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PROBIOTICATION OF SWEET ORANGE JUICE USING LACTOBACILLUS STRAINS

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A study was carried out for the production of probiotic sweet orange juice using two lactobacillus strains viz., *Lactobacillus delbrueckii* ssp. bulgaricus and *Lactobacillus plantarum*. Encapsulated and free strains were used to prepare the probiotic juice. Prior to juice extraction sweet oranges were treated with activated charcoal solution and lye peeled to prevent delayed bitterness in the juice. Encapsulation of strains was done using a mixture of sodium alginate and guar gum by extrusion technique and the probiotic beads were added to the juice and incubated at 37 °C for 10 hrs. The probioticated juices were studied for sensory acceptability and cell viability for 4 weeks at 4 °C. Microencapsulation of probiotic strains was found to be suitable in maintaining the cell viability in the juice, since viable cells were approximately 9.00 log cfu/ml even after a storage period of 4 weeks. During storage it was found that the viable cell count and sensory score for juice containing encapsulated strains was higher than juice with free strains.

Keywords: Probiotic sweet orange juice, *Lactobacillus delbrueckii* ssp. bulgaricus, *Lactobacillus plantarum*, Encapsulation, Cell viability

INTRODUCTION

Increasing awareness of health and wellness among people and across the age spectrum in the past two decades is fuelling interests in functional foods. Foods are no longer considered only in terms of taste and immediate nutritional needs but also in terms of their ability to provide specific benefits above and beyond their basic nutritional value. Functional foods targeted towards improving the balance and activity of intestinal milieu provides the largest segment of functional food market (Saarela *et al.*, 2000).

Probiotics are live microbial food ingredients, which provide the consumer with numerous health benefits by improving the intestinal microbial balance. However, probiotic foods available in the markets today, are usually in the form of fermented dairy products such as milk and yogurt and thus probiotication of fruit juices is beneficial as fruits and fruit juice based drinks are important components of the human diet. People choose fruit juices as a drink for many reasons, including relieving thirst, refreshment and nutritional benefits. Furthermore, fruits and vegetables do not contain any dairy allergens that might prevent usage by certain segments of the population (Luckow and Delahunty, 2004). Also, fruit and vegetable juices have an established market sector as a functional drink including calcium and vitamin fortified juices. It has been suggested that fruit juices could serve as a good medium for cultivating of probiotics (Mattila-Sandholm *et al.*, 2002).Different studies have been implemented to in- vestigate the suitability of fruit juices such as tomato, orange, grape, carrot, pomegranate, beet and cabbage juices as raw vegetables and fruits for probiotic drink production.

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Lactobacillus plantarum, L. delbrueckii, L. acidophi- lus, *L. casei* and *L. paracasei* have been used as probiotic cultures. Results have shown that all the strains of pro- biotic bacteria are capable of growth in the mentioned juices. Moreover, *L. plantarum, L. acidophilus* and *L. delbrueckii* have shown to be resistant to high acidic and low pH conditions during storage pe- riods at 4 °C for four weeks (Rasic, 2003; King *et al.*, 2007; Mousavi *et al.*, 2011; and Tamminen *et al.*, 2013).

The role of citrus fruits in providing nutrients and medicinal value has been recognized since ancient times. The fruits are known for their refreshing fragrance, thirst-quenching ability, and providing adequate vitamin C as per Recommended Dietary Allowance (RDA). In addition to ascorbic acid, these fruits contain several vitamins and phytochemicals**.** Sweet orange (*Citrus sinensis* L. Osbeck) is a widely grown sweet orange cultivar in India and most popular in Maharashtra, medium to large fruit, thin peeled, juicy nucellar selections available, acidity about 0.35-0.5 per cent when fully mature and hence sweet to ûat taste when stored, slightly acidic fruit with higher acidity and greenish-yellow color rind are better, early maturity (Ladaniya, 2008).

Viability maintenance of probiotic cells throughout food-processing, storage and gastro-intestinal transit is important for the microorganisms to reach the intended site of action in sufficient numbers. International Dairy Federation (IDF) has suggested that a minimum of 10^7 cfu/ml probiotic bacterial cells should be alive at the time of consumption per gram of the product. Probiotic stability in fruit and vegetable juice products is difficult to maintain during cold storage however probiotic encapsulation might solve this problem. Providing probiotic living cells with a physical barrier against adverse external conditions by microencapsulation is an approach currently receiving considerable interest. Microencapsulation helps to separate a core material from its environment until it is released. It protects the unstable core from its environment, thereby improving its stability, extends the core's shelf life and provides a sustained and controlled release (Franjione and Vasishtha, 1995; and Gibbs *et al.*, 1999).

Numerous studies have been carried out on probiotic foods and its health benefits and it is also being increasingly promoted by health professionals. Thus the objective of this study is to determine the suitability of the sweet orange juice as a probiotic drink. Also the present work studies the sensory quality and cell viability of juice with encapsulated strains and free strains during a storage period of 4 weeks.

MATERIALS AND METHODS

Probiotic Strains

The probiotic strains: *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus plantarum* were isolated and identified in Department of Food and Industrial Microbiology, College of Food Technology, VNMKV, Parbhani, Maharashtra and further confirmation of the results were carried out by genotypic identification (16S rRNA multiplex PCR analysis) at ARI Pune.

Preparation of Probiotic Cultures

The lactobacilli strains viz. *Lactobacillus delbrueckii* ssp.*bulgaricus* and *Lactobacillus plantarum* were individually activated in MRS broth at 37 ºC for 24 h and further subculturing was done. The starter culture was prepared by mixing equal amounts of cultivated broths and centrifuged at 4000 rpm for 10min.The harvested cells were washed twice with sterile water to remove the residual MRS media.

Sweet Orange Juice Extraction

Sweet oranges (cv. Mosambi) were procured from the local market of Parbhani, Maharashtra. The rind of the fruits were removed manually and the fruits were dipped in 1% activated charcoal solution and allowed to stand for 1hr.This was done to adsorb the bitter precursors from the fruit surface and the core. The fruits were then lye peeled by dipping it in boiling lye solution (1.5%) for 2 mins to remove the albedo section which is the major contributor of limonin precursors during juice extraction (Sheu and Marshall, 1993; and Teanpaisan *et al.*, 2015). Standardization of the concentration level and time of both the treatments were done based on the extent of broken segments after lye treatment, taste(bitterness reduction) and aroma of the juice. After lye treatment, the fruits were washed thoroughly in running tap water and the remaining alkali was neutralized by dipping in citric acid solution (1%) for 1min and washed again thoroughly. Juice was extracted without pressing the seeds and subsequently the juice was filtered using a strainer and the filtered juice is collected in a dispenser and pasteurized at 90 °C for 2 mins.

Chemical Analysis

Chemical parameters plays a significant role in the quality of the juice and also survival of probiotic cultures. In the

present investigation it was important to analyse the chemical properties of the sweet orange fruit to study the nutritional and chemical changes in the juice due to microbial activity and storage. The Total Soluble Solids (TSS) of probiotic juice, was determined using a hand refractometer (ERMA) corrected at 20 °C. The pH of the juice was measured using an electronic digital pH meter. Percent acidity was determined by titrating the sample against 0.1 N NaOH using phenolphthalein as indicator. Estimation of total sugars was performed by phenol sulphuric acid method and reducing sugars was determined by Nelson-Somogyi method. The non-reducing sugar was obtained by subtracting reducing sugars from total sugars. Ascorbic acid contents of the samples were obtained using 2, 6-dichlorophenol indophenol method.

Encapsulation of Probiotic Strains

Encapsulation of strains was done by extrusion method (Kore and Chakraborty, 2005). Probiotic culture at 10% (v/v) of the final juice was encapsulated using a combination of sodium alginate and guar gum at 1 and 0.8% (w/v) respectively. The cell suspension contained about $4.3x10^9$ cells per ml prior to encapsulation.10 ml of the probiotic culture was mixed with sterile sodium alginate-guar gum solution at a ratio of 1:2 and the mixture was placed in a sterile syringe and dropped into 0.3 M calcium chloride solution. Interaction between the two solutions led to formation of beads and the resultant beads were the resulting beads were then stored in 0.1% peptone solution at 4 °C.

Inoculation into Substrate

For preparation of probiotic sweet orange juice with free strains, the probiotic culture is added to the juice at 10% (v/v) inoculum level and incubated at 37 °C for 10 hrs. For preparation of probiotic juice with encapsulated strains, the washed beads were aseptically added to the pasteurized fruit juice and incubated at 37 °C for 10 hrs. Post incubation the probiotic juice samples were stored at 4 °C for a period of 4 weeks.

Organoleptic Evaluation

The sensory acceptance of probiotic sweet orange juice with free strains and encapsulated strains was performed after incubation and also at weekly intervals during storage at 4 °C.The sweet orange juice samples were evaluated for their sensory characteristics namely, color, taste, flavor and overall acceptability by a trained panel. A panel of 20 members were asked to rate the product on 9 point Hedonic scale with corresponding descriptive terms ranging from 1 = dislike very much, to 5 = neither like nor dislike, to 9 = like extremely well, i.e., higher sensory score indicated better overall acceptance. 9 'like extremely' to 1 'dislike extremely'.

Cell Viability Count

The viability of probiotic cells during storage is of paramount importance because for a probiotic food to confer health benefit the number of cells should be > 10^7 cfu/ml or gm at the time of consumption.Viable cell count of juice with free cells was analyzed at weekly intervals by the Standard Plate Count (SPC) method with MRS medium at 37 °C for 48 hrs. To determine the viability of encapsulated strains in the juice, the enumeration was done by releasing the entrapped strains from the capsules using the method suggested by Sheu and Marshal (Sandhu and Singh, 2001). The capsules were depolymerized by using a solution (28 ml of 0.2 M NaH_2PO_4 and 72 ml of 0.2 M Na_2HPO_4 adjusted to 200 ml with distilled water, pH 7.1 ± 0.1, sterilized). After incubation at 37 °C for 10 min, the mixture was vortexed at high speed for breaking the polymer and releasing completely the encapsulated culture into the buffer. The released cells were enumerated using MRS media at 37 °C for 24-48 hrs.

RESULTS AND DISCUSSION

Chemical Analysis

The samples were analysed quantitatively for their chemical compositions before and after probiotication. The data of various parameters viz. total soluble solids, pH, percent acidity, total sugars, reducing sugars, non reducing sugars and ascorbic acid content are presented in Table 1. Post incubation at 37 ºC for 10 hrs it was observed that the free strains reduced the TSS of the juice from 12ºBx to 11.4ºBx and the encapsulated strains reduced it to 11.6ºBx. The pH reduced for both the samples with increase in per cent acidity showing inverse relationship between pH and acidity. Martin-Diana *et al.* (2003) also reported that adding probiotic starter culture caused decrease in pH value of the beverage at the same time titratable acidity was found to be increased. Total sugars for juice with free strains and encapsulated strains were found to be 6.1 and 6.4 respectively showing that free strains used up more sugars for the same time. The ascorbic content of both the samples decreased to 40 mg/100 ml which may have been due to heat treatment during juice pasteurization.

Table 1: Chemical Analysis of Probiotic Sweet Orange Juice

Properties	Before Addition of Strains	Juice with Free Strains	Juice with Encapsulated Strains
TSS (ºBx)	12	11.4	11.6
% Acidity	0.41	0.82	0.77
pH	3.9	3.51	3.68
Total Sugars (%)	8.36	6.1	6.4
Reducing Sugars (%)	1.8	1.5	1.7
Non Reducing Sugars (%)	6.6	4.6	4.9
Ascorbic Acid (mg/100 ml)	47.6	40	40

Note: * Each value is an average of 3 determinations.

Organolpetic Evaluation

The sensory evaluation was performed to examine the acceptance of probiotic juice by consumer against its taste, flavor and overall acceptability characteristics.Sensory scores of the freshly prepared probiotic juice samples are demonstrated in Table 2. According to the score, there was no significant difference in overall acceptability of the freshly prepared probiotic sweet orange juice samples containing free and encapsulated strains. However, considering the slightly higher score for juice with encapsulated strains, it can be concluded that the prevention of excess utilization of sugars by encapsulated strains which controlled the pH and per cent acidity production at optimum level may have resulted in better acceptability of the sample. The panellists also experienced improvement in taste of both the juice samples after probiotication.

Table 2: Mean Sensory Score of Freshly Prepared Probiotic Sweet Orange Juice Samples

Sample	Color	Taste	Flavor	Overall Acceptability
Control	8.6	8.3	8.1	8.2
Juice with free strains	8.3	8.4	8.5	8.4
Juice with encapsulated strains	8.5	8.6	8.5	8.5
SE	0.13176	0.06455	0.05528	0.02357
CD @ 1%	0.5443	0.26665	0.22835	0.09737

Note: * 9 Point Hedonic Scale.

Table 3 shows the overall acceptability of the juice containing free strains and encapsulated strains during a storage period of 4 weeks at 4 ºC. The overall acceptability score reduced with increase in storage period for both the samples, but it was observed that the overall acceptability score for juice containing encapsulated strains was always higher than juice with free stains throughout the storage. Thus, encapsulation clearly appeared to be effective in maintaining the sensory quality of the juice. This may be attributed to the inhibition of unfavourable deterioration reactions due to encapsulation. Similar results were reported in a study by King *et al.* (2007) where sensory scores of tomato juice containing microencapsulated probiotics were higher than that of free cells during refrigeration storage. Although, juice containing probiotic beads is a new concept, panel members compared the product to those of commercial juices containing juice sacs and thus found it acceptable.

Cell Viability of Juice with Free and Encapsulated Strains During Storage at 4 ºC

The cell viability of probiotic juice samples during storage is shown in Table 4. Post incubation at 37 ºC for 10 hrs, the cell count of the juice with free strains was found to be higher indicating more utilization of sugars and better growth while the cell count of the juice with encapsulated

Table 3: Sensory Score (Overall Acceptance) of Sweet Orange Juice Containing Free and Encapsulated Probiotic Strains During Storage (4 ºC)

Time in Weeks	Juice with Free Strains	Juice with Encapsulated Strains
0	8.4	8.5
1	8	8.3
2	7.7	8
3	7.3	7.9
4	7	7.5

Note: * 9 Point Hedonic Scale.

Table 4: Effect of Refrigerated Storage (4 °C) on the Viable Cell Viability of Free and Encapsulated Probiotic Strains in Sweet Orange Juice

Time in Weeks	Juice with Free Strains (cfu/ml)	Juice with Encapsulated Strains (cfu/ml)
0	$3.5x10^9$	$3.0x10^9$
1	$6.3x10^8$	$3.1x10^9$
2	$5.8x10^7$	$4.7x10^9$
3	$2.9x10^7$	$2.6x10^9$
4	$3.3x10^6$	$1.5x10^9$

strains was comparatively lower. However, during storage the viable count in the probiotic beads increased from an initial number of $3.0x10^9$ to $4.7x10^9$ during second week and further declined to $1.5x10^9$ in the fourth week. It was found that the viable cell count of encapsulated strains was maintained at 10^9 cfu/ml even after 4 weeks of storage. Free strains gradually lost their viability with increase in storage period and by 4th week the cell count decreased to 10^6 cfu/ml. These results indicates that encapsulation of strains increased probiotic survivability in the juice as encapsulated strains showed better survival than free strains after a refrigerated storage (4 °C) of 4 weeks. The encapsulation provided a protective barrier from the low pH and high acidity of the medium which would have otherwise affected the survival of the strains as it did in case of free strains. A study by King *et al.* (2007) showed that immobilized cells of probiotic lactic acid bacteria retained more during the cold storage period of ten weeks in fermented tomato juice compared with free cells because the immobilized cells were protected from oxygen, high concentrations of substrate and products, and unfavorable conditions such as low pH and high acidity. Ding and Shah (2008) also reported that probiotics encapsulated in orange juice and apple juice are more durable than the free cells.

CONCLUSION

From the study it can be concluded that sweet orange juice is a suitable substrate for the culture of probiotics as the strains grew and survived well when added to the juice and it was also found to be organoleptically acceptable. However, probiotic sweet orange juice prepared with encapsulated strains was found to be more acceptable than juice with free strains with increase in storage time. Also, encapsulated strains showed better survival with a cell count of 9 log cfu/ml even after 4 weeks of storage when compared to free strains which reduced to 6 log cfu/ml by the end of the 4th week indicating that encapsulation helps in maintaining cell viability effectively. Further, it was also observed that probiotication of the juice helped in improving the taste of the drink as experienced by the sensory panellists.

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APPENDIX

Figure 1: Overall Acceptability of Probiotic Sweet Orange Juice with Free and Encapsulated Strains During Storage (4 °C)

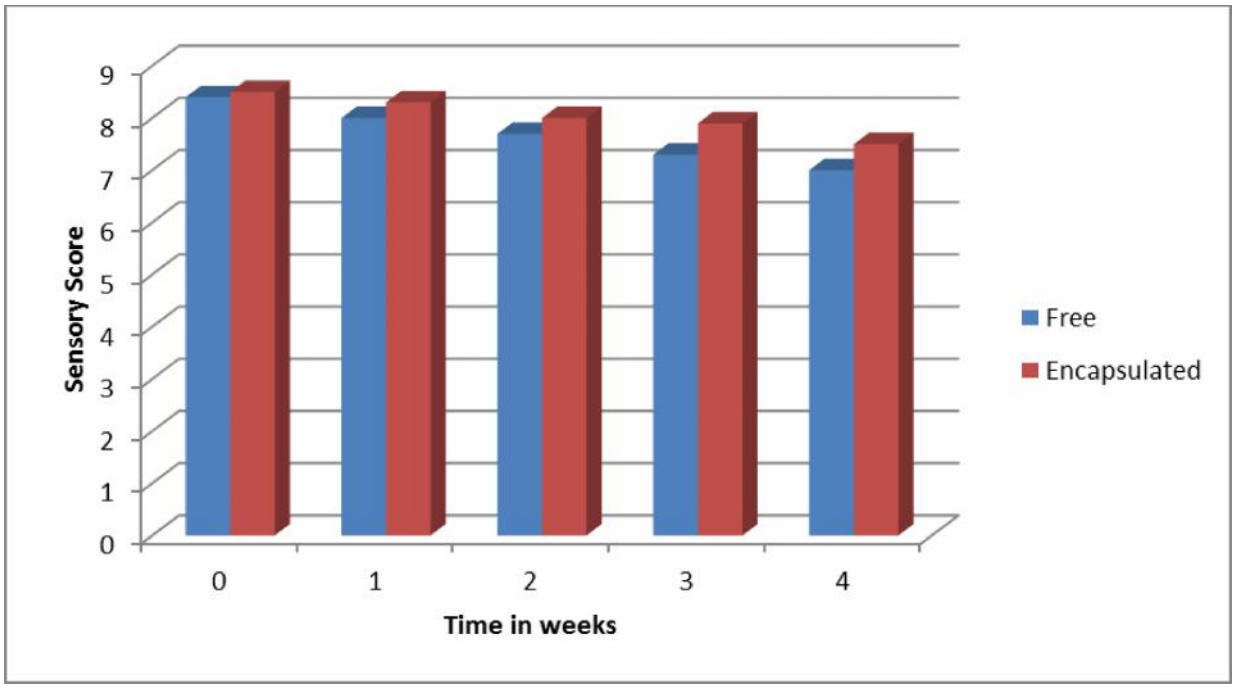


Figure 2: Cell Viability of Probiotic Sweet Orange Juice with Free and Encapsulated Strains During Storage (4 ºC)

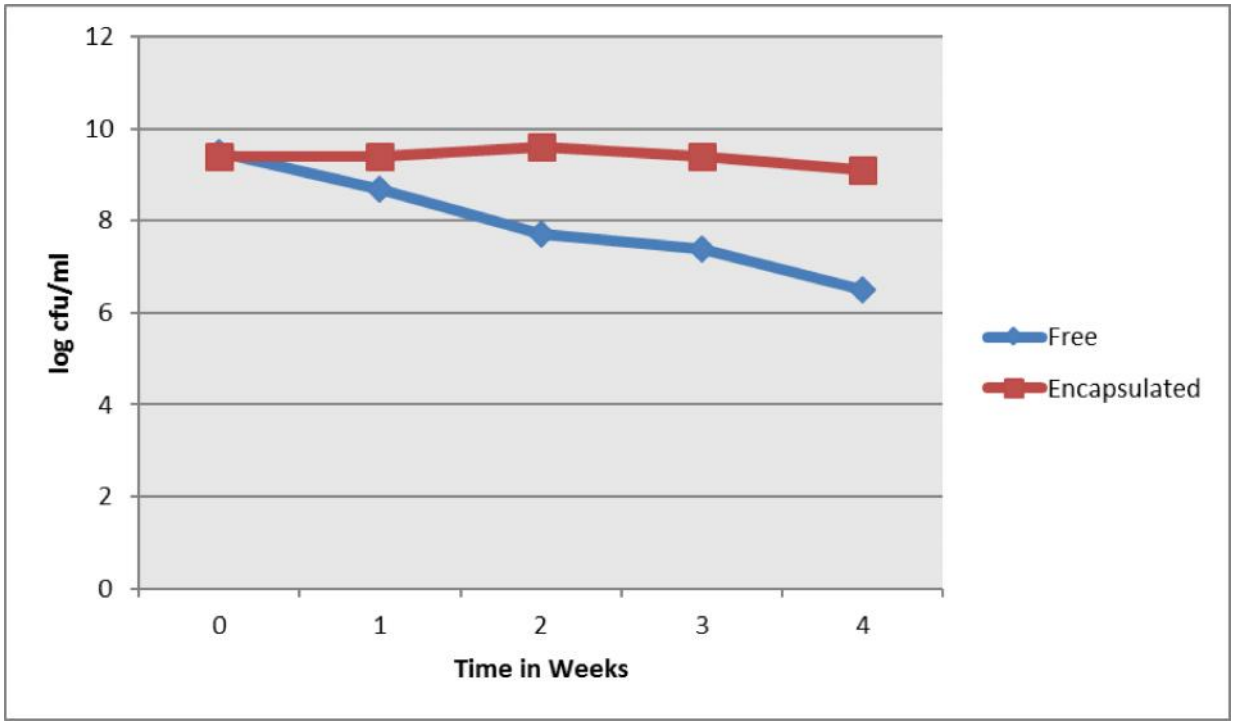
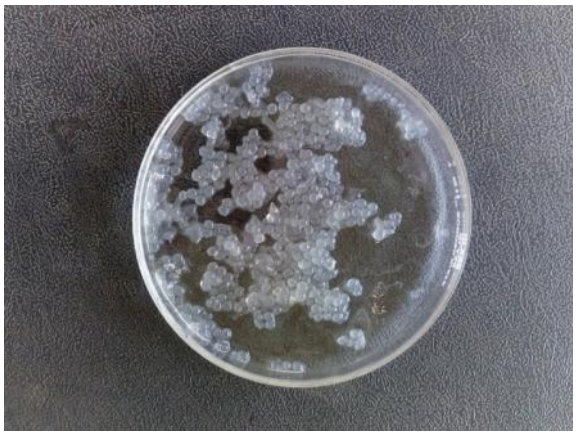


Figure 3: Lye Peeled Sweet Oranges

Figure 4: Probiotic Beads

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