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Enhancement of acid tolerance of entrapped probiotic bacteria by polyoxyethylene (80) sorbitan monooleate

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ABSTRACT

Recently there has been an explosion of probiotics as important nutraceuticals. In order for the probiotic organisms to exert a positive health effect, they have to reach the large intestine of the host, alive and in sufficient numbers. However, considerable losses in cell numbers of probiotics occurs in the stomach due to low pH. The objective of this study was to explore a method to enhance the survival of probiotic bacteria in adverse gastric conditions of low pH. Three strains of probiotic bacteria including Lactobacillus casei, Lactobacillus rhamnosus and *Lactobacillus bulgaricus were grown in De Man, Rogosa and Sharpe medium incorporated with 1 g* l^{-1} *polyoxyethylene sorbitan mono-oleate (Tween 80) and were subsequently immobilised using alginate. The viability of the cultures in acidic conditions was assessed over a 2 hour incubation period. Electron microscopy was used to determine the surface texture of the alginate beads before and after acid exposure. Inclusion of Tween 80 in the growth media revealed that it could improve the subsequent survival of encapsulated probiotic cultures up to 10-100 fold following 90 minutes of acid exposure.*

Keywords: Probiotics, acid tolerance, immobilisation, alginate, polyoxyethylene sorbitan mono-oleate.

INTRODUCTION

Nutraceutical, a term combining the words “nutrition” and “pharmaceutical”, is a food or food product that provides health and medical benefits, including the prevention and treatment of disease. These chemical components are derived from plants, foods and microbial sources, and provide medicinal benefits valuable to long-term health. Examples of these nutraceutical chemicals include probiotics, antioxidants, and phytochemicals. Nutraceuticals have become a mainstream supplement to our diet and research has shown evidence that these chemicals found in food are effective when processed effectively and marketed correctly.

Probiotics are live microbial food ingredients that provide beneficial effects on human health by improving the balance of intestinal microflora [1, 2, 3]. These live microorganisms transit the gastrointestinal tract and in doing so, benefit the health of the consumer [4]. These benefits include controlling serum cholesterol levels and intestinal infections, beneficially influencing the immune system, improving lactose utilization and anticariogenic activity [5, 6, 7]. *Lactobacillus* and *Bifidobacterium* are the main genera considered for human probiotic food use. The probiotics market offers great potential for manufacturers and has continued to gain momentum, despite the complex processing challenges of formulating products with these beneficial microorganisms. The beneficial effects of probiotics have been related to the release of bioactive molecules either directly produced by them or resulting from their enzymatic activities. For efficacy, it is recommended that probiotic bacteria should be delivered in high numbers in food products (more than 10^7 cells per millilitre or per gram of the product) [8]. In this regard, the ability of probiotics to tolerate acid is one of the requisite traits for survival during gastric transit and in fermented food products [9].

Conventional methods used to enhance the viability of probiotics, include selection of resistant strains, incorporation of micronutrients and immobilisation [10]. Immobilization refers to the entrapment of microorganisms within or throughout a polymer matrix for use in biotechnological applications. Providing probiotic living cells with a physical barrier against adverse external conditions is an approach currently receiving considerable interest. As the technique of immobilisation or entrapment has become more refined, immobilised cell technology has evolved into encapsulation of cells. Use of alginates for gel matrices is one of the most popular system of immobilization and encapsulation. Literatures and patents underline the widespread use and excellent potential of microparticulate systems of alginate for pharmaceutical and biopharmaceutical applications [11, 12, 13]. However, for probiotics, neither immobilisation or encapsulation method has resulted in desired numbers of stable, viable bacterial cells [14] and further studies need to be conducted to improve the efficacy of delivery and survival of probiotics in the gastrointestinal tract.

Bacterial membranes of *lactobacilli* are typically composed of straight-chain saturated, unsaturated and cyclopropane fatty acids. Polyoxyethylene sorbitan mono-oleate or (x)-sorbitan mono-9-octadecenoate poly (oxy-1, 2-ethanediyl) is routinely included in synthetic media for cultivation of lactobacilli, where it improves aerobic growth rates, glucosyltransferase secretion and glycine-betaine accumulation. Polyoxyethylene sorbitan mono-oleate (Tween 80) is a nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid **(Figure 1).**

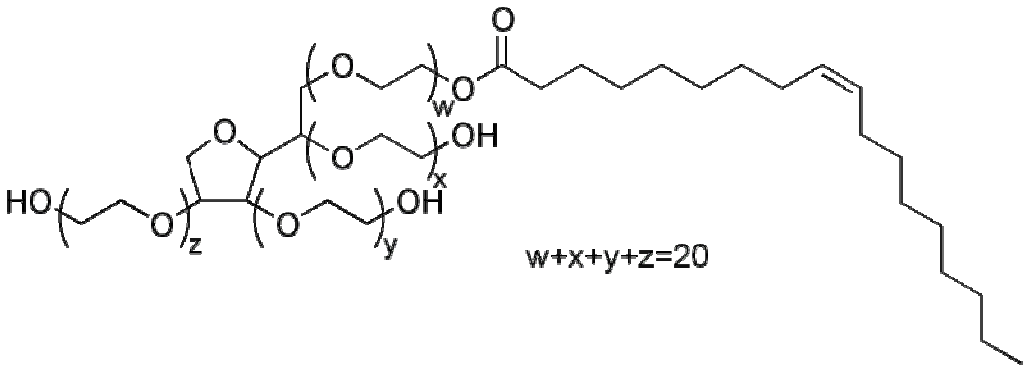


Figure 1: Chemical structure of Polyoxyethylene sorbitan mono-oleate

It has been reported that Tween 80 is important for alleviating the effects of stressful environments of lactobacilli, resulting in higher survival during storage in freeze-dried powders. A previous study by Ding et al (2009) has reported that *L. rhamnosus* GG grown in a medium supplemented with Tween 80 [polyoxyethylene sorbitan mono-oleate, which consists of up to 90% oleic acid], had oleic acid incorporated into its membranes and the free cells showed an approximate 1000-fold increase in survival when subsequently exposed for 90 min to simulated gastric juice, pH 2.5, compared to controls. The study showed the ability of increased membrane oleic acid to be reduced by H^+ to stearic acid. The resulting membrane had a more rigid structure, given its increased fatty acid saturation level.

In the present investigation, we tested the hypothesis that culturing lactobacilli with Tween 80, in combination with encapsulation of cells in a protective matrix can further improve their acid tolerance. Probiotic lactobacilli were grown in a medium containing Tween 80, subsequently encapsulated and checked for survival in simulated gastric juice. Previous studies have shown that probiotic viability after storage [15] and gastric transit is a strain-dependent characteristic , hence in this study we also report the acid tolerance of the three probiotic strains, *Lactobacillus rhamnosus Lactobacillus casei* and *Lactobacillus bulgaricus* after encapsulation.

EXPERIMENTAL SECTION

Probiotic bacteria and their cultivation

Lactobacillus bulgaricus was obtained from National Collection of Industrial Microorganisms (NCIM) Pune, India while *Lactobacillus rhamnosus* was obtained from National Chemical Laboratory (NCL), Pune, India. *Lactobacillus casei* was purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial technology, Chandigarh, India. Probiotic strains were maintained individually and grown in De Man, Rogosa and Sharpe (MRS) broth (Himedia, India) at 37^0C using 1% inoculum. Before use, probiotic organisms were activated by growing three times successively in MRS broth at 37^0C for 18 h.

Growing cells in presence and absence of polyoxyethylene sorbitan mono-oleate (Tween 80).

One hundred millilitres of MRS with and without Tween 80 broth was prepared and autoclaved at 121°C for 15 min. The concentration of Tween 80 was 1%. The broths were inoculated with overnight culture at 1% level and incubated at 37°C. After 24 h, the cultures were immobilised in an alginate matrix.

Immobilization of probiotics

Sodium alginate at 3% (w/v) was used as the encapsulating material for immobilisation of probiotics. All glassware, media and reagents used in the experiments were sterilized at 121°C for 15 mins before use. 3g alginate was added to 100ml distilled water in a volumetric flask and heated until completely dissolved. Alginate solution was then autoclaved and allowed to cool to 25^0C. To this encapsulating material 25ml of probiotic culture (grown in the presence / absence of Tween 80) was added. The alginate-cell suspension was extruded through a 21G syringe needle drop-wise into a 0.5 M calcium chloride ($CaCl_2$) solution. The drops solidified upon contact with the $CaCl_2$ solution, thus entrapping the cells. After 30 min of incubation in $CaCl_2$ solution at 4^0C to permit hardening, the beads were recovered by decantation. They were then stored in a sterile flask at 4^0C, in 0.01% peptone solution until further use.

Acid tolerance of entrapped cells

Encapsulated probiotic bacteria were inoculated in acidified MRS broth adjusted to pH 2 with 5M hydrochloric acid and incubated at 37^0C. Samples were taken for plate counts at 30, 60, 90, and 120 min intervals, for which bacteria were first released from capsules by sequestering calcium ions with 0.4 M phosphate buffer at pH 7. Plates were incubated at 37^0C for 72 hrs. Counts of encapsulated cells grown in presence/absence of Tween 80 and inoculated in MRS broth without acid were considered as controls. The acid tolerance of each strain was determined by comparing the plate count after exposure to acid with the control plate count.

All acid tolerance experiments were performed in triplicate and repeated three times. Mean values have been reported.

Morphology and diameter of the particles

To assess the change in surface texture of the beads after acid exposure, beads were removed from the MRS medium or acid solution and blot dried on paper towel, freeze dried and an environmental scanning electron microscope (ESEM) was used to take images.

RESULTS AND DISCUSSION

Viability of probiotics depends not only on developing or choosing the right technique in food processing but also on the survival of these bacteria during their passage through the human gastro-intestinal system. Though the advances in the field of nutraceuticals have been tremendous, there is scope, however, as to the technology of immobilisation of live probiotic bacterial cells inorder to further enhance the survival of cells. A modified method using calcium alginate for the encapsulation of probiotic bacteria has been investigated in this study inorder to improve the survival of *lactobacilli* in acidic gastric conditions.

Table 1: Viable count of *Lactobacillus rhamnosus* after acid exposure of encapsulated cells grown in the absence and presence of Tween 80

Time in mins	Count of viable encapsulated cells (CFU/ml)	
	In absence of Tween 80	In presence of Tween 80 (1 g l^{-1})
Control (without acid exposure)	2.5×10^{10}	2.5×10^{10}
30	3.42×10^{8}	4.83×10^{8}
60	4.35×10^{6}	9.83×10^{7}
90	3.3×10^{5}	1.34×10^{7}
120	1×10^{4}	8.85×10^{5}

Table 2: Viable count of *Lactobacillus casei* after acid exposure of encapsulated cells grown in the absence and presence of Tween 80

Time in mins	Count of viable encapsulated cells (CFU/ml)	
	In absence of Tween 80	In presence of Tween 80 (1 g l^{-1})
Control (without acid exposure)	2.85×10^{10}	2.85×10^{10}
30	2.52×10^{8}	3.95×10^{8}
60	8.75×10^{6}	1.78×10^{8}
90	4.42×10^{6}	1.49×10^{7}
120	3.15×10^{5}	3.99×10^{6}

In our study, the inclusion of Tween 80 (1 g/l) in the growth media, revealed that it could enhance the subsequent survival of encapsulated probiotic cultures up to 10-100-fold following 90 minutes of acid exposure (Tables 1, 2 and 3)

Table 3: Viable count of *Lactobacillus bulgaricus* after acid exposure of encapsulated cells grown in the absence and presence of Tween 80

Time in mins	Count of viable encapsulated cells (CFU/ml)	
	In absence of Tween 80	In presence of Tween 80 (1 g l^{-1})
Control (without acid exposure)	3.6×10^{10}	3.6×10^{10}
30	1.11×10^{9}	1.39×10^{9}
60	2.19×10^{7}	7.51×10^{7}
90	2.64×10^{6}	1.106×10^{7}
120	2.9×10^{5}	7.21×10^{6}

Encapsulated probiotic organisms grown in the absence of Tween 80 showed a steady loss in viability when exposed to acidic conditions while probiotic organisms grown in the presence of Tween 80 showed better viability following acid exposure. **Figure 2** shows the marked difference in the viability of *L. casei* after its growth in the presence of Tween 80 .

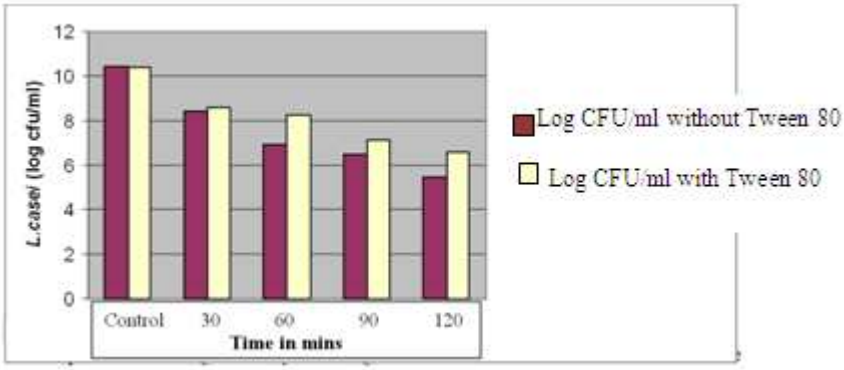
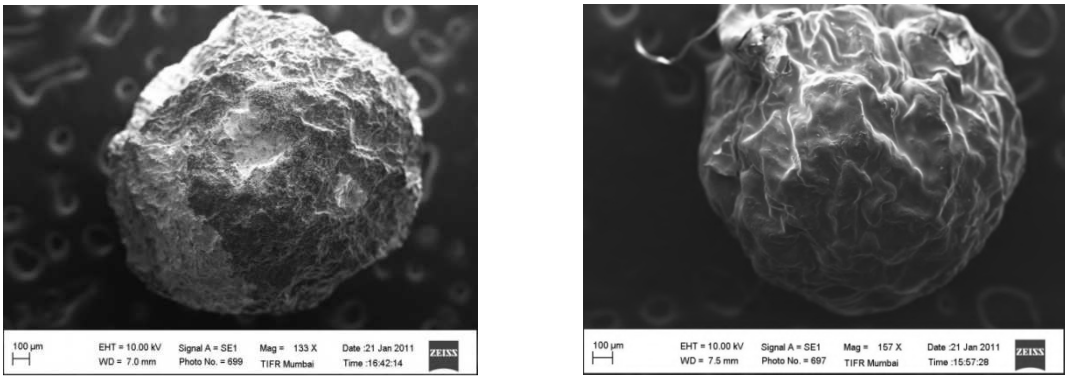


Figure 2: Graph showing the effect of acid exposure on encapsulated cells of *Lactobacillus casei* grown in the presence and absence of Tween 80



(A) **(B)**

Figure 3: ESEM images of alginate capsules. (A) Image of a capsule before exposure to acid. Note that the capsule is more spherical in shape. (B) Image of a capsule after exposure to acid. Note the change in capsule surface, which has become rough and irregular.

Though the results indicated a loss of viability of all strains in capsules; however, at 90 minutes of exposure, most probiotic organisms survived at $>10^7$ colony forming units (CFU) /mL and at 120 minutes of exposure most encapsulated probiotic organisms survived at $>10^6$ CFU/mL when grown in a medium with Tween 80. The results of this study thus concur with other similar studies where growing the cells in presence of Tween 80 in MRS broth has been found to increase the survival of free probiotic bacteria in acidic conditions [16]. Growth in the presence of sodium oleate, which is the active portion of Tween 80, modifies the fatty acid composition of *Lactobacillus* cells [17] and this change affects their acid tolerance.

Images from ESEM showing a marked difference in the surface texture between the alginate capsules, before and after acid exposure, are shown in **Figure 3A and 3B** respectively.

Initially alginate beads had a smooth surface texture without the presence of any pores or cracks, whereas the acid exposed beads showed a very rough and an uneven surface. Our results indicate that adding a coating material on the beads may create a more stable capsule and help to maintain the capsule's integrity during storage and exposure to acids. The encapsulation method used in this study did not result in uniform bead size, and hence additional experiments need to be designed using uniform bead size.

From a technological point of view, in order for probiotics to exert health benefits, the formulation should include selected microorganisms with the ability to survive at high levels during the industrial process and remain viable during gastrointestinal transit with unaltered properties for long periods of storage. The viability of encapsulated microorganisms was analysed after exposure to acidic conditions for two hours. *L. bulgaricus* and *L.casei* were found to be more acid tolerant ($>10^6$ CFU/ mL) compared to *L.rhamnosus* ($>10^5$ CFU/ mL).

In conclusion, our results indicate that the incorporation of polyoxyethylene sorbitan mono-oleate in the growth media enhances the subsequent survival of encapsulated cultures up to 100-fold following acid exposure compared with controls grown in the absence of Tween 80. In the future, combined use of Tween 80 and encapsulation could be further investigated as an approach to improve the survival of probiotic organisms during gastric transit.

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