

Effects of Yeast Freezing in Frozen Dough

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ABSTRACT

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The effects of freezing and frozen storage of bread dough and compressed yeast on bread quality were studied. Besides, the effects of compressed yeast freezing on cell viability, gas production and release of substances by the yeast cells were examined. Freezing and frozen storage of dough made with fresh yeast had more negative effects on baking quality than the addition of frozen yeast to dough. When the compressed yeast is frozen and stored at -18°C , the CO_2 production decreased, while the amount of dead cells, the total protein, and the total reducing sub-

stances leached from the yeast increased as the length of yeast frozen storage increased. SDS-PAGE showed that the substances leached from frozen yeast caused an increase in the solubility of some gluten proteins. On the other hand, size-exclusion chromatography (SEC) pointed out that the relative amount of two protein fractions of low molecular weight leached from frozen yeast increased for longer yeast frozen storage periods. The yeast leachates had an adverse effect on loaf volume.

In many countries, bread (crusty type) is usually consumed fresh, and it has a very short shelf life (one to two days) even when packaged. Frozen doughs can provide fresh products daily. The main consequences of bread dough freezing and frozen storage conditions are the production of bread with lower specific loaf volume, longer proof times, and stronger alteration of textural properties (Wolt and D'Appolonia 1984; Dubois and Blockolsky 1986; Neyreneuf and Van Der Plaet 1991; Inoue and Bushuk 1992; Rasanen et al 1997; Ribotta et al 2001).

In frozen dough manufacturing, yeast survival and gas retention are major problems (Hino et al 1987). Poor gas retention during proofing can result from damage of the three-dimensional gluten protein network. Dough weakening during frozen storage and successive freeze-thaw cycles has been attributed to the release of reducing substances such as glutathione from yeast during freezing (Kline and Sugihara 1968; Hsu et al 1979). Other workers (Varriano-Marston et al 1980; Wolt and D'Appolonia 1984; Autio and Sinda 1992) have suggested that the structural changes in freeze-thawed dough are not associated with the release of reducing substances from yeast cells but with a lack of gluten cross-linking. Berglund et al (1991) showed that the formation of ice-crystals in nonfermented dough stored for 24 weeks led to a disruption of the gluten matrix rendering a network separated from starch granules. While leached glutathione is certainly involved, the precise mechanism by which yeast contributes to increased slackening remains the subject of debate (Casey and Foy 1995).

The gassing power of yeast depends on the strain, the numbers of yeast cells, the cell activity, and the amount of fermentable sugars. Fast freezing reduces the gassing power (Autio and Sinda 1992; Gélinas et al 1993; Inoue et al 1994; El-Hady et al 1996) and the number of yeast cells (Lorenz 1974).

The objective of this study was to investigate the effect of yeast freezing on the cell viability, gas production, release of substances by the yeast cells and bread quality.

MATERIALS AND METHODS

Yeast Sample

Compressed yeast samples were provided by CALSA (Buenos Aires, Argentina).

Assays Performed with Fresh Yeast

Baking procedure and dough freezing. The recipe and breadmaking process followed here are those currently employed in Argentina in the preparation of bread. Its simplicity allows a clear observation of changes occurring during the processing of frozen dough. The dough formulation used in this study was 100% wheat flour (protein 12.9%, ash 0.75%, moisture content 13.4%), 3% compressed yeast, 1.8% sodium chloride, 0.2% sodium propionate, 0.015% ascorbic acid, and 63% of water. The water addition was based on a farinograph test using the 500 BU line. Ingredients were mixed in an L-20 mixer (Argental, Santa Fe, Argentina). Yeast and salt were separately dissolved in water and the remaining ingredients were added as solids. The resulting dough was allowed to rest for 15 min in a cabinet at 30°C and 70% rh and then the bulk dough was sheeted in a vf roller (Mi-Pan, Cordoba, Argentina) containing two rolls of 50×12.7 cm. The dough was then divided in 80-g pieces and hand-molded.

For nonfrozen dough, hand-molded pieces were proofed at 30°C (96% rh) up to maximum volume increment (Armero and Collar 1998) and baked at 200°C for 18 min.

For frozen dough, hand-molded pieces were immediately placed on individual trays, wrapped on polyethylene bags and frozen at -18°C in a freezer, and then stored at the same temperature. After the corresponding storage time, samples were thawed for 1 hr at 30°C (70% rh), proofed at 30°C (96% rh) up to maximum volume increment (Armero and Collar 1998) and baked at 200°C for 18 min.

Bread properties. Specific loaf volumes were determined by rapee-seed displacement and weight 24 hr after baking. Final proofing time and form ratio (maximum height/width in each piece) were measured. Two replicates were analyzed.

Survival ratio of yeast in frozen dough. Dough pieces stored in frozen state for 1, 40, 60, and 90 days were directly thawed for 45 min to 30°C . Yeasts from fresh and thawed dough were grown on potato dextrose agar (Difco Laboratories, Detroit, MI), adjusted to pH 3.5 with tartaric acid. The counts of surviving yeast in frozen dough were determined in triplicate according to Approved Method 42-50 (AACC 2000).

Assays Performed with Frozen Yeast

Yeast freezing. Compressed yeast was placed in polypropylene flasks and frozen at -18°C . Samples were assessed fresh and after 1, 40, 60, and 90 days of frozen storage, following thawing for 45 min at 30°C . Samples were tested for baking activity, gassing power, and percentage of dead cells, and then leached.

Gassing power. Gassing power was determined by suspending the yeast sample (2 g) in 100 mL of potato liquid broth (Dhingra and Sinclair 1985) containing 2 g of sucrose. This mixture was placed in a fermentation flask and kept under constant shaking for 90 min at 30°C . A flow of nitrogen gas was passed through the

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fermentation flasks, so conveying the carbon dioxide produced by yeast samples through gas dispersion tubes immersed on 2N NaOH in collecting flasks. Carbon dioxide was collected in two collecting flasks and quantified by back-titration of the NaOH with 1N HCl. Phenolphthalein and methyl orange were used as endpoint indicators. Two replicates were analyzed.

Dead cell percentage. Trypan blue method. Dead cells were assessed by suspending yeast (3.0 ± 0.0 g) in 100 mL of 0.1% (w/v) peptone. A 1-mL aliquot of this suspension was mixed with 9 mL of peptone. This procedure was repeated two more times. Then, a 200- μ L aliquot of the dilution was mixed with 200 μ L of Trypan blue (0.4% [w/w]). A drop of this suspension was examined under a microscope on a Neubauer hemocytometer. All stained cells were counted as dead. Two replicates were analyzed.

Baking activity. Frozen-thawed yeast was employed to prepare nonfrozen dough after the baking procedure above to assess the effect of yeast freezing and storage at -18°C on specific loaf volume and form ratio.

Leachates preparation. Leachates were obtained from compressed frozen-thawed and fresh yeasts. Yeast (20 g) was suspended in 200 mL of distilled water ($19 \pm 1^{\circ}\text{C}$) and shaken for 15 min at 20°C . The suspension was centrifuged for 10 min at $2,000 \times g$, and the supernatant was vacuum-filtered twice to remove yeast cells (Wolt and D'Appolonia 1984) first for a 1.0- μm retention filter, then for a 0.45- μm retention sterile glass filter.

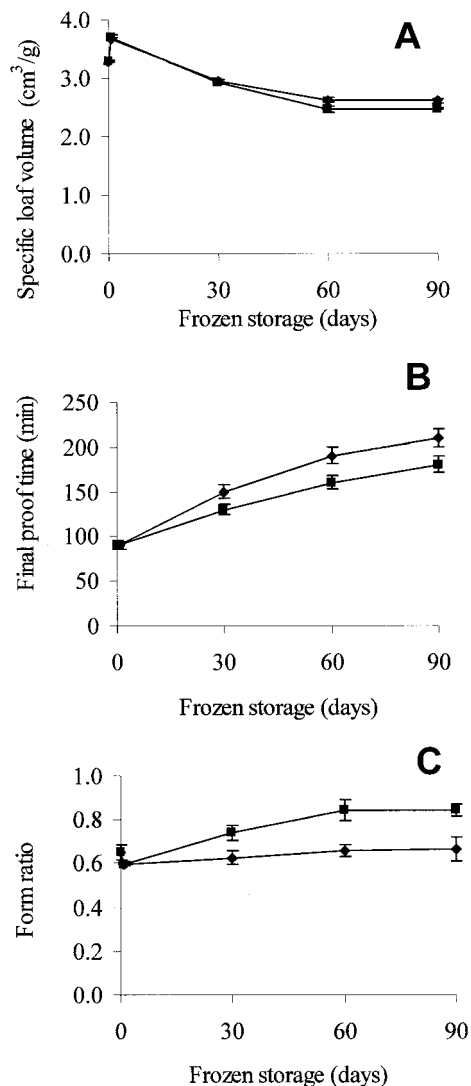


Fig. 1. Effects of freezing and frozen storage of dough (◆) and of compressed yeast (■) on bread quality.

Leachate Tests

Leachates analysis. Protein contents of the leachates were determined by the Kjeldahl method. Total reducing substances (TRS) in the leachates were determined according to Approved Method 10-01 (AACC 2000). Two replicates were analyzed and the results expressed as mean values \pm SD.

Baking activity. The baking procedure discussed above was used to measure baking activity. A fraction of the water of the original formulation was replaced by the leachate in the formulation. Volumes of leachate equivalent to the amount of material leached from 3% yeast (flour basis) were tested.

Preparation and analysis of gluten protein extracts. Protein extracts were prepared by incubating dry gluten (PANAR SA, Córdoba, Argentina) (125 mg) with 2.5 mL of leachates or water (referred to as water in the text) for 20 min. The suspension was centrifuged for 10 min at $2,000 \times g$ at room temperature, and the supernatant was heated for 3 min in a water bath at 95°C .

Gluten protein extracts obtained were analyzed by SDS-PAGE and size-exclusion chromatography (SEC).

SDS-PAGE. Leachates and protein extracts were loaded onto 12% SDS-PAGE, prepared according to Laemmli (1970). Gels were analyzed by densitometry in an Image Master VDS (Pharmacia Biotech). A blank lane was used to obtain the background signal. The volume of protein band (integrated absorbance, IA) was represented by the expression: $\text{IA} = (\text{mean intensity} - \text{background}) \times \text{band area}$. The proportions of protein fractions relative to total protein in the corresponding lane were quantified as IA from each band/total IA of the lane.

Size-exclusion chromatography (SEC). Filtered (0.22 μm) protein extracts of 200 μL were autoinjected into a Superdex 200 HR 10/20 column (Pharmacia Biotech) and using a FPLC system AKTA explorer 100 (Pharmacia Biotech). A 0.05M sodium phosphate buffer (pH 7.6) containing 0.15M NaCl was used as the eluent with a flow rate of 0.4 mL/min. The elution buffer was filtered through 0.45- μm filter and degassed under vacuum before use. Absorbance was measured at 210 nm (Singh et al 1990), whereas elution volumes and peak areas were obtained with software (Unicorn, v. 2.30, Pharmacia Biotech).

Statistical Analysis

The data obtained here were statistically treated by variance analysis (ANOVA) while the means were compared by the LSD Fisher test at significance level of 0.05, in both cases using the INFOSTAT statistical software (Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina). Relationships between measured parameters were assessed by Pearson's test (Conover 1999).

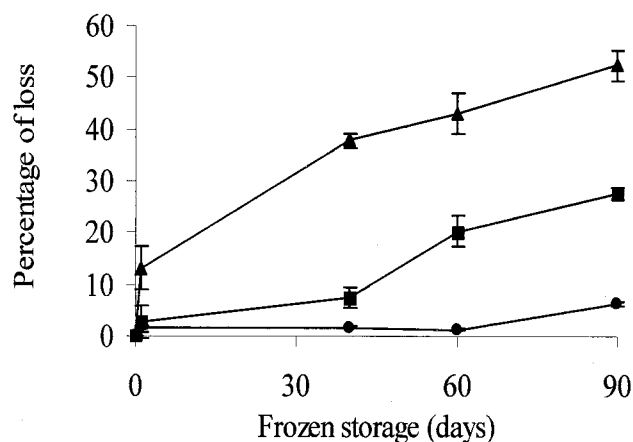


Fig. 2. Effect of freezing on CO₂ production and dead cells. Percentage of loss in CO₂ production (■), in dead cells due to compressed yeast freezing (●), and in dead cells due to dough freezing (▲).

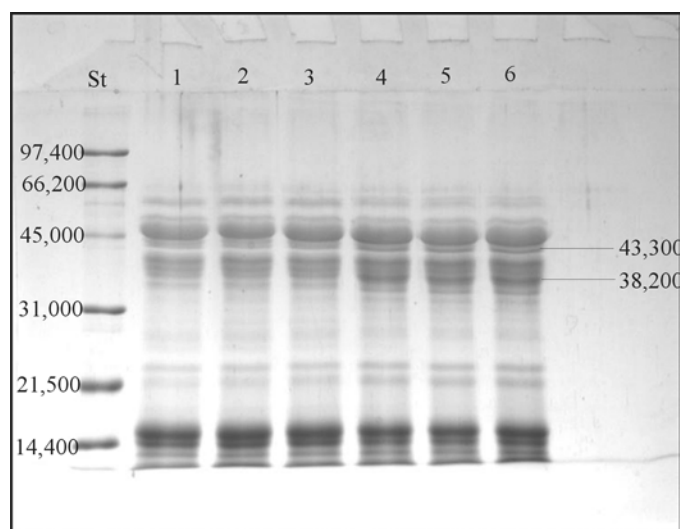


Fig. 3. SDS-PAGE patterns of soluble gluten proteins. Lanes: Molecular weight standard (St), gluten + water (1), gluten + fresh yeast leachate (2), gluten + leachate of compressed yeast frozen for 1 day (3), gluten + leachate of compressed yeast frozen for 40 days (4), gluten + leachate of compressed yeast frozen for 60 days (5), and gluten + leachate of compressed yeast frozen for 90 days (6).

RESULTS AND DISCUSSION

Effect on Bread Quality

The results of bread quality produced from nonfrozen dough made with frozen yeast and from frozen dough containing fresh yeast are shown in Fig. 1. In both types of bread, the specific loaf volumes decreased (Fig. 1A) and the final proof time increased (Fig. 1B) as the dough and yeast frozen storage periods increased. No significant difference was found in the specific loaf volume of these two types of bread, possibly because the proof time of frozen dough was longer than that of nonfrozen dough prepared with frozen yeast. The increase in proof time observed may be due to a loss of yeast viability and capacity to produce gas (Inoue and Bushuk 1992; Lorenz and Kulp 1995). The final proof time was higher for frozen dough than for nonfrozen dough made with frozen yeast.

Changes in dough elasticity were followed by the change in form ratio, which represents the ratio between maximum height to the width of each bread piece (Fig. 1C). The form ratio of bread baked from frozen dough remained almost constant, but it increased for nonfrozen dough made with frozen yeast.

The behavior of the final proof time and the form ratio showed that, in addition to the losses of yeast viability and capacity to produce gas, there was perhaps a loss of gas retention capacity and dough strength caused by protein matrix depolymerization during frozen dough storage (Ribotta et al 2001).

Effects of Yeast Freezing on Yeast Survival

Compressed yeast. Figure 2 shows the effects of yeast freezing on the CO₂ production and on the percentage of dead cells. The CO₂ production decreased significantly ($P < 0.05$) for longer storage periods. Loss in CO₂ production after 90 days of frozen storage reached 27.7%. This result agreed with Wolt and D'Appolonia (1984), Inoue et al (1994), and El-Hady et al (1996).

The Trypan blue method allowed viable and dead cells to be counted. Compressed nonfrozen yeast contained a significant amount of dead cells (6.6%) and longer frozen storage periods of yeast increased dead cells. Dead cell increment was significant ($P < 0.05$) after 90 days of storage (5.2%). Viable yeast losses through frozen storage did not explain the whole loss of gassing power. However, yeast freezing caused a loss in the number of yeast cells as well as losses in the capacity to produce gas in surviving cells.

TABLE I
Effects of Yeast Freezing on Leachate Composition

Leachates Analysis	Fresh Yeast	Yeast Frozen Storage (days)			
		1	40	60	90
Nitrogen ^a	0.028a ^b	0.014a	0.118c	0.085b	0.126c
Nitrogen extracted ^c	0.336a	0.311a	0.292a	0.287a	0.321a
TRS ^d	0.08a	0.37b	0.44c	0.66d	1.18e
pH	6.10	5.91	5.05	4.97	5.07

^a Leachate (mg/mL).

^b Values followed by the same letter in the same row are not significantly different ($P < 0.05$).

^c Nitrogen extracted from gluten minus nitrogen from leach (mg/mL).

^d Total reducing substances (TRS) in the leachate: mg of I₂/g of yeast determined by Approved Method 10-01 (AACC 2000).

Frozen dough. Freezing and frozen storage of dough caused significant losses in the number of viable cells (Fig. 2). The viable yeast cells decreased as the frozen dough storage increased, loss of viable cells reached 52.4% after 90 days of frozen storage.

These results agree with Lorenz and Kulp (1995), who suggested that freezing yeast in a dough system increased the susceptibility to cell damage compared with direct freezing of yeast because the yeast in a dough system was under osmotic pressure and in a state of active fermentation. In addition, in active fermentation, cells have a thinner plasma membrane than dormant cells; consequently, they become more susceptible to cell damage. Also, the organic compounds are concentrated by freezing of the aqueous phase, and this can cause autolysis of yeast cells (Stauffer 1993).

Effect of Yeast Freezing on Leachate Compositions

Table I shows the composition of the leachates from fresh and frozen yeast. Total nitrogen in leachates increased significantly ($P < 0.05$) after 40 days of frozen storage of the yeast. The total reducing substances (TRS) leached from the fresh yeast were present in very low levels, but increased significantly ($P < 0.05$) for longer periods of frozen storage for yeast. The TRS increment could be associated with sulfhydryl (SH) compounds released by dying yeast during the frozen storage of the dough as postulated by Kline and Sugihara (1968). The pH of leachates decreased as the frozen storage of yeast increased, indicating that the substances leached have an acid character. These results showed that yeast freezing released substances from dead yeast cells.

Leachate Tests

Influence on gluten protein solubility. The effect of leachate on dry gluten protein solubility was examined. The amount of soluble protein from gluten by the leachates did not increase with the frozen storage of yeast.

Soluble proteins from dry gluten by fresh and frozen yeast leachates were analyzed by SDS-PAGE to assess whether the substances released by frozen yeast affected the gluten proteins (Fig. 3, lanes 2–6). Additional extracts performed with water were included as control (Fig. 3, lane 1). The SDS-PAGE patterns of soluble protein extracts showed that the relative staining intensity of protein bands increased with the length of frozen storage of yeast increased, although the amount of soluble protein from gluten by the leachates analyzed by Kjeldahl procedure did not show such increase because of the higher sensitivity of SDS-PAGE. Densitometric analysis indicated that the relative amount of polypeptides with molecular weights of 43,300 and 38,200 increased from 40 days of frozen yeast storage (Table II). These results showed that the substances leached from frozen yeast caused a gradual reduction and increased the solubility of some gluten proteins.

Soluble proteins from dry gluten by fresh and frozen yeast leachates were also fractionated by SEC (Fig. 4). Leached protein from fresh yeast were fractionated in four peaks, p1', p2', p3', and p4' (dashed lines in Fig. 4B–D). Relative area of p1' and p4'

TABLE II
Effects of Yeast Leachates on Relative Amount (IA from each band/total IA of lane) of Soluble Protein (MW 43,300 and 38,200) from Dry Gluten

MW	Control ^a	Yeast Frozen Storage (days)				
		0	1	40	60	90
43,300	0.000	0.000	0.000	0.027	0.031	0.026
38,200	0.000	0.000	0.029	0.082	0.071	0.076

^a Water extraction.

TABLE III
Relationship Between the Relative Area of Peaks *pn* and *p5* and the Measured Parameters

Measured Parameters	<i>pn</i> Area (%)	<i>p5</i> Area (%)
SLV ^b from bread made with frozen dough (cm ³ /g)	-0.815	-0.983
SLV from bread made with frozen yeast (cm ³ /g)	-0.808	-0.985
Gas production loss (%)	0.949	0.955
Percentage of yeast dead by yeast freezing	0.994	0.829
Percentage of yeast dead by dough freezing	0.927	0.906
TRS (mg of I ₂ /g of yeast)	0.993	0.850
Nitrogen leached (mg/mL)	0.943	0.985

^a Five samples (0, 1, 40, 60, and 90 days of frozen storage) were used to establish the correlations.

^b Specific loaf volume.

(molecular weights of 5,800 and 300, respectively) became larger as the duration of yeast frozen storage increased.

Gluten proteins of water extract (control) were fractionated into five major peaks, as shown in Fig. 4A. Molecular weights obtained for peaks 1–5 were 116,500, 36,300, 7,800, 1,100, and 300, respectively. A new peak (*pn*) with a molecular weight of 5,800 appeared when the extracts were prepared with fresh or frozen yeast leachates (solid line in Fig. 4B–D). Peaks *pn* and *p5* of leached from frozen yeast corresponded to *p1'* and *p4'* of leached protein from fresh yeast, showing that the SEC patterns of the extracts prepared with yeast leachates did not change significantly with the frozen storage time. However, relative area of peaks *pn* and *p5* showed a high degree of correlation with the different measured parameters (Table III). The high correlation showed that the substances released by freezing yeast had an important effect on yeast behavior and frozen dough quality.

Effect on baking activity. To determine effects of reducing substances released by fresh and frozen yeast on bread quality, bread was made containing a fraction of leachates. Figure 5 shows the effects on specific loaf volume by incorporating the fresh and frozen yeast leachates into the baking test. Leachates from frozen yeast through 40, 60, and 90 days caused a significant ($P < 0.05$) decrease on loaf volume.

CONCLUSIONS

Freezing and frozen storage of compressed yeast at -18°C produced an increase of dead cells and caused losses of CO_2 production; baking quality reflected the lower specific loaf volume and longer proof times.

Freezing and frozen storage of dough had more negative effects on bread processing than the addition of frozen yeast to nonfrozen dough. This reflected the longer proof times, higher number of dead cells, and flatter bread loaf.

The fresh yeast showed a higher amount of dead cells but the leachate of the fresh cells held a lower amount of total reducing substances. As the length of frozen storage of the compressed yeast increased, the amount of both dead cells and reducing substances leached from the yeast cells increased.

The addition of yeast leachates to dry gluten affected the SDS-PAGE pattern of the gluten-soluble proteins, indicating that the substances leached from frozen yeast cells could affect the quality of

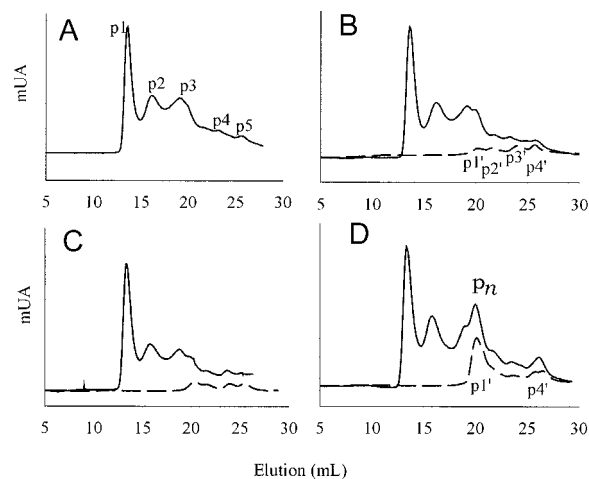


Fig. 4. Size exclusion chromatography of un-reduced leachate proteins (dashed line) and soluble protein extracts (solid line) from gluten proteins in water (A) and in leachates from fresh yeast (B) and in yeast frozen for 1 day (C) and 90 (D) days.

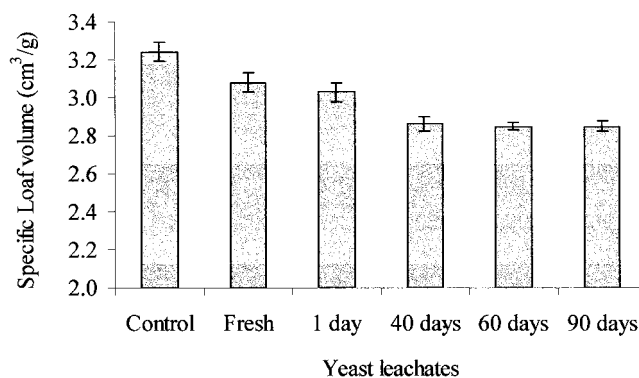


Fig. 5. Effects of leachates from fresh and frozen yeast on specific loaf volume. Control: formulation base. Fresh: leachate of fresh yeast. Leachates of compressed yeast frozen for 1, 40, 60, and 90 days.

frozen dough. As identified by SEC, the relative amount of two protein fractions of low molecular weight leached from frozen yeast increased for longer yeast frozen storage periods. Addition of leachate into the baking test confirmed these results.

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