ISOLATION, CHARACTERIZATION AND ENCAPSULATION OF *LACTOBACILLIUS SPP.* TO BE USED AS PROBIOTICS

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Highlights

Isolation of *lactobacillius spp* and probiotic characterization

Encapsulation of probiotic *lactobacillius spp*

Yogurt production

Abstract

Probiotics are live bacteria and yeasts that are useful for human health, especially human digestive system. The viability of probiotics is affected by many factors such as pH, water activity, storage conditions and processing during their storage. The objective of the present work was to isolate different lactic acid bacteria from milk and curd samples which may act as putative probiotics. 9 isolates were obtained from different samples by direct isolation method. The isolates were characterized with respect to their morphological and physiological features. It was found that the isolates L2, L3, L8 and L9 showed higher acid and bile salt tolerance as compared to others. Further, antimicrobial activity of the isolates L8 and L9 showed higher antimicrobial activity as compared to others. Hence, the isolates L8 and L9 were selected to act as putative probiotics useful for human. In order to enhance the life of probiotic bacterial cells, they were encapsulated by forming beads. This encapsulation technique for protection of bacterial cells resulted in enhanced viability of L8 and L9. The beads formulated thus enhanced the survival of bacterial cells count of 11.73 log cfu /g and 11.42 log cfu /g for L8 and L9 respectively. Henceforth, the isolate L8 showed higher yogurt production as compared to L9. Further, encapsulation of isolate L8 led to enhancement in the yoghurt production. Hence, encapsulation is an important technique for improving the shelf life of probiotic bacteria which can be useful in production of flavored yogurt.

Keywords: probiotic, encapsulation, yogurt, *Lactobacillus spp.*

Introduction

Probiotic means a microorganism introduced into the body for its beneficial qualities. Probiotics are live bacteria and yeasts that are useful for human health, especially human digestive system. Bacteria are usually thought of as germs causing diseases. But human body is full of bacteria, both useful and harmful. Probiotics are often called “helpful” bacteria because they keep human gut healthy. The term probiotic is used for ingested microorganisms associated with benefits for humans and animals. According to the world health organization (WHO) 2001, the definition of probiotic is live microorganisms which when administered in adequate amounts; confer a health benefit to the host. Probiotics are generally used to prevent diarrhea, gas and cramping caused by antibiotics. Antibiotics kill beneficial bacteria along with the bacteria that cause illness. A decrease in beneficial bacteria may lead to digestive problems along with other infections, such as vaginal and urinary infections. Probiotic intake may help to gain the lost beneficial bacteria. Probiotics may also be used to prevent infections in the digestive tract, control immune response (inflammation), as in inflammatory bowel disease (IBD). Probiotics are being studied for benefits in colon cancer, skin infections and irritable bowel syndrome (IBS).Health benefits have mainly been demonstrated for specific probiotic strains of different genera, viz. Lactobacillus, Bifidobacterium, Saccharomyces, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Bacillus, Escherichia, etc. Lactic acid bacteria (LAB) are an order of Gram-positive, low GC, acid tolerant, non-sporulating, rod or coccus shaped bacteria. These bacteria are usually found in decomposing plants and milk products, which produce lactic acid as the major metabolic end product of carbohydrate fermentation. Probiotic bacteria are used in production of functional foods and pharmaceutical products. However, the problems of probiotic application as a food additive in animal feed for livestock are the longevity of probiotic cells and the required

properties of probiotics during storage and in the intestinal tract A plausible solution to these problems is to encapsulate probiotic cells. For the protection of Probiotic cells encapsulation techniques are used. In order to achieve the desired health benefits, probiotic bacteria should be contained in the probiotic in higher viable count during their whole product shelf life, which is required for successfully production of foods Cell immobilization is done by various methods like cross linking, adsorption, covalent bonding, encapsulation and entrapment. Various methods used for encapsulation of microbial cells include extrusion, emulsion, freeze drying and spray drying. Extrusion is the oldest and the most common approach to make capsules with hydrocolloids.

MATERIALS & METHODS

Sample collection

The various milk and curd samples were collected from the some local market and cow stable from the area of Ganesh Sisodra Navsari, Gujarat-396427.

Isolation and screening of lactic acid bacteria from milk and curd samples

Take 1ml of sample and carried out serial dilution 10^{-1} , 10^{-2}, 10^{-3} up to 10^{-6} then from each dilution take 0.1 ml of sample and spread plated on specific medium of MRS agar plate (gm/l) containing peptone 10 g , beef extract 10 g, yeast extract 5 g, dextrose 20 g, Tween 80 1.0 g, ammonium citrate 2.0 g, MgSO4 5.0 g, MnSO4 0.100 g, sodium acetate 0.050 g and agar 20 g

and containing 1% $CaCo_3$ for lactic acid bacteria. Colonies forming a clear zone on the MRS agar plate were selected. Selected strains were characterized by physiological criteria, cell morphology was microscopically determined. (Lim *et al.*, 2009).

Acid tolerance of lactic acid bacteria

Inoculated (1%) into MRS broth that had been acidified to pH -2.5 (using Hcl) or non-acidified MRS broth, and incubated at 37°C for 2h. Then, the number of viable cells was determined by 0.1ml aliquots were spread evenly on MRS agar plates were incubated at 37°C for 24h, and the colony-forming units (CFU) were estimated (Chou and Weimer, 1999).

Bile salt tolerance of lactic acid bacteria

Inoculated (1%) into MRS broth containing 5% bile salt and non-bile salted MRS broth, incubated at 37°C for 24 h. Then, the number of viable cells was determined by 0.1ml aliquots were spread evenly on MRS agar plates were incubated at 37°C for 24h, and the colony forming units (CFU) were estimated (Lim *et al.*, 2009).

Antimicrobial activity

The agar well diffusion method was used for the activity. The nutrient agar with the inoculated 0.1ml culture of *E.coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* different plates poured in sterile Petri plates. After solidification, wells were perforated with a sterile 8 mm cork porer. The culture filterates (100µl) were placed into each well. The plates were incubated at 37°C for 24h. After incubation examined clear zones of inhibition. The diameters of the inhibition zones were measured (Papamanoli et *al.,* 2002).

Encapsulation of isolate L8 and L9

Inoculated isolates into MRS broth (50ml) incubated at 37°C for 24h. After incubation centrifuge at 10,000 rpm for 10 min. remove supernatant and dissolve cell palates in distilled water and add at a ratio of 1:5 (v/v) to alginate solution. Encapsulate extrusion method was performed by expression of the wall material-culture mixture through 10ml pipette drop-wise into CaCl2. Hardening time of beads 1h at 4°C. After dry at 40°C for 24h then drying store at 4°C (Woraharn *et al.*,2010).

Bio-chemical test

Overnight grown culture of the obtain isolates was inoculated in different biochemical media. The tubes were incubated at 37 C for 24h. After incubation the biochemical characteristic were observed. The obtain results were used to identified the putative bacteria by following Bergey's manual of determinative bacteriology (Holt et al., 1994).

Viability assay of cells in calcium alginate beads

Dry calcium alginate beads were dissolved in sodium phosphate buffered saline (pH-7.0). A serial dilution of this suspension was carried out. The cell suspension was enumerated by spread plate on MRS agar. The plates were then incubated at 37°C for 24h.colonies of bacteria were counted and converted to log CFU (colony forming units) (Woraharn *et al.*,2010).

Production of various flavored yogurt

Heat (pasteurize) the milk to 203°F (95°C) for 10-20 min. cool the pasteurized milk to 107-110°F (41-43°C) with place into cold water. Add starter culture (2%) in the cooled milk and mix them well. Place the inoculated milk in plastic containers and close them with lids. Incubate milk at 110°F (43°C) for 6-12 h. After incubation add 10% dry banana, chickoo and coconut powder respectively for flavored yogurt production (Mbaeyi-Nwaoha *et al.*, 2017).The advantages of using alginate as an encapsulating agent include non-toxicity, formation of gentle matrices with calcium chloride, simplicity in entrapping living microbial cells and low cost (Chavarri *et al*., 2003). Some important applications of microencapsulation in the food industry are stabling the core materials, controlling the oxidative reaction, providing sustained or controlled release, extending the shelf life and protecting components against nutritional loss, masking flavor, color and odors (Anal and Singh, 2007)

Results and Discussion

Isolation and screening of lactic acid bacteria

9 different isolates forming a zone of clearance on MRS agar plate were selected. Isolates L1, L2 and L8 were obtained from milk sample whereas isolates L3, L4, L5, L6 and L7 were obtain from curd sample. Table 4.1 describes the colony and cell morphologies of the obtained isolates. Martin *et al*. (2006) reported 2 types of *Lactobacillus* strains were obtained from milk samples. Maria *et al*. (2008) reported 8 isolates from 22 different milk samples. Arryo *et al*. (2010) reported 2 strains were obtained from different milk samples. As compared to these reports, 9 different isolates were obtained from only 2 milk and curd samples.

Acid tolerance

Before reaching the gastrointestinal tract, probiotic bacteria must first survive transit through the stomach and have their health promoting effects as metabolically viable active cells when they arrive in the colon. Therefore, isolates were screened for their acid tolerance. The 9 selected isolates showed acid tolerance activity shown in (fig.4.3) Out of the 9 isolates L1, L2, L3, L5, L6, L8 and L9 showed acid tolerance where as L4 and L7 could not survive the pH -2.5 . Lan-szu chou et.al. (1998) reported resistance to acidic pH-3.5 compare to present experiment resistance to acidic pH-2.5. In vitro studies Hood and Zottola, (1988); Charteris et al. (1998a) reported that enteric lactobacilli had a lower pH tolerance limit of 2.0 for several min. Erkkila and Petaja, (2000) reported the number of surviving bacteria was decreased from the inoculated level of to < 4 log cfu / ml at pH 1.0 and pH 2.0, whereas pH 4.0 and 5.0 did not affect the viability. Out of the 9 isolates L1, L2, L3, L5, L6, L8 and L9 showed acid tolerance at pH -2.5 surviving bacteria 4.50 log cfu/ ml.

Fig: **1 Acid tolerances of isolates.**

Bile salt tolerance

Probiotics should tolerate bile-salt because of its presence in high amount in the human stomach. Out of the 9 isolates, L1, L2, L3, L5, L6, L8 and L9 showed bile salt tolerance at 5% bile salt concentration (Fig 4.2). The obtained by Chou and Weimer (1999) reported 0.3% bile salt resistance isolates were obtained. The obtained by Papamanoli et al. (2003) reported 2.0% bile salt resistance. As reported, the isolate obtained in present studies showed 5% bile salt tolerance.

Fig: 2 Bile-salt tolerance of isolates.

Antimicrobial activity

Another essential condition for probiotic activity was the productive capacity of inhibitory substances against pathogenic strains. The isolates L8 and L9 showed higher antimicrobial activity against *E. coli, S. aureus*, *P. aeruginosa* as compared to other isolates (Fig.4.3). Papamanoli *et al*. (2003) reported 147 isolates showing antimicrobial activity against *L. monocytogenes* and *S.aureus.* Chuayana *et al*. (2003) reported Nestle yogurt probiotics were bactericidal for *S.aureus* and *P. aeruginosa* but inhibitory for *S. typhi*. Neslac probiotics killed *E. coli* and *S. typhi* while they were only inhibitory for *S.aureus* and *C. albicans*, probiotics inhibited *C. albicans*. papamanoli *et.al*.(2003) antimicrobial activity performed against *L. monocytogenes* and *S.aureus* compare to present experiment *microorganisms E.coli, S.aureus, P.aeruginosa* were used.

Fig: 3 Antimicrobial activity of isolate L8 and L9 against *E.coli, S.aureus, P.aeruginosa.*

Encapsulation of isolates L8 and L9

The microbial cells of isolates L8 and L9 were encapsulated with 2% sodium alginate for protection and enhancement of shelf life (Fig.4.4). As compare to Ozer et al. (2009) reported the viability of Bifidobacterium bifidum BB-12 and L. acidophilus LA-5 encapsulated in 3% sodium-alginate by extrusion method.

Biochemical characterization of isolates L8 and L9

Form the biochemical characteristic the isolate L8 was similar to *L. rhamnous* and isolate L9 was similar to *L. casei* according to bergey's manual of bacteriology (Holt *et al*., 1994)

Viability of encapsulated beads of isolates L8 and L9

Encapsulation was enhances the shelf life of microbial cells. The viability of these encapsulated microbial cells of isolates L8 and L9 was checked. Upon encapsulation the viability of microbial cells of isolates L8 and L9 increased till 35 days of storage at 4°C. The isolate L8 showed higher viability compare to isolate L9 was 11.73 log cfu/ g and 11.42 log cfu/ g respectively, as shown (Fig.4). Mandal et al. (2005) reported 5.60 log cfu/ g. Woraharn et.al. (2010) reported viability 11 log cfu/ g till 8 weeks storage.

Fig: 4 Viability assay of encapsulated isolate L8 and L9.

Yogurt production

Isolates L8 and L9 showed successfully yogurt production. During production of plain yogurt with Isolate L8 and L9 the L8 was selected for higher yogurt formation in lesser number times. Hence, Isolate L8 selected for further flavored yogurt production like chickoo, banana and coconut

Fig: 5 various flavored yogurt (A) Plain yogurt, (B) Chickoo yogurt,

(C) Coconut yogurt and (D) Banana yogurt.

CONCLUSION

In this study, 9 isolates were obtained from different milk and curd samples by direct isolation techniques. The isolates were characterized with respect to their morphological features. In order to check the potential of the obtained isolates to act as putative probiotics, their acid and bile salt tolerance abilities were checked. It was found that the isolates L2, L3, L8 and L9 showed higher acid (pH -2.5) and bile salt **Viability assay of encapsulated isolate L8 and L9.** (5%) tolerance as compared to others. Further, antimicrobial activity of the isolates against Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus was checked. It was found that the isolates L8 and L9 showed higher antimicrobial activity as compared to others. Hence, the isolates L8 and L9 were selected to act as putative probiotics useful for human health. In order to enhance the life of probiotic bacterial cells, they were encapsulated by forming beads using 2.0 % (w/v) sodium alginate solution. This encapsulation technique for protection of bacterial cells resulted in enhanced viability of the isolates L8 and L9. The beads form thus enhanced the survival of bacterial cells to the count of 11.73 log cfu /g and 11.42 log cfu /g for L8 and L9 respectively when stored at 4°C for 35 days. Further, the isolate L8 and L9 were used for yogurt production. Further, encapsulation of isolate L8 led to enhancement in the yoghurt production as compared to isolate L9. Hence, encapsulation is an important technique for improving the shelf life of probiotic bacteria which can be useful in production of flavoured yogurt.

Acknowledgments

This study was supported by a Department Of Microbiology, Naran Lala College of Professional And Applied Sciences, Navsari, Gujarat, India.

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Fig

Fig: **1 Acid Tolerances of Isolates.**

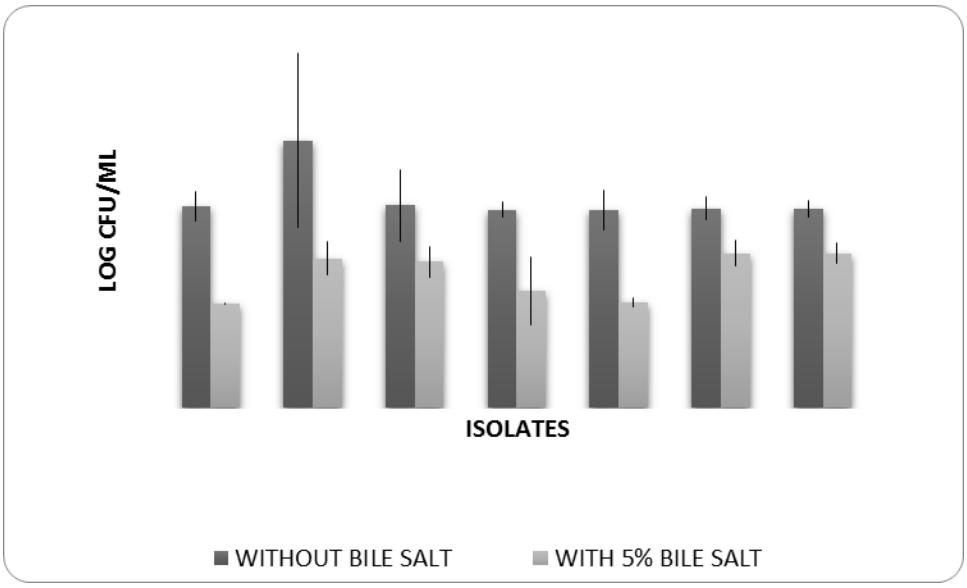

Fig: 2 Bile-Salt Tolerance Of Isolates.

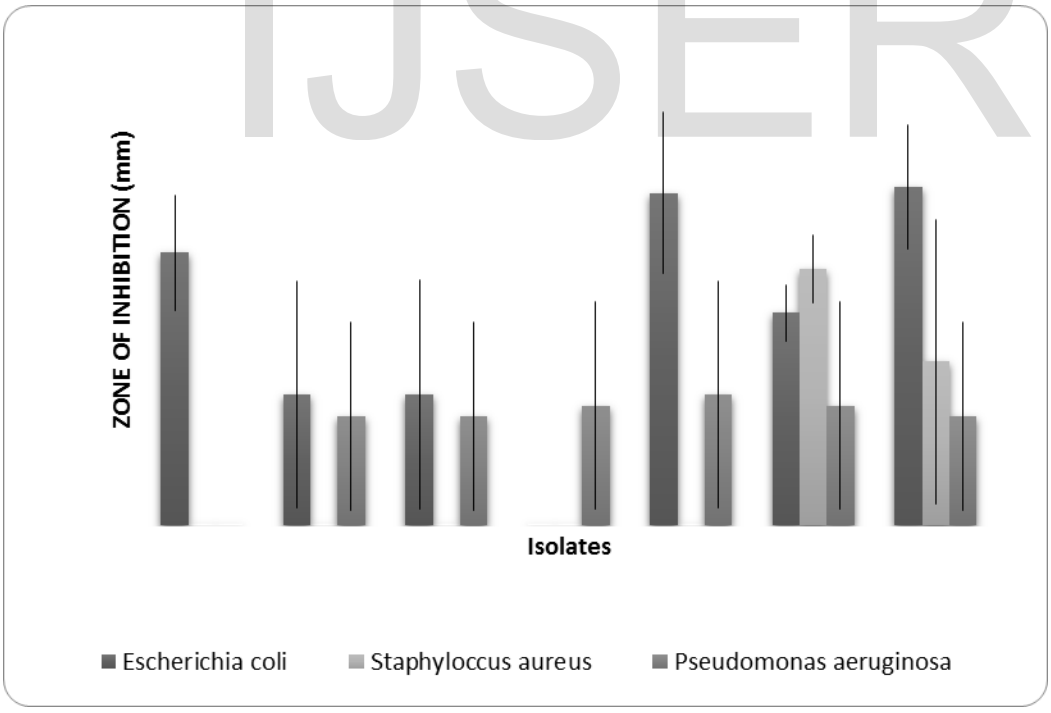

Fig: 3 Antimicrobial Activity of Isolate L8 And L9 Against *E.Coli, S.Aureus, P.Aeruginosa.*

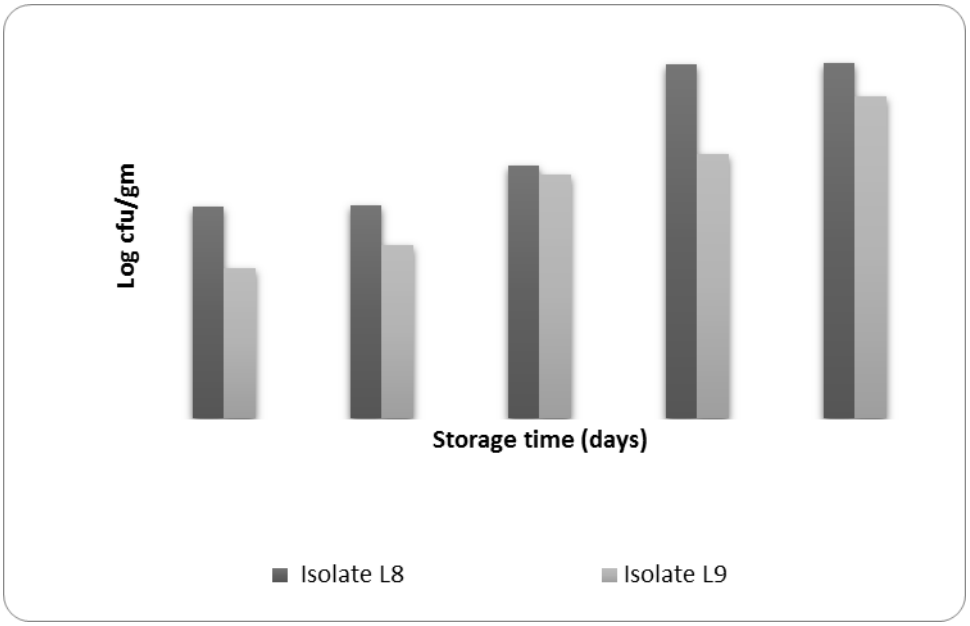

Fig: 4 Viability Assay Of Encapsulated Isolate L8 And L9.

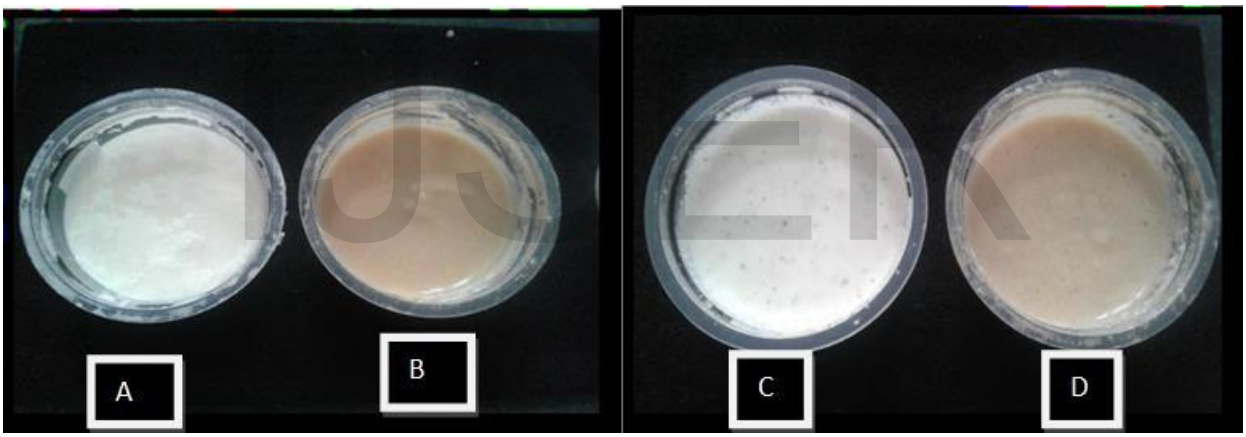

Fig: 5 Various Flavored Yogurt (A) Plain Yogurt, (B) Chickoo Yogurt,

(C) Coconut Yogurt and (D) Banana Yogurt.