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Sour milk production by wheat bran supported probiotic biocatalyst as starter culture

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ABSTRACT

In the present study, probiotic sour milk was produced by freeze-dried *Lactobacillus casei* ATCC 393 cells, free or immobilized on wheat bran. Wheat bran was used with and without lignin removal. The aim was to evaluate the possibility to produce sour milk using immobilized probiotic biocatalysts that could be removed after the end of fermentation and be used in a next fermentation batch. The effect of the biocatalysts on product quality was also evaluated during 30 days of storage at 4 °C. The aroma profile, organic acids production, residual sugar content and culture viability were monitored during that period. The immobilized bacteria on wheat bran were better protected in the acidic environment of the sour milk fermentations, compared to free cells. The microbial populations (>7.5 log cfu g^{-1}) were significantly higher in sour milks produced with the immobilized biocatalysts during the 30 day storage, while no growth of pathogenic microorganisms was observed. The microbiological, physicochemical, and sensory characteristics of the sour milks produced using the immobilized biocatalysts, indicated high commercialization potential of these products.

1. Introduction

Nowadays, functional dairy food is increasingly attracting the attention of consumers that wish their food to combine improved flavour, nutritional value and direct health benefits. To meet the market's needs, there is currently extensive research and development activity for novel dairy products containing probiotic bacteria, which can promote beneficial health effects. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2006). They include strains of the normal intestinal microbiota and usually belong to the *Lactobacilllus*, *Bifidobacterium*, *Streptococcus* and *Saccharomyces* genera (Marsh et al., 2014). In order to exert a health benefit to the consumer, it is suggested that concentrations higher than 10^6–10^7 cfu g^{-1} or mL^{-1} of these bacteria need to be contained in the probiotic food (Castro et al., 2015; Gourbeyre et al., 2011). Probiotic microorganisms are sensitive to various physicochemical stress exposures such as pH, acidity, temperature and preservatives. In order to improve the viability of probiotic bacteria during food production and storage, various methods such as cell immobilization and stress adaptation have been proposed (Champagne et al., 2005). Among lactic acid bacteria (LAB), *Lactobacillus casei* is one of the most commonly used in probiotic dairy products development due to its beneficial effects and increased resistance during low temperature storage. Specifically, the strain *L. casei* ATCC 393 has been used in many studies for food production such as yogurt (Sidira et al., 2013), bread (Plessas et al., 2007), cheese (Kourkoutas et al., 2006a) and sausages (Sidira et al., 2010) in order to confer probiotic properties.

The long-term survival of probiotic species in the human gastrointestinal tract depends on the presence of nourishing compounds in the diet, known as prebiotics. The most common type of prebiotics is dietary fibre, *i.e.* non-digestible polymeric carbohydrates such as beta-glucans (Gourbeyre et al., 2011; Chen et al., 2005). Cereals contain high amounts of dietary fibre with well established beneficial health effects (Manning and Gibson, 2004), including reduction of blood pressure, better weight control and proper intestinal function (Slavin, 2008). Wheat bran is a major by-product of wheat production rich in fibre, proteins, oil and other nutrients (Li et al., 2010). Therefore, it is a potentially ideal

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food-grade carrier for immobilization of probiotic cultures for use in dairy products production. However, its use as carrier for immobilized dairy probiotic cultures has not been reported.

Sour milk is a popular dairy product made by acidification of milk using various LAB. "Amasi" is an example of sour milk produced by milk inoculation with a commercial LAB starter culture resulting in a shelf life of 21 days in refrigeration (Gran et al., 2003). "Viili" is another type of fermented sour milk found in Finland with a ropy and gelatinous consistency produced from skim milk (Toba et al., 1990). Moreover, "Calpis" sour milk is a Japanese product containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, reported for reduction of blood pressure (Takano, 1998) and antihypertensive effect (Nakamura et al., 1995).

The aims of the present study were to: (1) use wheat bran (with and without lignin removal) as carrier for the immobilization of the probiotic strain *L. casei* ATCC 393; (2) evaluate the suitability of the immobilized biocatalysts for sour milk fermentation; (3) analyse the survival of the probiotic cells, the microbiological stability in general, and the sensory properties of the produced sour milks during storage. For comparison reasons experiments were also carried out using free *L. casei* cells.

2. Materials and methods

2.1. Culture preparation

The probiotic strain *L. casei* ATCC 393 (DSMZ, Germany) was grown in de Man, Rogosa, and Sharpe broth (MRS) (Fluka, Switzerland), for 48 h at 37 °C, and subsequently in cheese whey under the same conditions (Bosnea et al., 2009; Xu et al., 2016). Cheese whey (5% lactose, 0.8% protein) was obtained by a local cheese manufacturer (Chelmos S.A., Greece). It was initially boiled at 80 °C for whey protein precipitation, which was then removed by filtration using a cheese cloth. The whey pH was maintained at 5.0 by addition of Na_2CO_3 solution during incubation. *L. casei* cell mass was harvested by centrifugation at 5000 rpm for 10 min at 25 °C (Sigma Laborzentrifugen GmbH, Germany).

2.2. Immobilized biocatalyst preparation

Wheat bran, which was purchased by a local market, was used as carrier for the immobilization of *L. casei* cells. Prior to use, it was delignified by boiling with 1% NaOH for 3 h (Koutinas et al., 2012; Bekatorou et al., 2015). Delignified and non-delignified samples of wheat bran were sterilized at 135 °C for 15 min. Amounts of 500 mL of cheese whey were mixed well with 2 g of *L. casei* cells (wet weight biomass) and with 10 g of delignified or non-delignified wheat bran (dry weight). The mixture was incubated at 37 °C for 48 h for cell immobilization to occur (Bekatorou et al., 2015; Bosnea et al., 2009; Xu et al., 2016). The fermented liquids were drained away and the immobilized biocatalysts were washed twice with 50 mL of sterile Ringer's solution for removal of free cells. Both types of immobilized biocatalysts (made with delignified or non-delignified wheat bran), as well as free *L. casei* cells, were freeze-dried on a Freezone 4.5 freeze-drying system (Labconco, USA), at 5×10^{-3} bar and −45 °C, without addition of cryoprotectants (Bosnea et al., 2009).

2.3. Enumeration of immobilized probiotic cells

For the enumeration of the *L. casei* cells immobilized on wheat bran, 10 g of freeze-dried immobilized biocatalyst (with delignified or non-delignified wheat bran) were blended with 90 mL of sterile Ringer's solution (1/4 strength). The suspension was serially diluted (ten-fold), plated on MRS agar (Fluka, Switzerland) and incubated at 37 °C for 48–72 h. The cell counts were expressed as log cfu g^{-1} of wheat bran.

2.4. Sour milk production

The freeze-dried immobilized biocatalysts were confined into sterile pouches made from thin perforated nylon fabric, which were then immersed into homogenized and pasteurized cow's milk (3.5% fat, 13% total solids, 4.56% lactose, pH 6.5) for sour milk fermentation at 37 °C. The free, freeze-dried *L. casei* cells were dispersed into the milk, and sour milk fermentations were carried out as above (*Sour milk 1; S1*). Specifically, in 1-L glass cylinder bioreactors containing 500 mL of milk, 10 g of freeze-dried immobilized biocatalyst made with non-delignified (*Sour milk 2; S2*), or delignified wheat bran (*Sour milk 3; S3*) (final concentration in milk 10^8–10^9 cfu mL^{-1}), or freeze-dried free *L. casei* cells (final concentration in milk 10^7 cfu mL^{-1}), were added. When the pH reached 4.5 ± 0.05 (after about 20 h of fermentation), the immobilized biocatalysts were removed from the fermented milk and the products were kept for further analysis. All experiments were carried out in triplicate.

2.5. Microbiological analysis

All fermented samples were stored in glass containers at 4 °C for 30 days. For the enumeration of viable microorganisms, samples of 10 g were collected at various time intervals (0, 1, 7, 14, 21, 30 days). The samples were serially diluted in 90 mL sterile Ringer's solution (1/4 strength), homogenized (in a Bagmixer 400, Model VW, Interscience), decimal-diluted and plated on selective media. *L. casei* ATCC 393 was enumerated on MRS agar and incubated at 37 °C for 48 h. Total mesophilic aerobic bacteria were enumerated on Plate Count Agar (PCA) after incubation at 30 °C for 72 h. Yeasts and moulds were determined by plating on Potato Dextrose Agar (PDA) after incubation at 30 °C for 72 h. Total enterobacteria were enumerated on Violet Red Bile Glucose Agar (VRBGA) after incubation at 37 °C for 24 h, and coliforms were enumerated on Violet Red Bile Agar (VRBA) after incubation at 30 °C for 24 h. *Staphylococci* were enumerated on Baird Parker agar (BP) after incubation at 37 °C for 48 h. The original counts were expressed as log cfu g^{-1} of sour milk. All the above media (Fluka, Switzerland), were sterilized at 135 °C for 15 min before use.

2.6. Physicochemical analysis

The pH values of milk and fermented products were measured using a digital pH meter (Hanna HI99161). For lactose and organic acids determination, 5 g of sample were diluted with sterilized deionised water to 200 mL and homogenized. The mixture was centrifuged at 4125 rpm for 20 min (Shimadzu Application, No L213) and the supernatant was then filtered and analyzed. Lactose was determined by HPLC on a Shimadzu chromatograph (Shimadzu Corp., Japan) as described by Kourkoutas et al. (2006b), and the concentrations were calculated using standard curves.

Organic acids were analyzed on a Jasco LC-2000 Series HPLC system (Jasco Inc., Japan) equipped with a size-exclusion organic acid analysis column (Aminex HPX-87H, 300 × 7.8 mm i.d., 9 μm particle size, Bio-rad, France), fitted in a CO-2060 PLUS column oven, a PU-2089 pump, a AS 2050 PLUS