International Journal of Food Microbiology 237 (2016) 28–34

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Unconventional bacterial association for dough leavening

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ARTICLE INFO

Article history:
Received 18 December 2015
Received in revised form 18 July 2016
Accepted 10 August 2016
Available online 13 August 2016

Keywords:
Zymomonas mobilis
Lactobacillus sanfranciscensis
Dough-model system
Bacterial association
Yeast intolerance
Yeast-free products

ABSTRACT

The purpose of the research was to obtain innovative yeast-free doughs leavened by *Zymomonas mobilis* and *Lactobacillus sanfranciscensis*. *Z. mobilis*, as well as *Saccharomyces cerevisiae*, produces an equimolar mixture of ethanol and CO_2 through glucose, fructose or sucrose fermentation. In the present work, the inability of *Z. mobilis* to metabolize maltose has been circumvented by the addition of *L. sanfranciscensis* in the formulation. Indeed, *L. sanfranciscensis*, a heterofermentative lactic acid bacterium (LAB) typical of sourdough environment, hydrolyzes maltose releasing glucose which can be used by *Z. mobilis* for its metabolism.

Biomass samples of *Z. mobilis* subs. *mobilis* DSM 424 and *L. sanfranciscensis* DSM 20663 were grown separately in liquid media and then associated in a model dough. Leavening trials set up by using three different microbial combinations (*Lactobacillus*:*Zymomonas* 80 + 80 mg, 15 + 145 mg and 145 + 15 mg biomass, i.e. 1:1, 1:10 and 10:1 respectively on cell dry weight basis) evidenced CO_2 production levels (mL) higher than the mathematical sum of CO_2 produced by the single bacteria. In particular, when the biomass combination of *L. sanfranciscensis* and *Z. mobilis* was 1:1 (80 + 80 mg cdw) and 10:1 (145 + 15 mg cdw) a CO_2 production of 46.3–41.4 mL versus 26.7–28.5 mL was achieved. The calculated productivity gain showed positive performances of the microbial combination up to 180–240 min leavening. The subsequent efficiency loss may be due several factors, above all glucose shortage for *Z. mobilis*, as well as decrease of dough pH that can negatively affect both *Lactobacillus* and *Zymomonas* metabolism. As in traditional sourdoughs, *L. sanfranciscensis* was responsible for the souring activity with positive effects on both dough tasting and reduction of spoilage microbiota; *Z. mobilis* was instead responsible for most of the CO_2 production.

A bakery product leavened with the unconventional association *Z. mobilis* - *L. sanfranciscensis* will be addressed to people having adverse responses to the ingestion of bakery goods, thus providing innovation in the area of yeast-free leavened food.

1. Introduction

Lactic acid bacteria (LAB) together with yeasts, characterize most of the fermented food and they often interact in imparting the desired characteristics to the final product. In the area of leavened goods, such as in sourdough fermentation, LAB and yeasts contribute to acidification, flavor formation and dough leavening (De Vuyst et al., 2009; Moroni et al., 2009).

In recent years, adverse reactions resulting from ingestion of various food are on the rise: about 1 out of 5 adults (20%) reports food-related hypersensitivity reactions (Lied et al., 2010). Among these, the incidence of intolerance to baker's yeast is increasing in Western population (Mansueto et al., 2006). In a proportion of patients with Inflammatory Bowel Disease (IBD), *Saccharomyces cerevisiae* wall components have been recognized as antigens, and a cellular immune response to mannans, can be developed. Adverse reactions to baker's yeast often occur in patients with Crohn's disease, where anti-*S. cerevisiae* antibodies (ASCA) can be used as specific diagnostic markers (Brunner et al., 2007; Desplat-Jégo et al., 2007; Forcione et al., 2004): actually, ASCA have been described in up to 68% of patients with Crohn's disease (Reese et al., 2006). The incidence and prevalence of IBD are increasing with time indicating its emergence as a global disease (Melodeky et al., 2012). Moreover, these antibodies seem to be related also to other pathologies like autoimmune disorders (Muratori et al., 2003) and obesity (Salamati et al., 2015).

Z. mobilis is a Gram-negative bacterium classified as GRAS by FDA (Krishnan et al., 2000); it was first isolated from alcoholic beverages in tropical countries where it is used as fermenting agent in *Agave* sap, palm sap and sugarcane juice. It can also cause spoilage in beer, ciders and perries (Panesar et al., 2006). This microorganism can utilize only sucrose, glucose and fructose as carbon and energy sources through the Entner-Doudoroff (ED) pathway, giving ethanol and CO_2 as final products (Doelle et al., 1993; Sprenger, 1996; Zhang et al., 1995).

In a previous study, the possibility of using *Z. mobilis* as alternative to the commercial yeast *S. cerevisiae* was investigated. Results highlighted that for a significant dough leavening through *Zymomonas*, one of the

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http://dx.doi.org/10.1016/j.ijfoodmicro.2016.08.011
0168-1605/

above cited sugars must be present in the dough because maltose cannot be hydrolysed by the bacterium (Musatti et al., 2015).

In wheat flour, maltose concentration ranges from 1.7–3% w/w (Stear, 1990), while the sum of glucose, fructose and sucrose, the only fermentable sugars by *Z. mobilis,* is only 0.22–0.43% w/w (Martínez-Anaya, 1996).

In the present work a gradual release of glucose in a dough using the constitutive hydrolytic activity of *Lactobacillus sanfranciscensis* has been exploited. This LAB, an obligate heterofermentative key bacterium in type I sourdough (Gänzle et al., 2007; Picozzi et al., 2010), produces lactic and acetic acid from maltose and is responsible for the souring activity in sourdough bread (De Vuyst and Neysens, 2005; Gobbetti and Corsetti, 1997). *L. sanfranciscensis* hydrolyzes maltose, continuously provided by flour amylases, and accumulates glucose in the medium, which could be used by *Z. mobilis* for leavening the dough, analogously to maltose-negative yeasts in sourdough fermentation (Gobbetti, 1998). Here we report on the possibility of combining *Z. mobilis* and *L. sanfranciscensis* to obtain an unconventional bacterial association to be used in yeast-free dough leavening. To date this is the first investigation report dealing with such an unusual combination in the area of bakery goods.

2. Material and methods

2.1. Microorganism and culture conditions

Zymomonas mobilis subs. *mobilis* type strain DSM 424 (DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), originally isolated in Mexico from a fermented Agave sap to obtain pulque, an alcoholic beverage containing 4 to 6% (v/v) ethanol (Swings and De Ley, 1977), and *Lactobacillus sanfranciscensis* DSM 20663 were used in this study. *Z. mobilis* was weekly maintained in DSM liquid medium having the following composition (g/L): Bacto-peptone (Costantino SpA, Turin, Italy) 10, yeast extract (Costantino SpA) 10, glucose 20. Biomass production was carried out in IC G20 liquid medium (Musatti et al., 2015) having the following composition (g/L): Bacto-peptone 10, casein enzymatic hydrolysate (Costantino SpA) 10, glucose 20. For both media the pH was 6.8 and sterilization 112 °C for 30 min.

L. sanfranciscensis was maintained and cultivated in MRSm medium having the following composition (g/L): casein enzymatic hydrolysate 10, meat extract (Merck K GaA, Darmstadt, Germany) 5, yeast extract 5, K_2HPO_4 (Sigma Aldrich, Missouri, USA) 2.6, KH_2PO_4 (Sigma Aldrich) 4, NH_4Cl (Sigma Aldrich) 3, cysteine-HCl (Sigma Aldrich) 0.5, Tween 80 (Merck K GaA) 0.5, maltose (Merck K GaA) 10, pH 6.2 and sterilization at 112 °C for 30 min. After sterilization 1 mL/L of minerals and vitamins mix (sterilized by filtration) was added. The mix had the following composition (g/50 mL): $MgSO_4$ (Sigma Aldrich) 10, $MnSO_4$ (Sigma Aldrich) 1.9, thiamine (Merck K GaA) 0.01, niacin (Merck K GaA) 0.01, folic acid (Carlo Erba, Cornaredo, Italy) 0.01, pyridoxal (Sigma Aldrich) 0.01, pantothenic acid (BDH Chemicals, London, England) 0.01, cobalamin (Carlo Erba) 0.01.

Stock cultures of both microorganisms were stored at −80 °C in the same media (DSM for *Z. mobilis* and MRSm for *L. sanfranciscensis*) added with 20% (v/v) glycerol (VWR International, Leuven, Belgium).

2.2. Lactobacillus and Zymomonas biomass production

Z. mobilis was grown in 1 L flasks containing 600 mL of IC G20 medium, inoculated with a 5% (v/v) of a 9 h pre-culture in the same medium. Cultures were incubated at 30 °C for 14–16 h in stationary conditions.

L. sanfranciscensis was grown in 1 L flasks containing 600 mL of MRSm medium, inoculated with 2% (v/v) of a 24 h pre-culture in the same medium. Cultures were incubated at 30 °C for 16–24 h in stationary conditions.

The determination of the biomass growth was performed by centrifuging 200 mL of culture broth at 8600 ×*g* for 20 min at 5 °C in a Beckman J2-21 centrifuge. The recovered cell pellet was washed with deionized water, centrifuged again and then stored at 4 °C until use (the day after). In the meantime, part of the cell pellet was dried at 105 °C for 24 h to determine its cell dry weight (cdw) content.

2.3. Evaluation of microbial leavening performance in a model dough

CO_2 production level (mL) was determined by means of a model procedure adapted from Burrows and Harrison (1959) (Musatti et al., 2015). Briefly, sample biomass (160 mg cdw), 15 mL of distilled water and 20 g of commercial type 0 wheat flour (Manitoba) were mixed in a double chamber glass flask connected with a graduated burette. *L. sanfranciscensis* and *Z. mobilis* were added in dough separately or in different association ratio, always maintaining the total amount of biomass at 160 mg cdw: only *L. sanfranciscensis* or *Z. mobilis* (160 mg), 1:1 ratio (*L. sanfranciscensis* 80 mg + *Z. mobilis* 80 mg), 10:1 ratio (*L. sanfranciscensis* 145 mg + *Z. mobilis* 15 mg) and 1:10 ratio (*L. sanfranciscensis* 15 mg + *Z. mobilis* 145 mg).

CO_2 production (mL) was monitored by measuring the liquid level at appropriate intervals. The trend of CO_2 production was fitted employing the DMFit 3.5 software in order to estimate gas production rate (mL/min), lag leavening phase duration (min) and total amount of CO_2 produced (mL), according to the Baranyi and Roberts model (1994).

2.4. Evaluation of the microbial population in doughs

At appropriate leavening intervals, 5–8 g of dough were diluted in 45–72 mL sterile peptone water (10 g/L Bacto-peptone in distilled water, pH 6.8) and homogenized in a Stomacher 400 Circulator (Seward, Worthing, UK) for 5 min at 260 rpm. After decimal dilutions in the same solution, suspensions were plated in appropriate media. *L. sanfranciscensis* population was plated onto MRSm agar (MRSm broth added of 15 g/L agar) while *Z. mobilis* onto DSM agar (DSM broth added of 15 g/L agar). Plates were then incubated at 30 °C for 3 days in anaerobic conditions. Total bacterial count (TBC) was determined pour plating in Tryptic Soy Agar (TSA, Scharlab, Barcelona, Spain) after incubation at 30 °C for 48–72 h. Yeasts and moulds were determined pour plating in Yeast Glucose Chloramphenicol Agar (YGC-Scharlab) and incubated at 25 °C for 3–5 days. Counts were reported as logarithms of the number of colony forming units (Log CFU/g of dough), and means and standard deviations were calculated (n = 3).

2.5. Analytical determinations

Sugars (maltose and glucose) consumption, as well as ethanol, lactic and acetic acid produced during leavening were determined through an HPLC system (L 7000, Merck Hitachi) equipped with RI and UV (210 nm) detectors serially connected, using a (300–8 mm) SH1821 (Shodex, München, Germany) column, maintained at 50 °C and eluted with 5 mM H_2SO_4 at 0.5 mL/min. Aliquots of 2–4 mL of homogenized and appropriately diluted dough samples were centrifuged (Eppendorf 5804, 10,600 ×*g*, 10 min) and the obtained supernatants were filtered through a 0.45 μm syringe filter (VWR International, USA) before HPLC analysis. Date were referred to 1 g dough (mg/g dough).

Dough pH was monitored at different intervals on the integral undiluted dough sample (pH-meter Eutech Instruments pH 510).

2.6. Statistical analysis

Data were submitted to *t*-test and one-way analysis of variance (ANOVA) performed with SPSS software, version 21.0 (SPSS Inc., Chicago, IL, USA). When the effect was significant ($p < 0.05$), differences between means were assessed by Tukey-b test of multiple comparisons.