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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 a_w 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 a_w 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 a_w solution reduced this initial 5-day viability loss. Cultures grown in the alginate beads also had good stability in the 0.93 a_w 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 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 a_w 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.

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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 (2×) concentration; following addition of the alginate beads or the free cell suspensions, the mixtures gave a_w measurements between 0.91 and 0.97. The 2× 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 a_w readings of 0.97, 0.95, 0.93 or 0.91 following the 1:1 ratio mixing with beads. For the sucrose–NaCl 2× solutions 6, 12, 15 or 18 g per 100 ml of NaCl was added to a 40% sucrose solution. Some of the 2× 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 × *g*. The cell pellet was resuspended in 1% alginate to obtain a final concentration of 20×, which constituted a population of approximately 8 × 10^{10} 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 20× 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 NH_4OH 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 $CaCl_2$, 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 NH_4OH, using a Radiometer PHM84 – TTI Titrator – MNV 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 (a_w = 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 (Zurich, Switzerland). Accuracy of the probes was confirmed with saturated solutions of potassium nitrate (a_w = 0.936) and potassium sulfate (a_w = 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 a_w and composition of the preservation solution

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

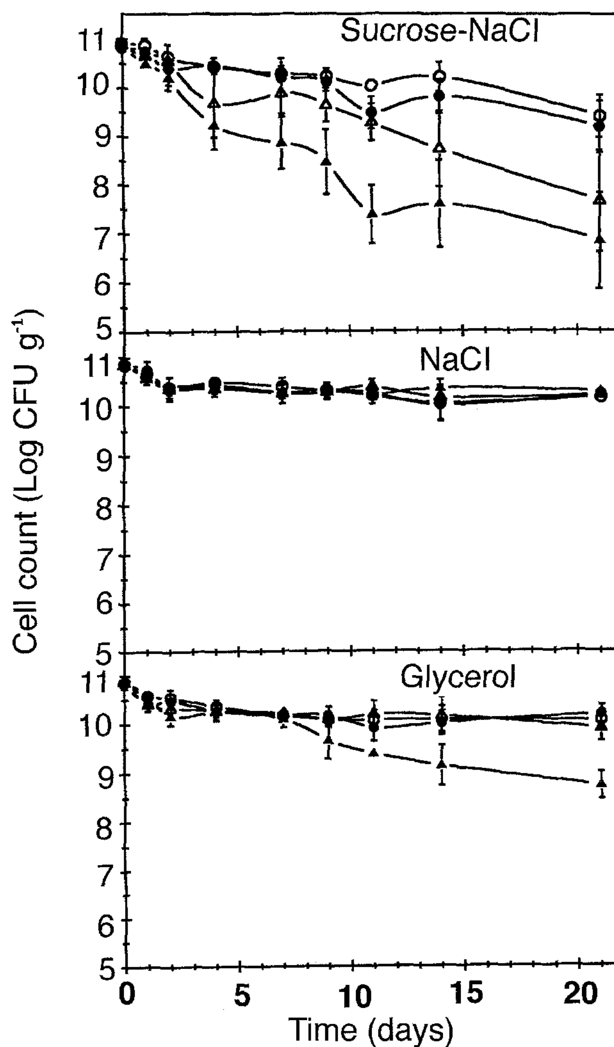

Fig. 1. Effect of water activity (a_w) and medium composition on the viability of immobilized *Lactococcus lactis* CRA-1 during storage at 4 °C. (○), a_w = 0.91; (●), a_w = 0.93; (△), a_w = 0.95; (▲), a_w = 0.97. Bars are 95% confidence limits.

effect of a_w differed between the various conservation solutions. With NaCl alone, the viability remained high at a_w values up to 0.97, while sharp losses in viability occurred with sucrose–NaCl solutions at this water activity level.

The pH dropped at least 0.4 units during storage (Table 1). The most important drops occurred at high a_w values and in sucrose-containing solutions where pH values of 5.0 could be obtained. This was not related to fermentation of the components in the preservation solution since *L. lactis* CRA-1 did not produce large quantities of lactic acid from sucrose or glycerol in fermentation trials carried out in M17 broth.

The lowest viabilities were generally found in samples having low pH. Since storage at low pH has been shown to be detrimental to lactococci [7,13], some assays were carried out with compounds added with the aim of stabilizing pH.

Effect of peptone and glycerophosphate

Sodium citrate and potassium phosphate could not be used to buffer the media since these compounds dissolved the alginate beads. Peptones and glycerophosphate did not have this drawback. Assays were also limited to water activities of 0.93 and 0.95 since it was found that little advantage was gained by lowering to 0.91 and high mortality was observed at 0.97 (Fig. 1).

Soytone (1%) and glycerophosphate (1%) were effective at keeping pH in the vicinity of 6.0 during storage in glycerol as well as in NaCl, and there was no effect of the state of the cells, free or immobilized in alginate beads, on the pH evolution patterns during storage (data not shown). Soytone was only slightly beneficial in glycerol at day 21 (Fig. 2), but population levels were 1 log higher with soytone after 35 days of storage as compared to controls (data not shown). The glycerol–soytone medium was thus deemed to have the best potential for long term storage of immobilized *L. lactis* CRA-1, and was used in the following studies with regeneration treatments.

The state of the cell suspension, free or immobilized, did not have a major impact on viability during storage except with soytone in NaCl. Therefore, with this particular procedure, immobilization did not confer an advantage over free-cell systems.

TABLE 1

Effect of a_w and composition of the conservation solutions on pH of the immobilized *Lactococcus lactis* CRA-1 cultures stored at 4 °C for 20 days. The initial pH was 6.0

Conservation solution	Water activity (a_w)			
	0.91	0.93	0.95	0.97
NaCl	5.6	5.6	5.5	5.5
Glycerol	5.5	5.5	5.4	5.4
Sucrose NaCl	5.4	5.4	5.3	5.0
NaCl + 1% soytone	ND[a]	6.2	6.2	ND
NaCl + 1% glycerophosphate	ND	6.0	6.0	ND
Glycerol + 1% soytone	ND	6.0	6.1	ND
Glycerol + 1% glycerophosphate	ND	5.8	5.7	ND

[a] ND = Not determined.

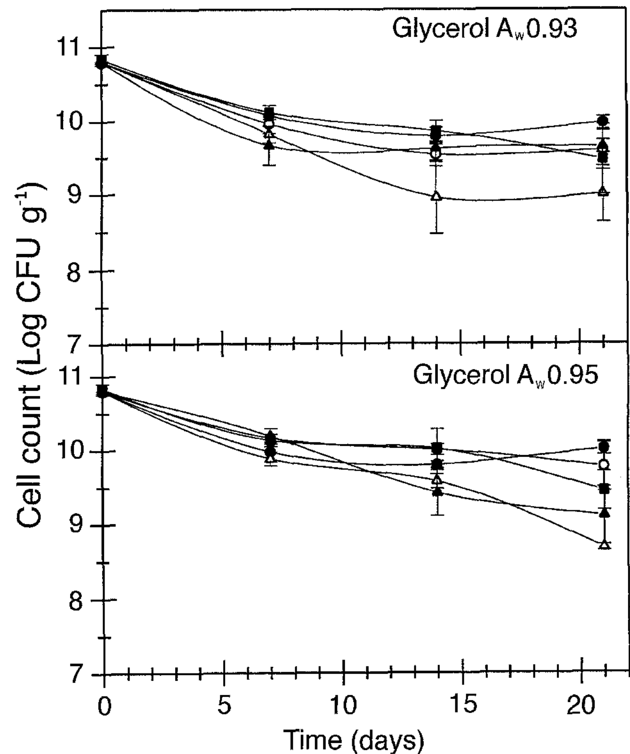

Fig. 2. Effect of buffer compounds, soytone or glycerophosphate, on viability of free (F) or immobilized (I) *Lactococcus lactis* CRA-1 during storage at 4 °C in solutions of glycerol at water activity (a_w) levels of 0.93 or 0.95. (■), Control (I); (●), Soytone (I); (○), Soytone (F); (▲), Glycerophosphate (I); (△), Glycerophosphate (F). Bars are 95% confidence limits.

As in the first series of experiments in glycerol or NaCl (Fig. 1), there was a drop in cell counts of approximately 0.5 log (70%) during the first 5 days, and a slower death rate afterwards. This is in contrast to the standard death curves observed during storage of liquid starters where a stationary phase is first observed, followed by exponential death [11]. The cultures were quite stable over 20 days at 4 °C following the initial mortality. Attempts were thus made to prevent this initial drop in viability.

Effect of post-immobilization regeneration

Dipping the beads into milk for 2 h immediately following immobilization of the centrifuge-concentrated culture reduced the subsequent death rate of the immobilized lactococci not only during the 0–5 day period, but also during extended storage in a 0.93 a_w glycerol–soytone solution (Fig. 3). During the first 30 days of storage at 4 °C, a 90% loss in viability was observed in the controls, while samples having received the 2-h regeneration treatment showed a viability loss of only 50% over the same period. This suggests that the immobilization procedure, added to that of centrifugation, stressed the cells and that regeneration can occur, as documented for *Salmonella* [1] and *Staphylococcus aureus* [10]. The process of immobilization in alginate beads is generally not lethal to cells [16], but no study has been performed on possible sub-lethal damages.

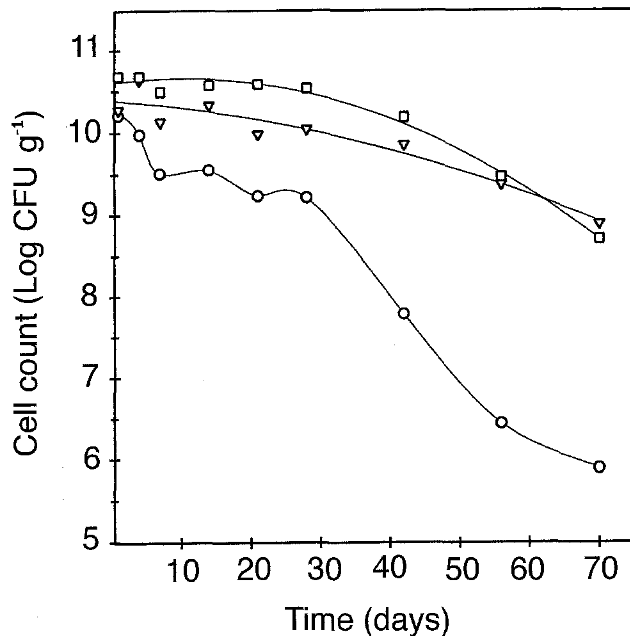

Fig. 3. Effect of the mode of preparation of immobilized *Lactococcus lactis* CRA-1 on their subsequent survival at 4 °C in a glycerol–soytone solution of a_w 0.93. (○), immobilization (control): cells placed in the glycerol–soytone solution immediately following centrifugation and immobilization in alginate beads; (▽), regeneration: prior to addition in the glycerol–soytone conservation solution, but immediately following centrifugation and immobilization of the cells, beads are incubated 2 h in milk at 30 °C; (□), bead-grown culture: cells grown in the alginate beads (24 h), subjected to the 2-h regeneration period in milk, and then added to the glycerol–soytone conservation solution.

Whatever the nature of the beneficial effect of the 2-h regeneration period, the only way it can be carried out without diluting the concentrated cell suspension is through ICT. The beads can be dipped in the regeneration solution and removed without dilution of the bacterial population, which would not be the case with free cells.

Bead-grown cultures

Concentrated lactic cultures can be obtained by incubating immobilized cultures in a suitable medium and allowing growth to occur inside the bead [5]. In this series of assays, the bead population reached 2.3×10^{10} CFU g^{-1}, following the initial 16-h fermentation, and increased to 4.7×10^{10} CFU g^{-1} after the 2-h regeneration treatment. This population density was similar to that of centrifuged cultures. When such bead-grown cultures were subsequently placed into soytone–glycerol solutions of 0.93 a_w, stability was very good for the first 30 days of storage (Fig. 3). The viability was still 78% of the initial population after 30 days of storage, but the death rate subsequently increased.

It was possible to prepare cultures of *L. lactis* that lost only 22% of their viable population over 30 days of storage at 4 °C. Although control of water activity is an important aspect of the process, ICT was necessary to obtain the highest stability.

The ICT-regeneration procedure could be used to stabilize concentrated suspensions of lactococci over intended periods of storage at 4 °C. Such a procedure would be useful in the case of a plant shutdown. It could also be applied by starter production firms that would provide immobilized lactic acid bacteria to plants using ICT for continuous inoculation of milk [12], organic acid production from whey [4] or concentrated probiotic cultures for specially frozen desserts [14]. It is also potentially used for direct-vat inoculation, following dissolution of the beads in citrate buffers. Since the culture would contain citrate, the application of this approach could be appropriate for fermented products such as sour cream or cottage cheese, where citrate is added to foster diacetyl production.

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