J. MICROENCAPSULATION, 1994, VOL. 11, NO. 2, 189–195

Microencapsulation of *Lactococcus lactis* subsp. *cremoris*

B. C. LARISCH†, D. PONCELET†, C. P. CHAMPAGNE‡ and R. J. NEUFELD*†

† Department of Chemical Engineering, McGill University, 3480 University St, Montréal, Québec, H3A 2A7, Canada
‡ Centre de Recherche et de Développement sur les Aliments, Saint-Hyacinthe, Québec, J2S 8E3, Canada

(*Received 8 October 1992; accepted 12 November 1992*)

Lactococcus lactis subsp. *cremoris* was microencapsulated within alginate/poly-L-lysine (alg/PLL), nylon or crosslinked polyethyleneimine (PEI) membranes. Toxic effects were observed with solvents and reagents used in nylon and PEI membrane formation. Alg/PLL encapsulation resulted in viable and active cell preparations which acidified milk at a rate proportional to the cell concentration, but at rates less than that of free cell preparations. At 4×10^8 colony-forming units (cfu/ml milk), encapsulated cells took 17 per cent longer than free lactococci to reduce the pH of milk to 5·5. Similar activities of free and microencapsulated cells may be attained at higher cell concentrations (10^9 cfu/ml milk). The rate of lactic acid production was approximately 2 mmol/h at an encapsulated cell concentration of 4×10^8 cfu/ml.

Immobilization of lactic starter cultures is of interest to the dairy industry since it facilitates cell removal, improving the control of the fermentation process (Champagne and Côté 1987, Champagne 1990). Since cells can be recovered and reused, high inoculation rates are possible reducing fermentation time (Champagne and Côté 1987, Champagne *et al.* 1988 a, 1989). In general, immobilization tends to stabilize cells, potentially enhancing viability and stability in the production, storage and handling of lactic cultures (Kim *et al.* 1988). In some food formulations, immobilized cells are more active than free cells (Kearney *et al.* 1990).

Microencapsulation involves immobilization within an ultrathin, semipermeable membrane. Cells are retained within the encapsulating membrane, potentially reducing cell loss as observed when lactic cultures were immobilized within alginate beads (Champagne and Côté 1987, Kolot 1988). Low molecular weight materials, including milk sugars and metabolic products, are free to diffuse through the membrane. One potential benefit may be in the protection of production cultures from phage contamination (Champagne *et al.* 1988 b, Steenson *et al.* 1987).

Alginate has been widely applied in the immobilization of lactic acid bacteria (Linko 1985) due to the simplicity of the technique, and gentle conditions. However alginate gels are unstable in high-phosphate media (Morin *et al.* 1992), and cell release from the gels is undesirable in some applications (Champagne *et al.* 1992). Thus other immobilization techniques are of interest.

Microencapsulation via interfacial polymerization can be problematic for whole cell immobilization due to the use of toxic solvents and extremes of pH during membrane formation. The objective of the present study was to microencapsulate

*To whom correspondence should be addressed.

0265–2048/94 $10·00

Lactococcus lactis within nylon, polyethylenimine and poly-L-lysine/alginate membranes. The effects of the solvents and monomers used in microencapsulation on the viability and activity of the immobilized cells were of particular interest.

Materials and methods

Cultures and culture media

A commercial strain of *Lactococcus lactis* subsp. *cremoris* CRA-1 was obtained from the culture collection of the Centre de Recherche et de Développement sur les Aliments, Saint-Hyacinthe, Québec. Reference slants were established using Elliker Broth (Difco, Detroit) and 1·5 per cent (w/v) Bacto-Agar. The cells were propagated from the reference slants by resuspension in 10 ml Elliker Broth at 22°C for 48 h, transfer of 0·1 ml to sterile Elliker Broth (50 ml) and incubation at 22°C for 24 h to 4×10^8 colony-forming units (cfu)/ml. A cell pellet obtained by centrifugation (9100*g* for 20 min) was resuspended in cell-free culture broth to 4×10^9 or 4×10^{10} cfu/ml prior to encapsulation.

Microencapsulation within alginate/poly-L-lysine

Cells were encapsulated within poly-L-lysine (Sigma, St Louis, MO, USA) membranes formed on alginate microspheres, produced at room temperature by an emulsification technique (Lencki *et al.* 1989). Cell paste (1 ml) was dispersed into a solution of 2 per cent (w/v) sodium alginate, 0·1 per cent (w/v) sodium citrate, and 1 per cent (w/v) calcium citrate (25 ml). The suspension was emulsified into 125 ml Canola Oil (Canada Packers, Montreal, Canada) by mechanical mixing for 5 min. To the water in oil emulsion formed, 0·667 per cent (v/v) glacial acetic acid in Canola Oil (30 ml) was added. After 15 min gelification, water (200 ml) was added with gentle mixing, permitting the microspheres to partition to the aqueous phase. The clear oil was decanted, and the microspheres filtered off, washed and immersed in 0·02 per cent (w/v) poly-L-lysine (PLL) solution (100 ml) for 20 min to permit membrane formation. Microencapsulated cells were used immediately.

Microencapsulation within nylon membranes

Cells were encapsulated within nylon membranes by suspending a cell paste in 4 volumes of a solution of 4·4 per cent (w/v) 1,6-hexanediamine, 10 per cent (w/v) polyethyleneimine and 1·8 per cent (w/v) sodium borate (pH 8·7). Six millilitres were emulsified in 50 ml ethyl benzoate facilitated by 1 per cent (v/v) span 85 by mechanical mixing for 2 min. Polymerization was initiated by the addition of 0·02 per cent sebacoyl chloride in ethyl benzoate (10 min). After 1·5 min, ethyl benzoate (100 ml) was added to stop the reaction. The organic phase was decanted and the microcapsules washed with 50 per cent Tween 20 and filtered off.

Microencapsulation within polyethyleneimine membranes

A cell paste was encapsulated within polyethyleneimine membranes (PEI), by suspending the cells in 4 volumes of a solution of polyethyleneimine (5 per cent) in 0·45 M-Tris (hydroxymethyl)methylamine buffer, pH 8·0. Five millilitres were emulsified in cyclohexane facilitated by 2 per cent (w/v) span 85 (50 ml) by mechanical mixing for 2 min. Polymerization was initiated by the addition of 0·02 per cent sebacoyl chloride in cyclohexane (10 ml). After 3 min the reaction was quenched with cyclohexane (100 ml). The organic phase was decanted and the microcapsules washed with 50 per cent (v/v) Tween 20 and filtered off.

Viability tests

Cell contact tests were performed by incubating equal volumes of cell suspension and reagent for 10 min at 22°C. Following dilution with sterile peptone water, plate counts were conducted on Elliker agar. Plates were incubated for 3 days at 22°C.

Viable cell concentrations within the microcapsules were measured by disrupting the spheres with a tissue grinder and performing plate counts.

Acidifying activity

The acidifying activity of the immobilized cultures was evaluated using standard activity tests (Stadhouders and Hassing 1980). The activity of an inoculum of free lactic culture (1 ml) or microencapsulated culture (50 ml) was monitored in 100 ml of reconstituted non-fat dry milk (9 per cent). The milk was continuously stirred and the pH monitored at 22°C. The time for the pH to drop to 5·5 from an initial value of 6·7 ($t_{5·5}$) was used as the lactic acid activity of the cells.

Lactic acid production

The culture was inoculated to several replicate flasks in the manner described above, and the cumulative amount of lactic acid produced by the culture with time determined by sacrificing a flask and titrating the mixture with 0·1 M NaOH to the original milk pH (6·7).The rate of lactic acid production was then monitored in the same flask at a controlled pH of 6·7 by metering 0·1 M NaOH for a period of 30 min. The rate of acid production remained constant during the 30-min period.

Results

Microencapsulation of lactic cultures

Optimum conditions for the immobilization of the lactic cultures were those described in Materials and methods. Yield and visual appearance of the microcapsules were considered with individual, uniform and spherical microcapsules desired. The strength of the encapsulating membranes was considered acceptable if they were able to withstand filtration without rupturing. The microcapsules appeared spherical, with intact and smooth membranes. The diameters ranged from 50 μm to 1 mm, depending on the membrane material and formulation conditions.

Cell viability following microencapsulation

The reagents used to formulate alginate/poly-L-lysine (alg/PLL) microcapsules were seen to be non-toxic, based on cell contact tests. The water-soluble reagents used in nylon and PEI membrane formation were non-toxic with the exception of PEI itself. Only 51 per cent of the cells survived the 10 min contact. The organic-soluble reagents used in nylon and PEI membrane formation together with the solvent cyclohexane, were highly toxic.

Microcapsule homogenates were plated with 10^6 and 10^8 cfu/ml of microcapsules observed within the alg/PLL and PEI microcapsules respectively. Cells microencapsulated within nylon membranes were non-viable. Acidification was not observed with cells encapsulated within PEI membranes, to 4×10^8 cfu/ml of milk.

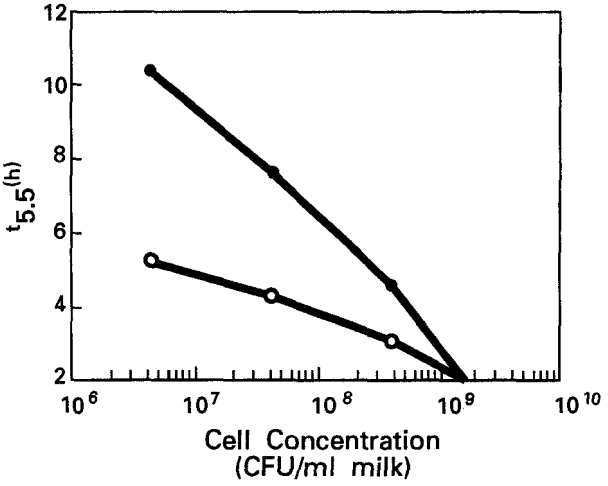

Figure 1. Time to reduce milk pH from 6·7 to 5·5 ($t_{5.5}$) for free (○) and alginate/polylysine-encapsulated (●) cells. Varying amounts of capsules were added to milk to achieve the desired initial cell density.

Activity of microencapsulated cells

Cells encapsulated within alginate-PLL were tested for acidifying activity in comparison to free cells (figure 1). The $t_{5.5}$ decreased with increasing inoculum of free cells, ranging from 3 to 6 h in comparison to 12 h for uninoculated milk. When varying concentrations of cells within alginate-PLL microcapsules were used, the $t_{5.5}$ ranged from 10·5 to 3·5 h. The time required both to begin lactic acid production and to achieve the final lower pH was markedly shortened by the higher inoculum within the capsules. Increasing the concentration of cells within the alg/PLL microcapsules had a more pronounced effect than free cells in reducing the $t_{5.5}$ value; consequently the difference in the $t_{5.5}$ values for free and encapsulated cells decreased as the cell concentration was increased. Extrapolation of the data in figure 1 indicates a possible point of convergence at a cell concentration of $1{\cdot}7 \times 10^9$ cfu/ml of milk.

The time-course of lactic acid production by *Lactococcus* was measured for free and encapsulated (alg/PLL) cells (figure 2). After 5 h the lactic acid produced by the free cells was approximately 7·5 times that of the encapsulated cells. In the latter case the overall rate of acidification after 5 h was approximately 2 mmol/h.

Acid production was also monitored at a constant pH of 6·7 by continuous addition of base (figure 3). Throughout the experiment, acid was produced by the free cells at a rate greater than the alg/PLL encapsulated cells for equivalent cell concentrations. After 4 h of fermentation at fixed pH, the lactic acid production by the free cells began to decrease while the production rate of the encapsulated cells continued to increase (figure 3).

Discussion

Three membranes were considered for the microencapsulation of *Lactococcus lactis*. An optimization of the procedures and reagents involved in each technique was largely based upon qualitative evaluations. For example, weak membranes would not tolerate repeated filtration, and poor yield of microencapsulation was evident by visual inspection. The diameters of the microcapsules were in the

micron size range, considerably smaller than those achieved with other immobilization procedures (1–5 mm; Hulst *et al.* 1985, Kierstan and Bucke 1977). One benefit of the smaller diameter is in enhanced rates of mass transfer. A previous study has shown that bead diameters of 300 μm result in immobilized cell activity similar to that of free cells (Neufeld *et al.* 1991).

Solvents and reagents commonly used for membrane formation in microencapsulation are potentially toxic, as are the pH levels of the polymerization reactions. During encapsulation, the cells in aqueous suspension do not directly contact the organic reagents during emulsification and subsequent interfacial

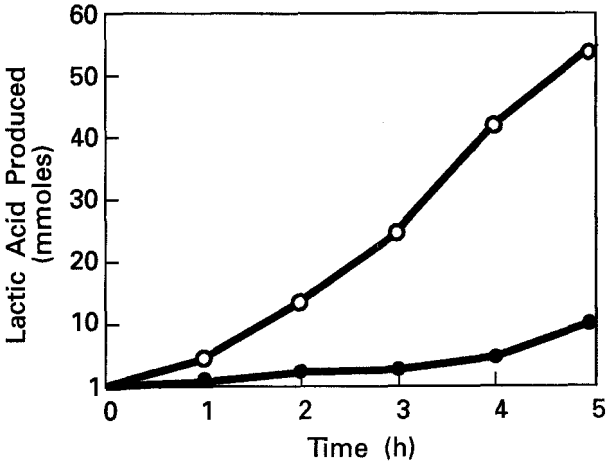

Figure 2. Cumulative amounts of lactic acid produced by free (○) and alginate/polylysine-encapsulated (●) lactococci were determined by sacrificing replicate flasks and titration to pH 6·7. Initial cell concentration in both cases was 4×10^8 cfu/ml of milk. Cells were introduced to 100 ml milk in 1 ml of inoculum (free cells), or 50 ml of microcapsules.

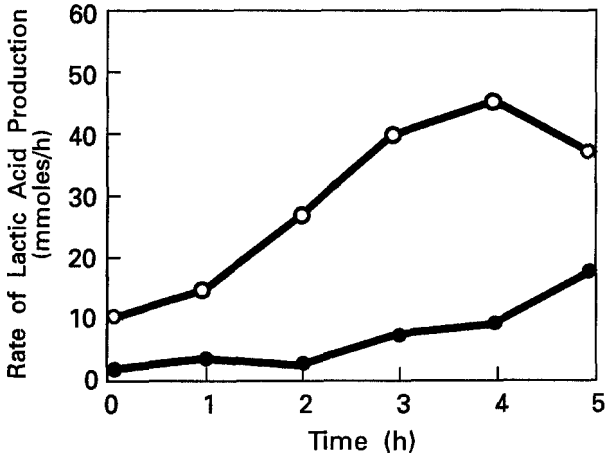

Figure 3. Rate of lactic acid production at pH 6·7 by *L. lactis* immobilized within alginate/polylysine (○) and by free cells (●). Fermentation described in figure 2 was terminated at the time intervals noted above, the milk titrated to pH 6·7 and the acidification rate monitored at a fixed pH of 6·7 over a 30-min period.

reactions, thus organic phase toxicity is of lesser concern. The toxicity of the organic phase may be minimized by an appropriate choice of solvent. Solvents which have both low polarity and high molecular weight have been shown to be less toxic to bacterial cells (Brink and Tramper 1985).

Alginate was previously stabilized by crosslinking with cationic polymers such as polyethyleneimine and polypropyleneimine, or polyethyleneimine and glutaraldehyde (Marx 1989). This treatment also serves to prevent the loss or leakage of cells. The formation of a membrane around the beads and the spraying of the beads with glutaraldehyde (Kolot 1988) have been proposed as stabilizing techniques to minimize cell release. Bead coating with poly-amino acids, as used here, is of interest due to improved biocompatibility, and potential for application in the food industry (Champagne *et al.* 1992).

Cell viability was observed in PEI and alg/PLL encapsulated cells while acidification of milk was observed only with alg/PLL encapsulated lactococci. The $t_{5\cdot5}$ of the encapsulated cells was similar to that previously observed when lactic acid bacteria were immobilized within large-diameter alginate beads at similar cells densities (Champagne and Côté 1987).

The difference in the activity of the free versus immobilized cells in the present study decreased with increasing cell concentrations. Extrapolation would suggest that sufficiently high concentrations of encapsulated cells would demonstrate similar activity to identical concentrations of free cells, implying that at higher cell loadings the membrane and gel matrix had no effect on the production and release of lactic acid, and that the cells were unaffected by the encapsulation procedure. Growth of cells is also known to occur within the beads. The culture used in the present study exhibited generation times between 1 and 1·5 h at 30°C (Champagne 1990), similar to the 1·2 at 30°C reported by Cogan (1980). When the cells were immobilized within alginate beads at cell densities of 10^9 or less, growth rates were slightly lower than with free cells at 30°C, but similar at 21–23°C (Champagne *et al.* 1992, Morin *et al.* 1992). Thus it is expected that within the time-course of the lactic fermentations described in the present study, levels of cells density between the free and encapsulated cells were comparable.

The higher rate of lactic acid production with free than immobilized cells may be due to higher local acidity within the microenvironment of the cells, due to mass transfer limitations, inhibiting activity.

Increasing the concentration of the encapsulated cells would reduce the time to drop the pH, while retaining the active biomass for repeated use. Cell stability with repeated use is an important consideration in commercial application. Activities of lactic cultures immobilized in alginate beads have been reported to be stable in five successive fermentations (Champagne *et al.* 1992). The potential for contamination under these conditions exists, but can be minimized by sterilization of the reagents, and encapsulation under aseptic conditions. The treatment of alginate beads with ethanol or short heat shock, can help to eliminate a surface contaminant (Champagne *et al.* 1992). The use of alginate/poly-L-lysine is likely to be the favoured method of encapsulation for use in the food industry, as the reagents are non-toxic and currently rated as food-grade materials.

Acknowledgements

The authors acknowledge the financial assistance of the Fonds pour la formation de chercheurs et l'aide à la recherche (FCAR) of the Province of Québec, as part of

the Programme des actions spontanées, and for graduate scholarship assistance to one of the authors (B.C.L.).

References

BRINK, L. E. S., and TRAMPER, J., 1985, Optimization of organic solvent in multiphase biocatalysis. *Biotechnology and Bioengineering*, **27,** 1258–1269.

CHAMPAGNE, C. P., 1990, Inhibition of psychrotrophic bacteria in raw milk by immobilized lactic acid bacteria. *Biotechnology Letters*, **12,** 771–776.

CHAMPAGNE, C. P., and CÔTÉ, C. B., 1987, Cream fermentation by immobilized lactic acid bacteria. *Biotechnology Letters*, **9,** 329–332.

CHAMPAGNE, C. P., CÔTÉ, C. B., and GOULET, J., 1988 a, [Fermentation of lacto-serum by immobilized cells of *Lactobacillus helveticus*]. *Canadian Institute of Food Science and Technology Journal*, **21,** 403–407.

CHAMPAGNE, C. P., CÔTÉ, C. B., and GOULET, J., 1989, Whey fermentation by immobilized cells of *Propionibacterium shermanii*. *Journal of Applied Bacteriology*, **66,** 175–184.

CHAMPAGNE, C. P., GAUDY, C., PONCELET, D., and NEUFELD, R. J., 1992, *Lactococcus lactis* release from calcium alginate beads. *Applied and Environmental Microbiology*, **58,** 1429–1434.

CHAMPAGNE, C. P., GIRARD, F., and MORIN, N., 1988 b, Bacteriophage development in an immobilized lactic acid bacteria system. *Biotechnology Letters*, **10,** 463–468.

COGAN, T. M., 1980, [The lactic mesophilic yeasts. A review], *Le Lait*, **60,** 397–425.

HULST, A. C., TRAMPER, J., BRODELIUS, P., EIJKENBOOM, L. J. C., and LUYBEN, C. A. M., 1985, Immobilized plant cells: respiration and oxygen transfer. *Journal of Chemical Technology and Biotechnology*, **35,** 198–204.

KEARNEY, L., UPTON, M., and MCLAUGHLIN, A., 1990, Meat fermentations with immobilized lactic acid bacteria. *Applied Microbiology and Biotechnology*, **33,** 648–651.

KIERSTAN, M., and BUCKE, C., 1977, The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels. *Biotechnology and Bioengineering*, **19,** 387–397.

KIM, H. S., KAMARA, B. J., GOOD, I. C., and ENDERS, G. L., 1988, Method for the preparation of stable microencapsulated lactic acid bacteria. *Journal of Industrial Microbiology*, **3,** 253–257.

KOLOT, F. B., 1988, *Immobilized Microbial Systems: Principles, Techniques, and Industrial Applications* (R. E. Krieger, Malabar, FL).

LENCKI, R. W. J., NEUFELD, R. J., and SPINNEY, T., 1989, Method of producing microspheres. US Patent 4,822,534.

LINKO, P., 1985, Immobilized lactic acid bacteria, in *Enzymes and Immobilized Cells in Biotechnology*, edited by A. I. Lasken (Benjamin/Cummings, Menlo Park, CA), pp. 25–36.

LUCAS, S., and REYROLLE, J., 1989, [Study on a series of lactic mesophilic fermentations. Balance of cells during the first stage of leaven production]. *Lait*, **69,** 121–130.

MARX, J. L., 1989, *A Revolution in Biotechnology* (Cambridge University Press, Cambridge).

MORIN, N., BERNIER-CARDOU, M., and CHAMPAGNE, C. P., 1992, Production of concentrated *Lactococcus lactis* cultures in calcium alginate beads. *Applied and Environmental Microbiology*, **58,** 545–550.

NEUFELD, R. J., PELEG, Y., ROKEN, J. S., PINES, O., and GOLDBERG, I., 1991, L-malic acid formation by immobilized *Saccharomyces cerevidiae* amplified for fumarase. *Enzyme and Microbial Technology*, **13,** 991–996.

STADHOUDERS, J., and HASSING, F., 1980, Description of a method for determining the activity of cheese starters. International Dairy Federation document 129, Brussels, pp. 9–11.

STEENSON, L. R., KLAENHAMMER, T. R., and SWAISGOOD, H. E., 1987, Calcium alginate immobilized cultures of lactic streptococci are protected from bacteriophage. *Journal of Dairy Science*, **70,** 1121–1127.