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A novel approach for improving yeast viability and baking quality of frozen dough by adding biogenic ice nucleators from *Erwinia herbicola*

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ABSTRACT

Freezing deteriorates the baking quality of frozen bread dough by causing lethal injury to yeast cells and depolymerization to the gluten network. To investigate the potential of biogenic ice nucleators in frozen food applications, the effect of extracellular ice nucleators (ECINs) from *Erwinia herbicola* on the baking quality of frozen dough upon three freeze/thaw cycles were investigated. With addition of ECINs to the activity of 2.4×10^6 units per gram of dough, hardening of bread crumb caused by three freeze/thaw cycles was alleviated by about 50% compared to the control. Additionally, the bread from frozen dough with added ECINs showed 50% larger specific volume compared to the control. The mechanism of cryoprotective effects from ECINs was possibly that ECINs helped in preserving the viability of yeast cells during freeze/thaw cycles. ECINs were able to improve the viability of log-phase and stationary-phase yeast cells in suspensions by about 100 and 10 fold, respectively, and viability of yeast in the frozen dough by 17%. This study revealed the potential of ECINs as a cryoprotectant for applications in the food and biotechnology industries.

1. Introduction

Ice nucleation materials function as heterogeneous ice nucleators to minimize the supercooling of water. Ice nucleation proteins (INP) have been found in the leaves of plants such as winter rye and prunus, in animals such as turtles and lichens, and in some microorganisms such as some strains in the genera of *Pseudomonas*, *Erwinia* and *Xanthomonas* (Zachariassen and Kristiansen, 2000).

The food application of INP-expressing (*Ina*+) bacterial cells and cell-free ice nucleators was comprehensively reviewed (Li and Lee, 1995). Notably, addition of the *Ina*+ cells was shown to significantly raise the ice nucleation temperature and shorten the time of freezing (Hwang et al., 2001; Li et al., 1997; Zasypkin and Lee, 1999). Extracellular ice nucleators (ECINs) from *Erwinia herbicola* revealed a protective effect on gel-forming capacity of fish by stabilizing fish actomyosin from freezing denaturation (Li and Lee, 1998; Zhu and Lee, 2007). *Ina*+ bacterial cells were also reported to improve the efficiency of freeze drying of soy sauce and soybean paste (Watanabe and Arai, 1987) and to modify the texture of raw egg white, bovine blood, soybean curd and milk curd after freezing (Arai and Watanabe, 1986).

Introduction of freezing technique to the bakery industry was revolutionary. For the manufacturer, freezing storage largely extends the shelf life of dough, which allows the industry to expand their sales from local to nationwide. For the consumer, frozen dough offers convenience in addition to warm bakery goods that have comparable qualities to freshly prepared ones. However, long term freezing storage is harmful to the qualities of frozen dough (Ribotta et al., 2001). The freezing damage to yeast was referred as the primary factor contributing to the quality loss (Rosell and Gomez, 2007). During freezing storage, yeast cells are injured, and consequently release glutathione which weakens the dough by cleaving disulphide bonds in the gluten. Additionally, the longer proofing time and smaller bread volume were also due to the freezing-injured yeast cells losing their capability for gas production. Severe temperature fluctuation during the storage and transportation also leads to increase of ice crystal size and consequently aggravates the frozen damage to yeast (Rosell and Gomez, 2007). Traditionally, to counteract the released reducing substances such as glutathione, oxidants like potassium bromate

Abbreviations: ANOVA, analysis of variance; ECINs, extracellular ice nucleators; HMW, high-molecular-weight; INA, ice nucleation activity; Ina+, ice nucleation protein-expressing; INP, ice nucleation proteins; LMW, low-molecular-weight; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; w/, with; w/o, without.

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are added into yeast-leavened frozen dough. However, the use of potassium bromate has been banned in many countries and also declined in the U.S. in recent years due to the potential harm to consumers (Selomulyo and Zhou, 2007). Alternatively, selection and isolation of freeze-tolerant yeast strains attract much scientific effort (Shima et al., 2010). Also, in order to compensate the deteriorating effect of freezing, many researchers focused on developing other improvers, such as emulsifiers, enzymes, hydrocolloids and ice-structuring proteins to stabilize the dough network (Al-Dmoor et al., 2009).

Previous study suggested that after three successive freeze/thaw cycles, frozen dough revealed significant quality deterioration (Rosell and Gomez, 2007). In this study, the same freeze/thaw method was adopted to investigate the protective effect of ECINs on yeast-leavened frozen dough. Firstly, ECINs were blended into frozen bread dough which was baked after repeated freeze/thaw cycles. The qualities of these breads were characterized and compared to the bread from frozen dough without ECINs and fresh dough. Furthermore, to investigate the mechanism of ECINs' protective effects, the viability of yeast and structure of the gluten network after freeze/thaw cycles were also examined. This study revealed the cryoprotective application of ECINs in frozen bread dough.

2. Materials and methods

2.1. Materials

Malt extract broth and malt extract agar (BD, Franklin Lakes, NJ), sucrose (Fisher), sodium chloride (Fisher), L-serine (Aldrich), L-alanine (Aldrich), were used as received. Deionized water was used throughout the experiment. All purpose flour (10% protein, 73% carbohydrate, 12% moisture, General Mills Inc., Minneapolis, MN), Fleischmann's® ActiveDry™ bakery yeast (ACH Food Companies, Inc., Cordova, TN) and shortening (92% fat, GreatValue®, Wal-Mart Stores, Inc., Bentonville, AR) for making dough were purchased from a supermarket.

2.2. Preparation of extracellular ice nucleators

The *E. herbicola* subsp. *ananas* was obtained from the American Type Culture Collection (ATCC Cat. No. 11530) and routinely cultured in yeast extract medium consisting of 2.0% yeast extract, 1.0% sucrose, 0.2% L-serine, 0.2% L-alanine, 0.086% K_2SO_4, and 0.14% $MgSO_4$ under 18 °C.

Extracellular ice nucleators (ECINs) were isolated from *E. herbicola* cells which were harvested by centrifugation at 10,000 × *g* for 20 min and then re-suspended in 20 mM Tris/HCl pH 8.0. Sonication of cells was done on ice, 10 s with 10 s interval for 3 times. Cells and debris were removed by centrifugation at 10,000 × *g* for 20 min and supernatant was subjected to filtration through a 0.45 μm filter. The filtrate was put on ultracentrifugation at 160,000 × *g* for 2 h and the pellet was re-suspended in 20 mM Tris/HCl with 20 mM $MgCl_2$, pH 8.0. Lyophilized ECINs in the form of powder were stored at −20 °C.

Ice nucleation activity (INA), defined as the activity units at −8 °C or higher was determined by the droplet-freezing assay (Vali, 1971). The detailed procedure was described previously (Zhu and Lee, 2007).

2.3. Dough preparation and baking procedure

A basic dough recipe was adopted from AACC method 10-10B (AACC, 2000). Two doughs were prepared for each condition. For each dough piece, 450 g flour, 252 g water, 7 g yeast, 27 g sucrose, 6.75 g NaCl, 27 g shortening were mixed and kneaded in a KitchenAid® PRO 500 mixer under 4 °C to minimize the activity of yeast. Each dough was about 720 g. Optionally, ECINs (ice nucleation activity: 6.9×10^6 units/mg) was dispersed in water and then mixed into dough to 0.35 mg per gram of dough. After mixing, dough was sheeted and rolled into 20 cm long cylinders, immediately followed by freezing at −20 °C for 48 h and were then thawed in a 4 °C refrigerator for 24 h as one freeze/thaw cycle. After three freeze/thaw cycles, dough pieces were fermented at 30 ± 2 °C under 80 ± 5% relative humidity for 60 min (fresh dough) or 2½ h (frozen dough). The dough was baked in 21.6 × 11.2 × 6.9 cm aluminum pan at 205 °C for 30 min. After baking, bread was cooled to room temperature and packed into plastic bags. Bread was left at ambient temperature and analyzed within 24 h.

2.4. Measurement of bread qualities

The method for measuring bread properties was adopted from AACC method 74-9 (AACC, 2000). Bread loaves were weighed and the loaf volume was measured by rapeseed displacement. Each bread loaf was cut into 25 mm slices. At least four slices were characterized for the texture profile analysis carried out with a TA.XT2 texture analyzer (Stable Micro Systems, Ltd., London, UK), using a 40 mm cylindrical acrylic probe. The bread slices were compressed at a speed of 1.7 mm/s to a total distance of 10 mm (40% strain) and withdrawn at the same speed. The following textural parameters were recorded from the force–distance curves: firmness (*g*, force at 25% strain), fracturability (*g*, the force when detecting the first significant peak during compression), and resilience (%, area during the withdrawal of the penetration divided by the area of the first penetration). In the following experiments, three slices from one bread were characterized and each was measured in triplicate. The pH of bread was recorded in a suspension of 1 g bread homogenized in 10 ml dH_2O. Water content was measured using a Denver Moisture Analyzer IR-200 (Denver Instrument, Bohemia, NY). Photos of cross-sections of bread slices were taken at resolution of 300 dots per inch and analyzed in gray scale (0–255). Image analysis was performed using the ImageJ v.1.45s (NIH, US) by the threshold method developed previously (Sapirstein et al., 1994). The parameters (number of cells, cell sizes, cell perimeter and ratio of gas cell area to total analyzed area) were calculated within a 4 cm × 4 cm square in the center of crumb by the ImageJ software. Crumb color was measured using a Minolta CR-200 Colorimeter (diffuse illumination with 0° viewing angle, Konica-Minolta, Ramsey, NJ) for values of *L* (lightness, white-black), *a* (color-opponent dimension, red-green) and *b* (color-opponent dimension, yellow-blue). The color difference, d*E*, was calculated as:

$$dE = \sqrt{(dL)^2+(da)^2+(db)^2}$$

where d*L*, d*a*, and d*b* were the differences for *L*, *a*, and *b* values between the sample and the reference (a white ceramic plate having *L* value of 93.4, *a* value of −1.8, and *b* value of 4.4). Pigment in crumb was extracted according to the AACC method 14-50 (AACC, 2000), by mixing 2 g crumb sample in 10 ml water-saturated *n*-butanol for 16 h. The mixture was then centrifuged and the absorbance at 436 nm ($A_{436\ nm}$) was measured in a Cary-50 spectrophotometer (Varian Instruments, Walnut Creek, CA).

2.5. Yeast survival counting in saline suspension

Yeast cells were grown in malt extract broth at 30 °C under shaking. When the OD_{600} of the broth reached 1.0, yeast cells at the early log phase were harvested. When the OD reached 3.5, yeast