Efficient Citrus (*Citrus unshiu*) Byproduct Extract-Based Approach for *Lactobacillus sakei* WiKim31 Shelf-Life Extension

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ABSTRACT: Lactic acid bacteria produce various bioactive compounds widely used in human healthcare. However, studies on cryoprotective agents for the efficient storage of lactic acid bacteria after freeze-drying are still lacking. Here, we report the shelf-life extension effects of a highly efficient and eco-friendly cryoprotective agent and a cold adaptation method on *Lactobacillus sakei* WiKim31. Cold adaptation of *L. sakei* WiKim31 increased exopolysaccharide expression in response to abiotic stress. As a possible cryoprotective agent, the citrus byproduct (CP) contains a variety of sugars, amino acids, and cations, exhibiting high antioxidant activity. *L. sakei* WiKim31 powders formulated with CP or a mixture of soy powder (SP) and CP exhibited high cell viability at 58.3 and 76.3%, respectively, after 56 days of storage. These results indicate that CP can be efficiently used as a novel cryoprotective agent either alone or in combination with SP to improve the storage conditions of *L. sakei* WiKim31 and preserve it longer.

1. INTRODUCTION

Lactic acid bacteria (LAB) are a heterogeneous group of bacteria that have been widely used in fermented food production, disease treatment, and cosmetic ingredients, accounting for their growing demand in human healthcare.\(^1\) In addition, they are the most feasible resources for various industrial applications, and they contribute to the growth of the global bioeconomy. The global probiotic market was worth over 44.2 billion US dollars in 2019 and is expected to grow at a compound annual rate of 7.7% by the end of 2025 based on recent market research.\(^2\) For the industrial application of LAB, recent studies have focused more on LAB functionality analysis, large-scale production, and shelf-life extension.

Drying technologies, such as fluidized bed drying, freeze-drying, spray-drying, and vacuum drying, have been used to improve the shelf-life of LAB.\(^3\) Among these, freeze-drying, an effective method for water removal, is widely used to produce high-viability LAB powders. However, freeze-drying may have negative effects on cell membrane lipids or the structure of sensitive proteins due to the high osmotic pressure.\(^4\) Cold adaptation, which induces the formation of unsaturated fatty acids in the plasma membrane of bacterial species, has been widely used to enhance cryotolerance.\(^5\) Thus, various cryoprotectants and cold adaptation methods have been tested to improve cell viability during freeze-drying.

As of 2019, the global production of all citrus fruits was estimated to be 158 million tons.\(^5\) China produced 44.1 million tons of citrus fruits in the same year, accounting for approximately 27.8% of the global production. More than 40 million tons of citrus waste is generated after juice extraction; although citrus waste contains large quantities of beneficial byproducts, such as soluble sugars, cellulose, and essential oils, these are discarded in the absence of a biorefinery process for the production of value-added materials.\(^6,7\) From this perspective, the utilization of agricultural waste is essential for environmental protection. The citrus (*Citrus unshiu*) byproduct (CP) contains many phenolic compounds, including didymin, hesperidin, narirutin, nobiletin, and tangeretin.\(^9\) These compounds exhibit high antioxidant activity, indicating potential for use in cryoprotection.\(^10\) Oxidative stress is significantly associated with the loss of viability in freeze-dried LAB.\(^11\) This is because excessive production of reactive oxygen species at extremely low temperatures leads to the oxidation of lipids, proteins, DNA, and RNA.\(^12\) Antioxidants, such as ascorbic acid, flavonoids, glutathione, and vitamins can prevent oxidation by lowering reactive oxygen species levels.\(^13–15\)
Low-molecular-weight sugars, such as glucose, glycerol, sucrose, trehalose, mannitol, and sorbitol, have been successfully used as cryoprotective agents for LAB during freeze-drying. As CP contains high glucose, fructose, and sucrose levels, it may be suitable as a cryoprotective agent. Therefore, a practical strategy for the utilization of CP in the production of biobased chemicals is required. Herein, we report a new approach for the utilization of CP as a cryoprotective agent to improve the shelf-life of *Lactobacillus sakei* WiKim31. The antioxidant activity of CP was evaluated, and its active ingredient levels, including sugars, amino acids, and cations, were analyzed. The synergistic effect of the combination of soy powder (SP) and CP on LAB shelf-life was assessed. In addition, microscopic observations were performed to evaluate the changes in LAB viability due to cold adaptation.

2. RESULTS AND DISCUSSION

2.1. Chemical Composition of CP. During freeze-drying, excipients (amino acids, sugars, polymers, polyols, surfactants, chelating complexes, and inorganic salts) are usually used to stabilize proteins and prevent their structural perturbations. Thus, the shelf-life extension capacities of various cryoprotective agents on microorganisms have been evaluated. In this study, we evaluated the potential of CP as a cryoprotective agent for *L. sakei* WiKim31 as CP can be used to produce valuable bioactive compounds.

The results for the validation parameter in the determination of sugars are shown in Table 1. During freezing, sugar molecules in the cryoprotective agent interact with the lipid bilayer to maintain plasma membrane integrity. Thus, a cryoprotective agent must have a high sugar content to protect LAB during freeze-drying. CP (dry matter) showed a sugar level of 63.4%; this included glucose (29.6%), fructose (11.3%), arabinose (7.6%), sucrose (7.3%), rhamnose (3.0%), xylose (2.8%), galactose (2.7%), and mannose (1.9%) (Table 2). The sufficient levels of sugar in CP might play a significant role to extend the shelf-life of *L. sakei* WiKim31.

2.2. Amino Acid Contents of Cryoprotective Agents. Various factors have been modified to improve the stability of LAB during storage. Amino acids are important as they are building blocks for proteins and act as cryoprotective agents in commercial protein formulations. In this study, we demonstrated the correlation between amino acids and cell viability during *L. sakei* WiKim31 freezing. SP (2.5%; w/v) mainly contained asparagine, arginine, leucine, glutamic acid, and serine at 806.8, 411.6, 402.5, 373.9, and 308.3 μM, respectively, whereas CP (2.5%; w/v) contained asparagine, proline, serine, and aspartic acid at 11,141.9, 971.9, 724.6, and 504.9 μM, respectively (Table 3). Proline, an amino acid with antioxidant and osmoprotectant properties, has very high solubility and osmotic pressure, and it accumulates in response to environmental stress, such as low temperatures. In a previous study, proline was shown to improve freeze stress viability, and a high proline level was found to enhance freezing tolerance. In addition, amino acids, such as alanine, glycine, lysine, serine, and 4-hydroxy proline, elicit a significant stabilizing effect during lyophilization and preserve enzyme activity by more than 95%. CP contains significant quantities of asparagine, which plays an important role in amino acid homeostasis, apoptosis suppression, and cell survival. These results indicated that amino acids might play a
significant role in improving the stability of L. sakei WiKim31 during storage. Moreover, proline and asparagine in CP might have synergistic effects when combined with SP.

2.3. Cation Levels in Cryoprotective Agents. Intra-cellular cations participate in cell homeostasis and regulate several activities ([Ca^{2+}], like intracellular pH ([Na^+])), numerous cellular functions and enzyme activity ([Mg^{2+}]), normal cell functions ([K^+]), as well as intracellular signaling and protein functions ([P^+]). Therefore, a sufficient cation concentration could increase the cell viability. We detected Ca^{2+}, K^+, Mg^{2+}, Na^+, and P^+ at 20.3, 763.9, 64.2, 198.6, and 80.3 mg/L, respectively, in SP and at 53.7, 268.5, 9.1, 14.5, and 10.4 mg/L, respectively, in CP (Table 4). Intracellular cations (Ca^{2+} and Mg^{2+}) with cryoprotective effects can extend the shelf-life of freeze-dried LAB by minimizing the damage caused by osmotic pressure. Particularly, cold adaptation can increase cation flux by activating cation channels. Therefore, a mixture of SP and CP can be used as a cryoprotective agent to improve the viability of L. sakei WiKim31 by supplying each deficient cation.

2.4. Antioxidant Activity. 2.4.1. DPPH Radical Scavenging Activity. The DPPH assay is commonly used to measure the antioxidant activity. DPPH is relatively stable and only weakly reacts with free radicals. It can mainly be reduced using phenolic compounds. The DPPH radical scavenging activities of ascorbic acid (SP), CP, and their mixture were 21.6, 50.0, and 71.3%, respectively (Figure 1A). CP showed a higher DPPH scavenging activity than the SP because CP tissues have higher phenolic and flavonoid compound levels than SP. In addition, hesperidin, which can efficiently prevent SARS-CoV-2 infection and replication by interacting with the main SARS-CoV-2 protease receptors, is abundant in CP (PDB: 6Y84).

2.4.2. Ferric Reducing/Antioxidant Power Assay. Ferric reducing/antioxidant power (FRAP) is widely used to evaluate antioxidant activity based on the reduction of the colorless ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to deep-blue ferrous tripyridyltriazine (Fe^{2+}-TPTZ). The FRAP values for SP, CP, and the SP−CP mixture were 10.0, 24.9, and 34.6 nM.

Table 3. Amino Acid Contents in Cryoprotective Agents

| (unit: μM) | SP          | CP          |
|------------|-------------|-------------|
| alanine    | 29.7 ± 0.3  | 91.7 ± 1.7  |
| arginine   | 411.6 ± 5.8 | 222.9 ± 4.0 |
| asparagine | 806.8 ± 18.1| 11,141.9 ± 118.6 |
| aspartic acid | 211.6 ± 0.6  | 504.9 ± 6.4  |
| cysteine   | ND          | ND          |
| glutamic acid | 373.9 ± 1.0  | 28.4 ± 0.9  |
| glycine    | 228.5 ± 8.8 | 66.6 ± 8.7  |
| histidine  | 26.8 ± 0.8  | ND          |
| isoleucine | 22.6 ± 0.6  | 7.9 ± 0.2   |
| leucine    | 402.5 ± 7.0 | 3.3 ± 0.1   |
| lysine     | 166.9 ± 2.5 | ND          |
| methionine | 39.1 ± 1.0  | 3.2 ± 0.3   |
| phenylalanine | 168.1 ± 5.3  | 34.1 ± 4.7  |
| proline    | ND          | 971.9 ± 3.9 |
| serine     | 308.3 ± 5.3 | 724.6 ± 15.9|
| threonine  | 109.6 ± 14.4| 114.1 ± 0.8 |
| tryptophan | 166.3 ± 6.0 | 92.7 ± 3.4  |
| tyrosine   | 154.7 ± 3.5 | 13.2 ± 4.9  |
| valine     | 37.7 ± 2.5  | 8.6 ± 2.7   |

The values represent the average of three replicates.
Table 4. Cation Contents of Cryoprotective Agents*  

| Cryoprotective Agent | Ca²⁺ (mg/L) | Fe²⁺ (mg/L) | K⁺ (mg/L) | Mg²⁺ (mg/L) | Na⁺ (mg/L) | P⁺ (mg/L) | Zn⁺ (mg/L) |
|----------------------|-------------|-------------|-----------|-------------|-------------|-----------|------------|
| SP                   | 20.3 ± 2.2  | 0.7 ± 0.1   | 763.9 ± 5.6 | 64.2 ± 8.0 | 198.6 ± 4.2 | 80.3 ± 8.3 | 0.7 ± 0.0  |
| CP                   | 53.7 ± 0.7  | 1.0 ± 0.0   | 268.5 ± 4.0 | 9.1 ± 1.0   | 14.5 ± 2.3  | 10.4 ± 2.1 | 0.4 ± 0.1  |
| Mixture of SP and CP | 61.3 ± 4.0  | 0.7 ± 0.1   | 993.2 ± 11.7 | 71.6 ± 9.5  | 205.4 ± 0.6 | 90.6 ± 11.8 | 1.1 ± 0.2  |

The values represent the average of three replicates. CP, citrus byproduct.

2.5. Transmission Electron Microscopy. Transmission electron microscopy (TEM) has been widely used to obtain structural information on LAB. Here, the effects of cold adaptation on the cell wall of L. sakei WiKim31 were observed and evaluated. Cold adaptation before freeze-drying has a positive effect on LAB cell viability.32 Here, L. sakei WiKim31 cold adaptation treatment compared to the non-adapted cells increased the expression of exopolysaccharides (EPSs) in the cell wall (Figure 2). In a similar study, cold adaptation was found to increase the surface-layer thickness of Lactobacillus brevis WiKim0069 cells by 26.7% compared with non-adapted cells.38 EPSs are extracellular carbohydrate polymers that are produced and secreted by LAB, such as Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, and Weissella sp., in response to abiotic stress (temperature, pH, salinity, and light intensity), which accumulate outside the cell wall to protect the cell from harsh environmental conditions.39,40 Thus, EPSs can increase the viability of L. sakei WiKim31 during freeze-drying. Furthermore, EPSs can be applied commercially in bioremediation, cosmetic, food, textile, and pharmaceutical industries.39,41,42

FeCl₃, respectively, based on the FeCl₃ standard curve (Figure 1B). Antioxidants in cryoprotective agents have many beneficial effects on human health as they remove oxygen free radicals.36 Furthermore, soybean increases the viability of Lactobacillus spp. VCC by 30% in camel-milk yogurts.37

Figure 1. (A) Antioxidant activity of SP, CP, and SP + CP mixture. (B) DPPH radical scavenging capacity ($F = 1,558.1; df = 3, 8; P < 0.05$). (C) Ferrous chelating ability ($F = 818.4; df = 2, 6; P < 0.05$).

Figure 2. TEM images of Lactobacillus sakei WiKim31 without and with cold adaptation. (A,B) L. sakei WiKim31 control (non-adapted) cells. (C,D) L. sakei WiKim31 cells cold-adapted at 4 °C. The thin layer at the surface of the cell wall represents exopolysaccharide (black arrow).
2.6 Viability of *L. sakei* WiKim31. Cryoprotective agents are used to protect structurally intact living cells and biological tissues from damage during freeze-drying. However, there is a need to develop efficient cryoprotective agents owing to the high production costs of commercial cryoprotective agents. Cold stress improves cryotolerance in microorganisms by inducing the accumulation of various metabolites. Right after the freeze-drying process, SP records a high viability of 90% or more for LAB species (*L. brevis* WK12 and *Lactococcus lactis* WK11) than commercial cryoprotective agents, such as skim milk, yeast extract, and trehalose. In the present study, the effects of cold adaptation and cryoprotective agents (SP and CP) on the storage stability of *L. sakei* WiKim31 were evaluated. The viability of *L. sakei* WiKim31 was measured according to the CP concentration, and a 5% CP solution was finally selected. The viability of *L. sakei* WiKim31 decreases at a concentration of 5% or more because essential oil in CP solution is thought to have a negative effect on viability. Immediately after freeze-drying, the viability of cold-adapted *L. sakei* WiKim31 increased by 53% compared with untreated *L. sakei* WiKim31 (Figure 3). The *L. sakei* WiKim31 powder with SP, CP, or the SP—CP mixture exhibited cell viabilities of 86.5, 75.0, and 92.9%, respectively (Figure 3). After 56 days of storage, the *L. sakei* WiKim31 powder with the SP—CP mixture showed the highest viability (76.3%); formulations prepared with distilled water, SP, and CP showed viabilities of 23.7, 60.3, and 58.3%, respectively (Figure 3). We confirmed that the combination of SP and CP improved cell viability. In a previous study, we found that the use of coffee residue extracts and SP powder as cryoprotective agents, with supercooling pretreatment, resulted cell viabilities of 48.8 and 81.2% for *Leuconostoc mesenteroides* WiKim32 and *L. brevis* WiKim0069, respectively, after 56 days of storage. Also, the viability of micro-capsulated *L. brevis* WK12 and *Lactobacillus lactis* WK11 with SP was 62.8 and 44.5%, respectively, after 56 days of storage. In addition, the viability of capsulated *Lactobacillus delbrueckii* subsp. *Bulgarius* cells with 8% trehalose, 6% skim milk, and 4% sodium ascorbate was 72% after 3 months of storage. Overall, CP contains a sufficient amount of complex elements, such as sugars, cations, amino acids, and phenolic compounds, to improve the storage stability of *L. sakei* WiKim31. Thus, CP can be used as a new cryoprotective agent either alone or in combination with SP to extend the shelf-life of *L. sakei* WiKim31. Furthermore, its use is economically advantageous and environmentally friendly.

3. CONCLUSIONS

In this study, the effects of CP, a cryoprotective agent, on the shelf-life of *L. sakei* WiKim31 were evaluated and suggested. CP contains a sufficient sugar content (63.4%), amino acids (proline and asparagine), and cations, exhibiting high antioxidant activity, which might play a significant role in improving cell viability. Moreover, the cold adaptation-induced increase in EPS expression can increase the viability of *L. sakei* WiKim31, and the highest viability (76.3%) was observed for the *L. sakei* WiKim31 powder formulated with a mixture of SP and CP after 56 days of storage. These results indicate that CP can be used as a cryoprotective agent either alone or in combination with SP during LAB freeze-drying to improve cell viability and storage stability.

4. MATERIALS AND METHODS

4.1 Raw Materials and Chemical Composition Analysis. CP was obtained from a mandarin field in Jeju, South Korea. CP was freeze-dried at −80°C for 5 days and then ground to smaller particles using an electric grinder. The CP particles were stored at −20°C until use. The soluble sugar content (sucrose, glucose, and fructose) in CP was analyzed using high-performance liquid chromatography for 40 min with a refractive index detector (2414; Waters, Milford, MA, USA) and a REZEX RPM (Phenomenex, Torrance, CA, USA) column (300 × 7.8 mm) at 80°C, and the injection volume was 10 μL; the mobile phase (deionized water) was added at a flow rate of 0.6 mL/min. Insoluble sugar contents (rhamnose, arabinoose, xylose, mannose, galactose, and glucose) in CP were analyzed using gas chromatography. Pretreatment and analysis were conducted using the method described by Choi et al. Also, the method validation was performed in accordance with AOAC’s guidance.

4.2 Cationic Ion and Free Amino Acid Extraction. Homogenized samples (0.25 g) were extracted using 10 mL of distilled water through ultrasonic irradiation for 15 min. The extract was centrifuged at 3,500 × g for 10 min and diluted with distilled water. Any particulate matter present in the solution was eliminated using a 0.45 μm syringe filter (Minisart SRP4, Sartorius, Goetting, Germany) before the solution was used for analytical analysis.

4.2.1 Free Amino Acid Analysis. Free amino acid content was determined using an amino acid analyzer (High-Speed Amino Acid Analyzer L-8900; Hitachi High-Technologies Corporation, Tokyo, Japan) attached to a Hitachi custom ion-exchange column (4.6 mm × 80 mm, packed with Hitachi custom ion-exchange resin, Hitachi High-Technologies Corporation). The analyses were conducted using the method described by Lu et al.

4.2.2 Cation Analysis. The cation levels (Ca2+, Fe2+, K+, Mg2+, Na+, and Zn2+) were determined using an ion chromatograph (Dionex ICS-5000; Thermo Scientific, Sunnyvale, CA, USA) equipped with a capillary cation exchange column (4.6 mm × 80 mm, packed with Hitachi custom ion-exchange resin, Hitachi High-Technologies Corporation). The analyses were conducted using the method described by Lu et al.
eluent generator (Dionex ICS 5000EG; Thermo Scientific) at a flow rate of 1 mL/min. The column oven was thermostatted at 30 °C before injection. Samples were filtered through a 0.45 μm syringe filter (Minisart SRP4, Sartorius, Goetting, Germany), and the injection volume was 25 μL. Instrumental control and data processing were performed using Chromleven System version 7 (Dionex; Thermo Scientific).

4.3. Antioxidant Activity. 4.3.1. DPPH Radical Scavenging Assay. DPPH free-radical scavenging activity was evaluated according to the method described by Geng et al., with slight modifications. Samples were prepared at a concentration of 2.5% (25 mg/mL), and then, 1,140 μL of 0.1 mM DPPH solution was added at 60 μL of the prepared samples or blank sample (distilled water). The reaction mixture was vortexed as follows: μL of the mixture was analyzed at 515 nm using ascorbic acid (100 μg/mL) as a reference. The scavenging activity was calculated as follows:

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\text{DPPH radical scavenging activity(%) = } \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100
\]

4.3.2. FRAP Assay. The FRAP assay was conducted using a FRAP assay kit (Abcam, ab234626; Caliph, MI, USA) in a 200 μL reaction mixture containing 152 μL of FRAP assay buffer, 19 μL of the FRAP probe, 19 μL of FeCl₃ solution, and 10 μL of the samples or the positive control (a mixture of FRAP positive control and FRAP assay buffer). The reaction mixture was incubated in the dark at 37 °C for 60 min, and the absorbance of the mixture was measured at 594 nm using a microplate reader. The antioxidant capacity was calculated using a ferrous standard curve, and the results were expressed as Fe²⁺ equivalents (μM).

4.4. Transmission Electron Microscopy. L. sakei WiKim31 cells were fixed using a mixture of 2% glutaraldehyde (v/v) and 2% paraformaldehyde (v/v) in 0.05 M cacodylate buffer (pH 7.2) at 25 °C for 4 h. The cells were then washed and post-fixed with 1% OsO₄ at room temperature for 1 h. The fixed cells were washed using phosphate buffer and dehydrated in graded ethanol series (30, 50, 70, and 100%). Thereafter, the cells were embedded in LR White resin (Sigma-Aldrich, St. Louis, MO, USA) at 50 °C for 24 h and sectioned using an ultramicrotome equipped with a diamond knife. Ultrathin sections were stained with lead citrate and uranyl acetate. A transmission electron microscope (JEM-2400F, Jeol, Tokyo, Japan) was used to visualize the cells.

4.5. Viability of L. sakei WiKim31. Exactly 10 g (dry weight) of CP was treated with distilled water for soluble extraction using an autoclave for 15 min at 121 °C and then centrifuged (8,000 rpm, 10 min) and filtered through a filter paper (Whatman no. 1, London) to separate the insoluble residues. The soluble extraction was used as a cryoprotective agent for the formulation of L. sakei WiKim31. L. sakei WiKim31 was cultured in an MRS medium at 30 °C for 24 h. The cells were washed and resuspended in distilled water at a concentration of 1 × 10⁸ cells/mL. Distilled water (control), SP solution (5%), CP solution (5%), or the mixture (5% SP and 5% CP solution) was added to the cell solution to adjust the concentration to 5 × 10⁸ cells/mL. The cells were exposed to cold stress for 2 h at 4 °C and were rapidly frozen at −80 °C for 6 h before freeze-drying (Freeze-dryer FDB; Operon, Gimp, Korea) at −120 °C for 48 h. The freeze-dried cells were stored at −20 °C for 56 days. The freeze-dried cells were periodically and randomly selected and serially diluted with sterile saline and then poured onto MRS plates. The plates were incubated at 30 °C for 48 h. The number of colonies on the plates was counted to measure the cell viability.

4.6. Statistical Analysis. Data were analyzed using IBM SPSS Statistics 19 (IBM Corp., Armonk, NY, USA). Tukey’s honestly significant difference test for the analysis of variance was used to determine significant differences among the different treatments. P < 0.05 was considered statistically significant.

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Notes

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