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Study of Bio-encapsulation of *Lactobacillus species* using cellulose

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Abstract

The present study was aimed to understand the better stability effects and higher tolerance range of cellulose encapsulated Lactobacillus in certain pH conditions over non-encapsulated Lactobacillus. The recommended dose of 10^6 to 10^9 cells/day was given by bioencapsulating 10^8 cells of Lactobacillus and CFU were assayed after 15 days and 30 days.The cellulose encapsulated Lactobacillus was found to be 80% and 90% viable after 15 and 30 days of storage respectively when compared to the nonencapsulated lactobacillus. From the stability test, the encapsulated capsule was found to be stable at low 2.5 pH and nonstable at 6.5 pH. The reason behind such stability is that the huge concentration of H+ ions does not affect the surface permeability and also the surface gets no threat from the OH- ions because the excess H+ ions shields the effect of the OH- ions from attacking the microcapsule. In higher pH conditions the concentration of OH- ions increase relatively and since the H+ ions cannot shield upto that effect as mentioned in the previous one, the capsular surface poses a threat and is bound to degrade and consequentially it fails to pass through the Gastrointestinal Tract of mammals. Bioencapsulation of lactobacillus with cellulose was also found to be antibiotic resistant and had a high tolerance range to phenol. From this study, it can be said that cellulose can be used to encapsulate Lactobacillus in place of alginate and cellulose encapsulated lactobacillus can pass through the GI tract of mammals and be released in the small intestine at much lower pH conditions.

Keywords: Probiotics, bioencapsulation, Lactobacillus, Probiotic stability and Cellulose

Introduction

Probiotics are functional food substances that contain LAB such as *Lactobacillus, Bifidobacterium* and *Enterococcus* species which on regular consumption improves intestinal health by inhibiting the growth and adhesion of pathogenic microorganisms. These live microorganism's secrets bacteriocins and maintain an acidic PH which retards the growth of toxin producing pathogens like *Helicobacter pylori (Sullivan and Nord, 2002 and Barbara Mombelli and Maria Rita Gismondo, 2000).*_Probiotics are well known for its potential properties like antibiotic resistance, resistance to phenol, acid tolerance, bile tolerance, bile salt hydrolase activity, certain species produces H_2O_2 and β-glycosidase and such properties has fascinated people to consume such preparation as a part of their food to promote health.Bioencapusulation of probiotic preparation are done by using prebiotic food components which are non-digestible dietary components *(Sanders and in't Veld 1999)* like Inulin, alginate, starch, chitosan etc that escapes digestion in small intestine and reaches colon to serve as a growth substrate for beneficial lactic acid bacteria *(Amparo et al., 2006)*. Usually alginate –starch beads are widely used for encapsulation because of its low cost, biocompatibility and its stability inside the gastro –intestinal system but such encapsulation is not recommended in European countries due to the side effects associated with alginate. This includes

decrease in blood albumin and hemoglobin level and sometimes to an extent of abortion. In this present study, cellulose is used as a prebiotic substance for encapsulation of probiotic living a microorganism *Lactobacillus acidophilus* because of its advantage over alginate-starch system that includes its ability to withstand hydrolysis by glycosidase enzyme and also the β 1-4 linkage of cellulose confer its resistance to the action of acids present in the digestive tract of mammals (*Ambika Shanmugam, 1982).* Hence cellulose can be preferred as an effective prebiotic substance for bioencapsulation of health promoting probiotic live microorganisms for effective targeted delivery system.

Materials and Methods

Sample collection

Curd was used as a sample to isolate *Lactobacillus* spp.

Sterilization and serial dilution

Petriplates, pipettes, test tubes containing distilled water and MRS medium were prepared and sterilized. After sterilization, 1 ml of curd sample was transferred into test tube containing 10 ml of water named as 10^{-1} and it was mixed throughouly and then the dilution was carried out to other test tubes ranging from $10^{-}2$ to $10^{-}7$ respectively. 0.1 ml of the diluted sample was taken from the test tubes 10^{-6} and 10^{-7} and was transferred to the Petri plates containing MRS media and spread plate technique was carried out .The plates with the bacterial culture was inverted and incubated at 37˚c for 24 hours *(Lansing Prescott, 2002).*

Isolation and identification of Lactobacillus spp

Discrete colony isolation

MRS medium was prepared and sterilized and 15 to 20 ml of the media was poured in the Petri plates and after solidification of the media, a loop full of culture was taken and quadrant streaking was performed. Then the plates were incubated at 37˚c for 24 hours (*Lansing Prescott, 2002).*

Gram's staining

A thin smear of the colony was prepared on the glass slide and the smear was heat fixed. After this, primary stain crystal violet was added and washed after one minute. The mordant dye Iodine was then added and washed after one minute. Then decolourizer alcohol was added and washed after 30 seconds. Finally counter stain Saffranin was added and washed. The slide was air dried and examined under microscope (*Lansing Prescott, 2002).*

Biochemical characterization

Biochemical characterization was carried out to determine the type of sugar the bacteria ferments and the final product that is produced at the end of fermentation. Lactose, glucose and sucrose broth suspended with bromo thymol blue was prepared and sterilized. Two sets of tubes were maintained for each sugar respectively. One tube was inoculated with *Lactobacillus spp*. and the second tube was kept as control. (Control tube).and these tubes were incubated at 37°C for 24 hours (Lansing Prescott, 2002).

Bacterial enumeration

The overnight mass multiplied *Lactobacillus* culture was centrifuged at 6000rpm for 15 minutes to get the bacterial cell pellets. This procedure was repeated for two times by washing the cell pellet with 0.9% of saline to remove all the impurities from the pellet *(Chan and Zhang, 2002).*

Quantitative determination of microorganism

Petri plates, pipettes, conical flask with 0.8% saline and MRS medium were prepared and sterilized. About one ml of centrifuged cells was transferred to the conical flask containing 99ml of sterile saline. This was homogenized mechanically by shaking the conical flask for few minutes and this dilutes the sample to 10^{-2} dilution. One ml of the sample was transferred to the second conical flask containing 99ml of sterile saline, which represents the 10^{-4} dilution and then for 10^{-6}. 0.1ml and one ml of the sample was transferred from the 10^{-4} and 10^{-6} dilutions to the Petri plates that contained MRS medium and spread plate technique was carried out. The plates were inverted and incubated at 37°C for 24 hours *(Lansing Prescott, 2002)*.

Determination of probiotic stability

Antibiotic resistance

The overnight culture of Lactobacillus was swabbed on the MRS media and the antibiotic disc like gentamycin, bacitracin were kept on the MRS agar and the plates were incubated at 37°C for 24 hours and determined for antibiotic resistance (*Saul Scheinbach, 1998).*

Phenol tolerance

The overnight culture of Lactobacillus was prepared. MRS broth with 0.4% of phenol was prepared and sterilized. Then one ml of the overnight culture was inoculated in the broth and the broth was incubated at 37°C for 24 hours. After incubation, 0.1 ml of the broth was inoculated on the plate containing MRS media to determine viability of Lactobacillus in 0.4% phenol. The plate was incubated at 37°C for 24 hours (*Saul Scheinbach, 1998).*

Bioencapsulation

Bioencapsulation is carried out with help of cellulose, the homo polysaccharide that entraps the bacterial cell in its matrix and protects the core from the environment factors. Empty capsules (capsule size 2), micropipette, vials, cellulose, laminar hood, Bunsen burner and sample with 2-3 × 10^7 of microbial load were taken for the bioencapsulation study. The vials were sterilized in autoclave. About 0.2 mg of cellulose was weighed and it was aseptically added to the vials. One ml of sample was pipetted out and added to the vials. After 30 minutes the vials were tapped gently and the content was filled in the capsule. Finally the capsules were stored at 4˚c for 15 days, after which it was examined for its viability *(Amparo et al., 2006).*

Stability of probiotic capsule

Stability at 2.5 pH

The 2.5 pH solution was prepared with hydrochloric acid (HCl) 35-38%, with specific gravity of 1.1835 and 1.16 g/ml and the capsule was put into 2.5 pH solution in a test tube and shaken gently. The test tubes were continuously observed at 15, 30, 45 and 60 minutes respectively.

Stability at 6.5 pH

The 6.5 pH solution was prepared in a test tube and the capsule was put into the test tube and shaken gently for 10 minutes. The test tubes were continuously observed at 10, 20 and 30 minutes.

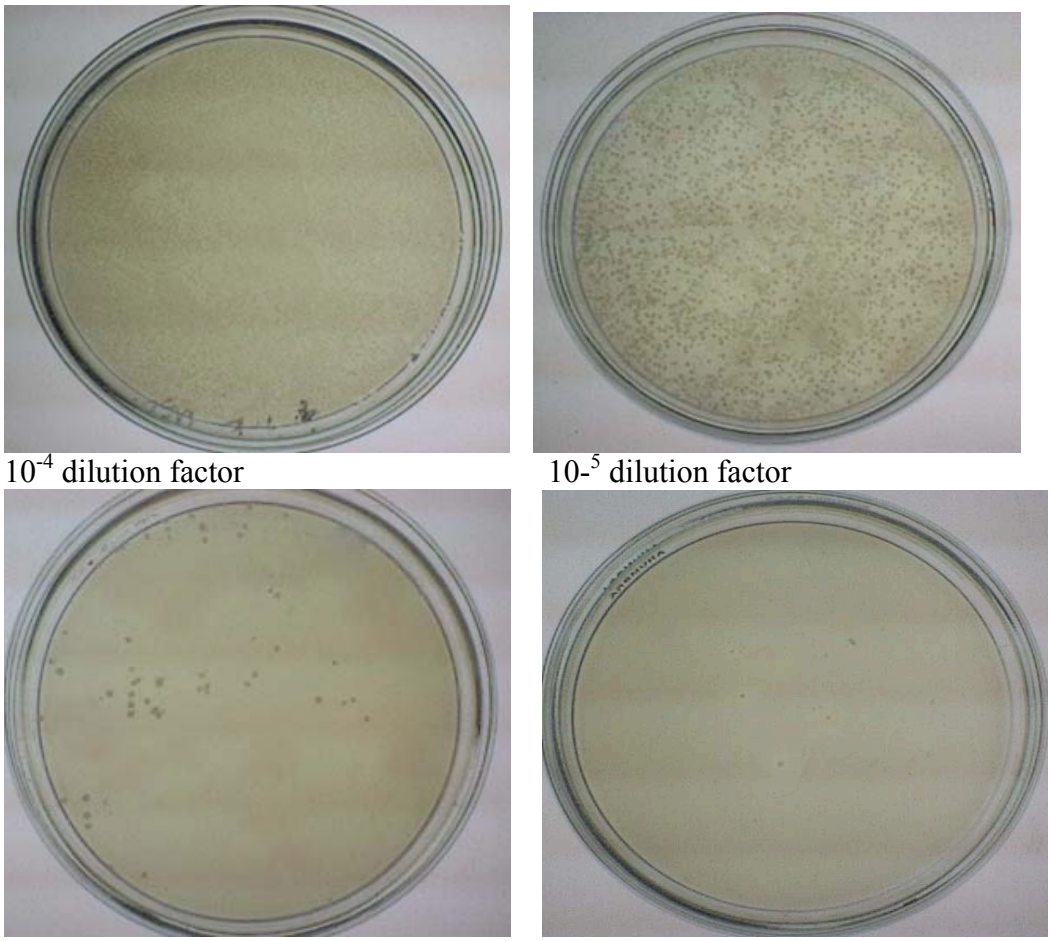


Fig 1.Serial dilution of curd sample

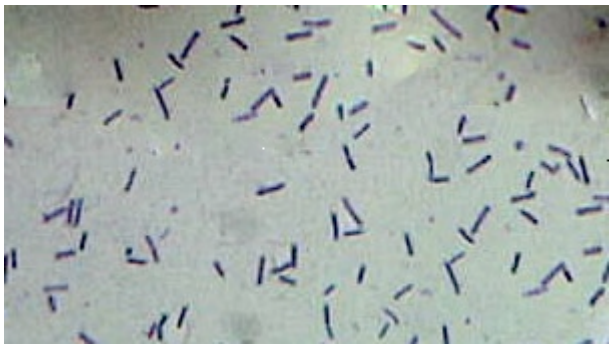
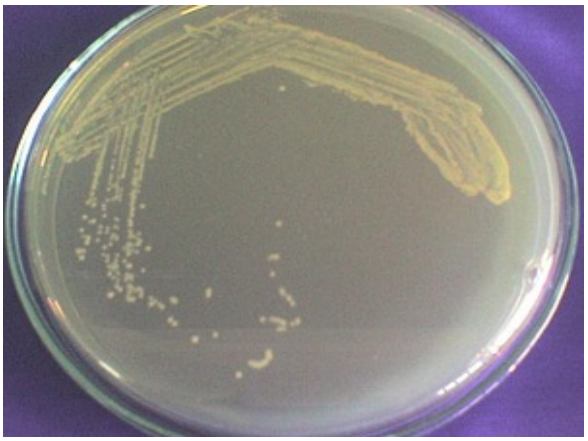
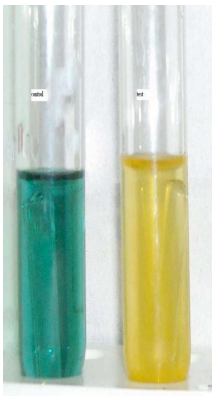


Fig.2 Gram's staining

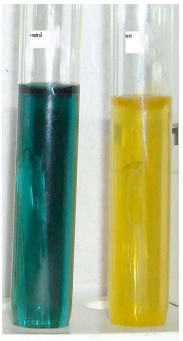


Discrete colony isolation



a. Glucose fermentation

b. Lactose fermentation

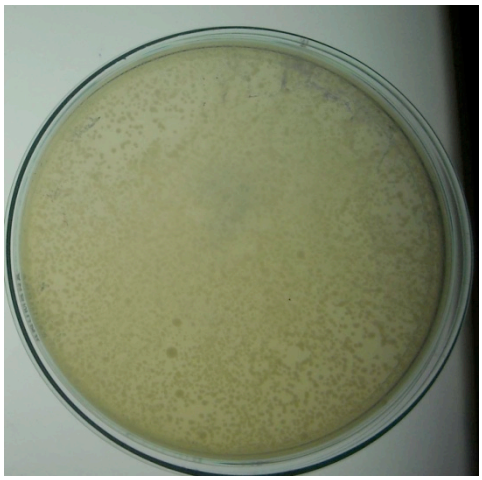


c. Sucrose fermentation

Fig. 3.Carbohydrate fermentation test

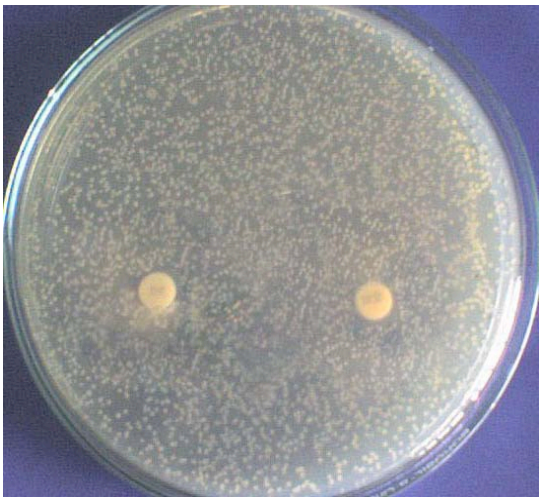
a.CFU before bioencapsulation
(Dilution factor 10^{-5})

b.CFU after bioencapsulation (15 days)
(Dilution factor 10^{-5})

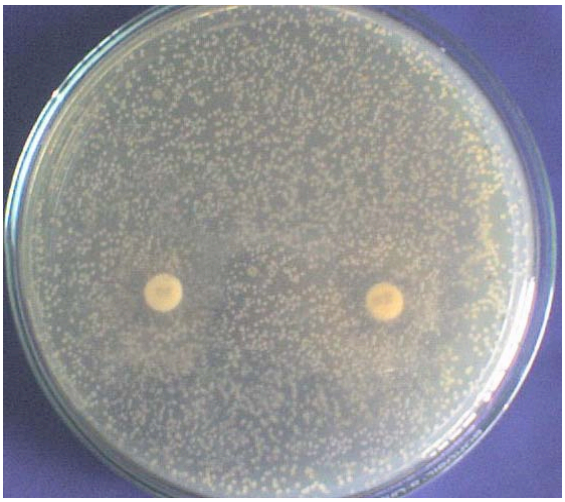


c. CFU after bioencapsulation (30 days)
(Dilution factor 10^{-5})

Fig. 4.CFU and Encapsulation analysis

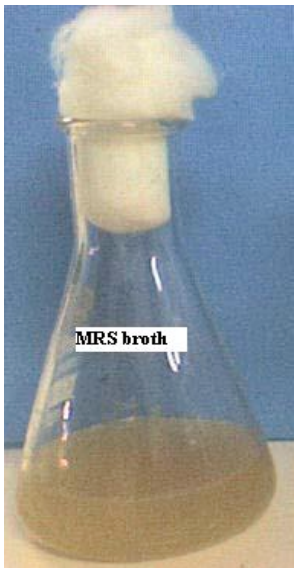


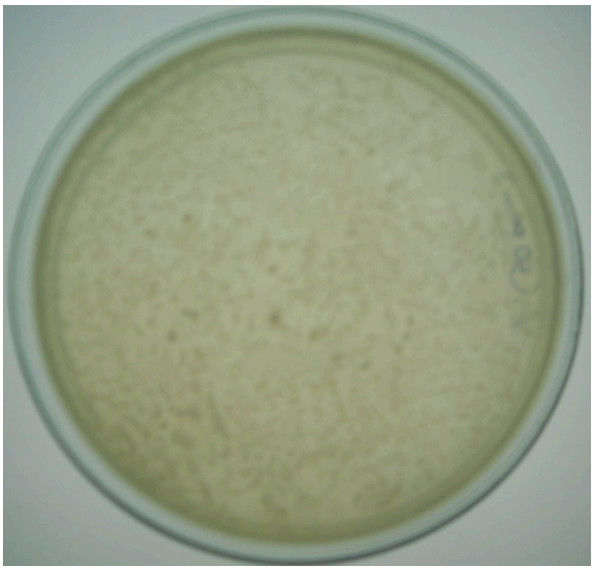
Antibiotic resistance
(6.1, Before bioencapsulation)



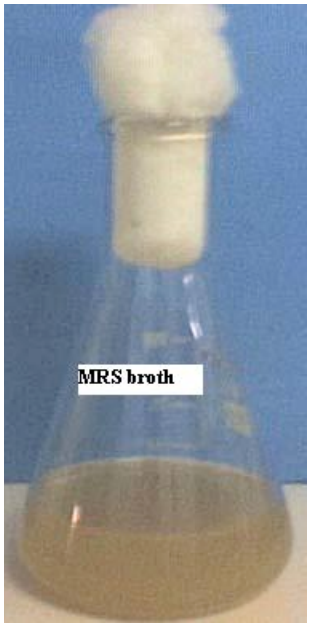
Antibiotic resistance
(6.2, after bioencapsulation)

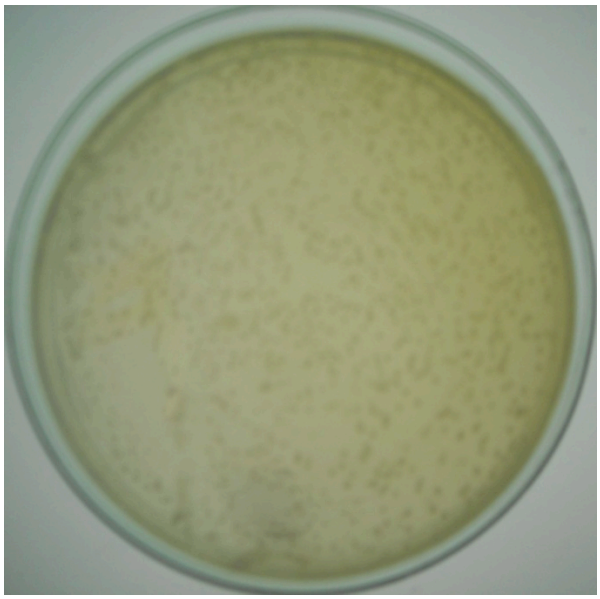
Fig. 5.Antibiotic resistance analysis





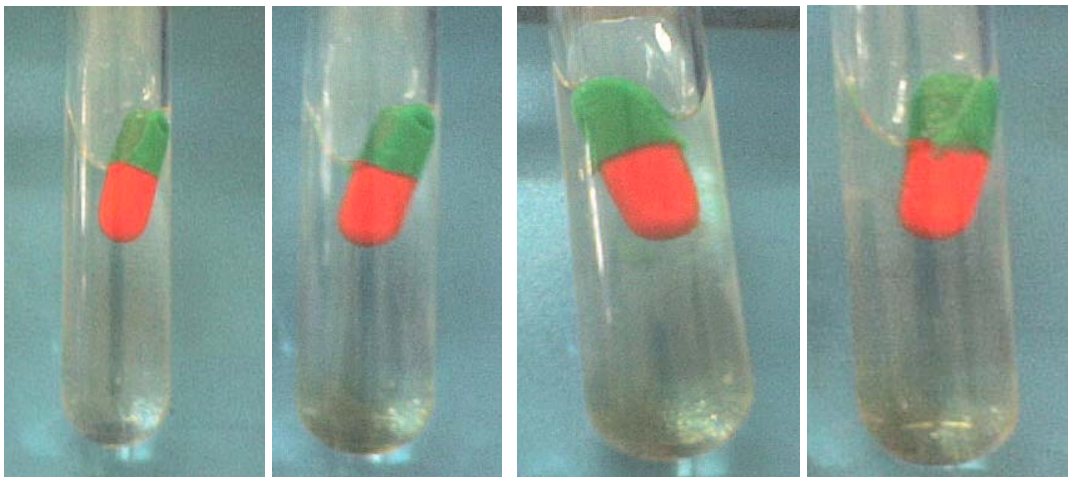
Phenol tolerance (Before bioencapsulation)
a. MRS broth (0.4% phenol)





Phenol tolerance (After bioencapsulation)
b. MRS broth (0.4% phenol)

Fig. 6.Phenol tolerance analysis



At pH 2.5

At start 30 minutes 60 minutes 90 minutes

At pH 6.5

At start 10 minutes 20 minutes 30 minutes

Fig. 7.Stability of probiotic capsule

Activity test

The bioencapsulated cells were released from its matrix into phosphate buffer and its activity is assayed to confirm its efficiency after encapsulation. The assay was carried out by analyzing CFU, antibiotic resistance and phenol tolerance.

Phosphate buffer preparation

Phosphate buffer was prepared mainly for the release of bioencapsulated cells from its matrix and its pH was maintained at 6.5.

CFU analysis

CFU analysis was carried after 15 days and 30 days to check the viability of cells that were encapsulated. The content of the capsule was emptied in the phosphate buffer (pH 6.5) and shaken well for 15 minutes for the dispersal of the cells into buffer. One ml was pipetted out and serially diluted. From 10^{-5} dilution, 0.1 ml was pipetted out and inoculated in MRS agar and incubated at 37°C for 24 hours (*Saul Scheinbach, 1998)*

Antibiotic resistance

After 15 days, the content of the capsule was emptied in the phosphate buffer (pH 6.8) and shaken well for 15 minutes for the dispersal of the cells into buffer. A sterile swab was dipped in the buffer and swabbed on the MRS agar. The antibiotic disc such as gentamycin and bacitracin were placed on the agar plates and the plates were incubated at 37°C for 24 hours (*Saul Scheinbach, 1998).*

Phenol tolerance

The content of the capsule was emptied in phosphate buffer (pH 6.5).MRS broth with 0.4% of phenol was prepared and sterilized. About one ml of sample from buffer was inoculated in the broth and the broth were incubated at 37°C for 24 hours and assayed for its bacterial count *(Saul Scheinbach, 1998).*

Results and Discussion

Serial dilution

Plates inoculated with 10^{-4} and 10^{-5}dilutions on MRS showed creamy white mucoid colonies OF Lactobacillus**. (Fig1)**

Gram's staining

The gram's staining procedure was carried out and the smear was viewed under the microscope at 100x (oil immersion). Purple colored rods were observed. Thus, CVI complex formed on the cells were not removed by decolorizer and so the bacterial cell wall was made of peptidoglycan layer, which was the characteristic feature of Gram positive bacteria**.(Fig 2)**

Biochemical characterization

The incubated tubes were observed after 24 hours. The media inoculated with *Lactobacillus spp*. turned its color from green to yellow. Thus, the sugars were fermented by the bacteria and acid end products were produced which lowers the pH, causing the pH indicator to change color from green to yellow (*Gerald W. Tannock, 1999***). (Fig 3)**

Quantitative determination of microorganism

The plates were observed after 24 hours of incubation and the numbers of colonies were counted by Quebec colony counter.

S. No	Dilution factor	Number of colonies
1	10^4	Too numerous to count
2	10^5	278
3	10^6	38
4	10^7	Too few to count

The CFU was calculated by using the formula,

$$\frac{\text{Number of cells} \times \text{Dilution factor}}{\text{Volume of Sample}} = \frac{278 \times 10^5}{0.1}$$

$$= 2.78 \times 10^8 \text{ cells/ml}$$

The minimum therapeutic dose per day for probiotics was suggested to be 10^6 to 10^9 cells and thus one ml of the centrifuged culture containing 2.78×10^8 cells can be used for the bioencapsulation of probiotics that provides minimal dose per day. **(Fig 4)**

Determination of probiotic stability of *Lactobacillus*

Antibiotic resistance

The plates were observed after 24 hours of incubation. The zone of inhibition was not seen around the antibiotic disc as shown in Thus, it was concluded that *Lactobacillus* ssp was found to be resistant to gentamycin and bacitracin. **(Fig 5)**

Phenol tolerance

The plate incubated was observed for the colony formation. Creamy white mucoid colonies were observed on the plate, which indicate *Lactobacillus* spp. could tolerate phenol concentration. Thus testing for the resistance to phenol generated the potential for the survival of *Lactobacillus* in gastrointestinal conditions. **(Fig 6)**

Stability of probiotic capsule

Stability under pH 2.5

The powder inside the capsule solidified into shape similar to that of original capsule and it was observed that the solidified powder was not disrupted even the solidified powder was shaken continuously in the same solution for 30, 60 and 90 minute. This result indicates that, following ingestion, the capsule can go through the stomach without disruption

Stability under pH 6.5

The major part of the capsule was disrupted within 30 minutes and the entire capsule was totally dissolved after 45 minutes. The capsule was totally dissolved at pH 6.5; the pH

prevails in the small intestine and the bioencapsulated cells were released from the matrix. **(Fig 7)**

Activity test

The activity test was carried out to check the efficiency of *Lactobacillus* after encapsulation. The activity confirmation can be carried out by comparing the activity of encapsulated *Lactobacillus* with non encapsulated *Lactobacillus.*

CFU analysis

The plates were observed for CFU after 15 days and 30 days and the number colonies formed on the plates were counted by Quebec colony counter.

CFU after 15 days,

$$\text{CFU (cells/ml)} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of sample}}$$

$$= 250 \times 10^5 / 0.1 \qquad = 2.5 \times 10^8 \text{ cells/ml}$$

CFU after 30 days,

$$\text{CFU (cells/ml)} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of sample}}$$

$$\text{CFU (cells/ml)} = 220 \times 10^5 / 0.1 = 2.2 \times 10^8 \text{ cells/ml}$$

$$\text{Viability (\%)} = \frac{\text{CFU after bioencapsulation}}{\text{CFU before bioencapsulation}} \times 100$$

S.No	CFU before bioencapsulation (cells/ml)	CFU after bioencapsulation		Viability (%)
		Days	CFU (cells/ml)	
1	2.78×10^8	15	2.5×10^8	90%
2	2.78×10^8	30	2.2×10^8	80%

The bioencapsulated capsule contains dose which meets the daily recommended dose of probiotics and thus the number of colonies gives the number of live bacteria that grows under the incubation condition after the bioencapsulation.

Conclusion

This experiment was performed for the isolation of lactobacillus from curd sample using MRS medium and by Grams staining and biochemical test. Several observations were made after the curd making lactobacillus was microencapsulated with cellulose. The cellulose encapsulated lactobacillus was found to be 80% and 90%viable after 15 and 30 days of storage respectively when compared to the nonencapsulated lactobacillus. From the stability test, the encapsulated capsule was found to be stable at low 2.5 pH and nonstable at 6.5 pH. This is a sheer indication that the acid resistant property is induced on the surface of the capsule which makes the system stabilized and resilient to certain other vigorous pH changes. The encapsulated lactobacillus with cellulose also found to be antibiotic resistant such as gentamycin and bacitracin and tolerant to phenol. From this study, it can also concluded that the cellulose can be used to encapsulate the Lactobacillus in place of alginate and cellulose encapsulated lactobacillus can pass through the GI tract of mammals and be released in the small intestine considering the stability of cellulose encapsulated lactobacillus at pH 2.5 and 6.5 respectively. Hence this procedure can be applied in various process rather than using alginate encapsulation.

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