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RESEARCH ARTICLE

STUDIES ON PROBIOTIC PROPERTIES OF *Lactobacillus plantarum* AND DEVELOPMENT OF PROBIOTIC INSTANT-MIX FRUIT POWDER

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ABSTRACT

The study focuses on developing a ready use product with the advantage of the probiotics supported with the prebiotics. The approach taken was that of the ones used in the flavour industry that is the encapsulation technique. The spray drying was adopted for encapsulation. The technique is found to be encouraging and needs further standardization as a successful product for the market

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INTRODUCTION

Currently, there is a trend towards a healthier way of living, which includes a growing awareness by consumers of what they eat and what benefits certain ingredients have in maintaining good health. Within the functional foods movement is the small but rapidly expanding arena of probiotics - live microbial food supplements that beneficially affect an individual by improving intestinal microbial balance. They could be combined with other healthful ingredients or simply used to complement the natural functional attributes of whole foods. However, it is important to note that any postulated benefit from consumption of probiotics should be accepted as facts only after extensive testing in human clinical studies (Rolfe, 2000). The technological aspects for selection of strains should include the following criteria:

1. strains claimed to be present in a product should survive in relatively high viable cell numbers
2. retain metabolic activity
3. provide desirable organoleptic qualities (Holzapfel *et al.,* 1998.).

One of the main selection criteria for probiotics has been competitive exclusion of pathogens. *Lactobacillus plantarum* is one of the lactic acid producing bacteria that have been used for centuries for the preservation of human food.It is known to produce antimicrobial substances, e.g. plantaricin, that are active against certain pathogens.

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It is claimed by the company (Probi A ,Sweden) that *L. plantarum* 299V is particularly valuable regarding its adherence to gastrointestinal cells. Probiotics and Prebiotics simultaneously present in a product are called synbiotics. Such a combination aids survival of the administered probiotics and facilitates its inoculation into the colon.

Viability

Viability of probiotic bacteria in a product at the point of consumption is an important consideration for their efficacy, as they have to survive during the processing and shelf life of food and supplements, transit through high acidic conditions of the stomach and enzymes and bile salts in the small intestine. The consumption of probiotics at a level of 108-109 cfu/g per day is a commonly quoted figure for adequate probiotic consumption, equating to 100 g of a food product with 106-107 cfu/g (Kebary, 1996; Lee and Salminen, 1996; Dave and Shah, 1997c). The viability of probiotic bacteria can be improved by methods such as immobilization, appropriate selection of acid, and bile resistant strains, use of oxygen impermeable containers, stress adaptation, and others. However, cell immobilization on a food-grade support is essential for food production. In addition, aroma and taste play a significant role in customer acceptance.

Immobilization and Microencapsulation

The terms immobilisation and encapsulation were used interchangeably in most reported literature. While encapsulation is the process of forming a continuous coating around an inner matrix that is wholly contained within the

capsule wall as a core of encapsulated material, immobilisation refers to the trapping of material within or throughout a matrix. Ingredients may react with components present in the food system, which may limit bioavailability, or change the colour or taste of a product. In many cases, microencapsulation can be used to overcome these challenges. Viability loss of probiotics in food products (especially fermented types) and acidic-bile conditions of gastrointestinal tract has always encouraged researchers to find new efficient methods of viability improvement. Encapsulation of probiotics in a biodegradable polymer matrix has a number of advantages. Once entrapped/ encapsulated in matrix beads or in microcapsules, the cells are easier to handle than in a suspension or in slurry. The number of cells in beads or microparticles can be quantified, allowing the dosage to be readily controlled. once the matrix beads/microcapsules have been dried, a further surface coating can be applied. This outer layer can be used to alter the aesthetic and sensory properties of the product and may also be functional, providing an extra level of protection to the cells. In addition, the coating layer can have desirable dissolution properties, which permit delayed release of the cells or release upon, for example, a change in pH. Various polymer systems have been used to encapsulate probiotic microorganisms to protect against low pH and high bile concentrations and to enhance physical stability during downstream processing. Food-grade polymers such as Alginate, Chitosan, Carboxymethyl cellulose (CMC), K-Carrageenan, Gelatin and Pectin are mainly applied, using various microencapsulation technologies.

Spray Drying

Spray drying is the most commonly used microencapsulation method in the food industry, is economical and flexible, and produces a good quality product (Dziezak,1988).For immobilising living bacterial cells, the capsule size is crucial and should be carefully controlled.

Additives

Maltodextrin as carrier in juice dehydration by spray drying is one of the most utilized substances due to its physical properties, such as high solubility in water. Several researchers recommend the use of Arabic gum (E414) as carriers in fruit juice process by spray drying, mainly for its properties of emulsification, low viscosity and high solubility in water. Potassium Ferro Cyanide (E536) is used as an anti-caking agent. Sodium Citrate (E331) is added as an acidity stabilise.

MATERIALS AND METHODS

Preparation of Pure Culture

200 ml of Lactobacillus MRS broth is prepared in the conical flask and Commercially available *L.plantarum* (Darolac^{TM}) is added into it and kept in shaker for overnight at 120 rpm. It is streaked on MRS agar plates the next day for confirmation. To qualify as a potential probiotic, several probiotic evaluation tests were performed.

Gram staining

Gram staining is an empirical method of differentiating bacteria into two large groups based on differences in their cell walls.

Survival under conditions stimulating the human GI tract

In order for a probiotic strain to exert its beneficial effects on the host, it must be able to traverse the GI tract with little loss to viability.

Acid resistance test

The stomach has a pH typically ranging from 1.0 during fasting to 4.5 after a meal. Lactobacilli and related species are capable of growth at a pH of 4.5. Culture was then inoculated in 9ml of sterile MRS broth whose pH was adjusted to 2 & 2.5 with 5N HCL. At 0th hr and 4th hr 1ml of sample was serially diluted with sterile saline solution to neutralize the medium acidity and inoculated on MRS agar plates.The agar plates were incubated for 24-48 hrs at 37 ℃ and colonies were counted using a colony counter. The reduction in viable colonies after exposure to low pH for 4hrs as compared to control was considered as criteria for acid tolerance.

Bile salt tolerance test

Bile salts serve to reduce cholesterol levels, emulsify lipids and fat soluble vitamin and aid in the reduction of bacterial flora found in the small intestine and biliary tract. Thus, the test strains are required to be tolerant to bile salts in order to adhere to and inhabit the small intestine. Similar procedure was followed here as that of Acid Tolerance test except that Bile salts are used here instead of a strong acid. The reduction in viable colonies after exposure to Bile salts for 4hrs as compared to control was considered as criteria for bilesalt tolerance.

Digestive enzymes (Protease, Lipase, Amylase) resistance test

Stomach is a mixture of several digestive enzymes produced by the exocrine cells of the pancreas. It is composed of amylase, lipase and protease. They digest foods only at alkaline pH. The test strains were exposed to all the three enzymes individually and also to the mixture at pH 8.0 to find out if they were tolerant. Broth cultures of 2ml volume were centrifuged at 10000 rpm for 2 min. The supernatant was drained and pellet was re-suspended in 2ml of sterile PBS (pH 7.2) and centrifuged at 10000 rpm for 1 min. To the pellet obtained, PBS of pH 8 [supplemented with 1mg/ml Amylase/Lipase/Protease and Mixture] was added until the turbidity matched that of McFarland Standard 2. The suspension was incubated at 37°C for 3 h. It was spread plated at 0 and 4 h on MRS agar plates. The plates were incubated at 37°C for 24 h in anaerobic conditions with 10% CO2. Colonies were enumerated after incubation.

Hemolytic activity test

Haemolysis refers to the breaking open of red blood cells and the release of haemoglobin into the surrounding fluid. A crucial safety requirement for probiotic strains is that they do not cause the haemolysis of RBCs. Human blood was taken with EDTA and saline solution was added. It was then centrifuged at 1000 rpm for 3 min to remove the upper serum part. Saline water is added and repeat the step until the clear solution was observed. The clear solution obtained was mixed

with blood agar base and plated. The broth cultures were streaked quadrantly on Blood Agar base. The plates were incubated at 37°C for 24 hr and observed for zones of haemolysis.

Antibiotic sensitivity test

To determine the sensitivity or resistance of the strains to the antibiotics, the Kirby-Bauer method was followed (Maragkoudakis *et al.,* 2006). Bacterial cultures were swabbed onto the MRS agar plates using a sterile swab. Antibiotic discs were placed on the inoculated media plates. The plates were incubated for 24 h in an incubator at 37°C. Plates were then observed for zones of inhibition around the antibiotic discs.

Antimicrobial activity test

Some strains of lactic acid bacteria produce bacteriocins which are antimicrobial compounds. They are also capable of limiting pathogen growth by means of the organic acids and other metabolic end products they generate. The test strains were examined for the extent of their antimicrobial activity against the pathogens *S.aureus, P.mirabilis, K.pneumoniae, E.faecalis S.epidermis Beta-Streptococci sp., and Pseudomonas sp* by the well-diffusion method.

Microencapsulation techniques

Alginate: 2% of sodium alginate was prepared and beads were prepared with th help of syringe and dripped into CaCl2 solution It was then inoculated with microbial culture in 1:1 ratio and mixed with commercially available mango juice and incubated 0℃. After serial dilution of the mixture, spreading was done on agar and incubated for 24hr at 37℃

***k*-Carrageenan**

A similar proecedure was followed here. Commercially available Pecgel-50 was used here. 2% of k-Carrageenan was prepared and beads were prepared with th help of syringe and dripped into KCL solution. It was then inoculated with microbial culture in 1:1 ratio and mixed with commercially available mango juice and incubated 0℃. After serial dilution of the mixture, spreading was done on agar and incubated for 24hr at 37℃

Mango peel powder

Mango peels were dried in hot air oven for 30min and ground to a fine powder. The peel powder was heated at 70℃ and then it was filtered using muslin cloth. It was then inoculated with microbial culture in 1:1 ratio and mixed with commercially available mango juice and incubated 0℃. After serial dilution of the mixture, spreading was done on agar and incubated for 24hr at 37℃

Acacia Gum

1% of Acacia Gum was prepared. It was then inoculated with microbial culture in 1:1 ratio and mixed with commercially available mango juice and incubated 0℃. After serial dilution of the mixture, spreading was done on agar and incubated for 24hr at 37℃.

Spray drying of mango juice containing probiotics and additives

Mango pulp was mixed with 3% maltose dextrin, 0.3% of acacia gum, 3% of maltose, 0.57% of potassium ferrocyanide and sodium citrate. It was then filtered with muslin cloth to remove any suspended particles that may block the spray nozzles. The filtered mixture was then mixed with microbial culture and distilled water was added to make it more viscous. Spray drying was done at inlet temperature 110℃ and flow rate at 200 cc/min through an automated suction pipe. Powder is collected at the outlet.

Assays to be done after spray drying of mango juice containing probiotic and additives

CFU

Microbial count in the sample was analysed by plating the sample on Lactobacillus MRS agar.

Physical tests

Moisture Content

Mass lost after 5 to 10g powder in an oven controlled temperature (105℃) during 4hrs.

Solubility

Solubility was checked by dissolving the powder directly in water.

Organoleptic Tests

Odour

The odour of the powder is detected by smelling it directly from the sachet.

Taste

The taste of the powder is detected by taking a pinch of it into the mouth

Colour

Visual detection for appealing is made through direct eye.

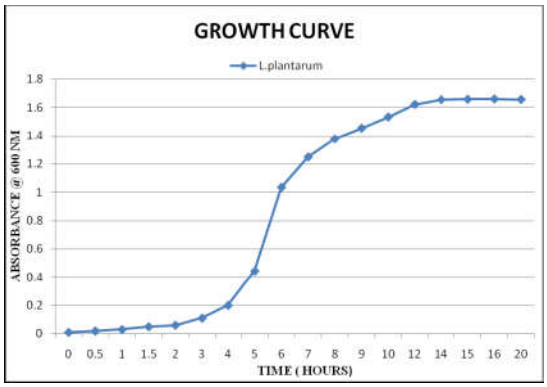
RESULTS AND DISCUSSUIONS

Gram staining

Morphological colonies on MRS agar plate were subjected for gram staining. The colonies were gram positive in nature.

Determination of Growth curve

Growth curve was plotted for the micro-organism. It was observed that Lag Phase was from 0th to 5th hour and Log Phase-6th to 14th hour and Stationary Phase-15th to 20th hour and Death Phase – after 23rd hour.



Lag Phase – 0 to 5th hour ; Log Phase-6th to 14th hour; Stationary Phase-15th to 20th hour; Death Phase – after 23rd hour

Tolerance to acid

The strains were exposed to bile salts and plated on MRS agar at 0th, 3rd and 5th hr. On performing a colony count, it was found that there were more colonies at 0th hr and less number of colony at 5thr.

TEST	TIME(Hrs)		
	CFU At 0th Hr	CFU At 3rd Hr	CFU At 5th Hr
ACID	Too Numerous	48	44

Tolerance to bile

The strains were exposed to bile salts and plated on MRS agar at 0th, 30 min and 4th hr. On Performing colony count , it was found that there were more colonies at 0th hr and less number of colony at 5th hr.

	TIME (Hrs)		
TEST	CFU at 0th Hr	CFU at 30 min	CFU at 5th Hr
BILE	Too Numerous	46	33

Resistance to digestive enzymes (amylase, protease, lipase and combination of all three)

The strains were tested for their tolerance to digestive enzymes. Colonies were observed with a slight reduction in the number.

	TIME (Hrs)	
Enzyme	CFU at 0th Hr	CFU at 4th Hr
Amylase	Too Numerous	88
Lipase	68	28
Proteinase	Too Numerous	84
Mixture	64	23

Test for haemolytic activity

The plates were observed for any sign of haemolysis. All strains were found to be gamma-haemolytic, i.e., no zones of haemolysis were formed for 24 h,

Antibiotic sensitivity test

The strains were subjected to the Kirby Bauer method to determine their sensitivity to 9 different antibiotics. Following incubation the zones of inhibition formed with the various antibiotics were compared with standard charts.

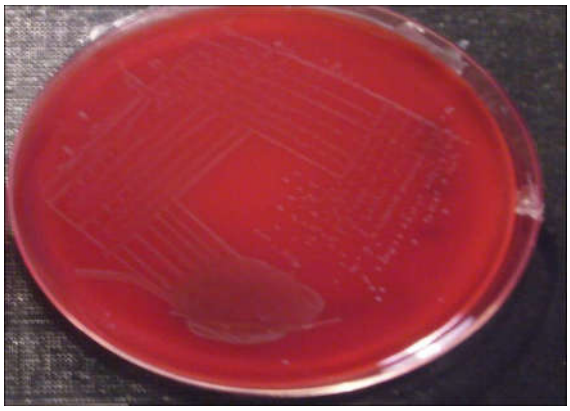


Fig. Heamolytic Activity

The strains were resistant to Streptomycin, Vancomycin, Trimethoprim and Gentamicin.

Antibiotic Disc	Zone of Inhibition
Ampicillin	I
Bacitracin	I
Gentamycin	I/R
Kanamycin	I
Streptomycin	R
Trimethoprim	R
Tetracyclin	S
Pencillin-G	I
Cholarphenicol	S
Rifampicin	I
Vancomycin	R

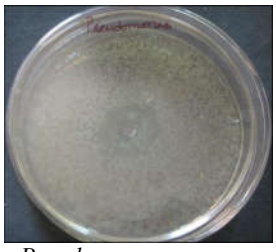
S-Sensitive; I-Intermediate;R-Resistant

Antimicrobial Activity

The well diffusion method was carried out for the sample strains to determine their inhibitory effect on seven pathogens namely, *S.aureus, P.mirabilis, K.pneumoniae, E.faecalis, S.epidermis , Beta-Streptococci sp., Pseudomonas sp* . Zones of inhibition were formed on plate containing *S.aureus, P.mirabilis, K.pneumoniae, E.faecalis, Beta-Streptococci sp.,* and no zone of inhibition is seen in *S.epidermis and Pseudomonas sp.*

Pathogen	Zone Of Inhibition(mm)
S.aureus	22
P.mirabilis	19
K.pneumoniae	15
E.faecalis	16
S.epidermis	24
Beta- Streptococci	14
Pseudomonas spp.	20

P.mirabilis



Pseudomonas sp.

Encapsulation Techniques

CFU was performed and results were interpreted as follows:

Encapsulation Material	Dilution	
	10 -5 CFU	10-7 CFU
Alginate	20	11
K-Carrageenan	3	1
Acacia Gum	5	4
Mango Peel	39	19

Alginate beads

Assays performed on powder

CFU: The count was too low to be released as a product according to FAO standards.

Physical Tests

Moisture content: There was almost no moisture in the sample.

Solubility: The powder was sparingly soluble in normal water but dissolved well upon boiling

Organoleptic Tests

Color: Brownish yellow in color.

Odour: Mango flavoured smell and a slight charred smell was also present.

Taste: Mango tasting. Again a slight charry taste was present.

DISCUSSION

Importance and variety of probiotics and food probiotic products on the one hand, and successes achieved by applying the microencapsulation process for the purpose of maintaining of probiotic cells viability on the other hand, have necessitated extensive research in the field of probiotic foods. We have investigated on *L.plantarum* with respect to metabolic and functional properties considered for the viability and the activity in GI tract and the stomach. For the selection of highly potent probiotic strains, safety and functionality properties such as antibiotic resistance, adhesion to intestinal cell lines, antimicrobial activity are highly important and should be studied using reliable *in vitro* screening methods. One of the safety requirements for a probiotic strain is that it should not have β-haemolytic activity. All the strains were gamma-haemolytic when grown on human blood agar plates, thereby suggesting that they have no undesirable interaction with our red blood cells. Some of these probiotics also produce bacteriocins, substances which kill harmful microbes. In our study, the supernatants of several strains inhibited the growth of the pathogenic organisms *S.aureus, P.mirabilis, K.pneumoniae, E.faecalis S.epidermis Beta-Streptococci sp., Pseudomonas sp*, using the well diffusion assay. Inhibition was seen against five species *S.aureus, P.mirabilis, K.pneumoniae, E.faecalis Beta-Streptococci sp.* With *S.aureus* having highest zone of inhibition and *K.pneumoniae* least. There was no inhibition seen against *S.epidermis and Pseudomonas.*

Factors that help to explain the inhibitory effect of *Lactobacilli* on pathogens:

1. The competitive crowding out of other microorganisms by implantation on the mucous surfaces and villi, thereby decreasing coliforms by as much as 90%.
2. The production of a broad-spectrum antibiotic, which is inhibitory to both viral and bacterial pathogens. An example of this is acidophilin from *L. acidophilus*.
3. The production of hydrogen peroxide, which is extremely toxic to some pathogenic ecosystems.
4. Decreasing the pH through the production of lactic acid. Many pathogenic intestinal organisms thrive in a neutral environment.

The mechanism through which probiotics may antagonise pathogens involves production of antimicrobial compounds such as lactic acid, acetic acid, hydrogen peroxide and bacteriocins. The inhibitory action of LAB is mainly due to accumulation of main primary metabolites such as Lactic Acid, Ethanol, and Carbon Dioxide. Aditionally, LAB are also capable of producing anti microbial compounds such as formic and Benzoic Acids, Hydrogen Peroxides, Diacetyl, Acetoin and Bacteriocins. The advantages of using Mango as medium for growth is that there was high viability seen when *L.plantarum* was inoculated in the mango pulp. Apart from that literature suggests that the natural polymers present in mango pulp and the fibre content in it would act as prebiotics on which the probiotic organisms feed. Mango peel which was naturally prepared scrapping out the mango pulp, was found to be the best micro encapsulating material. Alginate also had very good micro encapsulating capacity next to mango peel powder. Acacia gum was found to have a significant effect but the mechanism is still not known. And k-carrageenan gave very poor results in micro encapsulating the Lactobacillus cells. Mango peel is generally the waste generated from all the canneries and it can be effectively used now as microencapsulating agent. Pectin which is the polymer present in the mango peel could be the possible micro encapsulating agent of the cells. More detailed studies have to be done in order to establish theory regarding the exact mechanism going on during the microencapsulation process. Advantages of using mango peel are that its very cheap and generally thrown as waste and its natural and hygienic which would attract all the consumers. It was found that various processing parameters such as outlet air temperature, TS level in feed, compressed air pressure (feed rate) and blend proportions have

profound effect on the output and quality of powder obtained during spray drying. Initially two trials were performed.

Economics

Considering the utility charges like spray drying, power supply, water supply etc to be constant the economic value is low and affordable. Pure cultures can be readily prepared from the probiotic strains in bulk which can be used in the preparation at a lower cost. Mango is a versatile fruit which is available throughout the year with proper storage conditions and its available in bulk in India. Chemicals like Ca-Alginate or k-Carrageenan can be substituted by the novel microencapsulator mango peel powder which is natural as well as cheap. More research need to be performed of using natural ways to micro encapsulate these probiotics at an affordable price. Additives like Maltodextrin(Coating agent), Trehalose, Potassium Ferro Cyanide (anti caking agents) and Sodium Citrate (Acidity regulator) are food grade and must be included which are not very costly.

Conclusion

The potential probiotic properties of *L.plantarum* was explored. The market can still be expanded if probiotic strains that can alleviate a serious health problem are found or constructed. Probiotic properties like acid tolerance and bile tolerance and antibiotics and antagonistic property against pathogens and thermo-tolerance (from literature) successfully implied the use of *L.plantarum* for spray drying. Apart from Mango juice several other indigenous fruits and also vegetables can be tested for the probiotic survival in them. But mango has the added advantage of higher fibre content which acts as prebiotics to the probiotic micro organisms present. Microencapsulation have a major role during the spray drying because of its protective action on the microbes. Mango peel powder which is cheap and natural have proved to be the best microencapsulator suggesting its application widely, where a standardisation of process is required. Microbes survived after the spray drying and they were significant enough to be supplemented as probiotic functional food. Our study concludes that further optimisation of the conditions regarding the microencapsulation and spray drying have to be performed for a better yield.

Future Prospects

Future research could be concentrated on the aspects such as applying more efficient encapsulation materials or improving the common used ones; studying correlations between process factors and microencapsulation effectiveness in different products; optimization of the process factors in order to reach the highest viability and the most satisfactory sensory properties of the products along with the lowest cost and improving or inventing new methods relevant to evaluation of microencapsulation efficiency, particularly under *in vivo* conditions. If the health benefits are well documented and easy to understand, it is likely that even the consumers will accept a genetically engineered probiotic strain.Of course, much remains to be done about the development of effective combinations of pre- pro- and synbiotics.

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