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RESEARCH NOTE

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Characterization of Low Temperature-adapted *Leuconostoc citreum* HJ-P4 and Its Dextransucrase for the Use of *Kimchi* Starter

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Abstract *Leuconostoc citreum* HJ-P4 is a strain isolated for *kimchi* fermentation with its low temperature-adapted growth feature and its high dextransucrase activity. The detailed characteristics of cell growth and dextransucrase activities were investigated at various environmental conditions such as temperatures, pHs, salts, and raw ingredients. This strain showed almost 2-fold higher maximal cell concentration (X_{max}) than that of the type culture *Leuconostoc mesenteroides* B-512F at 10°C. The X_{max} of the strain was maximum at pH 7 and the cell growth was inhibited by salts in a dose-dependent mode up to 7%. Addition of pepper (<6%), garlic (<10%), and ginger (<2%) in *kimchi* gave no inhibition effect on the growth of HJ-P4. Dextransucrase synthesized by this strain retained over 80% of its maximum activity at 10°C showing a comparable cold-adapted feature to its host microbe. This culture can be used as a starter culture in the industrial *kimchi* production giving desirable functions and predominance at low temperature.

Keywords: *Leuconostoc citreum*, dextransucrase, low temperature, *kimchi*

Introduction

Traditional food fermentation usually relies on naturally inoculated (inherent) microbial flora resulting in variable and uncontrolled quality of products (1). In Korea, for its taste and quality control, a leading *kimchi* producer has begun to use *Leuconostoc mesenteroides* as a starter culture. However, until now few cultures were developed for the use of commercial *kimchi* fermentation, while in the dairy and meat sectors suppliers of lactic cultures have developed numerous starters. Thus, efforts for the development of useful and effective lactic acid bacterial inoculants for *kimchi* products are required. Particularly, considering the short period of manufacturing and distribution process of commercial *kimchi* for the institutional food services which is distributed at low temperature and consumed just after arrival, low-temperature adapted starter cultures with desirable properties are of great importance.

Dextransucrase (EC 2.4.1.5.) elaborated from *Leuconostoc* can be used to synthesize prebiotic oligosaccharides (2). The enzyme transfers the glucosyl moiety of sucrose to other carbohydrates (acceptors) by linking α-(1→6)-glucosyl bond (3) and the reaction product, a series of oligosaccharides, are used as food additive due to its physicochemical properites in foods and prebiotic effect on the intestinal bacteria (4). Thus, by using the enzyme reaction of *Leuconostoc* genus, a new strategy has been proposed to produce oligosaccharides in *kimchi* or fermented milk so called as a synbiotic synthesis of oligosaccharides during lactate fermentation (5,6). In the *kimchi* manufacturing process, simple addition of sucrose and maltose in the ingredients achieved high conversion yield into isomaltooligosaccharides by the reaction of dextransucrase secreted from the inherent *Leuconostoc* genus. This method provides an innovative process for the manufacture of health promoting *kimchi* containing prebiotic oligosaccharides. For this process, a low temperature-adapted starter culture, *Leuconostoc citreum* HJ-P4, was isolated (7). For industrial application of this strain, detailed characterization of this culture under the *kimchi* manufacturing condition is necessary.

Therefore, in this study, the effects of environmental factors were investigated on the growth and dextransucrase activities of HJ-P4, in terms of temperatures, pHs, salts, and raw ingredients of *kimchi* like pepper, garlic, and ginger.

Materials and Methods

Strains and media *Leuconostoc citreum* HJ-P4 (KACC 91035) was used to examine the effects of environmental conditions on *kimchi* starter strain. *Leuconostoc mesenteroides* NRRL B-512F was used as a control strain for comparison, due to its wide uses in the study of *Leuconostoc* genus and dextransucrase secreted by this strain. For the production of dextransucrase, the S-medium was used (8), where 0.5-1 mg/mL Tween 80 was added for enzyme stabilization during separation. General chemicals including dextranase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vegetables including cabbage, radish, red pepper, ginger, and garlic were purchased from the local grocery stores.

Characterization of physiological properties of HJ-P4 To measure the growth rate of *Leuc. citreum* HJ-P4 under various conditions, 100 mL of sterilized de Man Rogosa and Sharpe (MRS) broth was inoculated with 1 mL of pre-cultured broth and incubated at 28°C (if not noted) with 120 rpm of shaking speed. The cell growth was monitored by detecting optical density at 550 nm and the dry cell mass (g/L) was calculated using the predetermined

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correlation curves with optical density. In order to investigate the effect of ingredients, various concentrations of pepper, garlic, and ginger were added in MRS broth. The cell counting of *Leuconostoc* was performed by plate-spreading onto MRS agar and incubating at 28°C for 48 hr.

Assay of dextransucrase activity Dextransucrase purification was conducted by Miller's procedure (9) after minimal modification. Dextransucrase activities were measured by assaying changes in the fructose concentration in 20 mM of Na-acetate buffer solution (pH 5.2) 13 mL containing 1.5 M of sucrose 2 mL, 1 mM of $CaCl_2$, and 0.02% of NaN_3. The fructose concentration was measured using 3,5-dinitrosalicylic acid (DNS) method (10). One unit of dextransucrase was defined as the amount of enzyme used to produce 1 μmol of fructose/min at 28°C. Optimum temperature of purified enzyme was determined by measuring activities at various temperatures.

Results and Discussion

Effects of environmental factors on cell growth ***Temperature effect***: Fermentation conditions for *kimchi* are usually lower than room temperature and it is necessary for the starter strain to become a major population soon after inoculation, leading the whole fermentation process at the temperature. Therefore, detailed growth rates of HJ-P4 strain at various temperatures were measured at 10, 20, 30, and 37°C after incubation in MRS broth. As presented in Fig. 1, at 10°C the strain grew up to 0.8 (g/L) cell mass (X_{max}) resulting in about 2-fold higher X_{max} than that of the type culture *Leuc. mesenteroides* B-512F (Fig. 1A). This result revealed that HJ-P4 be a low temperature-adapted strain which would be able to become a predominant strain during the low temperature incubation period of commercial *kimchi* fermentation.

pH effect: To investigate the acid-tolerance of HJ-P4, growth at different pHs was observed after 10 hr of incubation at 28°C on MRS broth adjusted with 1 N HCl or 1 N NaOH to pH 3, 4, 5, 6, 7, 8, and 9. As presented in Fig. 1B, HJ-P4 showed an optimal pH at 7, and a typical sensitivity against acidic condition, declining linearly along with pH changes under pH 7. HJ-P4 was determined to be acid-labile strain like other species belonging to the same genus.

Salt effect: To measure the salt-tolerance of the strain, growth at different salt concentrations was observed after 1 day of incubation at 28°C on MRS broth with 1, 2.5, 3.5, 5, and 7%(w/v) NaCl. As salt concentrations increased, the cell mass of HJ-P4 reduced, and at 7% NaCl the cell growth was completely inhibited (Fig. 2C). In case of B-512F, it showed a comparable salt-tolerance with HJ-P4 with slight variation. These results were consistent with the previous report that lower salt concentrations (<6%) were favorable for the growth of *Leuconostoc* strains, meanwhile higher salt concentrations were for *Lactobacillus* strains (11). Considering the average salts content in *kimchi*, 2.5% in regular cabbage *kimchi* and 1% of *dongchimi* (watery radish *kimchi*), this strain was supposed to grow well enough in general *kimchi* products.

Effects of raw ingredients: To be used as a starter culture for *kimchi* fermentation, the growth of strain should not be inhibited significantly under the fermenting conditions,

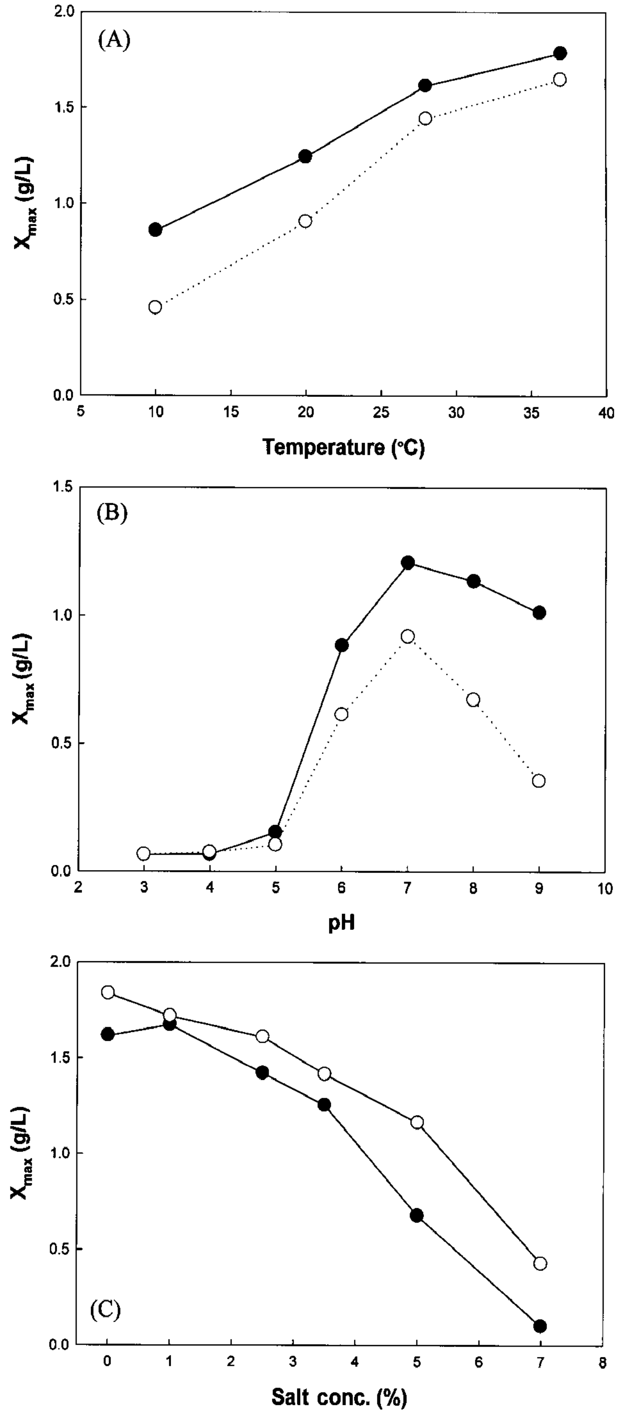

Fig. 1. Effects of temperature (A), pH (B), and salt concentration (C) on the growth of reference strain, *Leuc. mesenteroides* B-512F (○) and HJ-P4 (●) strain.

particularly from several major ingredients of *kimchi*. For this, cell growth of HJ-P4 was measured in the media including different ingredients contents; 1, 2, 3, 4, and 6%(w/v) of pepper, 2, 4, 6, 8, and 10%(w/v) of garlic, 0.05, 0.1, 0.5, 1, and 2%(w/v) of ginger (Fig. 2). The unique hot taste of *kimchi* which differentiate it from sauerkraut and pickled cucumber originates from red pepper. Capsaicin from pepper or red chilli was reported to give broad inhibitory effects on almost microbial growth including *Listeria monocytogenes*, *Bacillus subtilis*, and yeast to various

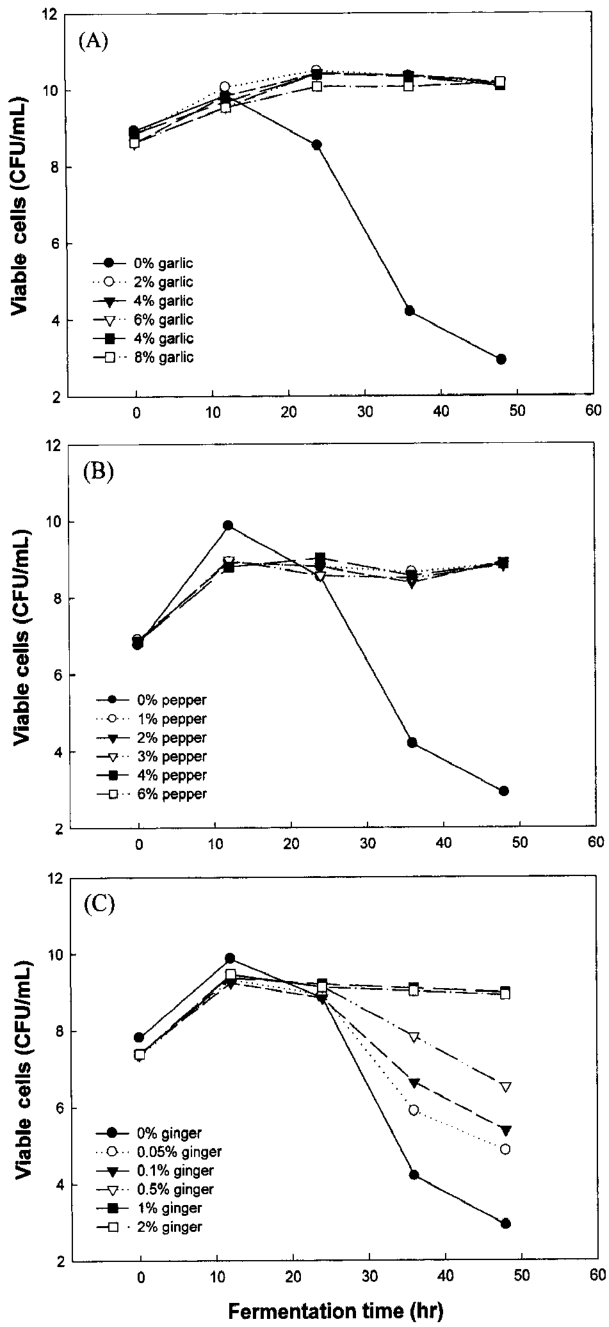

Fig. 2. Effects of various *kimchi* ingredients on the growth of HJ-P4 strain. Growth at different ingredients were observed after 48 hr of incubation at 28°C on MRS broth added with 0 (●), 2 (○), 4 (▼), 6 (▽), 8 (■), and 10% (□) (w/v) garlic (A); 0 (●), 1 (○), 2 (▼), 3 (▽), 4 (■), and 6% (□) (w/v) pepper (B); and 0 (●), 0.05 (○), 0.1 (▼), 0.5 (▽), 1 (■), and 2% (□) (w/v) ginger (C), respectively.

degrees (12,13). Accordingly, red pepper powder was mixed in the culture medium and cell growth of HJ-P4 was monitored for 48 hr (Fig. 2A). In case of control with no red pepper, HJ-P4 revealed a typical growth pattern of *Leuconostoc* genus in MRS broth showing an initial cell propagation and slower cell death due to possible over-acidification in broth. However, in cases of cultivation with red pepper, a remarkable result was observed, in all samples including up to 6% of pepper, after reaching maximum level of cell mass, the cell population of HJ-P4 was retained through out the fermentation period. A similar result was already reported in the previous study (14) observing a growth-stimulating effect of pepper on lactic acid bacteria (LAB) at over 3.5% of concentration. Considering the usual dosage (<3%) of red pepper in *kimchi*, it was found that, regardless of antimicrobial activity of capsaicin, HJ-P4 grows well and maintains its predominance in a general *kimchi* containing red pepper for an extended period of time. The facts that the growth of *B. subtilis* was strongly inhibited (15) but *Staphylococcus aureus* was not affected (16) implicate that the antimicrobial specificity of capsaicin on microbial growth vary depending on genus.

Other distinctive ingredients of *kimchi* could be garlic (*Allium sativum* L.) and ginger (*Zingiber officinale* Roscoe). Since garlic and ginger extracts were also known to exhibit a broad antibiotic spectrum (17,18), the antimicrobial effects of garlic and ginger on the growth of HJ-P4 were investigated and the results are presented in Fig. 2B and 2C. The cell growth profiles in the media containing garlic or ginger provide interesting results like red pepper, that, when HJ-P4 reached the stationary phase after 10 hr incubation, cell population was maintained through out the fermentation without growth inhibition. As more ginger was added up to 2%, HJ-P4 retained its maximum cell mass along the fermentation. Cho *et al.* (19) reported a same result that LAB population increased more at higher garlic concentrations during initial period of the fermentations. Also, Yi *et al.* (20) reported that ginger and red pepper powder showed positive effect on the growth of *Leuc. mesenteroides*. Garlic has long been used for medicinal purpose as well as food flavoring, most recently for its cardiovascular, antineoplastic, and antimicrobial properties with its active sulfur compound, allicin (21). Garlic extract exhibits a broad antibiotic spectrum against both Gram-positive and Gram-negative bacteria (17). Garlic extract containing allyl alcohol was known to inhibit the growth and respiration of *Candida albicans* by giving oxidative stress (22). Besides, ginger, one of the most widely used spices, has been applied frequently not only in foods as flavoring but also in oriental medicine. Its several constituents including gingerol, shogaol, and zingerone have been shown to exert antimicrobial activity (18). For manufacture of *kimchi* product, optimal content of garlic was not standardized yet varying on reports (23), however our results implicate that general addition of garlic(<10%) and ginger(<2%) would not inhibit the cell growth of HJ-P4 in *kimchi*.

Effects of environmental factors on dextransucrase activity HJ-P4 showed a fast growth rate at low temperature and thus we examined characteristics of its dextransucrase as well. To obtain a purified enzyme from HJ-P4, an ion-exchange chromatography was used (9). Sodium dodecyl sulfate- polyacrylamide gel electrohoresis (SDS-PAGE) analysis showed a purified single protein band (about 180 kDa) in the fractions containing enzyme activity after 1 M NaCl gradient elution of the ion exchange column (data not shown). When the enzyme activities were measured at various temperatures (Fig. 3), the enzyme revealed a comparable cold-adapted feature to its host strain, showing over 80% of maximal activity at 10°C (Fig.

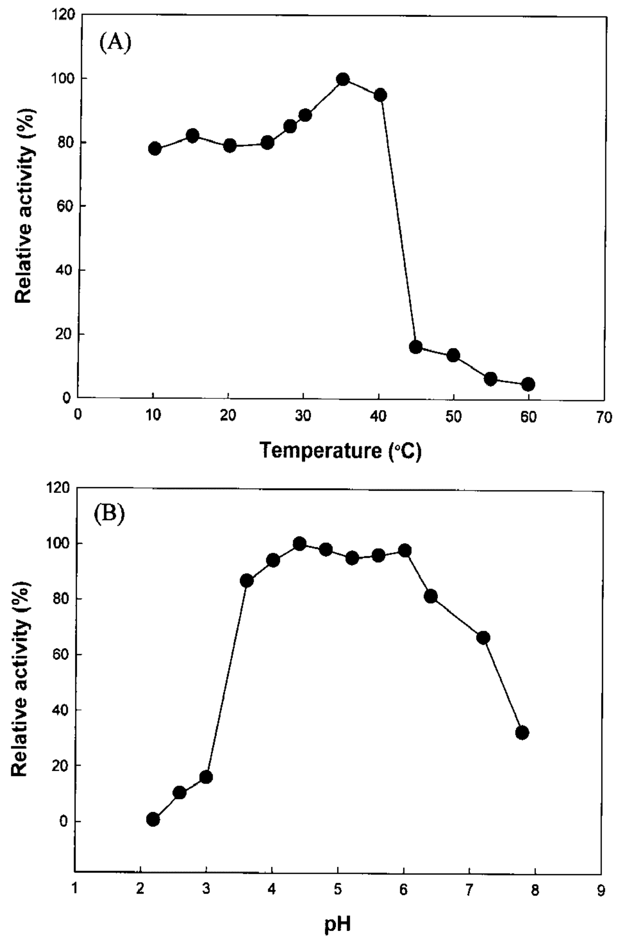

Fig. 3. Effects of temperature (A) and pH (B) on the purified dextransucrase activity.

3A). Next, the pH profile of purified dextransucrase was analyzed by measuring activities at various pH conditions (Fig. 3B). The enzyme showed over 80% of activities in between pH 3.7 and 7.5, revealing its general pH sensitivity like its host strain. This feature enables the enzyme to be used for the low temperature enzyme reaction to synthesize poly- or oligo-saccharides during *kimchi* fermentation. It means that, by addition of this strain as a starter with sucrose and maltose in *kimchi*, synbiotic synthesis of health-functional isomaltooligosaccharides at low temperature would be possible, particularly during the distribution period of commercial *kimchi* at below 10°C. This method (so called as 'acceptor reaction') provides another merits to *kimchi* producers such as prevention of dextran-polymer (giving unfavorable mouthfeel) synthesis and enhancement of sweetness level due to fructose residues released from sucrose after trans-glucosylation reaction of dextransucrase during *kimchi* fermentation.

In conclusion, *Leuc. citreum* HJ-P4 strain was evaluated for the use as starter culture under several environmental conditions of *kimchi*. This strain showed a fast growth rate and high dextransucrase activity at low temperature. Dextransucrase synthesized by this strain retained over 80% of its maximum activity at 10°C showing a comparable cold-adapted feature to its host microbe. This feature was considered as an important requirement of the starter culture for commercial *kimchi* manufacturing process and distribution. When this strain was cultured with various concentrations of ingredients, cell growth was inhibited by salts in a dose-dependent mode up to 7%(w/v), however, pepper, garlic, and ginger gave no inhibition effect on the growth of HJ-P4 under the usual dosage in *kimchi* and even they gave a growth factor-like effect. These strains are supposed to be useful as a function added-starter in the manufacture of lactate-fermented foods not only *kimchi* but also sauerkraut or pickled cucumber.

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