J. Chem. Tech. Biotechnol. 1993, 56, 259–263

Microencapsulation of *Lactococcus lactis* within Cross-Linked Gelatin Membranes

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(Received 24 June 1992; accepted 1 October 1992)

Abstract: Lactococci were encapsulated within gelatin membranes cross-linked with toluene-2,4-diisocyanate at an oil/water interface. Reagent toxicity was avoided by use of vegetable or silicone oil as a dispersant, and by minimizing cell exposure to the water insoluble cross-linking agent during membrane formulation. Growth of cells within the gelatin microcapsules was observed during fermentation and acidification activity of the encapsulated cells increased with subsequent reuse. A microencapsulated cell density of 10^9 cfu cm^{-3} resulted in acidification of milk to pH 5·5 within 2·8 h, similar to that achieved in free cell fermentations.

Key words: *Lactococcus lactis*, microencapsulation, gelatin.

1 INTRODUCTION

Lactic acid cultures are mainly used in the dairy industry to acidify milk in the manufacture of cheese and yoghurt. In some specialty products such as fermented creams used as dressings for cottage cheese and in the production of cultured butter, cream coagulation due to over-acidification is avoided by stopping the fermentation at approximately pH 5·5. Removal of immobilized cultures from the cream would reduce or eliminate residual activity in the refrigerated product, extending its shelf-life. Also immobilization of lactic acid bacteria would facilitate recovery of the cultures and permit recycle or use in a continuous process.[1] High cell densities in reactors may be achieved, and immobilization improves culture stability[2] while providing protection from bacteriophage.[3,4] Lactic cultures have been previously immobilized by entrapment in alginate[1] and kappa-carrageenan/locus bean gum beads.[5] The advantage of immobilization using natural polymers is that the reagents are non-toxic and the procedures are gentle to the microorganisms. However cell release from such beads[5] is undesirable in some applications.[1,6–7] Alginate beads containing lactic cultures have been coated with a poly-L-lysine membrane[6,8] to minimize cell release. The membranes were ineffective in substantially reducing accumulations of free cells in batch fermentations.[6] There is a need for stronger membranes to prevent cell leakage. McKnight *et al.*[9] used chitosan as a coating for calcium alginate beads, resolubilizing the alginate core leaving a thin, chitosan membrane bound, microcapsule.

An alternative microencapsulation technique which involves a single step process is interfacial polymerization. Micron-size capsules may be formed in which the only significant barrier to diffusion of substrates and products is the surface membrane. The interfacial polymerization technique involves the formation of an emulsion with an aqueous suspension of the biocatalyst as the discontinuous phase and an organic solvent as the continuous phase.[10] The droplets contain one monomer, and the reaction is initiated when a second monomer, soluble in the continuous organic phase, is added to the emulsion.

The inherent strength of membranes formed by covalent bonding may reduce cell release rates. Larisch[8] examined the membrane formation by interfacial polymerization of nylon and polyethylenimine for the encapsulation of lactic acid bacteria. Viability and activity of the cultures were severely reduced.

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J. Chem. Tech. Biotechnol. 0268-2575/93/$06.00

Polymers such as proteins can be cross-linked to form an encapsulating membrane[11] and non-toxic solvents such as mineral and vegetable oils have been successfully used as the organic phase for droplet dispersion.[8,12]

The aim of this study was to encapsulate *Lactococcus lactis* within interfacially cross-linked gelatin membranes.[11,13] Toxic reagents were replaced where possible and exposure to the reactive cross-linking agent was reduced. This study represents the first report of live cells immobilized by a process of interfacial polymerization.

2 MATERIALS AND METHODS

2.1 Culture

Lactococcus lactis subspecies *cremoris* was provided in lyophilized form by Institut Rosell Inc., Montreal. Bacto-Elliker broth (Difco Laboratories, Detroit, Michigan) or 12% (w/v) sterilized reconstituted skim milk powder were used as the growth media. Cells (0·1 g in 100 cm^3 medium) were rehydrated and cultured in Bacto-Elliker broth for 18 h at 22–25°C, harvested at 5000 rpm for 20 min, washed with 0·9% saline and recentrifuged. Plate counts on Bacto-Elliker broth supplemented with 1·5 w/v% Bacto-agar (Difco Laboratories) were used to enumerate viable cells following incubation at 32°C for 48 h.

2.2 Microencapsulation

A 24% gelatin solution, buffered with 0·45 mol dm^{-3} Na_2CO_3 at pH 9·8 was held at 35°C. Cells (0·5 g wet weight cm^{-3} gelatin solution) were suspended in the warm gelatin and the slurry was emulsified in sunflower seed oil or 10 cs Dow Corning silicone oil containing 2% Span 85. The ratio of gelatin–cell suspension to oil was 1/10. Mixing was provided by a sheet lattice[14] impeller at 300 rpm for 3 min to stabilize the emulsion. The cross-linking agent (toluene-2,4-diisocyanate dissolved in 10 cm^3 oil) was added to the emulsion to yield a final concentration of 34 mmol dm^{-3} oil. After 15 min, the reaction mixture was diluted to 1/4 the concentration with a surfactant solution (2% (w/v) laboratory detergent in water; Micro, Cole Parmer) and oil was decanted after another 15 min. The microcapsules were filtered from the aqueous phase and rinsed with distilled water.

2.3 Size and analysis

Volumetric size distributions of the microcapsules were determined using a particle size analyzer (Malvern Instruments) according to the log normal distribution model. The mean diameter and arithmetic standard deviation [$\sigma_a = 0{\cdot}5\ (d_{84} - d_{16})$] were calculated from the cumulative distribution curve.

2.4 Activity assay

Cell activity was monitored by following the reduction in pH of milk at 30°C using the equivalent concentration of 9·5 mg cell wet weight per cm^3 milk. Microencapsulated cultures were recovered by filtration from the acidified milk (clarified using sodium citrate) and re-assayed in fresh milk. Loss of microcapsule mass due to shrinkage and normal loss during filtration and handling operations was noted in repeated assays. The mass of microcapsules reported represents the actual measured mass prior to each assay. Resulting error in estimation of cell loading within the microcapsules could be as large as an order of magnitude.

2.5 Evaluation of viability after encapsulation

The viable count after encapsulation was measured by homogenizing the microcapsules, and plating the resulting homogenate. The microcapsules were broken using a 40 cm^3 tube and pestle homogenizer (Cole Parmer) for 15 min at 1800 rpm. Beyond 15 min, no substantial increase in the released cells was observed.

3 RESULTS AND DISCUSSION

3.1 Free cell activity

Lactococcus lactis subsp. *cremoris* was assayed for acidifying activity in milk to serve as a basis for comparison with microencapsulated cells. Figure 1 shows typical pH profiles of free cell fermentations. At the highest inoculum levels, the pH dropped immediately and rapidly. Lower cell levels resulted in longer initial delays prior to the onset of the decrease in pH. The rate of pH decrease at all levels of inoculum appear similar following an initial delay period. Cells were enumerated at the beginning of each assay and the time to reduce the

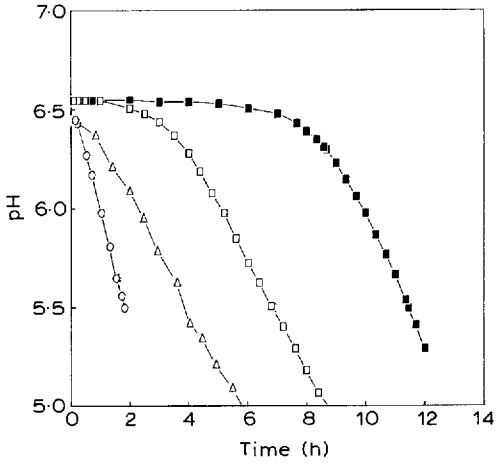

Fig. 1. Free cell activity at $3{\cdot}0 \times 10^4$ (■), $2{\cdot}7 \times 10^7$ (□), $5{\cdot}4 \times 10^7$ (△) and $2{\cdot}2 \times 10^8$ (○) cfu cm^{-3}.

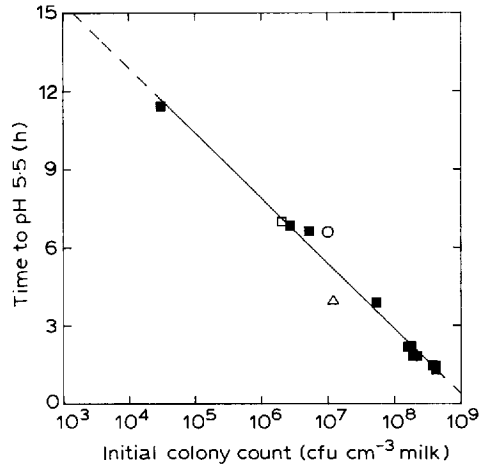

Fig. 2. Relationship between initial colony count and time to reduce the pH of milk to 5·5. Linear regression is based on data from present study. (■) Present study; (△) Champagne and Côté,[1] pasteurized cream; Christopherson and Zottola,[15] 13 w/w% (□) and 7·6 w/w% (○) reconstituted skim milk.

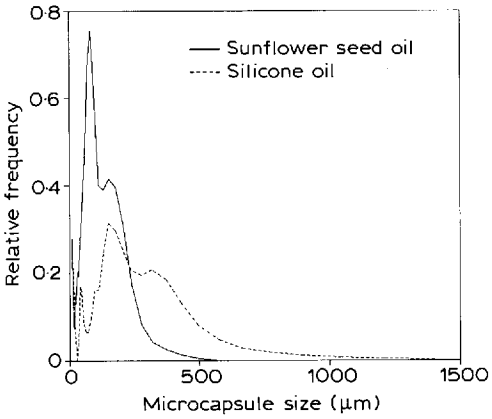

Fig. 3. Volume distribution curves for gelatin microcapsules produced in sunflower seed and silicone oils.

pH of milk to 5·5 ($t_{5.5}$) was recorded in each case. The assay with $2{\cdot}2 \times 10^8$ cfu cm^{-3} milk (9·5 mg cell wet weight cm^{-3} milk) resulted in a $t_{5.5}$ of 1·8 h. The $t_{5.5}$ values were correlated with the initial cell counts and compared to data appearing in the literature. As seen in Fig. 2, the free cell activity assays correlate well with literature data and fit the following linear correlation:

$$t_{5.5} = 22{\cdot}9 - 2{\cdot}50[\log_{10}(\text{cfu cm}^{-3}\text{ milk})]$$

This correlation is useful for predicting the time to reduce the pH of milk to 5·5 given an initial cell concentration.

3.2 Size analysis of microcapsules

Typical size distributions of microcapsules produced in sunflower seed and silicone oils are illustrated in Fig. 3. Volume distributions for both batches of microcapsules have a similar shape except that microcapsules produced in silicone oil have an extended tail towards the large diameter size of the distribution. The mean diameters were 124 ± 74 μm and 271 ± 168 μm for microcapsules formed in sunflower seed and silicone oils respectively. Corresponding specific surface areas were 736 and 407 cm^2 cm^{-3} of microcapsules. Thus a 45% decrease in surface to volume ratio occurred when vegetable oil was replaced with silicone oil.

3.3 Activity and viability of cells after encapsulation

Due to its reactive nature, the cross-linking agent was assumed to be the most toxic reagent in the microencapsulation procedure. The concentration of diisocyanate was reduced from 34 to 4·7 mmol dm^{-3} organic phase to minimize possible toxic effects. Weaker but intact membranes were formed at the lower concentration of diisocyanate.

Cell viability after encapsulation was tested on microcapsules prepared using sunflower seed oil. The microcapsules were homogenized and the released cells enumerated. Microcapsules produced at both 4·7 and 34 mmol dm^{-3} diisocyanate resulted in viable cell counts of 5×10^4 cfu g^{-1} cells encapsulated, compared to 2×10^{10} cfu g^{-1} cells in the non-encapsulated controls. A substantial reduction in viable cell count was observed with no benefit to reducing the concentration of cross-linker. Acidification activity was also similar at both levels of diisocyanate. Because the microcapsules were more resistant to breakage at the higher concentration, 34 mol dm^{-3} toluene diisocyanate was used to cross-link the gelatin in subsequent experiments.

Slightly larger microcapsules were obtained when sunflower seed oil was replaced with silicone oil. Figure 4 shows an improvement in the microencapsulated cell activity obtained by forming the microcapsules in silicone oil. Cells encapsulated using sunflower seed oil reduced the pH to 5·5 in 14·6 h whereas those encapsulated using silicone oil resulted in a $t_{5.5}$ of 8·2 h representing a 44% difference. The higher specific surface area of the smaller microcapsules would increase the probability of contact with the membrane during formation due to closer proximity to the interface. Desoize *et al.*[16] noted that the cytotoxicity of the encapsulation process was related to the degree of cross-linking and possible direct contact with the cells.

3.4 Sequential fermentations of microencapsulated cultures

Microscopic examination during fermentation indicated an increase in the intracapsular cell density. If the microcapsules were washed and re-assayed, the acidification rate was increased in comparison to the initial assay. Recycling of the microcapsules for a third fermentation resulted in a further enhancement of the activity for cells microencapsulated in silicone oil. Three sequential fermentations resulted in $t_{5.5}$ values of 14·6,

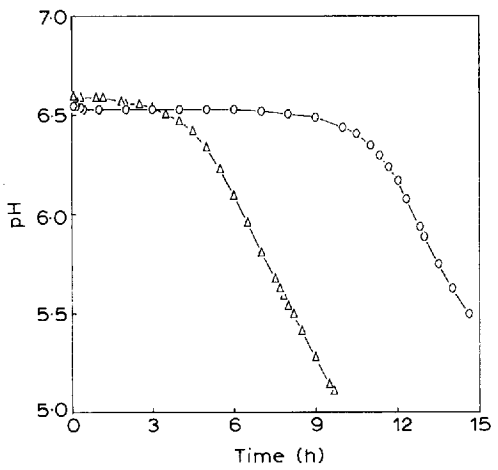

Fig. 4. Effect of organic phase on encapsulated cell activity. (○), Sunflower seed oil; (△), silicone oil.

5·1 and 6·4 h respectively for cells encapsulated in sunflower seed oil, and 8·2, 6·0 and 2·8 h for cells encapsulated in silicone oil. A $t_{5.5}$ of 2·8 h is similar to that observed for alginate entrapped bacteria. Champagne and Côté[1] observed a $t_{5.5}$ of 2 h at an initial cell concentration of $1{\cdot}6 \times 10^8$ cfu cm^{-3} pasteurized cream.

In examining lactic acid bacteria entrapped in alginate beads, Tipayang and Kozaki[17] found that cell numbers and acidification rates increased then stabilized during sequential fermentations. Champagne *et al*[4] also observed increased lactic acid production rates during sequential fermentations with entrapped bacteria. Lacroix *et al.*[5] incubated lactic acid bacteria entrapped in a kappa-carrageenan/locust bean gum mixture to increase the number of viable entrapped cells.

3.5 Estimation of viable cell counts in microcapsules

The activity assay used to measure acidification of the encapsulated cells may also serve as a monitor for cell viability. Using the regression equation developed from the free cell experiments, the number of encapsulated cells for each assay were estimated and the cells per gram of microcapsules calculated. Table 1 summarizes the results from activity assays of two batches of microcapsules formed in sunflower seed oil at different concentrations of diisocyanate, and assays of two batches formed in silicone oil at the same diisocyanate concentration. The volume of milk used in the assays described in Table 1 was adjusted so that 9·5 mg encapsulated cells cm^{-3} milk were used in each assay. As is evident from the estimated cell counts, a substantial loss in activity and viability appears to result following encapsulation. The second and third sequential fermentations showed improved activity and viability. In the case of microcapsules produced in silicone oil, the activity and viability of the encapsulated cells during the third successive fermentation approached that of free cells at an initial cell concentration of 9·5 mg cm^{-3} milk. Estimated cell counts per gram of microcapsules were initially higher for capsules produced in silicone oil than those produced in sunflower seed oil, but similar levels of cells (10^9 cfu g^{-1} microcapsules) were attained after one or two fermentations.

Champagne *et al.*[7] obtained cell densities of approximately 6×10^{10} cfu g^{-1} of alginate beads with *L. lactis* and Lacroix *et al.*[5] attained cell densities of 4×10^{11} cfu cm^{-3} following growth in kappa-carrageenan/locust bean gum gel with *Lactobacillus casei*, levels higher than that attained in the microcapsules. Further growth may be expected in the microcapsules

TABLE 1
Estimated Colony Counts for Encapsulated Cultures

Batch	*Sequential assay number*	$t_{5.5}$ *(h)*	*Estimated colony count (cfu cm^{-3} milk)*	*Estimated colony count (cfu g^{-1} capsules)*
Sunflower seed oil (34 mmol dm^{-3} cross-linking agent)	1	14·6	2×10^3	2×10^4
	2	5·1	2×10^7	6×10^8
	3	6·4	4×10^6	4×10^8
Sunflower seed oil (4·7 mmol dm^{-3} cross-linking agent)	1	13·6	5×10^3	4×10^4
	2	5·8	7×10^6	4×10^9
Silicone oil (34 mmol dm^{-3} cross-linking agent)	1	8·2	8×10^5	6×10^6
	2	6·0	6×10^6	3×10^8
	3	2·8	1×10^8	3×10^9
Silicone oil (34 mmol dm^{-3} cross-linking agent)	1	8·8	4×10^5	2×10^6

since stationary phase had not yet been achieved after three successive fermentations.

4 SUMMARY

Viability and acidification activity of *L. lactis* was demonstrated following immobilization within cross-linked gelatin microcapsules. Contact with the cross-linking agent, toluene diisocyanate was minimized by a reduction in the reaction agent concentration to 34 mol dm^{-3} during capsule production, and by minimizing the surface to volume ratio of the microcapsules. A lower viscosity oil (silicone) used in the dispersion step during microcapsule formation reduced the surface to volume ratio by 45%, yielding a 44% increase in activity over capsules produced in a higher viscosity oil. After encapsulation, activity and viability of the immobilized culture was increased by cell growth within the capsules. In a third sequential fermentation of cells encapsulated using silicone oil, a $t_{5.5}$ of 2·8 h was attained, similar to that of others using freshly prepared alginate bead immobilized cells. This study represents the first report of live cells immobilized using an interfacial cross-linking technique.

ACKNOWLEDGEMENTS

Institut Rosell (Montreal) kindly donated the lyophilized cultures. The research was supported by the Natural Sciences and Engineering Research Council of Canada through a graduate research scholarship to CLH and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche of the province of Québec as part of the Programme des actions spontanées.

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