the screened strains was measured by culturing the strains in MRS medium (pH 7.0) supplemented with 1% (w/v) arginine at 37°C for 48 h. Culture broth was separated from cells by centrifugation (5,000×g for 20 min at 4°C). Ornithine was extracted essentially as described by Baum *et al*. (1996) with modifications. Briefly, 800 µl of mixed organic solvent solution (methanol:chloroform: water=12:5:3) was added to 200 µl of cell-free supernatant or 200 mg of cells. The aqueous supernatant layer containing ornithine was obtained by centrifugation (13,000×g for 15 min at 4°C). 600 µl of solution (chloroform:water=1:2) was added to the bottom layer solution to extract further the residual ornithine and centrifuged and the supernatant was mixed with the 1st aqueous supernatant layer. The combined supernatants were re-centrifuged to remove the remnant impurities and then freeze-dried, re-suspended in water, filtered through a 0.45-µm PVDF membrane (Millipore, USA), and analyzed by HPLC (Waters, USA) after 6-aminoquioly-N-hydroxysuccinimidyl carbonate (AQC) derivatization. To separate the derivatives, a 3.9×150 mm AccQ·Tag™ (Nova-Pak™ C18, Waters) column at 37°C was used, along with mobile phases (AccQ·Tag™ Eluent A and 60% acetonitrile) with a 1.0 ml/min flow rate and a fluorescence detector (Waters) as previously described (Yu *et al*., 2009). The ornithine contents were calculated using an ornithine standard (Sigma Chemical Co.) based on a standard curve.

Identification of ornithine-producing *Weissella* strains

Identification of the ornithine-producing strains was done using 16S rDNA sequence analysis as described (Yu *et al*., 2009). For PCR, genomic DNA was isolated from the selected strains using a DNeasy® Blood & Tissue kit (QIAGEN, Germany) and purified according to the manufacturer's instructions. To clone 16S rDNA of the isolated strains, PCR amplification was performed using the primers 5'-AGAGTTTGATCMTGGCTCAG-3' (Forward) and 5'-ACGGGCGG GTGTGTRC-3' (Reverse). For the PCR amplification, 100 ng of template DNA, 0.5 µM of each primer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0) containing 0.1% Triton X-100 and Taq polymerase 0.025 U/µl were used with 35 cycles of denaturation, annealing, and extension at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min 30 sec, respectively, as previously described (Yu *et al*., 2009). The cloned 16S rDNA was sequenced using the dideoxynucleotide termination procedures based on synthetic oligonucleotide primers and dsDNA Cycle System (Perkin Elmer, USA). The nucleotide sequences were analyzed using the CLUSTAL W (1.81) program. The partial 16S rDNA sequences of the isolated strains were compared with those of other strains in the Entrez PubMed database (www.ncbi.nlm.nih.gov) for determination of the similarity index.

Characterization of ornithine-producing *Weissella* strains

Fermentaion potential of various sugars listed in Table 1 was determined using the API 50 CHL kit (bioMérieux, France) by following the manufacturer's instructions. Catalase activity was determined by bubble production in 3% (v/v) H_2O_2 and CO_2 gas production was measured using Durham tubes. To test the effect of pH on the growth of *Weissella* strains, MRS media were prepared using 100 mM sodium acetate/100 mM acetate buffer (pH 4.0-6.0) and 100 mM Na_2HPO_4/100 mM HCl buffer (pH 7.0-8.0) and the strains were cultured at 37°C for 12 h. To test the effect of temperature, the strains were cultured in MRS medium (pH 7.0) at the temperature range of 4-40°C for 12 h. Tolerance of NaCl was examined by culturing the strains for 24 h at 37°C in MRS medium (pH 7.0) containing 0-8% NaCl. Growth of the strains was determined by measuring culture turbidity at 600 nm every 6 h. Production of citrulline in the cells and medium during the growth of the selected *Weissella* strains was monitored using the L-citrulline kit K660 (Immundiagnostik AG, Germany) by following the manufacturer's instructions. The *Weissella* strains were cultured in MRS medium (pH

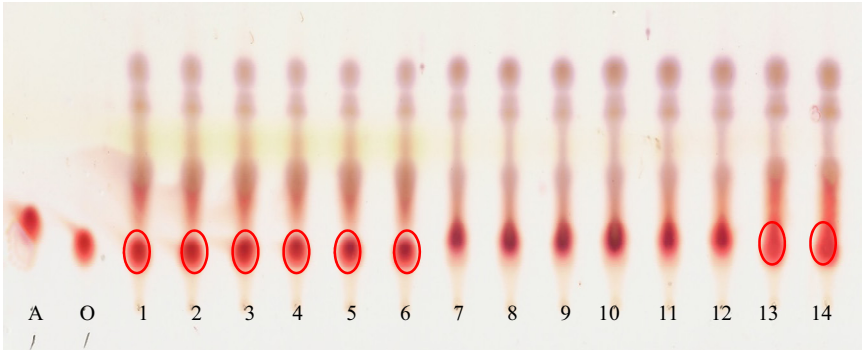

Fig. 1. TLC analysis of lactic acid bacteria cultures for ornithine production. The strains were cultured in an MRS liquid medium with 1% arginine. The strains with circles are selected as candidate lactic acid bacteria, *Weissella* species for the ornithine production. Lanes: A, spot of standard arginine; O, spot of standard ornithine; 1-14, spots of lactic acid bacteria cultures.

Table 1. Comparison of biochemical characteristics of *W. koreensis* strains

Characteristics	MS1-3	MS1-14	OK1-6	KCCM 41517
Gram stain	+	+	+	+
Catalase	-	-	-	-
Cell shape	Rod	Rod	Rod	Rod
CO_2 producton	+	+	+	+
Spore formation	-	-	-	-
Assimilation test				
D-ribose	+	+	+	+
D-xylose	-	-	+	+
D-galactose	+	+	-	-
D-glucose	+	+	+	+
D-fructose	+	+	+	+
D-manose	+	+	+	+
D-cellobiose	+	+	-	-
D-maltose	+	+	-	-
D-lactose	+	+	-	-
D-trehalose	+	+	-	-
D-tagatose	+	+	-	-
D-melezitose	+	+	-	-
D-manitol	+	+	-	-
D-sorbitol	+	+	-	-
D-melibiose	-	-	-	-
L-arabinose	-	-	+	+
L-arabitol	-	-	-	-
L-sorbose	-	-	-	-
Amygdalin	+	+	-	-
D-adonitol	-	-	-	-
Arbutin	+	+	-	-
Esculin	+	+	-	-
Gentiobiose	+	+	-	-
Glycerol	+	+	-	-
Inositol	-	-	-	-
N-acetylglucosamine	+	+	+	+
Potassium gluconate	+	+	+	+
Salicin	+	+	-	-
Sucrose	+	+	-	-
Growth in the presence of NaCl				
1% NaCl	+	+	+	+
2% NaCl	+	+	+	+
3% NaCl	+	+	+	+
4% NaCl	+	+	+	-
5% NaCl	+	+	+	-
10% NaCl	-	-	-	-

+, positive; -, negative
MS1-3, MS1-14, OK1-6, KCCM41517, *W. koreensis* MS1-3, *W. koreensis* MS1-14, *W. koreensis* OK1-6, *W. koreensis* KCCM41517 strains, in order.

7.0) supplemented with 1% (w/v) arginine at 37°C for 48 h and culture broth was separated from cells by centrifugation (5,000×g for 20 min at 4°C). Cells were washed three times with distilled water for removal MRS broth media. The washed cells were freeze-drying, in that order it were resuspended in lysis buffer (20 mM sodium phosphate, 1% Triton X-100, 0.02% lysozyme, pH 7.0) at 0.5 g/ml and incubated at 37°C for 30 min. Then the cells were homogenized (60 sec×5) using the Fisher sonic dismembrator 300 (ARTEK Systems Corporations, USA). The suspension was centrifuged at 15,000×g for 15 min at 4°C. The cell free medium and sonicated cell supernatant were used for the measurement of citrulline.

Results

Isolation of lactic acid bacteria from natural sea salts

The ornithine producing capacity of lactic acid bacteria strains isolated from natural sea salt was accessed using TLC. Through this method, 8 strains with ornithine producing potential were selected (Fig. 1). Among the selected strains, the ornithine producing potential of MS1-3 and MS1-14 was relatively high based on the data from HPLC (data not shown). According to the results derived by accessing the formational and biochemical characteristics of these two strains, they were gram-positive and short rod type bacteria, while producing carbon dioxide under anaerobic condition. The isolates fermented D-ribose, D-galactose, D-lactose, D-maltose, D-cellobiose, D-tagatose, D-trehalose, sucrose, D-melezitose, gentiobiose, D-glucose, while failing to decompose D-melibiose, inositol, L-sorbose (Table 1).

Identification of the strains

For the identification of the selected strains, 16S rDNA sequencing was conducted. The 16S rDNA sequencing of MS1-3 and MS1-14 strains, which were two of the selected strains from natural sea salt, showed 99.5% and 99.8% sequence homogeny respectively with *W. koreensis* OK1-6, while showing 99.5% and 99.6% homogeny with 16S rDNA sequence of *W. koreensis* S5623 (Access no. AY035891), which were selected from kimchi (Lee *et al*., 2002; Yu *et al*., 2009). Summarizing those results and referring to the identification method of lactic acid bacteria, like Sharp *et al*. (1996), those strains were identified as *Weissella koreensis* strain and named *Weissella koreensis* MS1-3 and *Weissella koreensis* MS1-14. *W. koreensis* MS1-3 and *W. koreensis* MS1-14 were discovered to have almost identical 16S rDNA sequence (99.9%), implying that they were from the same branch. The *W. koreensis* MS1-14 strain was deposited in Korean Culture Center of Microorganisms (KFCC11491P).

Characteristics of *W. koreensis* MS strain

The selected strains grew in the temperature range of 30-37°C and pH range of 6.5-10.0 in MRS medium, and the optimum temperature and pH for growth were 37°C and pH 7.0, respectively (Fig. 2).

The level of ornithine production in those strains was accessed through quantitative analysis using HPLC. All the strains used in this experiment produced ornithine and the amount of intracellular ornithine produced was *W. koreensis* OK1-6 (113.7 mg/100 g), *W. koreensis* MS1-14 (105.2 mg/100 g), *W. koreensis* KCCM41517 (102.5 mg/100 g), and *W. koreensis* MS1-3 (71.8 mg/100 g) (Fig. 3). The amount of extracellular ornithine produced was *W. koreensis* OK1-6 (8.9 mg/100 ml), *W. koreensis* KCCM41517 (8.9 mg/100 ml), *W. koreensis* MS1-14 (4.6 mg/100 ml), and *W. koreensis* MS1-3 (4.5 mg/100 ml) (Fig. 3). Although there was a time difference, through a 48 h culture, *W. koreensis* MS1-14, *W. koreensis* KCCM41517, and *W. koreensis* OK1-6 strains metabolized

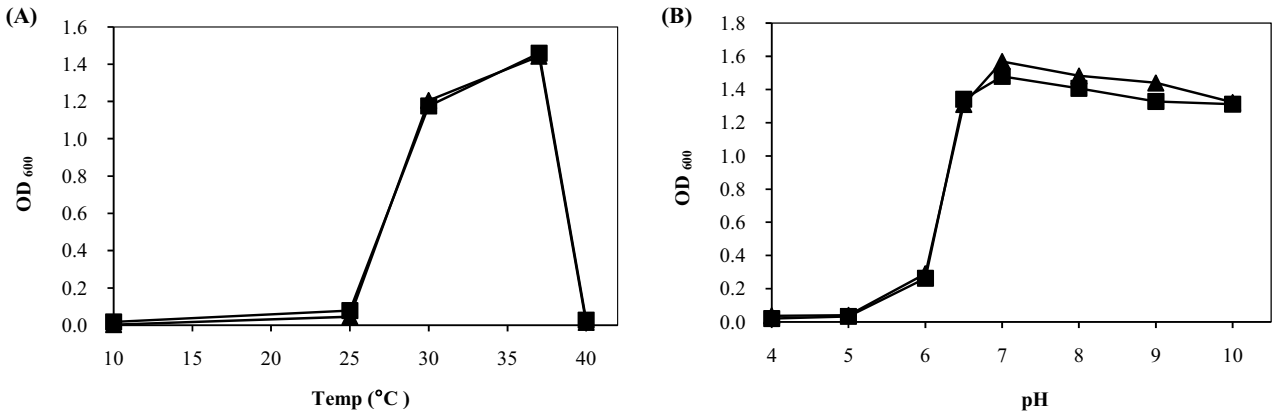

Fig. 2. Effects of temperature (A) and pH (B) on the growth of *W. koreensis* MS1-3 (■) and *W. koreensis* MS1-14 (▲). Growth was estimated by monitoring at OD_{600} after 12 h culture.

almost all the 1% arginine added (data not shown). In order to test their ability to transform arginine to ornithine via the arginine deaminase (ADI) pathway, the selected strains were studied for their ability to produce citrulline. Citrulline was formed and metabolized at higher rate during the exponential growth phase (Fig. 4).

Figure 5 shows the result derived by comparing and analyzing the level of salt resistance after adding NaCl with various concentration levels to MRS medium (pH 7.0, 37°C). *W. koreensis* MS1-3, *W. koreensis* MS1-14, and *W. koreensis* OK1-6 showed a growth rate of over 80% in 6%-NaCl added medium, implying they are moderately halophilic. However, the cell growth of *W. koreensis* KCCM41517 strain was not observed at the NaCl concentration of 5%, showing that its level of salt resistance was the lowest among the compared strains.

Discussion

Lactic acid bacteria possessing ornithine producing capacity were isolated from Korean natural sea salts, and their characteristics were then investigated. The isolated *Weissella koreensis* MS1-3 and *Weissella koreensis* MS1-14 strains possess the capacity to produce ornithine or ornithine and citrulline by decomposing arginine (Figs. 1, 3, and 4), suggesting that the ADI pathway is operative. The isolated *Weissella koreensis* MS1-3 and *Weissella koreensis* MS1-14 strains are also known to have 99.5% and 99.8% 16S rDNA sequence homogeneity with *W. koreensis* OK1-6, which was the recently isolated strain with ornithine producing capacity from Kimchi (Yu *et al*., 2009). However, the results showed that they could decompose sugar extensively which differentiated it from the strain discovered from Kimchi. For example, *W. koreensis* MS1-3 and MS1-14 strain decompose D-galactose, D-lactose, D-maltose, D-cellobiose, D-tagatose, D-trehalose, sucrose, D-melezitose, and gentiobiose, while *W. koreensis* OK1-6 and *W. koreensis* KCCM41517 strains fail to decompose those sugars (Table 1). *W. koreensis* MS1-3 and *W. koreensis* MS1-14 show similar salt resistance as that of *W. koreensis* OK1-6, however, the salt resistance with *W. koreensis* KCCM41517 strain was very

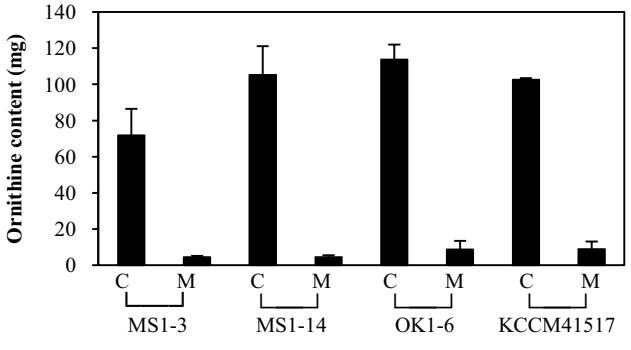

Fig. 3. Contents of ornithine produced by *W. koreensis* strains in MRS media containing 1% arginine. MS1-3, MS1-14, OK1-6, KCCM41517 stand for *W. koreensis* MS1-3, *W. koreensis* MS1-14, *W. koreensis* OK1-6, *W. koreensis* KCCM41517, respectively. Ornithine content was measured by HPLC as described in 'Materials and Methods' after 48 h cultivation of the strain. C, cell; M, medium. Ornithine content (mg), ornithine mg/100 g cell or ornithine mg/100 ml medium. Data expressed as Mean±SD from three independent experiments.

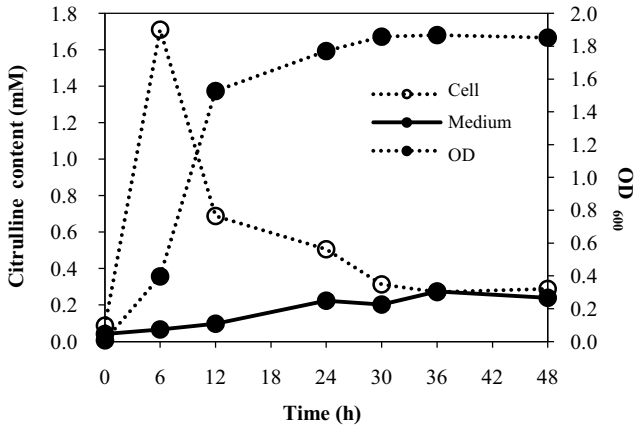

Fig. 4. Production of citrulline in the cells and medium during the growth of the selected *Weissella* MS1-3 strain. The *W. koreensis* MS1-3 was cultured in MRS medium (pH 7.0, 37°C) supplemented with 1% arginine.

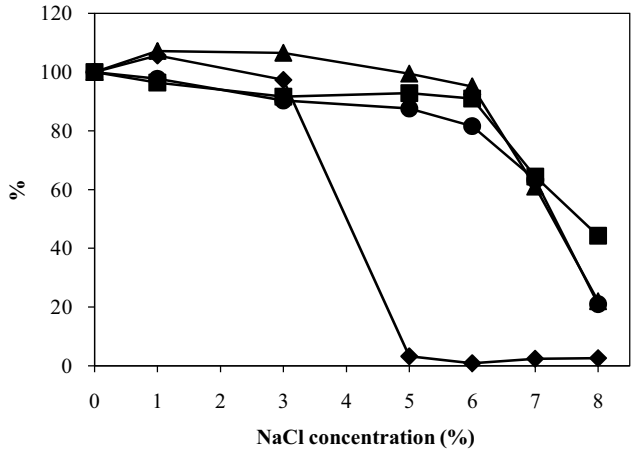

Fig. 5. Effects of NaCl on the growth of *W. koreensis* MS1-3 (■), *W. koreensis* MS1-14 (▲), *W. koreensis* OK1-6 (●), *W. koreensis* KCCM41517 (◆). Growth was estimated by monitoring culture turbidity at 600 nm after 24 h culture and expressed as % compared with 0% NaCl.

different (Fig. 5). Based on outcomes, it can be seen that there exists *W. koreensis* lactic acid bacteria strains that can produce ornithine in Korean natural sea salt similar to the ornithine producing strains discovered in Kimchi, but that there are some differences in the biochemical characteristics. In view of such, more *W. koreensis* strains having dynamic characteristics are expected to be found in those foods.

Ornithine is a type of medical amino acid that is produced by using arginine as a precursor. In the case of cheese, systematic studies on enzymes and microorganisms that are related to the arginine metabolism have been conducted (Liu *et al*., 2003). It is known that arginine is decomposed by arginase-urease (Kuensch *et al*., 1974) or arginine deaminase (ADI) (Liu *et al*., 2003) depending on the microorganisms producing ornithine. *Lactococcus lactis* subsp. *lactis* and *Lactobacillus buchneri* that originate from dairy products are known to transform arginine into ornithine through the ADI pathway (Crow and Thomas, 1982; Liu *et al*., 2003). The ornithine and citrulline formation in the isolated *Weissella koreensis* strains (Figs. 3 and 4) suggests that the arginine catabolism takes place via ADI pathway. Further studies to elucidate the molecular mechanism by which ADI pathway enzymes in the isolated *Weissella koreensis* MS1-3 and *Weissella koreensis* MS1-14 strains involve arginine catabolism may be needed.

The metabolism of amino acids promoted by lactic acid bacteria has significance in terms of the quality and safety of fermented food, while being known to serve a critical role for deriving the energy under nutrient-limited environments (Arena *et al*., 1999; Nadra *et al*., 2003). If arginine can be completely transformed into ornithine by the lactic acid bacteria of fermented foods and the produced ornithine can be well preserved without being decomposed, it can become an excellent food, exhibiting both functionality and safety. Until today, there has been no report of the arginine decomposition coupled with ornithine production route promoted by lactic acid bacteria in Korean fermented foods. Further studies of this topic might facilitate the development of a strain that can be effectively used in Korean traditional fermented foods, such as Kimchi, sauce/paste, and salted fish.

There are many traditional Korean fermented foods that use salt. For example, various salted dishes, such as Kimchi, sauces/pastes, and salted fish use a lot of salt; especially, salted fish use large amounts of salt. Therefore, in-depth studies of the wellbeing function and safety of salt that is an essential part of those fermented foods have to be made in order to realize the globalization of Korean fermented foods. Recently, the excellence of fermented food that is produced by using natural sea salt has been revealed. In Kimchi made of natural sea salt without bittern, for example, the growth promotion of effective microorganisms was accelerated as compared to the Kimchi made of refined salt, and further the extract of Kimchi that used the natural sea salt was reported to display anticancer effects (Han *et al*., 2009). Also, the water extract of soybean paste that is made of natural sea salt was shown to suppress cancer cell proliferation and promoted its destruction as compared to the water extract of soybean paste that made use of refined salt (Chang, 2009). Based these and other results, it is expected that the Korean natural sea salt itself can become a source of effective microorganisms. As such, it can provide the effective microorganism that serves a role for suppressing the growth of pathogenic microorganisms. As the Korean traditional food, Kimchi, is known to be effective against SARS or avian influenza (Lee, 2007), the interest in the source of functional microorganisms in Kimchi has increased. It is thought that the functionality of Korean foods can be further elucidated by conducting research on the functionality and origins of the lactic acid bacteria that are abundant in Korean fermented foods.

In March 2008, salt was classified as a type of food after being considered a mineral for several decades in Korea. Accordingly, food businesses that had previously used refined salt were now able to use natural sea salt in the preparation and production of various foods. Korean natural sea salt is richer in minerals, including Ca, Mg, and K, as compared to highly refined sea salt, foreign rock salt or lake salt (Park *et al*., 2000). It is also known to have relatively lower sodium chloride (NaCl) content (Park *et al*., 2000). Especially, Korean natural sea salt is thought to be abundant in minerals and live microorganisms derived from the mud flat areas. This has inspired ongoing studies to scientifically demonstrate the benefits of Korean natural sea salt and to encourage its application in the food industry. It is also thought that the revitalization of the natural sea salt industry in conjunction with the salted fish and fermented food industries may be realized through the discovery of functional microorganism as well as through improved hygiene and safety, and lead to their worldwide acceptance.

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