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# PROBIOTICATION OF FRUIT JUICES BY *Lactobacillus acidophilus*

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**ABSTRACT:**

The use of probiotics has moved from concept to application. Studies were carried out to isolate and identify microorganisms for probiotic use. Selection of strains included various criteria such as agreement with bio-safety aspects, viability during storage, tolerance to low pH and antimicrobial activity. The strains were isolated from milk curd and compared with those culture procured from Microbial Type Culture Collection and Genbank, Chandigarh. The strains were subjected to acid tolerability. The acidified strains were encapsulated with Sodium Alginate and inoculated to different fruit juices that served as substrates for probiotic fermentation. The characters of the probioticated substrates were studied and were tested for shelf life. It was evidently proved that immobilized cultures remained viable over a long period of time and the probiotically fermented drinks were potentially inhibiting the pathogenic growth. Prospective studies on mechanisms of the probiotic activities may enable their new medical applications for lactose intolerant and diabetic patients.

Keywords: Probiotics, Fermentation, Immobilized cultures, Lactose intolerance.

## I . INTRODUCTION

Probiotics are live microorganisms that when consumed in enough amounts exert their health benefits. [1]. In recent years, fermentation of different fruit juice by probiotic lactic acid bacteria was studied by several authors [2,3] Results revealed that since fruit juices are rich sources of saccharides, they may be served as a suitable medium to cultivate probiotic lactic acid bacteria to enhance the health benefits of the food product. Probiotic bacteria are included as components of the starter cultures for non-dairy foods. [2]. Recent studies report that fruit juices could serve as suitable media for cultivating probiotic bacteria. [4]. The calcium and vitamin-fortified juices, which are consumed casually by the people for health benefits, are essential if the full benefits attributed to probiotics are to be experienced. This marks the peak sale of the fruit juices in the market.[5]. The advancement in technologies for fermentation, encapsulation and freeze-drying probiotic preparations with proven prophylactic and healing action in children and adults against colitis, including ulcerative colitis, gastritis, enteritis, ulcerative disease, intestinal infections, disbacteriosis and some cases of dyspepsia, have been created.[6].

The commonly consumed juices like watermelon, sapodilla, grape and orange were taken for the study as a proper medium for lactic

acid fermentation and the probiotic juices obtained could serve as a health beverage for consumers who are allergic to dairy products. The objective of this study was to increase the fermentation efficiency of *L. acidophilus* in juices by calcium alginate entrapment of the bacteria and check for the antagonistic activity and shelf life of probioticated juice.

## II. MATERIALS AND METHODS:

### 2.1 Isolation of Lactobacillus acidophilus

Lactic acid bacterium was isolated from milk in $10^{-4}$ and $10^{-5}$ dilution with the selective media de Man, Rogossa and Sharpe (MRS medium, Merck). The strain isolation was based on the colony appearance, at initial stages. The isolated strain was compared with that of the Standard Culture (MTCC *447), obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh. Bacteria were proliferated for 24 hours and further sub culturing was carried out after increasing the volume of the medium for the next 48 hours anaerobically at 37°C. [7]

### 2.2 Characterization:

The sub-cultured species were subjected to various biochemical tests. Various tests like grams' staining, catalase test, starch hydrolysis, hydrogen sulphide tests, litmus milk reactions, gelatin hydrolysis, casein hydrolysis, triple sugar ion tests were carried out and the species biochemical activity was measured. The active cultures were grown on the slants and plates of the modified or specified substrate to check the response of the bacillus cultures. The cultures were maintained at 37°C.[8]

### 2.3 Acid tolerance tests:

*L.acidophilus* is inoculated (1%) into the MRS broth acidified with concentrated hydrochloric acid at pH ranging from 2.0 to 5.0.The broth culture is incubated at 37ºC in a temperature controlled mechanical shaker incubator. After 24-32 hours of incubation, the samples from each flask are swabbed on MRS agar plates. The plates are incubated anaerobically at 37ºC and observed for the growth after 24 h to until 96 hours. Individual colonies are selected and recultured in acidified MRS agars for cross checks.[9]

### 2.4 Immobilization:

*L. acidophilus* is cultured statically in MRS broth. 50 ml of the cell suspension were mixed with an equal volume of a 2% sterile (121°C, 15 min.) alginate solution at 21°C. This mix was aseptically added drop by drop to 250 ml of a sterile 0.33 M CaCl solution. Alginate jellification occurred, entrapping lactic acid bacteria in the form of solid beads of 2mm in diameter. Beads remained in CaCl for one hour to permit hardening. CaCl was then removed by decantation and 100 ml of cold (7°C) MRS broth was added. Beads were stored at 4°C until utilized.[10]

### 2.5 Preparation of substrates:

The fruits sapodilla, grapes, orange and watermelon are purchased from the local market. The selected fruits are washed thoroughly with running tap water, rinsed with distilled water and blotted dry. The seeds are separated manually from the pulp. The juice is then extracted by hand pressing and straining the above prepared material through double fold muslin cloth. These juices are filter sterilized and used as substrates for further studies.

### 2.6 Inoculation on to substrates:

A 2% inoculum of *L. acidophilus* is added to the substrates respectively, for cell fermentation. Also, the immobilized cell culture is inoculated in the same set of substrates for immobilized cell fermentation. 100 ml juice(s) were mixed with immobilized cells following removal of the MRS broth by decantation. Inoculated juice was incubated at 37ºC for 80 h. Bacterial counts were immediately taken following the separation of immobilized cells in the fermented products on a comparison basis with the juice inoculated with starter cultures on classical methods.[11]

### 2.7 pH and turbidity:

The pH of the substrates after and before inoculation of the inoculum is checked. The transmittance and absorbance of the fruit substrates is measured using UV Spectrophotometer (Model U-2900/2910, Hitachi High tech, Japan) at 540nm before and after probiotication.

**2.8 Antagonistic Activity of Probioticated Substrate:**

The antagonistic activity of the probioticated substrate is studied against certain pathogenic species. To detect antimicrobial activity of the preparations, the following organisms grown in nutrient broth at 37°C for 24 h are used: *Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus.* Actively growing culture of the test organisms were mixed 2.5% ($2.5 \times 10^7$ cfu/ml) with melted nutrient agar and poured in sterile petri dishes and allowed to solidify. A 1 cm wide ditch was cut in the agar across the centre of the dish. The probioticated substrate was pipeted out into the ditch. When the mixture solidified, the plates were first incubated at 4°C for 60 min to allow the test material to diffuse in the agar and then incubated at 37°C for 18 h. After incubation, the diameter of the clear zone is measured in centimeters from the centre of the well.[12]

**2.9 Shelf Life Study:**

After 72 h of fermentation at 30°C, the fermented samples (25 ml) are stored at 4°C for 4 weeks. Samples are taken at weekly intervals, and viability of probiotic cultures in probioticated juice are determined and expressed as colony forming unit (CFU).

**III RESULTS:**

**3.1 Biochemical characterization**

The pure strains isolated were biochemically characterized.[**Figure 1**]. The colonies showed positive results for Gram's staining, thus confirming *Lactobacillus sp.* It could be inferred from the clear zone observed on starch hydrolysis that the organism produced starch splitting enzyme amylase. The organism is motile in nature as observed from the line of streak in hydrogen sulfide test. When subjected to citrate utilization test organism is capable of using citrate as carbon source that was identified by the presence of growth on the surface of slant accompanied by the blue coloration when subjected to citrate utilization test. The organism secretes proteases thereby exhibiting the zone of proteolysis when subjected to casein hydrolysis. The cultures remain liquefied thereby producing hydrolytic extracellular enzyme capable of degrading gelatin, demonstrating rapid gelatin hydrolysis. From TSI, red slant and yellow butt with gas production indicates the fermentation of glucose and reduced oxygen tension thereby grouping the organism under enterobacteriaceae. The microorganism enzymatically transform milk substrate into various metabolic end products due to which the culture medium for litmus milk reactions begins to lose body and produced translucent whey like appearance. *L. acidophilus* showed tolerance to broad range of pH **[Figure 3]** Growth was better when pH ranged from 3 to 4 and maximum at pH 3.5.

**3.2 Immobilization and inoculation**

The cell suspension obtained from MRS broth contained about $40x10^9$ cells prior to immobilization **[Figure 2]**. 50mL of this suspension was immobilized and inoculated in 100mL of fruit juices. Hence the inoculation rate of the bacteria could be probably estimated as $2.1 \times 10^9$ immobilized cells of *L.acidophilus* per mL of fruit juices. The viable cell number of immobilized cells was found to be higher than that of free cells. Immobilized cells which leaked during fermentation grew in the medium as free cells. Some diffusion of cells from the entrapped gel beads occurred during immobilized cell fermentation because of the growth of bacteria in the immobilized gel beads.

**3.3 pH and turbidity**

The pH and % transmittance of the substrates were measured before and after probiotication. The acidity of the substrates increased during probiotication. Grape juice was found to be better fermented product than the other substrates which might probably due to higher moisture and sugar content. **[Figure 4 & 5]**

**3.4 Antagonistic activity of probioticated substrates**

The inhibitory activity against *Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus* was seen in the zone of clearance in each plate obtained with each organism. The inhibition zone as well as the activity index was calculated and tabulated.**[Table-1]**.The values were represented as mean value of the duplicates

with the difference in the standard deviation. Sapodilla recorded the overall higher and constant activity indices against the three pathogens. Grapes recorded the highest against *E.coli*

**3.5 Shelf life study:**

Significant drop in the initial pH of the probioticated juices was observed. The viable counts of encapsulated cells were slightly decline; whereas, the viable counts of free cells were remarkably dropped during the storage **[Figure 6 & 7].** It was reported that acid production ability by lactic acid bacteria, especially during post-incubation (post-acidification), affected the cell viability of probiotic bacteria [13]. These results indicate that higher viable cell numbers are obtained during immobilized cell fermentation than during free cell fermentation. Better results were achieved by immobilized cell fermentation because immobilized cells were protected from oxygen and high concentrations of substrates and products and unfavorable conditions like low pH.

## IV DISCUSSION

The encapsulation of probiotics in alginate beads can protect the cells inside from the inhibiting compounds, for example acid and flavonoids, in fruit juices. The micro organisms that are encapsulated survive the digestive system of the host and colonize at the place where they can provide the benefits to the host. In the classical fermentation method, a 1% inoculation rate constitutes 1.2 x $10^7$cells per ml of substrate at the beginning of the fermentation. Thus, the utilization of immobilized cells permits much higher inoculation rates since cells can be recuperated and reutilized.

A rapid decrease in pH in the beginning of fermentation is of great importance for the quality of the end product [14]. The rapid increase in acidity minimizes the influence of spoilage bacteria. In the slowly acidified medium, lactic acid fermentation can be suppressed by butyric bacteria activity [15]

The inhibitory action of probiotic bacteria against the pathogens may be due to the accumulation of main primary metabolites such as lactic acid, acetic acids, ethanol and carbon dioxide. Additionally, they also produce secondary metabolites including anti microbial compounds such as formic acid, benzoic acid, hydrogen peroxide, diacetylacetin, and bacteriocin. The production levels and the proportions among these compounds depend on the strain, medium compounds and physical parameters Probiotics have shown to process inhibitory activities mostly towards G+ve pathogens and closely selected bacteria due to the bactericidal effect of protease sensitive bacteriocins.[16] The results of our present study agree with [17] who inferred that antimicrobial sub-stances produced by *Lactobacillus* have a great potential for inhibiting the growth of pathogenic microorganisms.

The level of microencapsulated probiotics in fruit juices was also above the therapeutic level ($10^7$ cfu/ml) throughout the storage. Though the fruit juices provided no better environment for the probiotic organism to survive, the probiotics encapsulated in calcium alginate beads survived for up to a month.

In conclusion the probioticated juices could differ in their antagonistic activities against the pathogens which could be due to the metabolite secreted by the lactic acid bacteria specially type of organic acids. Results obtained in this study will be helpful for developing an appropriate probiotic juice with more health benefits which could be served as a health beverage for vegetarians and consumers who are allergic to dairy products. Although the encapsulation method increases the survival of probiotics in fruit juices, the effect of probiotic beads on the sensory characteristic and consumers' conception should be analyzed further.

## REFERENCES

1. FAO/WHO. Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. Food and Agriculture Organization of the United Nations and World Health Organization [Report July 20, 2003]. http://www.fao.org/es/ESN/Probio/probio.htm .

2. Mousavi Z, Mousavi S, Razavi S, Emam-Djomeh Z, Kiani H. [2011] Fermentation of pomegranate juice by probiotic lactic acid bacteria. *World Journal of Microbiology and Biotechnology* 27:123-128.
3. Yoon KY, Woodams EE, Hang YD. [2006] Production of probiotic cabbage juice by lactic acid bacteria. *Bioresource Technology* 97:1427-1430.
4. Mattila-Sandholm T, Myllarinen P, Crittenden R, Mogensen G, Fonden R, Saarela M. [2002] Technological challenges for future probiotic foods. *Int Dairy J* 12:173–182.
5. Sheehan VM, Ross P, Fitzgerald GF. [2007] Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices. *Innov Food Sci Emerg Technol* 8:279–284.
6. Granato D, Branco GF, Nazzaro F, Cruz AG, Faria JA. [2010] Functional foods and nondairy probiotic food development: trends, concepts, and products. *Comprehensive Reviews in Food Science and Food Safety* 9(3):292-302.
7. Ayad EHE, Nashat S, El-sadek N, Metwaly H, Elsoda M. [2004] Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy product according to production and technological criteria. *Food microbiology* 21:715-725.
8. Bergey DH. [1934]. Bergey's manual of determinative bacteriology. *The American Journal of the Medical Sciences*, 188(2): 282.
9. de Porres E, de Arriola MC, Garcia R.[1985] Lactic acid fermentation of banana puree. *Lebensm Wiss Technol.*18:379-82.
10. Kearney L, Upton M, McLoughlin, A. [1990] Meat fermentations with immobilized lactic acid bacteria. Applied Microbiology and Biotechnology, 33(6): 648-651.
11. Claude P. Champagne, Carole B. Côté**.** [1987] Cream fermentation by immobilized lactic acid bacteria**.** *Biotechnology Letters* 9(5): 329-332.
12. Tagg JR., Dajani, A. S. and Wannamaker, L. W. [1976] Bacteriocines of Gram positive bacteria. Bacteriological Review 40: 722-756
13. Lankaputhra WEV, Shah NP. [1995] Survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the presence of acid and bile salts. *Cult. Dairy Prod. J.* 30:2-7.
14. Viander B, Maki M, Palva A. [2003] Impact of low salt concentration, salt quality on natural large scale sauer kraut fermentation. *Food Microbiology* 20:391-395.
15. Karovicová J, Kohajdová Z. [2003] Lactic acid fermented vegetable juices. *Horticultural Science* 30:152-158.
16. Sheela T, Kulothugan S, Saravanamuthu R. [2011] Antagonistic action of synbiotic carrot juice against diarrhoea causing organisms. *Indian J. Applied & Pure Bio* 26(1):15-22.
17. Soleimani, N. A., Kermanshahi, R. K., Yakhchali, B., & Sattari, T. N. [2010]. Antagonistic activity of probiotic lactobacilli against Staphylococcus aureus isolated from bovine mastitis. *African Journal of Microbiology Research*, 4(20): 2169-2173.

**Figure and Tables:**

Fig 1: Slant of L.acidophilus


Fig2: Immobilized Gel Beads

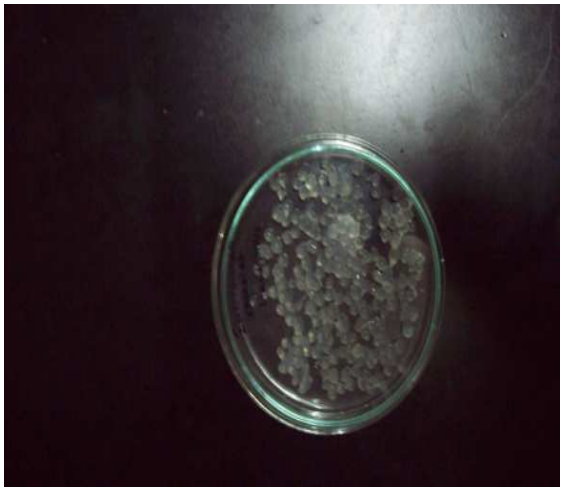


Fig 3: Effect of pH on Colony count

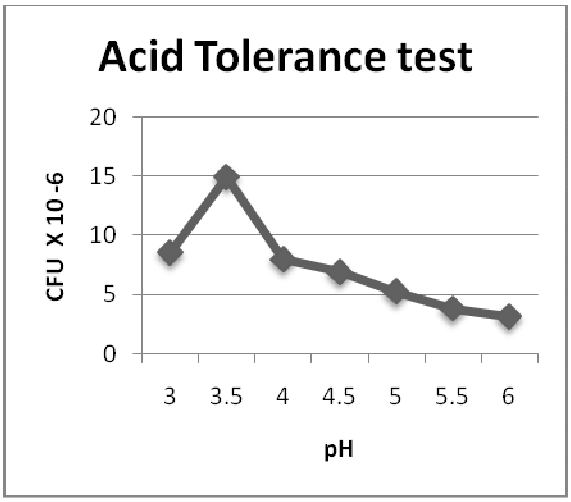



Figure 4: Effect of pH before and after probiotication

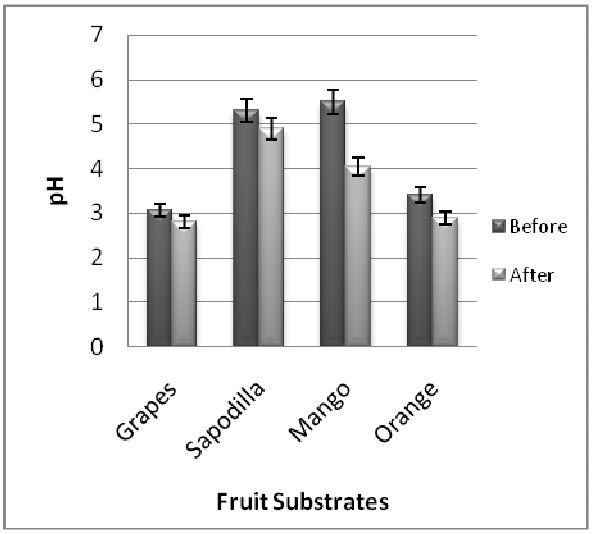



Figure 5: Effect of Transmittance before and after probiotication

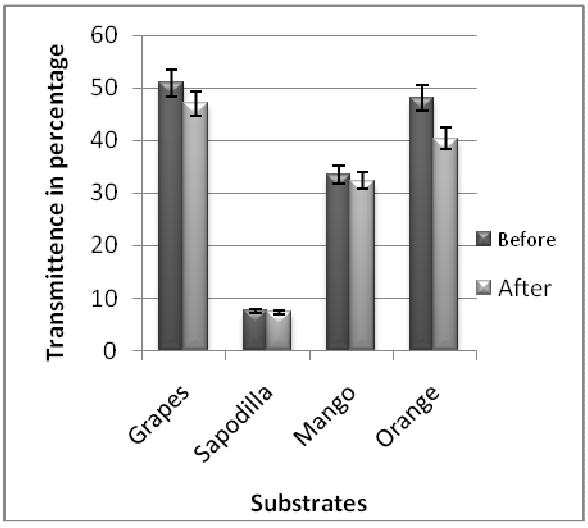



Figure 6: Shelf Life of the probioticated bewerages with immobilized beads.

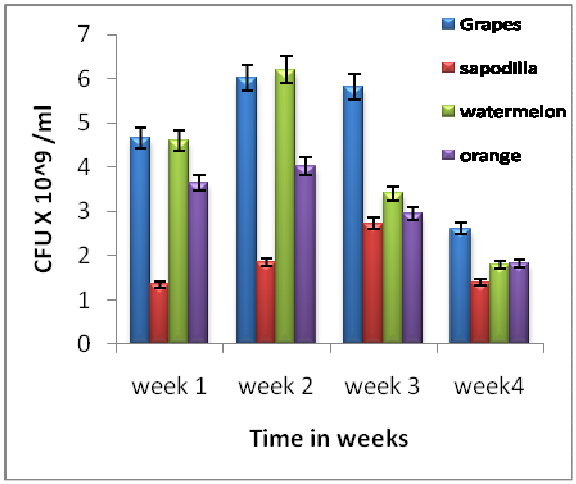
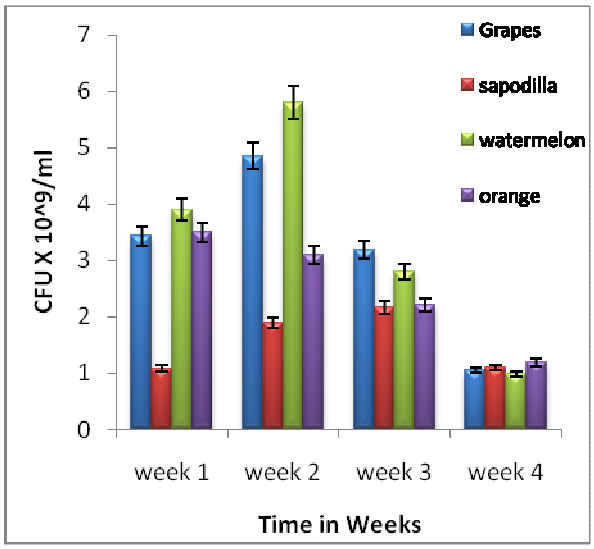



Fig 7: Shelf life of fermented products by free cell fermentation




| Substrate | *Staphylococcus aureus* | *Escherichia coli* | *Salmonella typhimurium* |
|---|---|---|---|
| *Grapes* | 0.516±0.013 | 0.886±0.003 | 0.586±0.013 |
| *Sapodilla* | 0.633±0.003 | 0.676±0.006 | 0.76±0.016 |
| *Watermelon* | 0.413±0.003 | 0.536±0.029 | 0.49+0.064 |
| Orange | 0.586±0.013 | 0.501±0.054 | 0.611+0.27 |
| Tetracycline (Positive control) | 18.73±0.27 | 13.77±1.74 | 16.23±0.86 |

**Table 1: Activity Index of the fermented product against pathogens**

The Positive control used was tetracycline The values are calculated as mean ± SD. AI of the fermented product is reported. Key: AI: Activity Index = Inhibition zone of the test sample divided by inhibition zone of a standard drug.