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Optimization of Ascorbic Acid-2-Phosphate Production from Ascorbic Acid Using Resting Cell of *Brevundimonas diminuta*

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Abstract With the aim to produce ascorbic acid-2-phosphate (AsA-2-P) from L-ascorbic acid (AsA, Vitamin C), nine bacteria conferring the ability to transform AsA to AsA-2-P were isolated from soil samples alongside known strains from culture collections. Most isolates were classified to the genus *Brevundimonas* by 16S phylogenetic analysis. Among them, *Brevundimonas diminuta* KACC 10306 was selected as the experimental strain because of its the highest productivity of AsA-2-P. The optimum set of conditions for the AsA-2-P production from AsA using resting cells as the source of the enzyme was also investigated. The optimum cultivation time was 16 h and the cell concentration was 120 g/l (wet weight). The optimum concentrations of AsA and pyrophosphate were 550 mM and 450 mM, respectively. The most effective buffer was 50 mM sodium formate. The optimum pH was 4.5 and temperature was 40°C. Under the above conditions, 27.5 g/l of AsA-2-P was produced from AsA after 36 h of incubation, which corresponded to a 19.7% conversion efficiency based on the initial concentration of AsA.

Keywords: Ascorbic acid-2-phosphate, ascorbic acid, *Brevundimonas diminuta*, 16S rRNA

L-Ascorbic acid (AsA, vitamin C) is used as an ingredient for various medicines, food additives, cosmetics, and antioxidants [1, 5, 18]. AsA is unstable in solution, especially when exposed to high temperatures, pH levels, metal ion and oxygen concentrations, and in the presence of some enzymes [1, 20]. Thus, more stable derivatives of AsA have been developed as substitutes, *e.g.*, ascorbic acid-2-phosphate (AsA-2-P), ascorbic acid-2-fructoside, 2-*O*-α-D-glucopyranosyl L-ascorbic acid, and ascorbic acid-6-palmitate [3, 4, 7, 9, 12, 15, 16]. AsA-2-P shows a remarkably high stability *in vitro*, and is easily converted into AsA *in vivo* by enzymatic dephosphorylation [13, 21]. Although several approaches to synthesize AsA-2-P by chemical methods have been attempted, it is still expensive to phosphorylate AsA at the specific C-2 position [19]. Therefore, the enzymatic phosphorylation of AsA is considered a more advantageous method because of its high specificity and comparatively low cost (Fig. 1).

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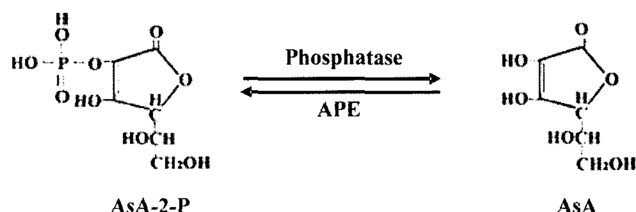

Fig. 1. Schematic diagram of the converting reaction between ascorbic acid and ascorbic acid-2-phosphate (APE: ascorbic acid phosphorylating enzyme).

In this study, many microorganisms were isolated and screened for this particular function. The culture condition was optimized for the production of AsA-2-P from AsA by the selected strain *Brevundimonas diminuta* KACC 10306.

MATERIALS AND METHODS

Chemicals

L-Ascorbic acid (AsA) was purchased from Showa Co. (Tokyo, Japan). Authentic ascorbic acid-2-phosphate (AsA-2-P) and disodium pyrophosphate were obtained

from Sigma Co. (St. Louis, U.S.A.). Luria-Bertani (LB) was from Difco Co. (Detroit, U.S.A.). All other chemicals used were commercially available and of analytical grade.

Microorganisms and Culture Conditions

Microorganisms obtained from the culture collection and isolated from soil samples were screened. The bacteria were inoculated onto an LB agar plate and incubated at 30°C overnight. A single colony was selected from each plate and transferred to a test tube containing 5 ml of LB medium with rotary shaking at 200 rpm for 24 h. Two ml of the starter culture broth was transferred into a 500-ml Erlenmeyer flask containing 100 ml of LB medium. This was followed by aerobic cultivation at 30°C for 16 h, shaken at 200 rpm. Growth was calculated by measuring the optical density at 600 nm using a spectrophotometer (SmartSpec Plus; Bio-Rad Co., Hercules, CA, U.S.A.).

Reaction Mixture Condition

The cells were recovered by centrifugation (7,000 ×*g*) at 4°C for 30 min. The pellets were washed twice with 0.9% saline solution and suspended in the standard reaction mixture. The reaction mixture was 2 ml and contained 100 mg/ml (wet weight) of resting cell, 200 mM AsA, 200 mM disodium pyrophosphate, and 50 mM sodium acetate buffer (pH 4.5). The reactions were performed at 30°C for 16 h with weak shaking (50 rpm) and the supernatant was assayed for the formation of AsA-2-P by High-Performance Liquid Chromatography (HPLC) and Thin-Layer Chromatography (TLC).

Analysis of Ascorbic Acid and Ascorbic Acid-2-Phosphate

The samples were analyzed with HPLC (Agilent 1100 Series; Agilent Co., Santa Clara, CA, U.S.A.) using a C-18 column (250 mm×4.6 mm, 5 µm; Thermo Co., U.S.A.) as previously described by Kwon *et al.* [11]. The solvent system was 100 mM KH_2PO_4 (pH 3.0) with a flow rate of 1 ml/min and a column temperature of 40°C. The compounds were detected by absorbance at 254 nm using a variable wavelength UV detector. The retention time of AsA was 4.2 min and AsA-2-P was 3.6 min. AsA and AsA-2-P were also detected by TLC (Cellulose F, Art. 5728; Merck, KG Darmstadt, Germany) developed with a solvent system of butanol:acetic acid:water (5:2:3, v/v). The presence of AsA-2-P could be detected by a deep-red coloration (480 nm) after spraying with 0.5% ferric chloride ethanol solution [14].

Identification of Isolates

The isolates were identified by 16S rRNA gene sequencing and phylogenetic analysis [6]. DNA was extracted using a commercial genomic DNA extraction kit (K-3032; Bioneer Co., Daejeon, Korea). The 16S rRNA gene was amplified using PCR (GeneAmp 9700; Applied Biosystems, Foster City, CA, U.S.A.) and the universal primer pair fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3'). PCR products were purified by using Multiscreen PCR (Millipore Co., Billerica, MA, U.S.A.) and sequencing reactions were carried out by using a DNA sequencing kit (BigDye Terminator Cycle Sequencing v2.0 Ready Reactions; Applied Biosystems,

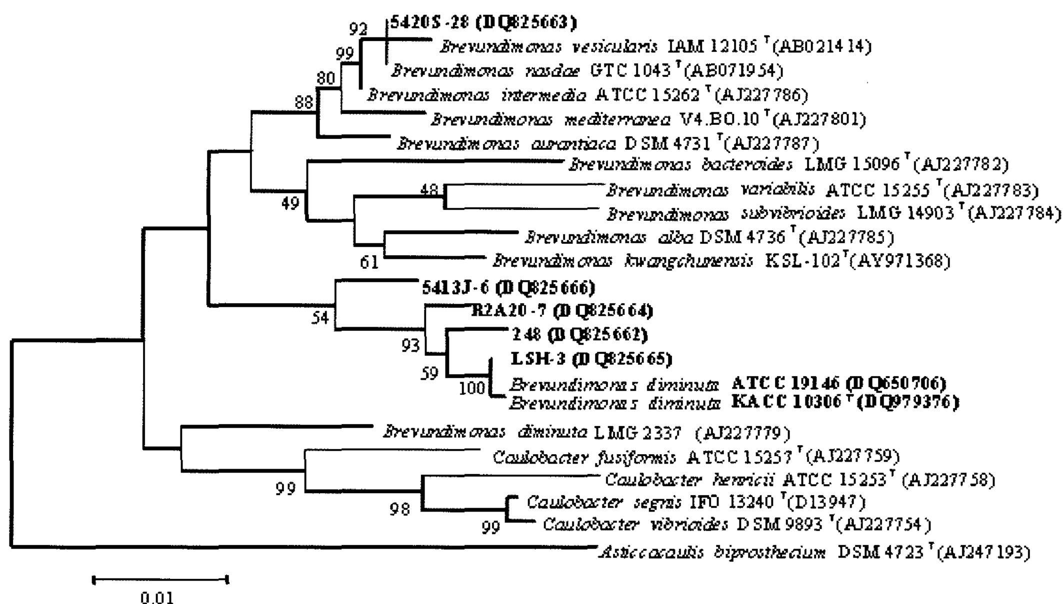

Fig. 2. Phylogenetic tree showing the relationship of the isolates and related strains on the basis of 16S rRNA sequences.
Bootstrap values (expressed as percentages of 1,000 replications) greater than 45% are shown at branch points. Bar scale, 1 nucleotide substitution per 100 nucleotides.

Foster City, CA, U.S.A.) on a Genetic Analyzer (ABI PRISM 3100; Applied Biosystems, Foster City, CA, U.S.A.) as described by the manufacturer. Phylogenetic analysis was performed by using the MEGA version 3.1 developed by Kumar *et al.* [10] after multiple alignment of the data by CLUSTAL W [22]. Distances were obtained using the Kimura two-parameter model [8] and clustering was performed using the neighbor-joining algorithm [17]. The stability of relationships was evaluated by performing bootstrap analysis based on 1,000 resamplings [2].

Nucleotide Sequence Accession

The 16S rRNA gene sequences of isolates and selected strains that showed high productivity of AsA-2-P were determined and subsequently deposited in GenBank. The accession numbers of the isolates and reference strains that are closely related to the selected strain are indicated in Fig. 2.

RESULTS AND DISCUSSION

Screening and Identification of Ascorbic Acid-2-Phosphate-Producing Microorganisms

In order to screen the isolates that produce AsA-2-P from AsA, we used reaction mixtures containing the resting cells that had been incubated at 30°C for 24 h. It seemed reasonable to assume that phosphorylation of AsA must be catalyzed by a type of kinase. On this assumption, we decided on screening conditions in which disodium pyrophosphate (ppi) was used as a phosphate donor. Nine bacteria capable of producing AsA-2-P were screened and partially identified by 16S rRNA gene sequencing and phylogenetic analysis. Among them, seven bacteria were classified to the genus of *Brevundimonas* (Fig. 2). In particular, *Brevundimonas diminuta* KACC 10306 showed the highest productivity of AsA-2-P. Therefore, we chose this bacterium as the model of AsA-2-P production to carry out subsequent experiments in this study.

Cell Growth and Ascorbic Acid Phosphorylating Activity

In order to determine the stage of growth with the highest phosphorylating activities, the growth of *Brevundimonas diminuta* KACC 10306 in LB medium was continuously monitored for a total of 24 h at intervals of 2 h (Fig. 3).

It was observed that the activity was highest during the stationary phase and decreased after 16 h. Thus, the threshold of phosphorylating activity was reached at 16 h for the cultures grown at 30°C. Cells were harvested at this stage of growth for subsequent experiments.

Effect of Cell Density

The effect of cell concentration on the AsA-2-P production was investigated by increasing the cell mass from 40 to 160 g/l of the reaction mixture. Increasing the cell mass enhanced the AsA-2-P formation from AsA, however, a cell concentration higher then 120 g/l inhibited the reaction. Thus, the optimum concentration was determined as 120 g/l.

Effect of Substrate Concentration

The effect of substrate concentration on phosphorylation was investigated by performing reactions at different concentrations of both AsA and disodium pyrophosphate (ppi). The effect of AsA concentration variation on the AsA-2-P production was investigated by setting the pyrophosphate concentration as 400 mM and increasing the concentration of AsA from 200 mM to 600 mM. The AsA-2-P production grew up to the highest value (21.2 g/l) with the increase of AsA concentration up to 550 mM (Fig. 4). Therefore, the concentration of AsA was controlled at 550 mM in subsequent experiments. The greatest production of AsA-2-P was attained when the pyrophosphate concentration was 450 mM (Fig. 5).

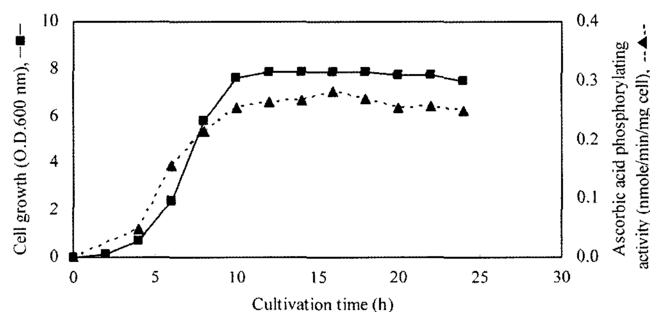

Fig. 3. Growth curve of *Brevundimonas diminuta* KACC 10306 and change in ascorbic acid phosphorylating activity.

Reactions were carried out for 24 h at 30°C in a reaction mixture containing 100 g/l (wet weight) of cell concentration, 200 mM ascorbic acid, 200 mM pyrophosphate, and 50 mM sodium acetate buffer (pH 4.5).

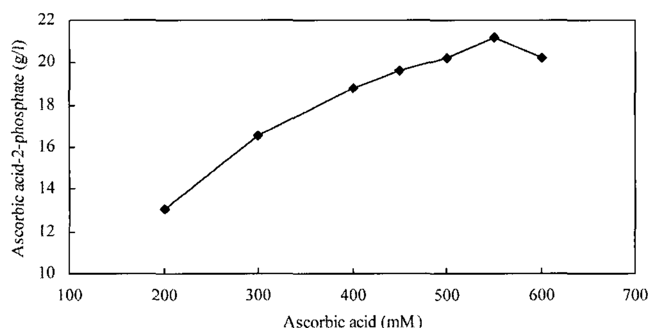

Fig. 4. Effect of ascorbic acid concentration on ascorbic acid-2-phosphate production.

Reactions were carried out for 24 h at 30°C in a reaction mixture containing 120 g/l (wet weight) of cell concentration, 400 mM pyrophosphate, and 50 mM sodium acetate buffer (pH 4.5).

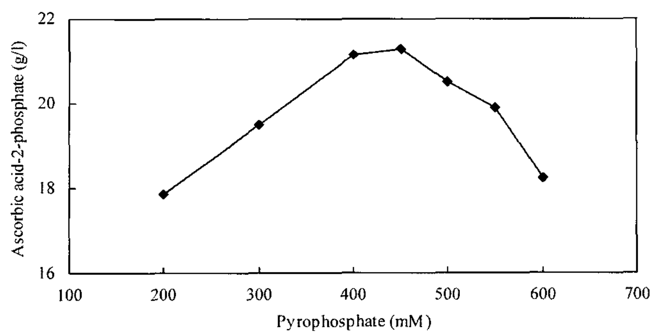

Fig. 5. Effect of pyrophosphate concentration on ascorbic acid-2-phosphate production.
Reactions were carried out for 24 h at 30°C in a reaction mixture containing 120 g/l (wet weight) of cell concentration, 550 mM ascorbic acid, and 50 mM sodium acetate buffer (pH 4.5).

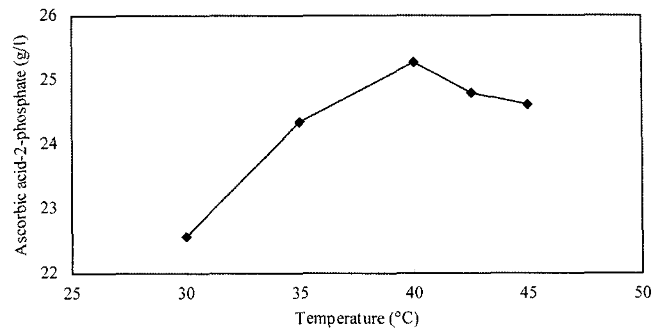

Fig. 7. Effect of temperature on ascorbic acid-2-phosphate production.
Reactions were carried out for 24 h in a reaction mixture containing 120 g/l (wet weight) of cell concentration, 550 mM ascorbic acid, 450 mM pyrophosphate, and 50 mM sodium formate buffer (pH 4.5).

Effect of Buffer Type and Concentration

In order to investigate the effect of buffer type and its concentration on the reaction mixture that was used to convert the AsA to AsA-2-P, several reactions were carried out using four different buffers under acidic conditions. This test showed that the sodium formate was the most effective buffer and the optimum concentration of sodium formate was 50 mM. Under these conditions (pH 4.5), 22.6 g/l of AsA-2-P were accumulated after 24-h incubation at 30°C.

Effect of pH and Temperature

The pH and reaction temperature were found to be an important factor for the efficiency rates of AsA-2-P production. The highest production level was achieved at pH 4.5 in 50 mM sodium formate buffer (Fig. 6). In addition, the AsA-2-P production by resting cells was measured at 30, 35, 40, 42, and 45°C to evaluate their effect on the production (Fig. 7). The highest amount of AsA-2-P accumulated occurred when the reaction mixture was reacted at 40°C and could be obtained by 25.3 g/l through 24 h of cultivation.

Time Course of AsA-2-P Production with Resting Cells

The time course of AsA-2-P production from AsA and pyrophosphate using the resting cells was investigated under the previously optimized conditions. The AsA-2-P production continuously increased until it stabilized at 36 h (Fig. 8).

In conclusion, with the optimized condition, the AsA-2-P production for the first 36 h was 27.5 g/l (108.6 mM) and its conversion rate was 19.7%, which corresponds to the theoretical mole ratio of ascorbic acid (550 mM). This result demonstrates a higher production rate of AsA-2-P compared with the 17.71 g/l of AsA-2-P production using resting cells of wild strain, previously reported by Kwon *et al.* [11]. In addition, considering the similar AsA-2-P productivity of phylogenetically related strains of the selected strain, our findings suggest that this particular enzymatic ability is a major characteristic of *Brevundimonas diminuta.* In the future, the study for the purification and characterization of specific enzyme relating to the production of AsA-2-P could serve as good achievements to increase the yield of AsA-2-P production and the conversion efficiency.

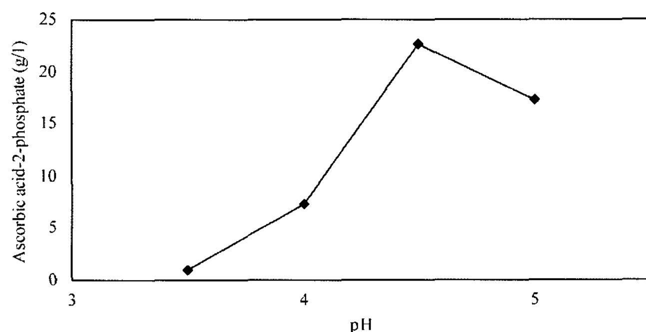

Fig. 6. Effect of pH on ascorbic acid-2-phosphate production.
Reactions were carried out for 24 h at 30°C in a reaction mixture containing 120 g/l (wet weight) of cell concentration, 550 mM ascorbic acid, 450 mM pyrophosphate, and 50 mM sodium formate buffer.

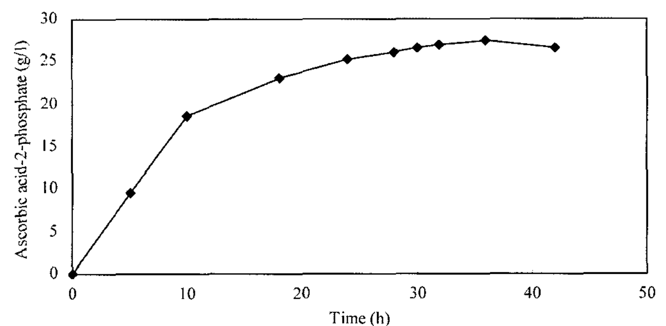

Fig. 8. Time course of ascorbic acid-2-phosphate production.
Reaction was carried out at 40°C in a reaction mixture containing 120 g/l (wet weight) of cell concentration, 550 mM ascorbic acid, 450 mM of pyrophosphate, and 50 mM sodium formate buffer (pH 4.5).

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