RESEARCH ARTICLE

Use of antifungal lactic acid bacteria (LAB) for bread preservation

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ABSTRACT

Fungal spoilage of food and feed is a common and global phenomenon. The global food industry sector is under constant pressure from both consumers and regulatory bodies to provide high quality fresh food with minimal processing. The consumers prefer safe preservative-free products. Fungal spoilage is the main cause of economic loss in the bakery industry. In present study, the antifungal starter culture was developed using selected antifungal lactic acid bacteria (LAB) and was used for bread preservation. The best biopreservation effect was obtained with 50% antifungal slurry SL-AL2 containing *Lactobacillus ingluviei* against *Penicillium* sp., which was more effective than 0.4% Calcium propionate (Pca), a common preservative used in bakery products. The antifungal starter culture SL-AL2 extended the shelf life of packaged bread to 19 days, more than 4 times longer shelf life than breads prepared with only 0.4% PCa.

Key words: Lactic acid bacteria (LAB), Antifungal activity, Antifungal starter culture, Biopreservation.

INTRODUCTION

Food and feed spoiling moulds and yeasts cause great economic losses worldwide. In addition to economic losses, food spoilage also represents a health hazard for consumers, especially when it is contaminated with mycotoxigenic molds (Legan, 1993).

Bakery products are the important staple foods in most countries. These products are subjected to spoilage problems which include physical, chemical and microbial spoilage. Since the most common factor of bakery products is water activity (a_w = 0.94 - 0.97) and a pH of approximately 6, mould growth is of major concern in bakery products.

The fungal spores are killed during baking and the airborne moulds recontaminate the baked goods during the processing of bread such as cooling, slicing, wrapping and storage operations. The most common spoilage moulds isolated from bakery goods belong to the genera *Rhizopus, Mucor, Penicillium, Aspergillus*, *Monilia, Endomyces, Cladosporium* and *Fusarium*. Several different approaches have been

adopted to control mould growth in bread, including the addition of propionic acid and its salts, benzoates, sorbates, modified atmosphere packaging, irradiation and pasteurization of packaged bread etc. In present work, the efficacy of antifungal LAB isolates as bio-preservative in bread making was evaluated

MATERIALS AND METHODS

Formulation of the antifungal starter culture (slurry): The strains AL2 (*L. ingluviei*) and IB2 (*Weissella confusa*) used in this study were obtained from lassi and idli batter respectively. Before experimental use, the strains were grown twice in de Man Rogosa Sharpe (MRS) broth without acetate at 30^0 C for 24 h. The cultures were used as single cultures and inoculated (15% v/v) in a mixture of 250 g of wheat flour, 5 g sucrose, 12.5 g of skimmed milk, 0.75 liters of tap water. After homogenization, the pH was adjusted to 5.90 $\pm$ 0.15 with Na_2HPO_4, and fermentations were allowed to proceed at 30^0 C for 24 h with continuous stirring at 100 rpm. Each resulting semisolid fermented product was named slurry (SL) and was used in the dough formula.

LAB growth was determined by the plate dilution method using MRS agar, the plates were incubated at 30^0 C for 48 h, and results were expressed as log CFU per milliliter. The rate of slurry acidification was determined by measuring pH and total titratable acidity (TTA) (Potentiometric method with Domic solution [0.1 N NaOH] using Phenolphthalein as an indicator) in 10-ml samples. Results were expressed in milliliters of Domic solution needed to achieve a pH of 8.3 to 8.6.

Dough fermentation and bread manufacture:
The lactic slurries were used for the production of wheat bread. The doughs were prepared as follows: 1,000 g of commercial wheat flour, 10 g of NaCl, 20 g of sucrose, 50 g of skim milk, 20 g of fat, and 0.5 liters of tap water. To incorporate the fermented slurry, tap water was partially replaced (50%) in the dough preparation by equal amounts of the slurry fermented by each of the selected LAB isolates. A commercial strain of *Saccharomyces cerevisiae* (baker's yeast) was used as the leavening agent. Dough containing yeast only (Y-dough) and doughs with yeast plus 0.4 % (wt/wt) Calcium propionate (PCa) (YCP-dough) were used as controls.

The doughs were individually placed in aluminum pans for fermentation (2 h, 30^0 C). The pH was determined using a pH meter (Hanna Instruments). The leavening power, determined by fermenting a 50-g portion of dough in a beaker at 30^0 C for 2 h, was calculated as V (cm^3) = Vf – Vi, where Vf and Vi are the final and initial leaven volumes, respectively. After fermentation, the doughs were baked in oven (160^0 C for 60 min), and the bread loaves were cooled at room temperature for 90 min. The volume of the baked breads (length by width by height) was expressed in cm^3. The bread loaves were inoculated (1 ml per 100-g loaf) with a conidial suspension (10^4 conidia per ml) of fungi; then they were packed into polyethylene bags and stored at room temperature. The bread shelf life was defined as the time (in days) for moulds to become visible on the surface of the packaged loaves. Observations were performed daily.

RESULTS AND DISCUSSION

The chemical and microbiological characteristics of the slurries obtained with each LAB strain (SL-AL2 and SL-IB2) are summarized in Table 1.

After 24 h of fermentation, pH values for both the slurries were found to be approximately same i.e. 3.8 for SL-AL2 and 3.9 for SL-IB2 and cell counts were 9.1 and 9.4 log CFU per ml for slurries SL-AL2 and SL-IB2 respectively. SL-AL2 yielded the higher value for TTA (14.8 ml of 0.1 N NaOH per 10-ml sample) and titratable acidity in terms of lactic acid was 1.33 Gm% for this slurry. TTA value for SL-IB2 was 14.5 and yielded 1.30 Gm% lactic acid.

Each fermented slurry was subsequently used (50% vol/vol) to produce doughs (YLB doughs) that were identified with the different LAB strains. Dough without incorporation of slurry (Y dough) and dough with 0.4% wt/wt Calcium propionate as a chemical preservative (YCP dough) were used as controls. All doughs were prepared with commercial yeast (Baker's yeast). The pH values and leavening power was calculated for each dough type.

Doughs prepared with fermented slurries (YLB doughs) showed significantly higher leavening power and attained lower pH (5 to 5.6) as compared to Y-doughs (pH 6.5). YLB-IB2 dough had the highest leavening power (115.46 cm^3). Results are presented

in Table 2. Differences in shelf life were also seen for breads prepared with only Yeast (Y), SL and PCa. Y-breads were spoiled within 2 days of ambient storage.

Fungal growth was initiated on Day 4 after preparation in bread sample with yeast and 0.4% calcium propionate (PCa) as chemical preservative as well as bread sample with yeast and SL-IB2 slurry as starter culture, which indicates that biopreservation effect obtained with 50% antifungal slurry containing SL-IB2, was as effective as 0.4% calcium propionate (PCa). Promising results were observed with breads prepared from doughs containing SL-AL2 (50% v/v) where no visible fungal growth was observed up to 19 days, which was very much comparable with the preservative activity exhibited by Calcium propionate where shelf life was found to be more than 4 times longer than breads prepared with only 0.4% PCa.

Table 1: Characteristics of the slurries fermented with Lactic Acid Bacteria

Slurries[a]	**Colony Counts**[b] (log CFU per ml)	**pH**	**TTA**[c]	**Titratable acidity in**[d] **terms of Lactic acid (Gm%)**
SL-AL2	9.1	3.8	14.8	1.33
SL-IB2	9.4	3.9	14.5	1.30

[a] The slurries (SLs) were fermented with LAB isolates AL2 and IB2 at 30^0 C for 24h. Semiliquid slurries contained an inoculated mixture: Wheat flour, 250 g; Sucrose, 5 g; Skimmed milk, 12.5 g; and Tap water, 0.75 liters

[b] Colony counts of LAB expressed as log CFU per ml of slurry

[c] TTA – Total titratable acidity, reported as milliliters of 0.1 N NaOH needed to achieve a pH of 8.3 to 8.6 in 10-ml samples

[d] Titratable acidity in terms of Lactic acid, is calculated using the formula:

$$\textbf{Lactic acid Gm \%} = \frac{0.1 \text{ X B.R. X } 9}{\text{Volume of the sample}}$$

Where, 0.1 → Normality of NaOH
B.R. → Burette reading
9 → Equivalent weight of Lactic acid

Table 2: Characteristics of doughs prepared with fermented slurries as a starter culture and without fermented slurries i.e. controls – Y dough and YCP dough

Dough Type[a]	**Initial Volume (V_i) cm^3**	**Final leaven Volume (V_f) cm^3**	**Leavening**[b] **Power (V_f - V_i) cm^3**	**pH**	**Volume of baked doughs cm^3**
Y dough	88.51	138.54	50.03	6.5	324
YCP dough	111.61	165.48	53.87	5.5	323
YLB-AL2 dough	92.36	173.18	80.82	5.2	432
YLB-IB2 dough	115.45	230.91	115.46	5.6	435

[a] All doughs were prepared with commercial yeast (Baker's yeast). When no PCa (Calcium propionate) was used (0% PCa), the resulting control dough was designated as Y dough.

YCP dough: PCa, Calcium propionate, a chemical preservative added to the dough at 0.4% (wt/wt).Slurries SL-AL2 and SL-IB2 were incorporated by partially replacing (50%) tap water in the dough preparation by equal amounts of the slurry fermented by each of the selected LAB strains

[b] The leavening power, determined by fermenting a 50 g portion of dough in a beaker at 30^0 C for 2 h, was calculated as V (cm^3) = V_f - V_i, where V_f and V_i are the final and initial leaven volumes respectively.

Table 3: Effect on preservation of bread inoculated with mould spores

Target mould	Visible mould growth observed on the surface of packaged loaves* (Days) Dough types					
	I] Bread prepared with Slurry SL-AL2 as starter culture			II] Bread prepared with Slurry SL-IB2 as starter culture		
	Y	YLB-AL2	YCP	Y	YLB-IB2	YCP
Aspergillus flavus	Day 2	Day 2	Day 3	Day 2	Day 3	Day 3
Aspergillus fumigatus	Day 2	Day 2	Day 3	Day 2	Day 2	Day 3
Aspergillus niger	Day 2	Day 3	Day 2	Day 2	Day 3	Day 2
Fusarium sporotrichioides	Day 3	Day 7	Day 7	Day 3	Day 3	Day 7
Penicillium sp.	Day 2	Day 7	Day 5	Day 2	Day 2	Day 5

* Day on which mould growth was initiated on the surface of packaged loaves

(A) **(B)**

Fig. 1 : The extent of growth and sporulation of *A. flavus* (A) and *A. niger* (B) was highly reduced in bread with SL-AL2 slurry as compared to Y-Doughs

The overall extent of growth and sporulation of moulds *A. flavus* and *A. niger* was highly reduced in presence of AL2 (Photograph 1) as well as IB2 as the starter culture. The results were promising with both LAB strains against *Aspergillus niger*, as fungal growth was initiated on Day 2 after preparation in bread sample prepared using Calcium propionate as a preservative; whereas the fungal growth was initiated on Day 3 in bread sample with slurries SL-AL2 and SL-IB2 as a starter culture. Use of these slurries lengthened shelf life by one day with respect to controls prepared using only yeast and yeast plus PCa as chemical preservative. A remarkable antifungal activity was observed in bread sample with yeast and SL-AL2 as the starter culture against *Fusarium* and *Penicillium sp.*, wherein mould growth of was delayed up to 7 days. It was noteworthy that, the bread sample prepared with Calcium propionate showed initiation of *Penicillium* growth on Day 5, whereas in presence of AL2, growth was delayed up to 7th day. LAB AL2 inhibited the growth of *Fusarium* and *Penicillium* and lengthened the shelf life one to three fold with respect to bread prepared using only *Saccharomyces cerevisiae*. The extent of fungal growth as well as sporulation was highly limited in the bread sample with LAB IB2, as compared to bread prepared with only yeast as a starter culture, however no significant results were obtained with LAB isolate IB2 against *Fusarium.* Also, no significant antifungal activity was observed with the LAB isolates AL2 and IB2 against *A. fumigatus* (Table 3).

DISCUSSION

LAB play an important role in sourdough starter, in improving the flavor, taste and shelf life of final product. During the last decade, the main technological advancements in the baking industry have been shortened leavening time and the availability of ready-to-use starter cultures. Although baker's yeast (*S. cerevisiae*) reduces the time of bread making, the finished bread is less flavorful than bread prepared using specific strains of LAB (Brandt, 2007). The

antifungal slurries (SL-AL2 and SL-IB2) developed in this study contains the LAB strains *Lactobacillus ingluviei* and *Weissella confusa* respectively along with low-cost ingredients that are compatible with the food matrix (wheat dough). Since the slurry was developed to partially replace tap water in the dough, no major changes in the conventional process of bread making are introduced.

In present study, YLB doughs showed significantly higher leavening power than Y-doughs. Dough with SL-IB-2 had the highest leavening power (115.46 cm^3). Isolate IB2 (*Weisella confusa*), an obligate hetero-fermenter, produces CO_2 as one of the end-products. This indicates that, the ability of *S. cerevisiae* to produce CO_2 was enhanced in the presence of lactic starters. These results verify that the LAB played an important role for improving the leavening ability of yeast. These results are in good accordance with those reported by Martinez- Anaya et al. (1990), Gobbetti (1998) and Häggman and Salovaaraa (2008).

The YLB-doughs attained a significantly lower pH (5 to 5.6) than Y dough (pH 6.5), which may be due to production of organic acids during fermentation by LAB strains. This result is in agreement with Arendt *et al.* (2007) and Iacumin *et al.* (2009).

LAB strain *Lactobacillus ingluviei* tested was found to be active against *A. niger, F. sporotrichioides* and *Penicillium,* however, *Weisella confusa* was found to be active only against *A. flavus* and *A. niger.* The fungal growth was delayed as well as effects on sporulation were observed by addition of slurry to the doughs, an effect that was dependent on the LAB strains involved. The antifungal effectiveness of slurries at 50% were comparable with breads prepared with 0.4 PCa (wt/wt), as a chemical preservative. The slurries (SL-AL2 and SL-IB2) prepared from each lactobacilli strain also delayed mould growth, thus allowing an extended shelf life as compared to Y-breads prepared without preservatives. Also, extent of sporulation of moulds was found to be affected when slurries were used as starter culture. Use of SL-IB2 produced the biopreservative effect (4-days shelf life), which is similar to that obtained using 0.4% PCa. The best biopreservative effect was observed with SL-AL2 slurry, where no visible fugal growth was observed upto 19 days. The antifungal effect of the slurries was mainly related to the production of various organic acids by lactic acid bacteria and presence of PLA and OH-PLA (which was observed in our previous study (data not published), thus favoring the undissociated active fraction of the acids (Piper, Calderon, Hatzixanthis and Mollapour, 2001). After fermentation, the dough attained a low pH (pH 5.0 – 5.6), which favored the undissociated fraction of the organic acids and the antifungal effect of the slurry. The anti-mould activity of LAB against moulds have been recently reviewed by Hassan and Bullerman (2008). Studies have shown that, LAB strains in sourdough breads offer very good protection against a wide range of mould spoilage organisms including *P. corylophillum, P. expansum, E. fibuliger, A. niger and F. graminearum* (Lavermicocca et al., 2000). Recently *Lb. rossiae* LD108 and *Lb. paralimentarius* PB127 were used in the production of bread and panettone, and found to prevent growth of *A. japonicus* with shelf lives ranging from 11-32 days as compared to bread prepared with baker's yeast dough (Garofalo et al., 2012).

In our study, supplementing traditional doughs prepared with commercial yeast, with slurries prepared using lactic acid bacteria significantly delayed the mould spoilage of bread and therefore this property can be exploited to enhance shelf life of bread.

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