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Biosynthesis of L-Ascorbic Acid by Microorganisms in Kimchi Fermentation Process

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Abstract

Kimchi is an important source of various vitamins, minerals, dietary fiber, organic acids and other nutrients. In order to get a basic information for developing vitamins-rich funtional kimchi, we investigated microorganisms which are capable of synthesis of vitamin C in kimchi system. Microorganisms isolated from aliquots of kimchi were screened and cultured by using MRS or nutrient agar medium. L-Ascorbic acid produced by microorganism in medium was measured with high performance liquid chromatography. As the result, we isolated two bacteria strains N7 and N5202 producing L-ascorbic acid from the kimchi system. Morphological and Gram staining experiments showed that N7 was Gram positive *bacilli*, while N5202 was Gram negative. There were also several bacteria that were considered to synthesize erythorbic acid which is an analog of ascorbic acid. These results suggested that vitamin C-rich functional food could be developed by using the kimchi microorganisms.

Key words: kimchi, fermentation, microorganism, L-ascorbic acid, *HPLC*

INTRODUCTION

Kimchi, which is a traditional fermented Korean vegetable food, is an important source of various vitamins, minerals, dietary fiber, organic acids and other nutrients. Korean consumes about 90g of kimchi per day, and kimchi is a major source of the various nutrients(1). Kimchi fermentation is carried out by various microorganisms, mainly lactic acid bacteria, and other fermentation also processes by aerobic bacteria, yeasts, and molds occur simultaneously(2-4). Numerous biochemical changes of nutrients such as vitamins in the ingredients of kimchi occur during fermentation. Interestingly, some vitamins, particularly those of the vitamin B group and L-ascorbic acid(L-AsA) contents in kimchi, increase during kimchi fermentation compared to their initial level of kimchi preparation, and the contents are observed to be maximized in properly ripened kimchi (5,6). The observation strongly indicates that the vitamins could be synthesized during the fermentation process, however, very little is known about the change. In order to get a basic information for developing vitamin-rich functional kimchi, we investigated the presence of microorganisms which are capable of synthesis of L-AsA and its analogs in kimchi system.

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MATERIALS AND METHODS

Isolation of bacteria from kimchi

Aliquots of kimchi were homogenized and diluted with sterilized water to $10^7 \sim 10^9$ and smeared on the plate. Lactic acid producing bacteria was isolated using MRS medium. Other bacteria were isolated using nutrient agar medium. After colonies were formed, each colony was transferred to MRS broth or nutrient broth and incubated at 37°C for various time.

Preparation of sample for ascorbic acid

Aliqouts of cultured broth are centrifuged and the supernatant added with 2% HPO_3 solution at the same volume. All samples were stored at -70°C until ascorbic acid in the samples is analyzed.

Determination of L-ascorbic acid

L-AsA produced by microorgamism in a medium was

measured with high performance-liquid chromatography (HPLC) combined with UV detector at 254nm. μBondapak C_{18} or Spherical C_{18} column(40×150mm, i.d., Waters) and mobile phase, which consisted of methanol : 0.05M phosphate buffer containing 0.005M tetra-n-butylammonium bromide(20/70, v/v) were used(7). Sample was treated with ascorbate oxidase (ASOD, EC 1.10.3.3), which is specific for only L-ascorbic acid(8), confirming whether the peak is L-ascorbic acid. The flow rate used in this study was 0.7ml/min.

RESULTS AND DISCUSSION

The microorganisms involved in kimchi fermentation include about 200 species of bacteria and several yeasts isolated from kimchi(1). Since nutrient and MRS broth do not contain ascorbic acid, we used these mediums for screening microorganisms. From the screening of approximately 200 strains of bacteria in kimchi system, we isolated two bacteria strains N7 and N5202 which were found to grow in Nutrient agar plates and produce L-AsA.

The strain N7 was Gram positive *bacilli*, and N5202 was Gram negative *bacilli*(Data not shown). The N7 utilized cellulose, ribose, glucose, fructose, mannose and maltose, while the N5202 utilized glucose, fructose, maltose, lactose, as a carbon source(Table 1).

N7 and N5202 were grown with shaking at 30°C for

Table 1. Utilization of carbohydrate in N7 and N5202 strain isolated kimchi fermentation process

Carbohydrate in medium	Strains	
	N7	N5202
Glycerol	−	−
D-Arabinose	−	−
Cellulose	+	−
Ribose	+	−
D-Xylose	−	−
L-Xylose	−	−
Adonitol	−	−
Galactose	−	−
Glucose	+	−
Fructose	+	+
Mannose	+	−
Sorbitol	−	−
Maltose	+	+
Lactose	−	+
Sucrose	−	−

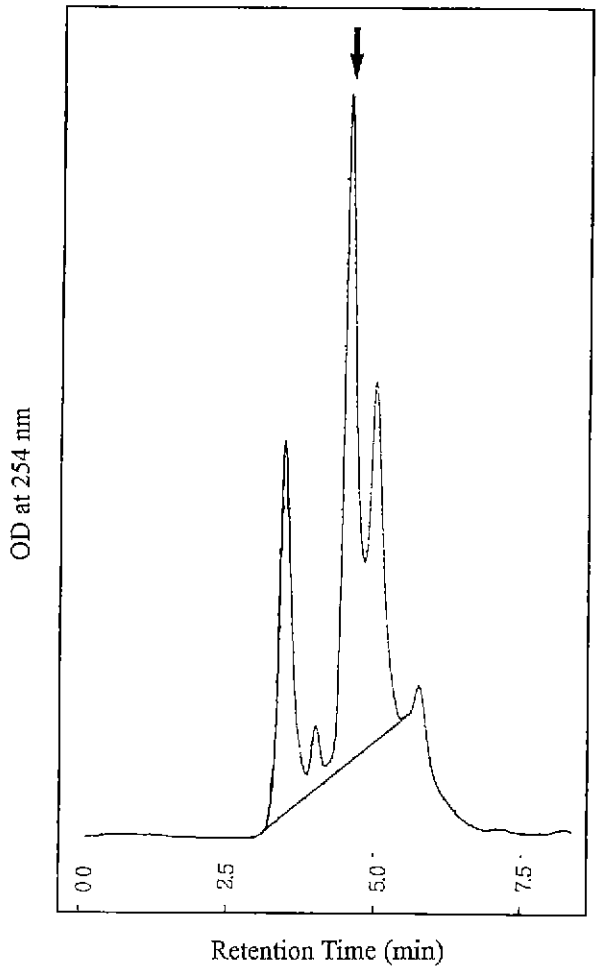

Fig. 1. HPLC chromatogram of L-ascorbic acid produced by N7.
L-AsA in medium was measured with HPLC combined with UV detector at 254 nm. μBondapak C_{18} or Spherical C_{18} column(40×150mm, i.d., Waters) and mobile phase which consisted of methanol : 0.05M phosphate buffer containing 0.005M tetra-n-butylammonium bromide(20/70, v/v) were used. ↓, L-ascorbic acid.

12, 24, 36, 72 hrs in a nutrient and MRS broth respectively. L-AsA produced into the medium by the bacteria was detected by HPLC. Fig. 1 and 2 show the chromatogram of the medium containing L-AsA produced by N7 and N5202. There was a peak which appeared with the same retention time of L-AsA standard peak. The peak was reconfirmed by treatment with ASOD, which is capable of oxidizing L-AsA, specifically. It was found that the peak on the chromatogram was completely lost when the sample was treated with the enzyme ASOD(Data not shown), indicating that the bacteria produced L-AsA into a medium. L-AsA

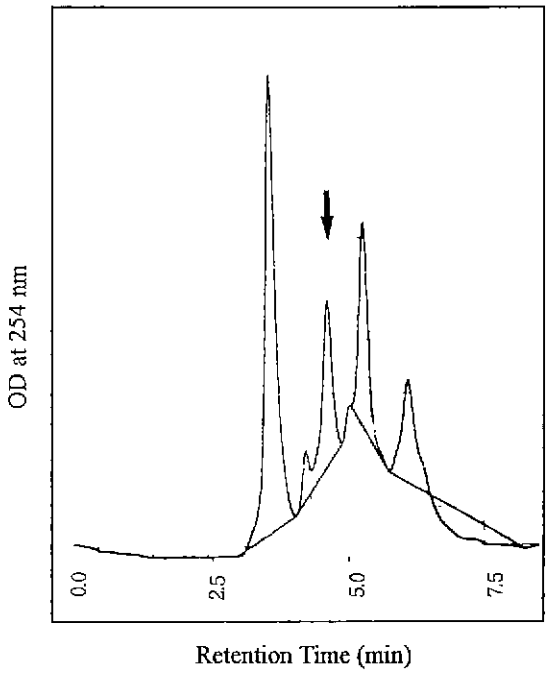

Fig. 2. HPLC chromatogram of L-ascorbic acid produced by N5202.
L-AsA in medium was measured with HPLC combined with UV detector at 254nm. μBondapak C_{18} or Spherical C_{18} column(40×150mm, i.d., Waters) and mobile phase which consisted of methanol : 0.05M phosphate buffer containing 0.005M tetra-n-butylammonium bromide(20/70, v/v) were used. ↓, L-ascorbic acid.

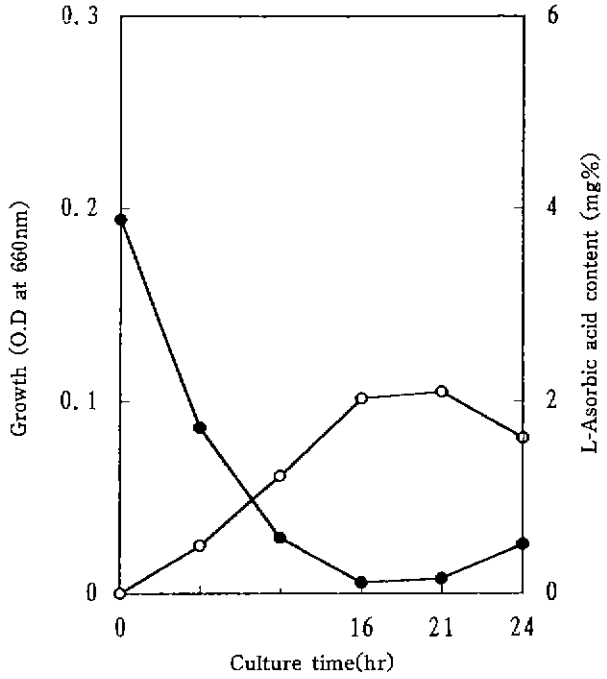

Fig. 3. Time course of the cell growth and L-ascorbic acid formation by N7 isolated during kimchi fermentation.
○ : Growth curve, ● : L-ascorbic acid contents

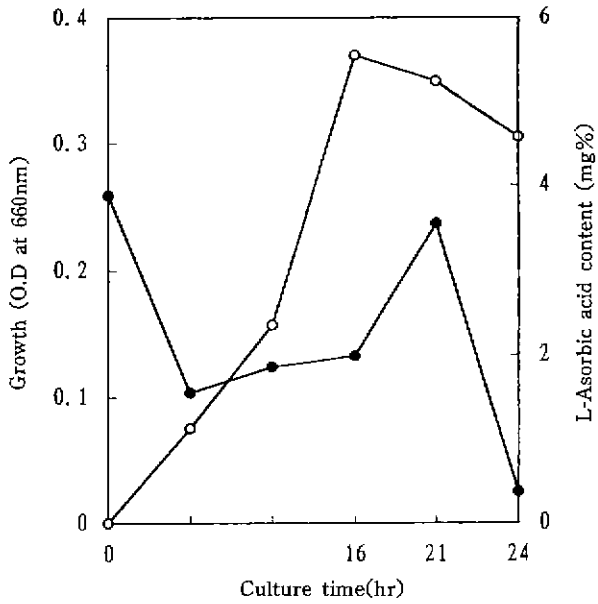

Fig. 4. Time course of the cell growth and time course of L-ascorbic acid formation by N5202 isolated during kimchi fermentation.
○ : Growth curve, ● : L-ascorbic acid contents

in a medium produced by N-7 and N5202 was 20mg% and 5mg%, respectively, when bacteria were cultured for 21 hr, which reached to its highest amount.

Fig. 3 and 4 show the growth curve and L-AsA content change of N7 and N5202 with function of time, respectively. Since L-AsA is unstable at the culture condition, it is very difficult to measure trace amount of L-AsA in the medium produced by the bacteria. In order to detect a small amount of L-AsA production, L-AsA was added to the medium with and without bacteria at the initial stage of culture and measured the change of L-AsA amount in medium for 0~24 hrs. L-AsA degradation occurred naturally in the medium was subtracted from the amount of L-AsA in the cell free system. As the result, L-AsA amount in the medium was found to increase with growing bacteria.

On the other hand, there were also some bacteria that were considered to produce L-AsA analogs, because the peaks appeared right before or next L-AsA peak, and the peaks disappeared when they were oxidized with bromine, but not with ASOD. It has been shown that animals and plants produce L-form analog (L-AsA) from D-form substrate, but suggested that microorganisms may not be able to produce L-form

from D-form substrate, and thus the reversion of substrate molecule may not occur(9). Therefore, the L-AsA analogs such as D-araboascorbic acid(D-erythrobic acid) might be produced by the microorganisms isolated from kimchi.

Some of microorganisms have been shown to be capable of synthesizing L-AsA or its analogs(10-13). For examples, *Saccharomyces cerevisiae* and *Lipomyces starkeyi* can produce L-AsA from D-glucose and L-galactono-gamma-lactone(11,12), and *C. guilliermondii*, *C. utilis*, *C. zeylanoides*, and *C. norvegensis*, *Penicillium cyaneo-fulvum* can produce D-erythroascorbic acid from D-xylose(9,13). Vitamins in the kimchi system may undergo various biochemical changes because of chemical reactions, enzymes, and microorganisms during fermentation and preservation. Therefore, besides the involvement of the microorganisms, some of enzymes or chemical reaction might alternatively be involved in the increased L-AsA contents during kimchi fermentation.

Our finding is for the first time to isolate microorganisms which are capable of biosynthesis L-AsA from kimchi system. From the results, we suggest that kimchi microorganisms might be useful for developing vitamin C-rich kimchi as a functional food.

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