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Selection of psychrotrophic *Leuconostoc* spp. producing highly active dextransucrase from lactate fermented vegetables

Hyun-Ju Eom, Dong Mi Seo, Nam Soo Han *

Department of Food Science and Technology, Research Center for Bioresource and Health, Chungbuk National University, Cheongju 361-763, South Korea

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Abstract

Leuconostoc is the major bacterial genus in the initial phase of the lactate fermentation of vegetables. The dextransucrase elaborated from this bacterium is used to synthesize dextran polymers or prebiotic oligosaccharides. To use *Leuconostoc* as a starter culture in the manufacture of the kimchi-like fermented foods at low temperature, we isolated microbial flora that showed fast growth rates and high enzyme activity under the test conditions. Nine hundred colonies of *Leuconostoc* were collected from kimchi, sauerkraut, and pickled cucumber using three consecutive selection media; after batch culture and enzyme activity assays, four strains were selected. Sequencing of 16S rRNA genes of the strains revealed that HJ-S7 and HJ-S13 were *Leuconostoc* (*Ln.*) *mesenteroides* and HJ-P4 and HJ-P5 were *Ln. citreum*. When compared to the type strain, *Ln. mesenteroides* B-512F, HJ-P4 showed a more than twofold faster growth rate and 20-fold higher enzyme activity during cultivation at 8 °C. These strains are suitable as oligosaccharide-synthesizing starters for the fermentation of not only kimchi but also sauerkraut and pickled cucumbers.
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Keywords: Dextransucrase; Kimchi; *Leuconostoc*; Oligosaccharides; Panose; Pickle; Psychrotroph; Sauerkraut

1. Introduction

Lactic acid bacteria (LAB) are the principal microorganisms responsible for the fermentation of vegetables such as cabbage, carrots, beets, and cucumbers, but the indigenous LAB flora varies as a function of the quality of the raw material, temperature, and harvesting conditions. Spontaneous fermentation thus leads to variations in the sensory properties of the products. It has been shown that the use of a starter culture helps to standardize fermentation by controlling the microbial flora (de Valdez et al., 1990). Although suppliers of lactic cultures have numerous starter cultures for the dairy and meat sectors, very few cultures designed for vegetable fermentations are available (Gardner et al., 2001). Thus, studies on the evaluation and development of effective LAB starters to rapidly initiate fermentation of vegetables are necessary.

Leuconostoc spp. are hetero-fermentative lactic acid bacteria and are the major bacterial population in kimchi and sauerkraut from the initial to the middle stage of fermentation (de Valdez et al., 1990; Lee et al., 1992). During these stages, these bacteria produce various constituents, such as lactic acid, acetic acid, alcohol, CO_2, and mannitol, all of which contribute to the flavor of fermented foods. The number of these bacteria is highest during the optimum ripening period. Dextransucrase (EC 2.4.1.5) elaborated by *Leuconostoc* spp. transfers the glucose moiety of sucrose to form dextran by releasing fructose as free residue and catalyzes the transfer of glucose from sucrose (donor) to other carbohydrates (acceptors) by linking an α–(1→6)–glucosyl bond (Robyt and Eklund, 1983). When the acceptor is a monosaccharide or disaccharide, a series of oligosaccharide acceptor-products is usually produced.

These oligosaccharides are useful as food additives because of their desirable physicochemical properties in foods and their prebiotic effect on intestinal bacteria (Tomomatsu, 1994). Oligosaccharides, consisting of a mixture of hexose oligomers with a variable extent of polymerisation, are food products with interesting nutritional properties. They may be naturally present in food, mostly in fruits, vegetables or grains, or produced by biosynthesis from natural sugars or polysaccharides and added to food products because of their nutritional properties or

* Corresponding author. Tel.: +82 43 261 2567; fax: +82 43 271 4412.
E-mail address: namsoo@chungbuk.ac.kr (N.S. Han).

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organoleptic characteristics. The extent of resistance to enzymic reactions occurring in the upper part of the gastrointestinal tract allows oligosaccharides to become 'colonic nutrients', as some intestinal bacterial species express specific hydrolases and are able to convert oligosaccharides into short-chain fatty acids (acetate, lactate, propionate, butyrate) and/or gases by fermentation. Oligosaccharides that selectively promote some interesting bacterial species (e.g. lactobacilli, bifidobacteria), and thus equilibrate intestinal microflora, are now termed prebiotics (see reviews, Delzenne, 2003; Chow, 2002).

We have proposed a new strategy to produce oligosaccharides in kimchi by using a simultaneous biocatalytic reaction during lactate fermentation (Han et al., 2002; Eom et al., 2003). In the kimchi manufacturing process, simple addition of sucrose and maltose to the ingredients has achieved a high conversion yield of isomaltooligosaccharides by the reaction of dextransucrase secreted by the inherent *Leuconostoc* bacteria. This method provides a simple and innovative process for the manufacture of health-promoting kimchi containing prebiotic oligosaccharides.

For the above process, starter cultures with high oligosaccharide productivity and fast growth rates at low ripening temperature are required. This research was carried out to obtain starter cultures of *Leuconostoc* spp. that could be added in the manufacture of kimchi. The requisite characteristics of the starters were fast growth rate, to allow the starter to become a major component of the LAB microbial flora, and high dextransucrase activity, for overproduction of oligosaccharides during incubation at generally low ripening temperatures. Starter strains of *Leuconostoc* spp. were isolated by following strategies: colony screening from kimchi, sauerkraut, and pickled cucumber using three consecutive selection media; selection of colonies that had dextransucrase activity by thin layer chromatography (TLC); selection of superior strains showing rapid growth rate and high dextransucrase activity at low temperature during batch fermentation; and identification of selected strains using 16S rRNA gene sequencing.

2. Materials and methods

2.1. Preparation and fermentation of vegetables

Kimchi is a traditional Korean lactate-fermented vegetable made from Chinese cabbage or radish mixed with various spices such as hot pepper, garlic, ginger, and onions (Cheigh and Park, 1994). A watery radish kimchi (Dongchimi-kimchi) was prepared, incubated at 10 °C as described previously (Han et al., 2002), and used as a source for the isolation of potential starter strains. Commercial kimchi products were also purchased from retail stores in Cheongju, South Korea. For sauerkraut preparation, shredded cabbage was salted in 2.5% brine solution, mixed with crushed garlic and onions, and incubated at 10 °C (Halász et al., 1999). For pickled cucumber, cucumbers placed in 5% brine solution were incubated at 10 °C for the first 3 days; after removal of the brine solution, the salted cucumbers were mixed with 1% salt solution, garlic, and onions and kept at the same temperature (Jang, 1997).

2.2. Experimental media

For selection of low-temperature-adapted Leuconostoc strains from fermented vegetables, lactobacilli MRS (Difco Laboratories; Detroit, MI, USA) agar medium, PES agar medium (Miyao and Ogawa, 1988) containing 2% sucrose in phenylethanol agar (Difco), and a novobiocin–leuconostoc selective (NLS) agar medium were used after modification (Choi et al., 1996). NLS agar was prepared by the addition of tryptone (10 g), yeast extract (1 g), beef extract (1 g), glucose (20 g), K_2HPO_4 (2 g), $MgSO_4$ (0.1 g), sodium acetate (5 g), ammonium sulfate (2 g), sorbic acid (0.5 g), sodium azide (0.0075 g), Tween 80 (1 g), agar (15 g), novobiocin (5 mg; filter sterilized), vancomycin (30 mg; filtered), and cysteine-HCl (0.5 g; filtered) per liter of distilled water. For the assay of dextransucrase activity, S-medium was used; it contained sucrose (24.7 g), peptone (4.2 g), yeast extract (4.2 g), K_2HPO_4 (20 g), $MgSO_4{\cdot}2H_2O$ (0.2 g), NaCl (0.1 g), $FeSO_4{\cdot}7H_2O$ (0.1 g), $MnSO_4{\cdot}H_2O$ (0.1 g), and $CaCl_2{\cdot}2H_2O$ (0.13 g) per liter of distilled water (Motomitsu and Robyt, 1998). For the detection of panose on TLC, SM-medium was used, which was prepared by adding maltose (24.7 g) to the S-medium.

Sucrose and standard chemicals were purchased from Sigma Chemical Co. (St. Louis, MO. USA). Maltose was obtained from Duksan Pharmaceuticals (Yongin, South Korea). Lactobacilli MRS broth was purchased from Difco. Cubic plastic jars with sealing lids were used as fermentation vessels (retail purchase). Vegetables, including cabbages, cucumbers, radishes, red pepper, green onions, and garlic, were purchased from a local grocery store.

2.3. Selective screening of Leuconostoc spp.

The diluted liquid from fermented vegetables was spread on lactobacilli MRS agar and incubated for 48 h at 28 °C; colonies that grew on the plates were identified as *Lactobacillus* spp. or *Leuconostoc* spp. They were transferred onto both PES and NLS agar and incubated for 48 h at 20 °C; colonies that grew on both plates were identified as *Leuconostoc* spp. (Miyao and Ogawa, 1988; Choi et al., 1996). PES agar medium was used for inhibition of gram-negative bacteria and NLS agar medium was used due to the high resistance of *Leuconostoc* spp. against vancomycin and novobiocin up to 500 μg/ml of the MIC (minimal inhibitory concentration). The formation of gel-like dextran made it difficult to isolate single colonies from the agar plate, therefore glucose rather than sucrose was used as the carbon source on NLS medium.

2.4. Assay of dextransucrase activity

Dextransucrase activities were measured by assaying changes in the fructose concentration in 20 mM Na-acetate buffer solution (pH 5.2, 13 mL) containing 1.5 M sucrose (2 mL), 1 mM $CaCl_2$, and 0.02% NaN_3. Aliquots (1 mL) of the reaction solution were taken every 30 min and mixed with 10% (v/v) pyridine to terminate the reaction. The samples were centrifuged (1000 ×*g*) for 5 min, and the liquid fraction was filtered through a 0.45-μm

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