Increasing the stability of immobilized *Lactococcus lactis* cultures stored at 4 °C

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SUMMARY

Immobilized cell technology was used to prepare concentrated cultures of *Lactococcus lactis* that lost only 22% of viability over a 30-day storage period at 4 °C. Concentrated cultures of *L. lactis* CRA-1 were immobilized in calcium alginate beads and added to glycerol, NaCl or sucrose-NaCl solutions in order to obtain aw readings ranging from 0.91 to 0.97. The suspensions were subsequently placed at 4 °C and viability (CFU g⁻¹ of bead) was followed during storage. Viability losses were high at aw readings of 0.95 and 0.97, and pH dropped significantly (up to one unit) in the unbuffered solutions. Addition of 1% soytone or glycerophosphate helped stabilize pH, and a beneficial effect on viability during storage was observed in the glycerol-soytone mix when the beads were added to the conservation solutions immediately following immobilization. When beads were added to the conservation solutions immediately following immobilization, a 70% drop in cell counts occurred during the first 5 days of incubation. Dipping the *L. lactis*-carrying beads in milk for 2 h before mixing with the glycerol-soytone 0.93 aw solution reduced this initial 5-day viability loss. Cultures grown in the alginate beads also had good stability in the 0.93 aw glycerol-soytone solution, where 78% of the population was viable after 30 days at 4 °C. The process could be used to store immobilized cells at a processing plant, or by suppliers of lactic starters who wish to ship cultures without freezing or drying.

INTRODUCTION

Immobilized cell technology (ICT) has been proposed to the dairy industry for the continuous inoculation of milk [12], organic acid production from whey [4,9], biomass production for the starter industry [5], and for the improvement of survival of culture bacteria in frozen desserts [14]. Industrial application of ICT has certain challenges, such as preservation of immobilized cells during plant shutdown for scheduled maintenance/sanitation or shipping of cultures from supplier to processor. Liquid cultures of lactococci are generally not very stable, and a 90% reduction in viable counts is typical when milk-grown starters are stored at 4 °C for 14 days [11]. This partially explains why the lactic acid bacteria (LAB) industry uses freezing or drying to preserve cultures destined for direct-vat-inoculation [15] as opposed to the yeast-making industry which provides fresh compressed yeasts for the baking industry [6]. Freezing and drying add to the cost of preparing preparing lactic starters, and shipping of frozen cultures is expensive. There is thus a need to develop techniques that enable preservation of the viability of liquid cultures of LAB upon storage at temperatures above freezing.

Studies on the conservation of fresh immobilized LAB have been limited to thermophilic organisms [2,3], and no data are available for immobilized lactococci. Water activity levels under 0.95 severely repress growth and metabolite production by LAB [8,17], but it remains to be determined if adjustment of aw could be used to control the metabolic activities of lactococci, and potentially enhance stability during storage at 4 °C. The aim of this study was to determine the effect of water activity on the stability of immobilized *Lactococcus lactis* during storage at 4 °C, and develop a method to increase the stability of such cultures during refrigerated storage.

MATERIALS AND METHODS

Microorganism

Stock cultures of *Lactococcus lactis* subsp. *lactis* CRA-1 were maintained on 12% reconstituted nonfat dry milk and transferred twice a week by inoculating milk (1% v/v) and incubating it for 16 h at 23 °C, thus reaching a pH of 4.6-4.8. The culture was held at 4 °C between transfers. Cultures destined for immobilization in alginate beads were prepared by inoculating 2 L of M17 broth (Oxoid, Nepean, Ontario, Canada) with the milk-grown culture (0.1% v/v) and incubating it for 20-22 h at 23 °C.

Media

Nonfat dry milk (Agropur, low heat type) was reconstituted at 12% (w/w) solids and sterilized at 115 °C for 10 min. Sodium alginate (BDH, Montréal, Quebec, Canada) solutions used for the formation of beads were prepared at 1% (w/v) and sterilized at 115 °C for 10 min. Various solutions of glycerol, NaCl or sucrose were tested for the conservation of immobilized cultures at 4 °C. The protective solutions were
initially prepared at double (2X) concentration; following addition of the alginate beads or the free cell suspensions, the mixtures gave aw measurements between 0.91 and 0.97. The 2X solutions contained 24, 43, 62 or 81 g ml⁻¹ glycerol, 8.6, 15, 21.2 or 27.6 g per 100 ml NaCl which gave the respective aw readings of 0.97, 0.95, 0.93 or 0.91 following the 1:1 ratio mixing with beads. For the sucrose-NaCl 2X solutions 6, 12, 15 or 18 g per 100 ml of NaCl was added to a 40% sucrose solution. Some of the 2X solutions were further supplemented with soytone (2% w/v) or glycerophosphate (2% w/v). All solutions were adjusted to pH 6.0 prior to sterilization (121 °C, 15 min).

Cell immobilization

Unless otherwise stated, the M17-grown cells were recovered by centrifugation for 10 min at 5000 x g. The cell pellet was resuspended in 1% alginate to obtain a final concentration of 20X, which constituted a population of approximately 8 x 10⁸ CFU ml⁻¹. Immobilization was carried out as described by Champagne et al. [5]. For assays with free cells, the pellet obtained following centrifugation was resuspended in sterile distilled water, with the same 20X concentration factor.

Storage

The cell suspensions were placed in sterile test tubes, one tube being prepared for each sampling time, and tubes were stored at 4 °C for up to 70 days.

In one series of assays, the immobilized cells were not immediately placed in the conservation solutions. A regeneration treatment was carried out by mixing 300 ml of milk with 15 g of beads, containing the concentrated cells, and incubating at 23 °C for 2 h under 30 r.p.m. agitation using a Bellco Quad drive system (Vineland, NJ, USA); pH was kept at 6.5 with periodic additions of 5 N NH₄OH controlled by a Radiometer system (Copenhagen, Denmark). Following this 2-h regeneration period, beads were separated from the milk, rinsed twice with 100 ml of sterile peptone 0.1%, and added to the conservation solutions at a 1:1 ratio.

Effect of biomass production in beads

Alginate beads (15 g) were prepared from a culture grown on M17 broth. They were added to 300 ml of milk supplemented with 3 ml of 0.1 M CaCl₂, and incubated for 16 h at 23 °C to enable growth of the bacteria inside the beads. During this fermentation, pH was maintained at 6.5 by adding 5 N NH₄OH, using a Radiometer PHM84 – TIT Innova valve system. A 2-h regeneration treatment previously described was also carried out. Beads were then weighed and distributed in the protective solution made of glycerol and soytone (aw = 0.93) at the usual 1:1 ratio, and stored at 4 °C. This treatment will be referred to as the ‘bead-grown culture’.

Analyses

Alginate beads were liquefied by aseptically adding the content of a test tube (2 g) to 99 ml of sodium citrate (1% w/v) and homogenizing the mixture in a Stomacher Lab blender 400 unit (Seward Medical, London, Ont., Canada) for 5 min at 22 °C. Free-cell samples were also mixed with the same amount of sodium citrate and homogenized for 5 min in order to maintain identical plating procedures. Bacterial counts of the liquefied beads or free-cell samples were obtained by plating appropriate dilutions (0.1% peptone) on M17 agar (Oxoid, Nepean, Ont., Canada) and incubating the plates at 30 °C for 48 h. pH measurements were carried out with a Radiometer PHM84 unit (Copenhagen, Denmark).

The water activity of the various solutions was determined by a Novasina hygrometer (Zürich, Switzerland). Accuracy of the probes was confirmed with saturated solutions of potassium nitrate (aw = 0.936) and potassium sulfate (aw = 0.973). The equilibrium time was kept constant at 90 min for standards and samples, and temperature was maintained at 25 °C.

RESULTS AND DISCUSSION

Effect of aw and composition of the preservation solution

In the aw range of 0.91–0.97, viability of immobilized L. lactis was highest at values of 0.91 and 0.93 (Fig. 1). The