MICROENCAPSULATION OF *LACTOCOCCUS LACTIS* SUBSP. *CREMORIS* FOR APPLICATION IN THE DAIRY INDUSTRY

by

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ABSTRACT

Microcapsules are comprised of particles or droplets surrounded by ultrathin polymeric membranes. The process of microencapsulation is currently used in the immobilization of biocatalysts, including enzymes and biological cells. A study of the possible use of encapsulated lactic acid bacteria in the dairy industry was performed for the purpose of comparing three methods of immobilizing *Lactococcus lactis* subsp. *cremoris*. A new immobilization method involving microencapsulation of *L. lactis* subsp. *cremoris* within a cross-linked polyethyleneimine membrane was developed. This technique was compared to existing alginate bead and nylon membrane microcapsule immobilization in terms of the microorganism viability and lactic acid production activity. The alginate bead, surrounded by a poly-L-lysine membrane, afforded the greatest viability and activity of the encapsulated cells. The nylon encapsulation procedure did not result in the maintenance of viable cells, and the polyethyleneimine encapsulation procedure did not provide evidence of lactic acid production by the cells.

The rate of lactic acid production by *Lactococcus lactis* subsp. *cremoris* encapsulated in alginate-PLL is less than that produced by unencapsulated or free cells. The difference diminishes with increasing cell concentrations. At a concentration of approximately 10^9 cells/ml of milk, the time required to acidify the milk to the point at which it is useful in the dairy industry is identical for both free and encapsulated cells.

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1.0 INTRODUCTION

1.1 Cell Immobilization

Immobilization offers an alternative to free cell growth. It can be defined as any technique which places a severe limitation on the free movement of the cell and thus aids in their recovery. In industrial processes, the cost of biological catalysts make up a significant proportion of the total cost involved in any conventional batch process. The retention of such catalysts has been shown to result in a 40% cost reduction in the production of L-aspartic acid by Escherichia coli [33]. The use of immobilized cells often facilitates the transition from a batch to a continuous process, thus reducing the reactor size required to achieve a given rate of production.

1.1.1 Historical Background

Enzymes were immobilized as early as 1908. The adsorption of enzymes on various solid supports was accomplished at this time by Michaelis and Ehrenreich leading to the Michaelis-Menten hypothesis of enzyme catalysis[25]. Until the 1930's, the dependence of adsorption on various parameters such as pH and concentration of enzyme was studied, leading to the development of the field of enzyme purification[25].

Microbial cells, being inherently more complex than enzymes, were immobilized only after pioneering work with enzymes had been accomplished. Using techniques similar to those developed for use with enzymes, the cells were initially adsorbed onto solid supports as a means of anchorage[25]. Successful industrial applications of immobilized cells have involved the conversion of

steroids, the production of vinegar, effluent treatment by trickling filters, and production of digoxin and digitoxin for the treatment of heart disease[4].

The advantage of immobilizing whole cells, as opposed to enzymes alone, is immediately obvious. The enzymes, while within the cell, retain their natural environment, and are thus more stable and easier to handle. Immobilization however, can involve many techniques other than adsorption. There are basically six forms of immobilization which are of interest in the production and harvest of cellular products. They are as follows: absorption, cross-linking with chemical reagents, chemical bonding to natural or synthetic polymers, cell flocculation (cross-linking of the microorganisms), entrapment (within a polymeric matrix), and microencapsulation (in artificial membranes or hollow fibres)[21]. It is in areas of entrapment and microencapsulation that a large amount of research is currently being conducted. These two techniques are more advanced than simple adsorption, and provide a means of anchorage, and also protection of the cells.

1.1.2 Entrapment

Research into methods of entrapment, in which the microorganism is incorporated into a polymeric material, began over 30 years ago. It was observed that destruction of some cells invariably occurred following entrapment within polyacrylamide gels due to the reactive nature of covalent gel formation. Although variable, one study demonstrated that 99% of Gram negative, and 30% of Gram positive cells were destroyed during immobilization[28]. It is clear that the encapsulation of living cells must be performed in a non-toxic medium. Gel-like polysaccharides which form non-covalent gels are extracted from marine algae.

These polysaccharides have the advantage of providing an environment for the microbial cells which does not differ greatly from the environment within the cell itself.

One of the methods most widely used for this purpose has been that of immobilizing the cells within beads of calcium alginate. The solid support is formed by ionic cross-linking between the carboxylic acid groups of sodium alginate to form the gel form of calcium alginate. Effectively, the divalent cation Ca++ displaces the monovalent Na+, resulting in gel formation. The calcium alginate entraps 60% of its weight in water and becomes gelatinous, solidifying around entrained microorganisms[25]. Research into the use of alginate and calcium alginate for the entrapment of various microbial species has been conducted since the early 1980's. It is ideal for this purpose, since the calcium alginate is very hydrophillic, and, due to the quantity of water available within the gel, is able to exhibit a high retention of activity.[25] Early work involved the cutting of the gel into cubes and allowing the entrapped microorganisms to replicate or carry out the desired bioconversions. Spherical beads were easier to form and more desirable from an engineering point of view. The technique for their formation involved dispensing the Na-alginate drop-wise from a syringe. Due to the difficulty in scaling up such a process, an emulsification technique for forming polysaccharide beads has since been developed.[24] It has been observed in gel-entrapped systems, that the bacteria are preferentially localized near the outer gel surface, where the nutrients are most readily available.[19] The greatest disadvantage of using calcium alginate lies in the fact that it is unstable when in the presence of phosphate salts and other chelating agents. Calcium alginate has been stabilized by further cross-linking with cationic polymers such as

polyethyleneimine and polypropyleneimine, or polyethyleneimine and glutaraldehyde[22]. This also serves to prevent the loss or leakage of cells. The formation of a membrane around the beads and other stabilizing techniques, such as the spraying with glutaraldehyde[20] have been proposed when it is desirable to limit cell release to a minimum. It has been shown that the addition of a poly-L-lysine membrane has been effective in the coating of alginate beads with entrapped mammalian (hybridoma) cells.[18]

Much of the research time devoted to alginate cell immobilization systems worldwide is spent on the encapsulation of hybridoma cells for the production of antibodies[18,23,26]. In 1978 Damon Biotech patented an encapsulation process, Encapcel®.[26] They were subsequently able to achieve higher antibody concentrations than with conventional cell suspension cultures Changes in cell physiology take place upon cell immobilization. Product formation has been found to be superior in gel immobilized cells to free cells once steady state had been reached. The respiration rate of fixed cells has also been found to be higher than with free cells. It has been hypothesized that these effects may be due to any of three phenomena. The support may activate the membrane effector which governs cell respiration, the support may modify the permeability of the membrane, or the environment surrounding the cells may be modified by the carrier. It is clear that any modification may have either a beneficial or a detrimental effect, although in general, the main effect upon yeast and bacterial has been to increase cellular activity.[19]

1.1.3 Microencapsulation

Research into the uses of the microencapsulation process began over 30 years ago. An artificial membrane bound microcapsule is, in some respects, similar to the living cell. Enveloping a living organism within a protective membrane, skin or shell, such as that of the plant cell or the bird's egg, is designed to provide a protective layer isolating the contents from a harsh environment. Encapsulation, microencapsulation, or formation of the artificial cell provides a mechanism to mimic and control what occurs in nature. It is possible to select materials for the capsule wall providing desirable mechanical properties and the possibility of controlling diffusion of substrate materials and products through the encapsulating membrane.

Early work in microencapsulation involved the formation of impermeable membranes. As a result, in order for the encapsulated substance to interact with the environment, the capsule had to be broken apart, usually by mechanical means. These capsules were used when controlled release of a particular substance was desired. By 1969, such microcapsules were being used in the cosmetics industry (perfumes), in the pharmaceutical industry for sustained release of drugs, for the protection and introduction of flavours and aromas in food, in medicine as detoxicants, in agriculture for slow release of fertilizers, and in the textile and printing industry as dye precursors.[28]

One of the first industrial uses of microencapsulation, which originated in 1953 with the work of Green, was the coating of paper with encapsulated ink, to produce carbonless copies[29]. Another widely accepted application was in the field of fertilizer manufacture. By instating a successive prilling and coating process, the industry was able to offer the benefits of avoiding high local concentrations of fertilizer and reducing the necessary number of applications,

thus reducing the toxicity.[29]

More recently, interest in the use of semi-permeable substances in forming the capsule wall began to accelerate. Encapsulation was seen as a method to be used in continuous fermentation processes, where the active reagent (enzymes or whole cells) would be held within microcapsules as the necessary substrates entered and products left the microcapsule. It was at this time that many forms of microencapsulation were found to be compatible with the human body. This enabled advances in the medical field towards the realization of smaller artificial kidneys, through the encapsulation of detoxifiers within semi-permeable membranes.[9] At same time research was directed towards the encapsulation of both plant and mammalian cells.

One method of microcapsule formulation which has remained at the forefront of immobilization technology for almost 30 years is that of interfacial polymerization. This method has been used to form nylon membranes for the microencapsulation of enzymes.[10] Most recent interest in microencapsulation for use in foods involves research conducted by Desoize and Levy.[12] They have formed microcapsules by cross-linking proteins, such as casein and pepsin. Although an inhibitory effect on encapsulated cells was observed, the encapsulation process proved successful.

1.2 Milk

Milk and milk products constitute approximately one fifth of the average food intake in America. Although mostly water (87%), milk contains 3.2% proteins (of which 80% is comprised of casein), 3.8% butterfat, and 4.8% carbohydrates.[1]

Bacteria occur naturally in milk, and can vary in number as a result of differing handling practices, diet of the cows, or occasional disease within the animals. As long as they are not overwhelming in number or are the result of disease within the cow, the bacteria are not harmful. In many instances, such as in the fermentation of milk to produce yoghurt and cheese, the presence of the microorganisms is desirable. Milk is an ideal growth medium for many microorganisms due to the large number of substrates it possesses which are available for fermentation. Raw milk contains lactose, fat, various proteins, and vitamins and minerals which serve to stimulate growth. In the manufacture of fermented milk products, such as yoghurt and cheese, cream is acidified to approximately pH 5.5 via the lactic acid culture introduced into the milk or cream product. Fermentation times of 2 to 4 hours are typical, but the cultures which remain within the cream continue to produce acid, often leading to an over-acidification of the cream. The immobilization of the cells would facilitate removal of the dairy culture once the desired pH is reached, avoiding subsequent curdling of the cream upon storage.

Until a few decades ago, milk was an important contributor to the transmission of disease, but presently in the western world, due to more stringent pasteurization requirements, as well as more rigorous herd inspection, milk is among the safest foods.

Raw milk will undergo spontaneous souring due to the production of lactic acid by Streptococcus lactis. This homofermentative process, meaning that only lactic acid is produced, causes the milk to curdle. The curdling of milk is observed at pH's of 5.4 or less, at which point the casein precipitates. Temperature must be regulated during any fermentation involving the monitoring

of pH, as the pH of milk decreases by 0.01 units/°C between 30 °C and 10 °C.[13]

1.3 Microorganisms

1.3.1 Psychrotrophic Microorganisms in Milk

Psychrotrophic microorganisms are defined as those organisms which multiply at a significant rate at temperatures below 7-10°C. They are present in most raw milk, most frequently as Gram-negative rods[11], and can remain in the milk through treatment to contaminate pasteurized milk. Many psychrotrophs are capable of producing proteases which are heat stable and able to survive the conditions involved in the production of UHT (ultra-high temperature) milk. It has been reported that a protease produced by one psychrotroph, a pseudomonad, was greater than 4000 times more heat resistant than the spores of *Bacillus stearothermophilus*. The proteases which remain in the milk affect milk quality and can be responsible for off-flavours and odours. Psychrotrophic microorganisms have also been thought to cause decreased yields of Cheddar and other cheeses. Inhibition of these natural contaminants may be achieved by lowering the pH using an inoculum of lactic cultures. Since the culture must be removed from the milk prior to further processing, the immobilization of the cells may provide a simple and inexpensive method for milk treatment, in a manner that may be easily performed on the farm.

1.3.2 Lactic Acid Bacteria in the Dairy Industry

Lactic acid bacteria are Gram positive, typically ovoid shaped microorganisms which grow in pairs, or short or long chains. They do not produce large colonies on ordinary media and are therefore easily analyzed by standard

plate counting methods.[31] Lactic acid bacteria have long been regarded as essential in the upkeep of human health[3], and due to their preference for and rapid propagation in milk environments, have been traditionally associated with milk and milk products. They metabolize lactose, which comprises approximately 4.8% of the milk. The lactic streptococci produce only the L(+) isomer of lactic acid.[15] Lactic bacteria are most commonly used on an industrial scale as starter cultures in the manufacture of both cheese and yoghurt. *Lactococcus lactis* subsp. *cremoris*, commonly referred to as *Streptococcus cremoris**, is a facultative anaerobe whose acid production in raw milk is enhanced by the presence of psychrotrophic microorganisms, discussed in section 1.3.1. It has been assumed that this enhancement is due to the use of the end products of the proteolysis by the lactic streptococci.[11] Lactic cultures are able to produce compounds which stimulate anti-microbial systems in milk cultures. It may be possible to introduce the lactic cultures to the raw unpasteurized milk during farm storage, thus extending the storage period or maintaining milk quality. If the cells are immobilized, removal before shipping and pasteurization may be facilitated.

Early immobilization of lactic acid bacteria, in 1975, was performed on Berl saddles, coated with gelatin and sprayed with glutaraldehyde.[20] Later, *Lactobacillus lactis* was first successfully entrapped within calcium alginate beads in 1980.[20] It has been established that the diffusion of small molecules, such as lactose and lactic acid, are not severely restricted when lactic acid cultures are encapsulated within alginate gels.[5]

Immobilized *Streptococcus cremoris* and *Lactobacillus bulgaricus* have been suggested in 1977 by Divies to be useful in the continuous prefermentation of milk for the manufacture of yoghurt.[20] It has also been determined that *L.*

* In 1986, was also listed by Bergey as *Streptococcus raffinolactis*

lactis subsp. *cremoris* immobilized in calcium alginate is unstable and releases cells, necessitating further treatment of the alginate beads.[20] One method of calcium alginate stabilization which has proved to be of interest, is the addition of a poly-L-lysine membrane around the bead.[18] As poly-L-lysine is used in the food industry, it is known to be non-toxic, and therefore lends itself to the encapsulation of cells.

2.0 OBJECTIVES

The main objectives of the study are as follows:

1. The feasibility of microencapsulating *Lactococcus lactis* subsp. *cremoris* within artificial membranes will be examined. This culture is used in the dairy industry for producing lactic acid in a variety of dairy applications.
2. The viability and activity of the microencapsulated cultures will be examined in a manner which is consistent with industrial criteria for lactic culture testing, and which will yield data for calculating rates and yield of lactic acid production on a milk substrate.
3. Three methods of microencapsulation will be examined: within poly-L-lysine membranes coating alginate beads, within nylon membranes, and within polyethyleneimine membranes.

Important criteria in the selection of a suitable form of microencapsulation include low toxicity of the reagents and solvents to maintain high viability of the cultures, and high mechanical strength of the polymeric membranes sufficient to tolerate filtration operations. For the purposes of high mass-transfer rates of lactic acid across the membrane, small diameter microcapsules are preferred, but the diameter must be sufficiently large to permit removal by a physical process.

3.0 MATERIALS AND METHODS

3.1 Culture Preparation

A commercial strain of *Lactococcus lactis* subsp. *cremoris* Hansen was supplied by Dr. Claude Champagne of the Centre de Recherches et de Dévelopment des Aliments (C.R.D.A.) in St. Hyacinthe. Reference slants were established using Elliker Broth(Difco Laboratories Inc., Detroit, MI) and 1.5 % (w/v) Bacto-Agar(Difco Laboratories Inc., Detroit, MI) The cells were propagated from the reference slants by resuspension in 10 ml of Elliker Broth at 22 °C for 48 hours. 0.1 ml of the 10 ml suspension was transferred to 50 ml of sterile Elliker Broth and incubated at 22 °C for 24 hours. After 24 hours, the concentration of bacteria was 4×10^8 CFU/ml. Weekly transfers as outlined above provided a fresh supply of culture for experimentation and monthly propagation from reference slants minimized the possibility of contamination or mutation of the experimental strains. A concentrated cell suspension was prepared for encapsulation by centrifugation at 10,000 rpm for 20 minutes. The cell pellet was resuspended in various volumes of its own cell free culture broth to achieve final inoculation levels of 4×10^9 and 4×10^{10} CFU/ml.

3.2 Milk

Unpasteurized milk powder was supplied by the C.R.D.A. in St. Hyacinthe. For all experiments, the milk was rehydrated at a concentration of 9.1% (w/w) in sterile peptone water (0.1% w/w). Bacto Peptone was supplied by Difco Laboratories Inc., Detroit, MI.

3.3 Microencapsulation of Lactic Acid Bacteria

3.3.1 Alginate-Poly-L-lysine Microcapsules

Alginate beads were used as support for the poly-L-lysine membrane, and were formed at room temperature by emulsion. Three liquid solutions were prepared. The first, the gelling solution, was prepared using 2 % (w/v) sodium alginate (Anachemia, Montréal, Québec), 0.1 % (w/v) sodium citrate (Anachemia), and 1% (w/v) calcium citrate (Anachemia). The second solution was the organic phase, comprised of 100% Canola Oil (Canada Packers, Montréal, Québec), and the third was the reactive organic solution, comprised of 0.667% (v/v) glacial acetic acid (Anachemia) in Canola Oil.

A cylindrical reaction vessel was used, containing 4 equally spaced perpendicular baffles, with a volume of approximately 175 ml. 125 ml of Canola oil were added to the vessel and a mesh impeller was set rotating at 200 rpm. Immediately before emulsification, 1 ml of bacteria was added to 25 ml of the gelling solution. These 26 ml of the gelling solution, containing the bacteria, were added to the mixing oil organic to create a water in oil emulsion. After 5 minutes of emulsification, 30 ml of the reactive organic solution was added. The pH of the alginate suspension was lowered due to the addition of the oil soluble acid, solubilizing some of the calcium citrate, resulting in gel formation. Once the beads were formed, the bacteria were trapped within an alginate-calcium matrix.

After 15 minutes of reaction, the contents of the reaction vessel were transferred to 200 ml of distilled water and mixed slowly. Upon separation of the alginate beads into the aqueous phase, the majority of the oil phase was decanted. A vacuum aspirator was used to remove the final traces of oil, and the beads were immersed in 100 ml of a 0.02% (w/v) poly-L-lysine (PLL) (Sigma,

St. Louis, MO.) solution for a period of 20 minutes. The charge difference between the alginate bead and the polycation resulted in the spontaneous formation of a membrane on the bead surface.

3.3.2 Nylon Membrane Formation [8]

Nylon membranes were formed around emulsified aqueous droplets containing the bacterial suspension. The emulsion was formed with an aqueous diamine solution dispersed within an organic solvent. The diamine solution consisted of 4.4 % (w/v) 1,6-hexanediamine (Aldrich, Milwaukee, Wis.), 10% (w/v) polyethyleneimine (Aldrich) and 1.8% (w/v) sodium borate (Fisher, Nepean, Ont.), as a buffer. The pH of the diamine solution was adjusted to 8.7 by addition of 12 N HCl prior to the addition of the cells. The ratio of cell slurry to diamine solution was 1/5 and the concentration of cells in the slurry was varied. The diamine solution - cell mixture (6 ml) was emulsified within 50 ml of ethyl benzoate (Aldrich) facilitated by 1% span 85. An emulsifier (1 % (v/v) span 85 (Atkemix, Brantford, Ont.)) was added to 50 ml of ethyl benzoate. The emulsion was mixed at 300 rpm within a 150 ml beaker using a 45 mm diameter frame impeller for 2 minutes. Polymerization was then initiated by the addition of 0.02% sebacoyl chloride (Aldrich) in 10 ml ethyl benzoate. After 1.5 minutes, 100 ml of ethyl benzoate were added to stop the reaction. The organic phase was decanted and the microcapsules washed with Tween 20 (A&C, Montréal, Québec) and filtered.

3.3.3 Polyethyleneimine Membrane Formation

The polyethyleneimine (PEI) microencapsulation procedure is similar to the nylon encapsulation procedure. It utilizes a crosslinking of the imine about the emulsified aqueous phase. The aqueous phase consisted of 5 % polyethyleneimine in 0.45M of Tris buffer. The pH was adjusted to 8.0 by addition

of 12 N HCl prior to the addition of the cells. 5 ml of the PEI/cell suspension (1 ml cells + 4 ml PEI and Tris) were emulsified within 50 ml cyclohexane facilitated with 2% (w/v) span 85. Mixing was provided by a mesh impeller, mounted within a 150 ml beaker, operating at 200 rpm. Following 2 minutes of emulsification, the polymerization reaction was initiated by the addition of 0.02 % sebacoyl chloride in 10 ml cyclohexane and mixed for 3 minutes. The reaction was quenched by the addition of 100 ml of cyclohexane. The organic phase was then decanted and the microcapsules were washed with a 50% (v/v) solution of Tween 20 and filtered.

3.4 PROCEDURE OF ANALYSIS

3.4.1 Viability Tests

The viability test was performed to determine the tolerance of *L. lactis* subsp. *cremoris* to the reagents and solvents used in the encapsulation procedures. 5 ml of cells, in suspension, were placed in contact with 5 ml of each reagent for a period of 10 minutes, after which the mixture was diluted in sterile peptone water by a factor of 10^6 in order to achieve final plate counts within the range of 30-300 colonies. 1 ml of the 10^6 dilution was plated on solid nutrient agar, and the colonies were counted following 3 days at room temperature. Colony forming units (CFU)/ml were compared with the values resulting from control cells which were not contacted with the potentially toxic reagent. A ratio of the two values, referred to as the "percent viability" was used to gauge the toxicity of each reagent.

The breaking of microcapsules to determine viable cell concentrations within the capsules was performed using a Fisher tissue grinder. The cells and

broken microcapsules were plated as outlined above.

3.4.2 Determination of Activity

Lactic acid activity of free cells was determined by inoculating 1 ml cell suspension in 100 ml of raw milk, rehydrated at 9.1% (w/w) (Section 3.2).

Microencapsulated cultures were assayed for lactic acid activity by suspending the capsules in 100 ml of 9.1% rehydrated milk (Section 3.2). The total suspension volume, for the alginate-PLL microspheres, was 150 ml. The milk was constantly stirred and the pH was monitored with a strip chart recorder. The change in pH through the fermentation, was used as an indication of the lactic acid production, and provided a comparison between free and encapsulated cells. The time for the pH to drop to 5.5 from an initial value of 6.7 was determined as a quantitative method of comparison. The lactic acid production activity of the natural microflora within the raw milk was monitored in the same manner to determine the level of background activity.

3.4.3 Measurement of Lactic Acid Production

Two measurements of lactic acid production were taken: the cumulative amount of acid produced in the milk, and the rate at which the acid was being produced. Encapsulated bacteria were incubated in raw milk at room temperature for varying periods of time. At hourly intervals, the mixture was titrated with 0.1 N NaOH to the pH of raw milk (6.7). This provided a measurement of the cumulative amount of lactic acid which had been produced by the cells during that time interval.

The rate of lactic acid production was monitored at pH 6.7 by metering 0.1 N NaOH to the milk in order to maintain the pH set-point. The assay was

repeated at hourly intervals and the activity measured for periods of one half hour. The samples used in these fermentations were identical to those discussed above, which were titrated with base to the pH of raw milk.

4.0 RESULTS

4.1 Microencapsulation Procedures

Conditions which were optimum for the immobilization of the lactic cultures were determined for each of the three encapsulation procedures as outlined in Table 4.1. The optimum conditions for encapsulation using alginate-PLL and nylon were determined as part of this study; previous work provided the optimum conditions for PEI membrane formation. Trends observed during the microencapsulation procedures are outlined in the following sections.

Table 4.1: Conditions for Lactic Culture Immobilization

Microencapsulation Method	Alginate-PLL	Nylon	PEI
Aqueous Phase Reagents	2% Na-alginate	4.4% diamine	5% PEI
	0.1% Na-citrate	10% PEI	0.45 M Tris
	0.1% Ca-citrate	1.8% borate	
Impeller Speed	200 rpm	300 rpm	200 rpm
Emulsification Time	5 min.	2 min.	2 min
Reaction Time	15 min.	1.5 min.	3 min.
Emulsifier	--------	1% span	2% span
pH		8 7	8 0
Temperature	Room T.	Room T	Room T

Various trends follow, which which were observed during enapsulation.

4.1.1 Alginate-PLL Microcapsules

The bead diameter of the alginate-PLL microcapsules was dependent on the mixing rpm during emulsification. It was also observed that a smaller impeller mesh resulted in smaller diameter beads. At 100 rpm, the beads were spherical, large and smooth. Higher rpm resulted in some bead deformation and smaller diameter beads. At 500 rpm, the majority of the beads were non-spherical. Also short reaction times (rt) resulted in agglomeration of the alginate beads, necessitating a minimum of 5 minutes for stable emulsion formation.

The alginate was gelled by the liberation of Ca^{2+} ions from the insoluble calcium citrate by the lowering of the reaction pH. Any excess calcium citrate was entrapped along with the cells once the beads were gelled.

The concentration of the alginate was varied from 0.5% to 4%. It was observed that the alginate concentration did not significantly affect the activity of the encapsulated *L. lactis* subsp. *cremoris*.

4.1.2 Nylon and Polyethyleneimine Microcapsules

The formation of the microcapsules was sensitive to the pH of the aqueous solution. At pH 8.5 and ambient temperature, there was no polymerization reaction. At a pH of 9.0, the reaction proceeded too rapidly, and large quantities of nylon were produced. The lower pH values resulted in microcapsules with weak membranes, which were easily broken. Higher pH levels resulted in strong membranes, but promoted agglomeration of the beads during polymerization. An attempt was made to replace the solvent phase in nylon membrane formation with sunflower oil. Although polymerization was

evident, and resulted in the formation of nylon membranes, there were no microcapsules present in the final mixture.

4.2 Viability Tests

Several of the solvents and reagents used in the microencapsulation procedures are potentially toxic to the lactic cultures. The toxicity of the individual reagents and solvents was assessed by conducting viability tests, and monitoring the percentage viability of the cultures following a contact time of approximately 10 minutes. Viable counts are compared in the tabulated data with controls run involving cells in sterile water only. The percentage survival values are in comparison to the controls with viable counts based on colonies formed on agar plates following a dilution of the culture by a factor of 10^6.

It may be seen in table 4.2 that the reagents used in the formation of alginate-poly-L-lysine microcapsules are essentially non-toxic, with % survival values in excess of 75%.

TABLE 4.2: Viability of Cells Following Contact with the Reagents used in the Formation of Alginate-PLL Microcapsules

Reagent	Tests*	Reference Count dilution 10^6	Final Count dilution 10^6	% Viability
Alginate Solution**	1	45 ±7***	43 ±7	96 ±29
	4	154 ±12	205 ±14	133 ±20
Poly-L-lysine, 0.02%	1	45 ±7	33 ±6	73 ±24
Sodium Citrate, 0.1%	1	45 ±7	37 ±6	82 ±26

* Number of plates counted at a dilution factor which provided ~ 30 - 300 colonies/plate

** 2% Sodium alginate, 0.1% Sodium citrate, 0.1% Calcium citrate

*** Determination of error: Error (plate count) = sqrt(plate count)

$$\text{Error (\% viability)} = \frac{(\text{final count})\times(\text{ref. error}) + (\text{ref. count})\times(\text{final error})}{(\text{ref})\times(\text{ref})}$$

Results of cell contact with the aqueous solutions and individual water soluble reagents used in the formation of nylon and PEI membranes are tabulated in Table 4.3. The aqueous diamine solution, at the pH used in the nylon encapsulation procedure, is moderately toxic to the cells. At the lower pH of 7.6, there was no viability observed at the dilution factor of 10^6. Of the three components of the diamine solution, only polyethyleneimine is toxic to moderately toxic; the diamine alone and the borate buffer, are non-toxic. The toxicity of the PEI may contribute to the moderately toxic effect of the diamine solution on the cells. The aqueous solution used in the polyethyleneimine encapsulation procedure, a mixture of PEI and Tris, was also moderately toxic to

the cells.

TABLE 4.3: Viability of Cells Following contact with the Reagents used in the Formation of Nylon and Polyethyleneimine Membranes : **AQUEOUS PHASE**

Reagent	Tests	Reference Count dilution 10^6	Final Count dilution 10^6	% Viability
Diamine Solution				
pH = 8.7	4	167 ±13	111 ±11	66 ±12
pH = 7.6 (4.4% diamine + 10% PEI * + 1.8% borate)	4	130 ±11	0 ±0	0 ±0
Diamine, 4.4%, pH 8.7				
1 min	1	449 ±21	471 ±22	105 ±10
10 min	1	449 ±21	513 ±23	114 ±10
10 min	4	109 ±10	135 ±12	124 ±23
10 min,pH 12	4	167 ±13	0 ± 0	0 ±0
PEI, 5 %	4	167 ±13	32 ±6	19 ±5
	4	109 ±10	56 ±7	51 ±12
PEI, 5% +Tris, 0.45M	4	154 ±12	86 ±9	56 ±11
Borate, 1.8%	4	167 ±13	171 ±13	102 ±16
	4	109 ±10	119 ±11	109 ±20

* Polyethyleneimine

Results of cell contact with the organic reagents used in nylon and PEI membrane formation are tabulated in Table 4.4. Solvents used in nylon membrane formation involve a mixture of cyclohexane and chloroform. Ethyl

benzoate, which was chosen as an alternative to the cyclohexane-chloroform mixture was also seen to be highly toxic. The reagents responsible for initiating the interfacial polymerization reaction were also toxic to the lactic acid bacteria. Both mineral oil and silicon oil were found to be non-toxic and were tested as possible alternatives to cyclohexane and chloroform. The actual membrane formation in these alternative solvents is the subject of another study.

Table 4.4: Viability of Cells Following contact with the Reagents used in the Formation of Nylon and Polyethyleneimine Membranes : **ORGANIC PHASE**

Reagent	Tests	Reference Count dilution 10^6	Final Count dilution 10^6	% Viability
Cyclohexane/ chloroform (4:1, v/v)	1	171 ±13	0 ±0	0 ±0
Cyclohexane	4	154 ±12	0 ±0	0 ±0
	4	130 ±11	5 ±2	4 ±2
Ethyl Benzoate	1	171 ±13	0 ±0	0 ±0
	4	154 ±12	0 ±0	0 ±0
	4	130 ±11	19 ±4	15 ±5
Sebacoyl dichloride in ethyl benzoate	4	167 ±13	0 ±0	0 ±0
Reactive Solution (e.b.* + sebacoyl + di+tri chloride)	1	171 ±13	0 ±0	0 ±0
	4	167 ±13	0 ±0	0 ±0
Mineral Oil	4	27 ±5	26 ±5	96 ±37
Silicon	4	27 ±5	36 ±6	133 ±48

* ethyl benzoate

When contacting the bacterial cells with organic solvents, such as those seen in Table 4.4, greater inconsistency was observed between tests conducted on separate occasions than was observed when testing the water soluble reagents. A proposed explanation is the effect which the form of contact has upon the bacteria. Since the bacteria and the organic solvents are immiscible, shaking the two together vigorously produces an emulsion, and thus provides more surface area over which the two may enter into direct contact. The organic solvent which exhibited the greatest differences in toxicity between successive tests was cyclohexane. On two separate occasions, survival of both 59 (±10) % and 0(±0) % were measured. In a further exploration of this phenomena, two samples of bacteria were placed in contact with cyclohexane. One sample was vigorously shaken, for 10 minutes; the second sample was shaken only once, and allowed to sit undisturbed for 10 minutes.

As can be seen in Table 4.5, the form of contact undergone between the cells and the reagent plays a significant role in the observable toxicity of the solvent. The unshaken sample was diluted by a factor of 1×10^6 in order to plate a countable number of colonies (30-300 colonies), whereas the shaken sample required less dilution by a factor of 10^2. An unshaken sample of cells may therefore survive contact with a reagent which proves to be toxic upon more intimate contact.

TABLE 4.5: Effect of Agitation Upon Cell Viability

Reagent	Tests	Reference Count dilution 10^6	Final Count	% Viability
			dilution 10^4	
1. Sample Shaken	3	212 ±15	400 ±20	2 ±0
			dilution 10^6	
2. Sample Unshaken	3	212 ±15	50 ±7	24 ±5

The contact of cells with individual reagents, as seen in Tables 4.2-4.4, provides a method of separating the effects of each reagent on the cells. However, the procedure is unable to mimic the conditions to which the cells are exposed during encapsulation. Broken microcapsules were plated to determine the effect of the encapsulation process on the cells. As seen in Table 4.6, cells microencapsulated within the nylon membranes were non-viable, whereas those within the alginate-PLL and PEI membranes were viable.

TABLE 4.6 Intracapsular Concentration of Viable Cells

Initial Concentration = 4×10^8 CFU/50 ml

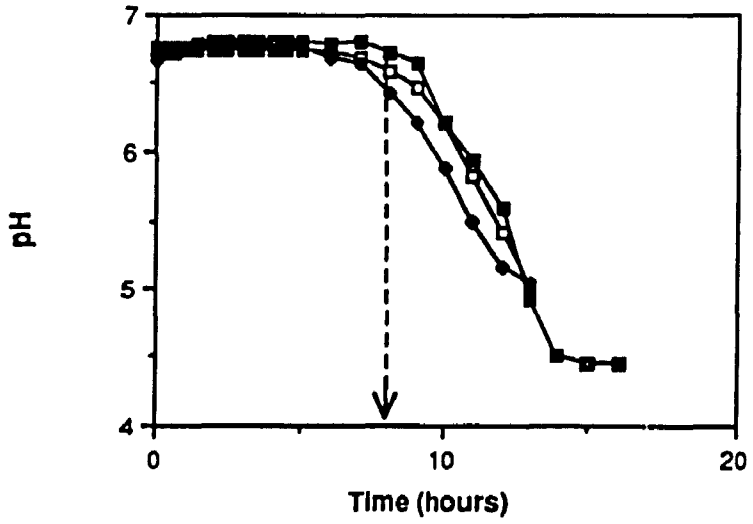
	Tests	Final Concentration [CFU/50 ml]
Alginate	2	5×10^7
Nylon	2	0
PEI	2	6.5×10^8

4.3 Activity Tests

Encapsulated and free cells were tested for their lactic acid production, and thus for their activity. Production of lactic acid was monitored by observing the pH drop, which provided an indication as to how rapidly *Lactococcus lactis* subsp. *cremoris* was able to acidify the milk. The reduction of the pH of raw milk, uninoculated with *Lactococcus lactis* subsp. *cremoris*, was chosen as a cell free control.

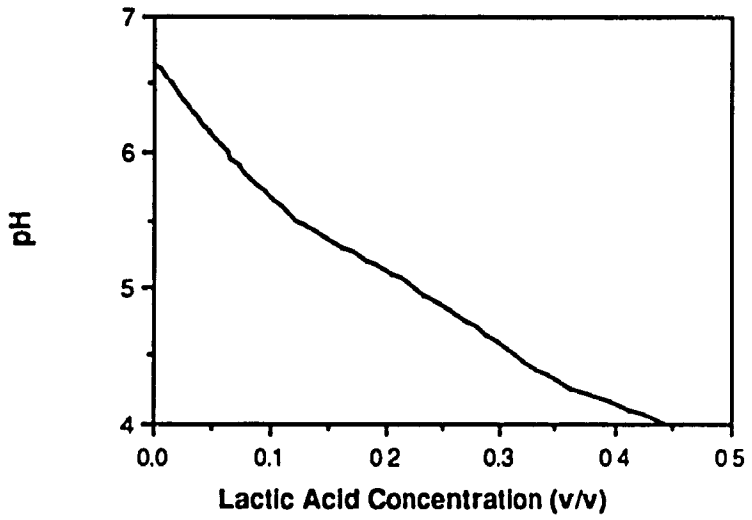
Raw milk at room temperature (20°C) undergoes a natural fermentation due to the presence of acid producing microorganisms. The pH begins to drop after a lag time of approximately 8 hours as is seen in Figure 4.1. The pH of the raw milk was initially 6.7, dropping to a minimum pH of 4.6 after 14 hours. The milk has fully curdled at the latter stages of the fermentation.

Fig. 4.1 : Reduction in milk pH via naturally present microflora. Data is presented for three different samples of raw milk. The vertical line denotes the average time required by the milk to begin the souring process.



The buffering capacity of the milk was examined by titration with lactic acid over the pH range of 4.0 to 6.7 as seen in Figure 4.1

Fig. 4.2 : Standard titration of milk using 0.1 N Lactic Acid.

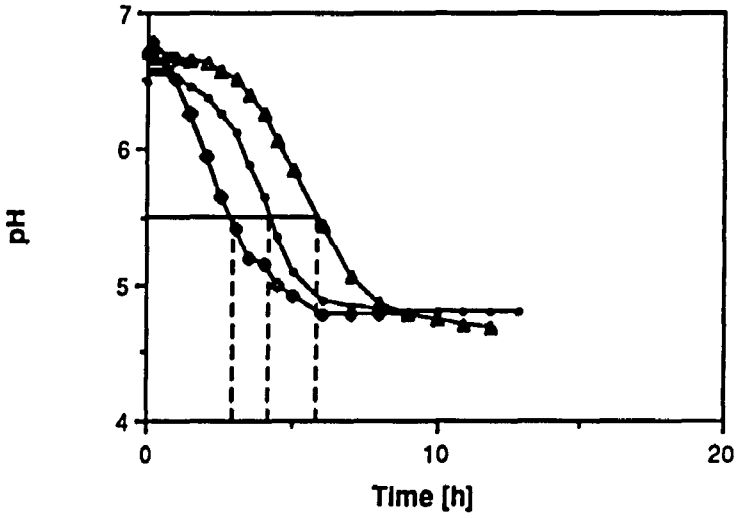


The relationship between lactic acid concentration and pH was approximately linear within the range of interest for milk fermentation. (pH 6.7 to 4.6) and linear within the range of pH used to assay lactic acid activity in the present study (6.7 to pH 5.5).

4.3.1 Activity Tests with Free Cells

Having established the pattern of pH reduction in raw milk, free cells were added to establish the effect of lactic acid production, by the cells, on the time required to reduce the pH of the milk.

Fig. 4.3 : Reduction in pH of raw milk following inoculation with *L. lactis* subsp. *cremoris* at concentrations of : $4x10^6$ (Δ), $4x10^7$ (•), and $4x10^8$ (◊) CFU/ml milk.

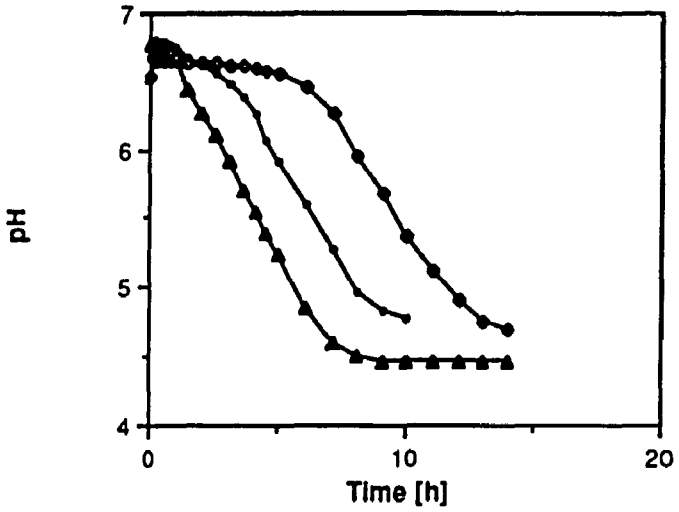


Raw milk, when inoculated with *L. lactis* subsp. *cremoris*, results in a pH reduction due to the synthesis of lactic acid as may be seen in Figure 4.3. The rate of decrease in the pH is similar with increasing amount of inoculum, although the lag period prior to the onset of the acid production phase was shortened with increasing inoculum concentrations. In all cases, the minimum pH attained was approximately 4.6, similar to that attained with raw milk. The $t_{5.5}$, defined as the time from the onset of fermentation until the milk pH drops to 5.5, was shortened with increasing inoculum, ranging from 3 to 6 hours. In comparison, the $t_{5.5}$ for uninoculated milk was approximately 12 hours.

4.3.2 Activity Tests with Encapsulated Cells

Alginate-poly-L-lysine microcapsules containing lactic acid bacteria were used to inoculate raw milk and the pH was monitored with time. The effect of varying concentrations of bacteria within alginate-PLL microcapsules is plotted in Figure 4.4. It may be seen that the higher concentrations of immobilized cells stimulated pH reduction within shorter periods of time. In all cases, the minimum pH attained was approximately 4.5 and the $t_{5.5}$ ranged from 10.5 to 3.5 hours, at the lowest concentration to the highest concentration of cells respectively. As was also observed during the fermentation of free bacteria (Figure 4.3), the lag period prior to pH reduction decreases with higher concentrations of cells. 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 and, in all cases, was shorter than the time required by naturally present microflora within the milk (Figure 4.1).

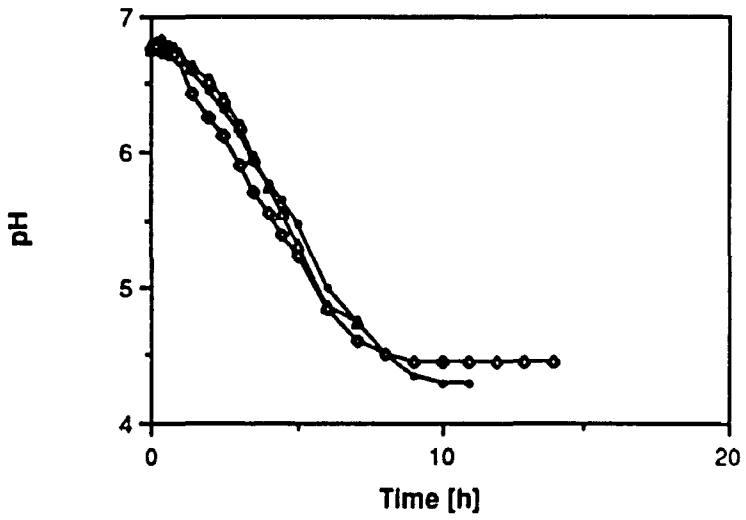
Fig. 4.4 : Reduction in pH of raw milk following inoculation with Alginate-PLL microcapsules containing 4 x 10^6 (◊), 4 x 10^7(•), and 4 x 10^8(Δ) CFU/ml.



Three replicates were performed as a check in all experiments with similar degrees of reproducibility observed. The data points of the replicates have been omitted from the figures for the sake of clarity.

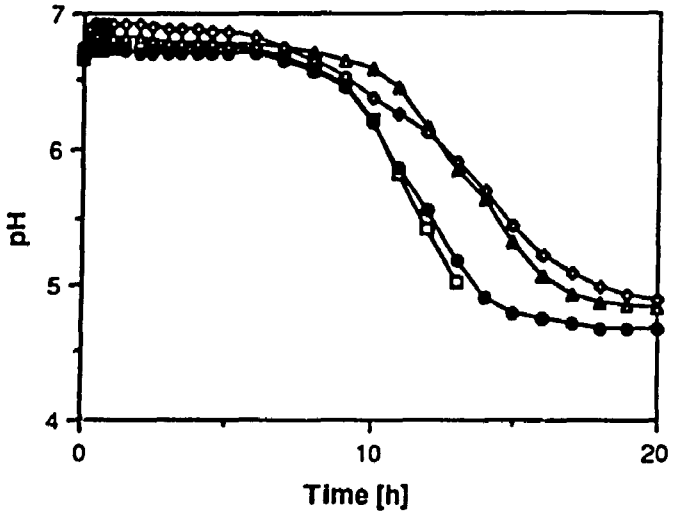
The reproducibility of the data is demonstrated in Figure 4.5 where the results of three replicate experiments, of milk inoculated with alginate-PLL entrapped cells, are plotted. It may be seen that there is no observable difference in lag time, and the $t_{5.5}$ ranges from 4.5 to 5 hours for the three replicates. Figure 4.5 has been included to demonstrate the good reproducibility of the data.

Fig 4.5 : Reduction of pH in milk due to lactic acid production by 4 x 10^8 CFU/ml milk. Cells were entrapped within alginate-PLL microcapsules. Data represents results of three replicate experiments



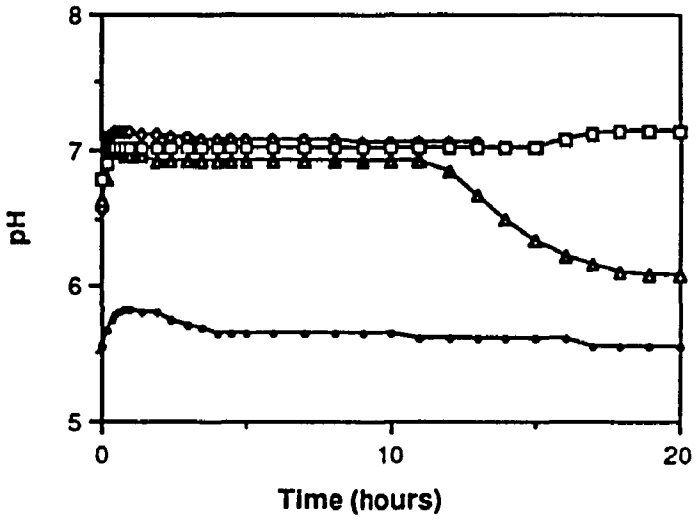
The second form of encapsulation investigated was that of immobilizing the cells within nylon membranes. The introduction of nylon microcapsules containing *L. lactis* subsp. *cremoris* into raw milk results in a pH reduction as illustrated in Figure 4.6. The time required for the pH to decrease to 4.6 (the pH corresponding to curdled milk) is longer than that required by raw milk. The $t_{5.5}$ values of nylon encapsulated cells, in comparison to the uninoculated control, were 14 and 12.5 hours respectively. The presence of bacteria within the microcapsules did not contribute to the souring ability of the milk. Uninoculated nylon microcapsules were also introduced into raw milk in order to determine their effect upon the fermentation. They exhibited no noticeable effect upon the course of pH reduction.

Fig. 4.6 : Time course of pH reduction in uninoculated raw milk (□), in raw milk containing uninoculated nylon microcapsules (●), and in milk to which nylon microcapsules were added containing the equivalent of 4 x 10^6 (◊) and 4 x 10^7 (Δ) CFU/ml milk.



It appears that cells microencapsulated within nylon microcapsules were not actively producing lactic acid. The microcapsules also appeared to buffer the milk or inhibit the natural activity of the indigenous microflora since the natural souring was slower in the presence of the microcapsules than it was in their absence.

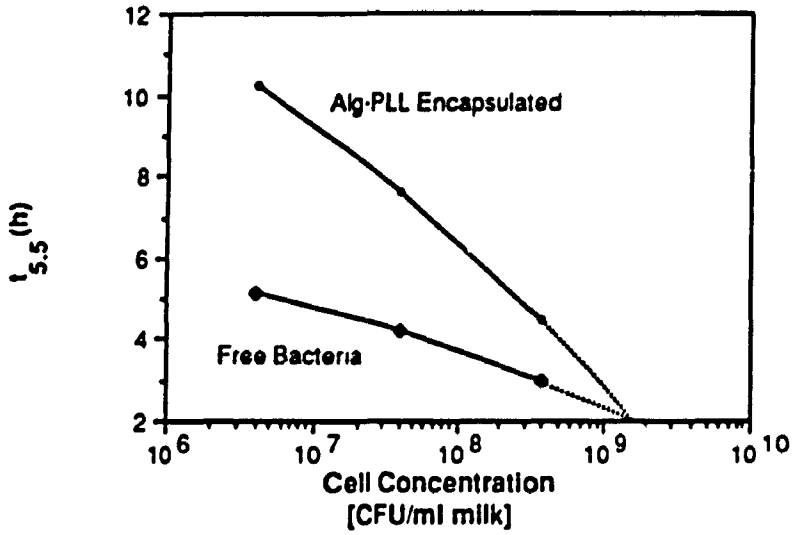
Fig. 4.7 : Changes in milk pH following the addition of uninoculated PEI microcapsules (◊), and PEI microcapsules inoculated with the equivalent of 4 x 10^6 (Δ) and 4 x 10^8 (□) CFU/ml milk. A similar time-course is presented where the initial pH of the milk was adjusted to 5.5 and the milk inoculated with PEI microcapsules containing the equivalent of 4 x 10^6 CFU/ml milk (•).



After addition of both inoculated and uninoculated PEI microcapsules to the milk, the pH increased rapidly from that of raw milk (6.5-6.7) to 7.0. Subsequent lactic acid production by either *L. lactis* subsp. *cremoris* or microflora naturally present in the milk was undetectable, as the pH of the milk remained constant at 7.0. Capsules with an effective inoculum of 4 x 10^8 CFU/ml of milk failed to cause any reduction in pH by the time that raw milk (Figure 4.1) was at the lower pH of 4.6. Lactic acid activity was also examined by lowering the milk pH to 5.5, following the introduction of PEI microcapsules with the equivalent of 4 x 10^6 CFU/ml of milk. Following a slight initial increase in the milk pH to 5.7, the pH remained relatively unchanged over the course of 20 hours

4.4 Evaluation of Alginate-Poly-L-lysine

Fig. 4.8 : Comparison of the time necessary for free (◊) and alginate-PLL encapsulated (•) bacteria to reduce the pH to 5.5.

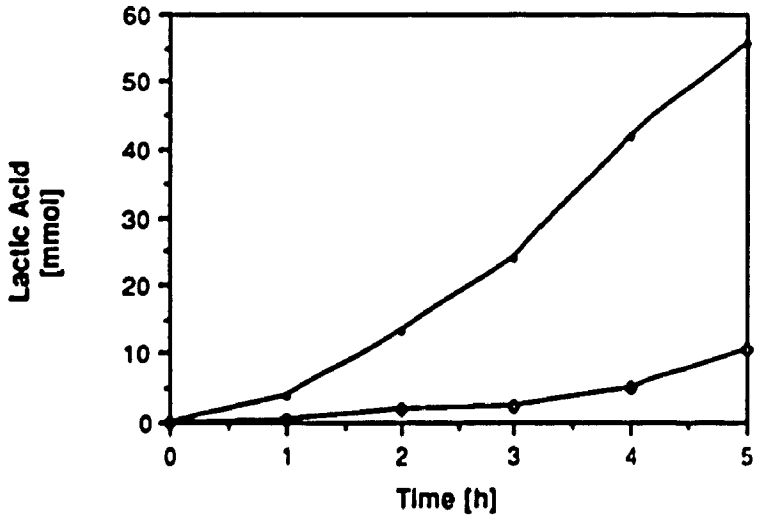


Points taken from Figures 4.3 and 4.4 were used to plot $t_{5.5}$ for three concentrations of encapsulated and free cells as illustrated in Figure 4.8. Increasing the concentration of cells within the alginate-PLL microcapsules had a more pronounced effect upon reducing the $t_{5.5}$ value, resulting in shorter fermentation times. The difference in the t5.5 values for free and encapsulated cells decreases as the cell concentration is increased. At the highest concentration, the $t_{5.5}$ for encapsulated cells approaches to within 2 hours of the $t_{5.5}$ for free cells. Extrapolation of the data curve to higher concentrations of cells indicates a possible point of convergence at a cell concentration of 1.7×10^9 CFU/ml milk.

The amount of lactic acid produced by *L. lactis* subsp. *cremoris* was measured following various periods of fermentation after inoculation of both free

and alginate-PLL encapsulated bacteria at initial concentrations of 4 x 10^8 CFU/ml of milk. (Figure 4.9) The quantity of lactic acid produced was determined by titrating with base to pH 6.7 following each sampling time interval. The amount of lactic acid produced by the free cells exceeded, by increasing amounts, that produced by the encapsulated cells. At the end of 5 hours, the lactic acid being released into the milk was still increasing for both free and encapsulated cells, and the amount of lactic acid which had been released by the free cells was approximately 7.5 times that of the encapsulated cells.

Fig. 4.9 : Cumulative amounts of lactic acid produced by inoculations of 4 x 10^8 CFU/ml of milk in free (•) and alginate-PLL encapsulated (◊) bacteria.



The data of Figure 4.9 can also be analysed with respect to the anticipated yield of lactic acid from the available lactose in the milk. Assuming a lactose concentration of 4.8%, the maximum amount of lactic acid producible through this

conversion (100%) is 56 mmol of lactic acid in 100 ml of milk. The yield of lactic acid, produced by free cells at a concentration of 4 x 10^8 CFU/ml of milk, can be seen in Table 4.7 to approach 100% at a fermentation time of 5 hours.

Table 4.7: Lactic Acid Fermentation by *Lactococcus lactis* subsp. *cremoris*

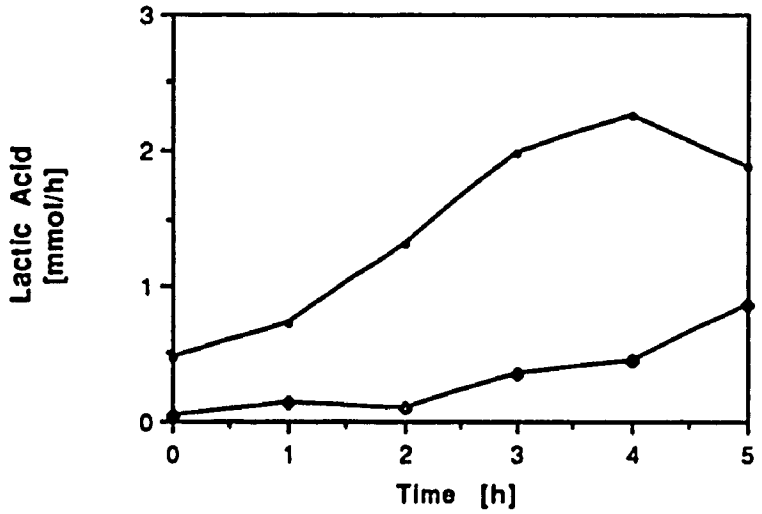
Time (hours)	% of Maximum Yield
0	0
1	7
2	24
3	43
4	75
5	99

This corresponds to the data of Figure 4.3 where, at a concentration of 4 x 10^8 CFU/ml of milk, the milk had reached a final pH of 4.6 in approximately 5 hours.

The rate of lactic acid production was monitored at a constant pH of 6.7 by continuous addition of base. Results are plotted in Figure 4.10. The lactic acid was produced by the free cells at a greater rate than by the alginate-PLL encapsulated cells. After 4 hours of fermentation, the lactic acid production by the free cells began to decrease while the production rate of the encapsulated cells continued to increase. As previously indicated in Table 4.7, the lactic acid fermentation of free cells was nearing completion, and production of lactic acid

was at the point of cessation.

Fig. 4.10 : Rate of lactic acid production at pH 6.7 by *L. lactis* subsp. *cremoris* immobilized within alginate- PLL (◊) at a concentration of 4×10^8 CFU/ml milk. Rates are compared to the activity of non-immobilized cells (•).



The tests of lactic acid activity have shown that, of the cells immobilized by the three methods of encapsulation investigated, only the bacteria encapsulated within alginate-PLL microspheres were able to approach the level of acidification achieved by free cells.

5.0 DISCUSSION

5.1 Microencapsulation Procedures

Three types of microencapsulation procedures were considered in the present study for the immobilization of *Lactococcus lactis* subsp. *cremoris*. An optimization of the procedures and reagents involved in each technique was largely based upon qualitative evaluations. For example, weak membranes could not tolerate repeated filtration operations, and poor yield of microencapsulation was evident by a visual inspection of the reactor components following membrane formation. A microscopic analysis of the microcapsules was performed to assess the approximate diameters, to look for membrane debris and to determine if the beads were spherical with smooth membranes.

5.2 Viability Tests

Solvents and reagents commonly used in the microencapsulation procedures are potentially toxic to the microorganisms being encapsulated. In addition, the pH of the polymerization reactions may not be tolerated by the cells. The solvents and reagents involved in the three immobilization techniques being considered were screened for possible toxic effects. Viability following contact was monitored using standard plate counts, giving rise to the number of colony forming units CFU/ml at a dilution factor of 10^6. The results were presented in terms of % viability in comparison to the control population of cells. In general, a reagent was considered non-toxic if 75% of the cells survived contact, moderately toxic for 25 to 75% viability, and toxic or highly toxic if less than 25% of the cells survived the viability test.

The objective in carrying out the viability tests was to rank the immobilization procedures in terms of their potential lethal effect on the cells, and in order to consider improvements to the immobilization procedures by using solvents and reagents which are less toxic to the cells of *Lactococcus lactis* subsp. *cremoris*.

The toxicity tests provided a method of individually testing the reagents and solvents for the toxic effect they may have upon the cells. The type of contact that the cells experienced during the test, however, may not be identical to the form of contact that they may experience during the microencapsulation operations. The observed viability after contact with these reagents may be lower than that which would be observed during encapsulation. Firstly, the effect of the water soluble reagents on the cells is more likely to play a greater role than the effect of the organic phase in the determination of the final viability of the cells. During encapsulation, the cells are in aqueous suspension and only contact the organic reagents indirectly during the emulsification procedures and subsequent interfacial reactions. It is therefore of lesser concern that the organic phase should prove to be more toxic to the cells in the toxicity tests, since these tests forced the cells into closer contact than they would experience during encapsulation. Secondly, the time of contact, held constant during the viability tests to provide a basis of comparison, varies during the encapsulation process. While the bacteria remain within the aqueous core material of the capsule for much longer than ten minutes, contact with the external organic solvent phase is restricted in the case of nylon and PEI, to less than ten minutes

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 than other solvents.[2]

When considering the reagents which resulted in 0% viability of the cells, it must be kept in mind that the plate counts were performed after the reagent-cell mixture had been diluted by a factor of 10^6. This does not eliminate the possibility of $<10^6$ cells surviving contact, but not registering as colony forming units on a plate after a dilution by a factor of 10^6. This final surviving number of bacteria, albeit low, is certainly not the same as a 0% survival, as would be indicated by a plate of 10^6 dilution, but is more accurately represented by "survival of $< 10^6$ CFU/ml ". In the interest of discovering a method of encapsulation which is of industrial use, however, the reagents which indicated "0% survival" were left as such, to denote those reagents which have a significant impact upon the bacteria.

Lactococcus lactis subsp. *cremoris* normally grows as short chains of cocci. Since one chain or one cell will both give rise to one CFU, the degree to which chains may be broken during the contact tests will affect the observed viability of the cells. Values of percent survival which exceed 100% may be explained by the disruption of the chains of cocci giving rise to more than one colony per chain of bacteria. The ease with which the chains of cells may be disrupted during the agitation which occurs during the toxicity test, may be affected by cell age, and other factors related to their physiological state.

It was also observed that placing the bacteria in contact with the organic phases (for example, cyclohexane) on repeated instances gave rise to differing values for the viability of the cells. This may be partially explained by the physiological state of the bacteria, influencing their resistance to the toxic material, the form of contact, and the ability of the cells to recover following

contact.

As was previously mentioned, the viability tests of individual reagents were unable to represent the environment to which the cells are exposed during encapsulation. With this in mind, the viability of cells within the microencapsulated product was examined by disrupting the microcapsules and performing plate counts on the released contents. There were no viable cells within the nylon membrane bound microcapsules, which may be expected in light of the toxicity of the organic solvents involved in the encapsulation procedure as discussed above. However, viable cells were observed within both alginate and polyethyleneimine microcapsules. It is perhaps unexpected that the polyethyleneimine microencapsulation procedure should appear to either retain a higher number of living cells or support a higher cell growth rate, since the previously mentioned toxicity tests revealed that the organic solvents involved in the making of PEI microcapsules were toxic to *L. lactis* subsp. *cremoris*. It was observed during the subsequent plating of the cells released from within the PEI membrane, that the resulting colonies were smaller in size than those of the control cells, suggesting an inhibition of growth due to the possible presence of reagents remaining within the microcapsule following encapsulation. It may also be possible that the cells were damaged or had not recovered fully from contact with the reagents during the encapsulation procedures.

Total viability was observed following immobilization within alginate-poly-L-lysine. This may be expected since the reagents were found to be non-toxic.

5.3 Activity Tests

5.3.1 Assay medium: Milk

Unpasteurized milk was used as the medium for the lactic acid activity tests. Natural contaminants present in raw milk, resulted in an acidification of the milk causing the pH to drop in the absence of inoculum. Typically, the pH of the raw milk would begin to drop after 7-8 hours reaching a pH of 5.5 following 12.5 hours incubation at room temperature. Since lactic cultures, when inoculated to the milk, initiated lactic acid production well in advance to that of the natural contaminants, it was felt unnecessary to sterilize the milk prior to testing.

5.3.2 Lactic Acid Activity by *Lactococcus lactis* subsp. *cremoris*

Lactic acid production was observed following the inoculation of raw milk with *Lactococcus lactis* subsp. *cremoris*. A decrease in pH was seen shortly after inoculation reaching a final minimum pH of approximately 4.6.

Increased lactic acid activity was observed with increasing levels of inoculum. This was evidenced by more rapid pH reduction with increasing cell concentration. The similar slope of each curve, regardless of the cell concentration, indicates the same rate of pH decrease. This would suggest that the rate of lactic acid production is the same, regardless of cell concentration. Since the lactic acid titration of milk is approximately linear, it may be concluded that the rates of lactic acid production are independent of cell concentration. It may also be possible that, at the lower initial cell concentrations, cell growth occurs at higher rates, thus resulting in the longer lag period prior to acid production. Eventually, as the cell concentration increases, the growth rate

decreases, and the resulting higher rates of acidification are observed to be similar. Taken together with the lactic acid titration of milk, which shows the relationship between lactic acid concentration and pH to be approximately linear, it may be concluded that the rate of lactic acid production are indeed similar.

5.3.3 Lactic Acid Activity of *Lactococcus lactis* subsp. *cremoris* Encapsulated Within Alginate-Poly-L-lysine

The production of lactic acid was observed in raw milk inoculated with alginate-PLL encapsulated bacteria. The bacteria were entrapped within the microcapsules and thus contained within a microenvironment with a higher cell concentration than was the case when non-immobilized cells served as the inoculum. This different environment may have an effect upon the lactic acid production of the cells, and the associated lag period. The lag time prior to the onset of lactic acid production was longer for the encapsulated cells than it was for the free cells. This may also be due to significant mass transfer resistance through the encapsulating membrane and gel bead, a longer recovery time for the bacteria following the encapsulation process, or the lag time of the bacteria.

5.3.4 Lactic Acid Activity of *Lactococcus lactis* subsp. *cremoris* Encapsulated Within Nylon Membranes

Lactococcus lactis subsp. *cremoris* immobilized within nylon microcapsules, at concentrations of both 10^6 and 10^7 CFU/ml of milk, were seen to be inactive. When broken microcapsules were plated, viable cells were not detected. Solvents with high molecular weight and low polarity may reduce the toxic effect of the encapsulating solvents on the cells, possibly permitting the application of nylon membranes for live cell immobilization. While it would

appear that nylon membrane encapsulation may not be suitable for cell immobilization, urease and other enzymes have been successfully microencapsulated using this technique.[10]

5.3.5 Lactic Acid Activity of *Lactococcus lactis* subsp. *cremoris* Immobilized Within Polyethyleneimine Membranes

Lactococcus lactis subsp. *cremoris* encapsulated within PEI microcapsules did not demonstrate lactic acid activity. In addition, it appears that the presence of the PEI microcapsules or perhaps unreacted PEI may inhibit the natural fermentation of the milk. The cells within the capsules are still viable, as evidenced by the plating of broken microcapsules, discussed previously. The encapsulation process or contaminating presence of unreacted PEI may inhibit lactic acid activity or damage the cells sufficiently to cause them to produce no lactic acid. Small diameter colonies observed during the viable cell counts may have resulted from the inhibitory affect of contact with the encapsulation reagents, or cell damage during the encapsulation procedure. Any inhibition of growth would likely be accompanied by an inhibition of lactic acid production. Further studies must be conducted to determine the reason for the absence of activity by the natural microflora within the raw milk.

5.4 Evaluation of Alginate-Poly-L-lysine Encapsulated Cells

Encapsulation of cells within alginate-poly-L-lysine yielded viable and active cells, as demonstrated by reduction in milk pH following inoculation. Cells of *Lactococcus lactis* subsp. *cremoris* did not survive nylon membrane encapsulation, and it appears that while viability was maintained within the PEI membranes, lactic acid activity was inhibited. As a result, only the alginate-PLL encapsulation procedure was examined in greater detail.

5.4.1 Lactic Acid Production by Alginate-PLL Encapsulated Cells

The $t_{5.5}$, or the time required for the cells to reduce the pH to 5.5 in milk was similar to previously observed rates of acidification observed with lactic acid bacteria immobilized within large diameter alginate beads. A $t_{5.5}$ value of 2 hours was observed at the Centre de Recherches et de Dévelopment des Aliments (C.R.D.A.) for the fermentation of cream, with immobilized lactic bacterial cell concentrations of 1.6×10^9 cells/ ml.[6] The $t_{5.5}$ value obtained in the present study is comparable, as extrapolation of the $t_{5.5}$ value at a cell concentration of 4×10^8 would indicate.

The difference in the activity of the free versus immobilized cells decreased with increasing cell concentrations. This would suggest that sufficiently high concentrations of encapsulated cells would demonstrate similar activity to the same concentration of free cells and would indicate that the membrane and gel matrix have no mass transfer effect on the production and release of lactic acid into milk.

5.4.2 Lactic Acid Production by *L. lactis* subsp. *cremoris* : Free vs. Encapsulated Cells

The results demonstrating viability and lactic acid production by the encapsulated cells suggest that the microbes were relatively unaffected by the encapsulation procedure. It is also possible that growth of the cells takes place within the beads, countering any inhibitory effect experienced by the cells during encapsulation, and eventually increasing the quantity of lactic acid produced. The increased amount of lactic acid produced by free cells as compared with encapsulated cells may be a result of the contact of the cells with the reagents or indicative of the recovery time required by the bacteria after the encapsulation process is complete.

5.4.3 Rates of Lactic Acid Production : Free vs. Encapsulated Cells

The rate of lactic acid production was seen to increase with time when the pH was held constant. The cells, which release lactic acid into the environment, may be automatically prompted to produce more, at a greater rate, when the conditions fail to become acidic. The rate of increase of lactic acid is greater for free cells as compared to encapsulated cells. This may be explained by higher local acidity within the alginate-PLL microcapsule and immediately surrounding the cells, which may serve to inhibit the activity of the cells. The mass transfer resistance of the microcapsules to the lactic acid has been determined to be negligible, as discussed in Section 5.4.1. It is therefore possible that the acid produced by the encapsulated cells is not dispersed through agitation, but accumulates within the microenvironment of the cells. This environment, which is more acidic than the bulk solution, may further inhibit production of lactic acid by

the cells.

In the final test, that of the rate of lactic acid procuction, the pH of the milk was held constant. While the maintenance of a constant pH enables one to regulate all variables outside of the cell's metabolic functions, it does not provide an accurate picture of the performance of the cell during an actual fermentation. During such a fermentation, it is likely that, as the environment surrounding the cell becomes acidic, the functions within the cell would change to accommodate the new situation. This would result in lactic acid production rates which would differ from those observed in this study. In addition, the effect of keeping the pH constant upon the bacteria is likely to be strongly affected by various physiological factors, such as cell age and generation. These same effects are likely to play different roles in the acidic environment produced during a milk fermentation.

During an actual milk fermentation to a pH of 5.5, the bacterial cells, although able to live in an acidic environment, are likely to become somewhat inhibited by the acid they produce. This study would tend to indicate, however, that any inhibition would occur subsequent to the time necessary to achieve a pH of 5.5. An industrial process, therefore, such as would be encountered within the cheese industry, whose goal it is to achieve a lowered pH under controlled conditions, would be able to do so with the certainty that the encapsulated cells would perform their task.

Industry would benefit from the knowledge that a desired pH can be reached in a known and controlled amount of time. With increased concentrations of encapsulated cells the time required to achieve the desired pH can be shortened, while retaining the active biomass for use in repeated

fermentations. Retention of cells not only saves in cost of biocatalyst, but also provides more control of the acidification procedure. In the cheese industry, where processes are based strongly in tradition, any method of providing a more scientific procedure would likely increase both productivity and yield of marketable cheese. When bacteriophage contamination is a potential problem, the use of alginate beads can serve to protect the cells from the phages, and allow for continued fermentations.[7]

The use of alginate-poly-L-lysine microcapsules is likely to be the favoured method of encapsulation for use in the food industry, as the reagents are non-toxic and are, in fact, currently rated as food grade materials. If cell release is undesired, the beads can be stabilized by addition of a poly-L-lysine membrane. It is likely that this method of immobilization will be able to provide the same production of lactic acid as has been traditionally achieved with free cells, and offers the advantage of providing the element of fermentation control and the opportunity to reuse the biocatalyst.

6.0 CONCLUSIONS

Lactococcus lactis subsp. *cremoris* was successfully immobilized within alginate-poly-L-lysine microcapsules, and within nylon and polyethyleneimine membranes. When immobilized in alginate-PLL, the cells were viable and actively producing lactic acid on a milk substrate. Encapsulation within polyethyleneimine membranes yielded viable cells, but lactic acid activity was not observed. The presence of the polyethyleneimine microcapsules within the milk also appears to inhibit the natural souring activity in the milk. The lactic cultures were no longer viable after encapsulation in nylon membranes, most likely due to the toxicity of the solvents during membrane formation.

It was established that the organic solvents involved in the formation of nylon and polyethyleneimine microcapsules are toxic to varying degrees, to *Lactococcus lactis* subsp. *cremoris*, whereas all the reagents involved in the alginate-poly-L-lysine encapsulation are essentially non-toxic

The preferable method of encapsulating *Lactococcus lactis* subsp. *cremoris* is currently that of entrapping the bacteria within a calcium-alginate complex, and coating the resulting bead with a poly-lysine membrane This technique results in high viability of the cells and high levels of lactic acid activity

The rate of lactic acid production by *Lactococcus lactis* subsp. *cremoris* encapsulated in alginate-PLL is less than that produced by unencapsulated or free cells. The difference diminishes with increasing cell concentrations At a concentration of approximately 10^9 cells/ml of milk, the time required to acidify the milk to the point at which it is useful in the dairy industry is identical for both free and encapsulated cells.

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