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Original Article

PREBIOTIC INFLUENCE OF *PLANTAGO OVATA* ON FREE AND MICROENCAPSULATED *L. CASEI*– GROWTH KINETICS, ANTIMICROBIAL ACTIVITY AND MICROCAPSULES STABILITY

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ABSTRACT

Objective: To study the enhancement of antimicrobial effects of probiotic *Lactobacillus case*i (*L. casei*) both in free and immobilized form and in the presence of a natural prebiotic-*Psyllium* seed husk.

Methods: Arabinoxylan was isolated from *Psyllium* seed husk. The isolated arabinoxylan was characterized using FTIR, TLC and HPTLC method. The growth kinetics of *L. casei* has been studied with and without arabinoxylan extracted from *Psyllium* husk. The antimicrobial activity of *L. casei* against *Escherichia coli* has been determined using glucose and arabinoxylan as substrates. Zone of inhibition in the presence of arabinoxylan has been observed. The probiotic has been immobilized through microencapsulation technique and the size distribution of the microcapsules has been microscopically determined. Effects of centrifugal force and high temperature stress on the stability of microcapsules have been studied.

Results: The values of kinetic parameters, μ_{max} and k_s have been determined to be 0.379/h, 0.3942 g/l and 0.08127/h, 0.3094 g/l for glucose in MMRS and Basal media respectively. In the case of arabinoxylan the zone of inhibition was 14.5 mm and for glucose it was observed 13 mm. 15 g of microcapsules have been obtained from 5 ml of cellular broth. The size of microcapsules was in the range of 0.1 mm-0.55 mm in which 0.25 mm of diameter were maximum size.

Conclusion: The presence of arabinoxylan enhances the growth and antimicrobial activity of *L. casei* both in free and immobilized forms. The retention of probiotic cells in fortified milk under freezing condition increases in presence of arabinoxylan. The size of microcapsules follows a normal distribution.

Keywords: Probiotics, Prebiotics, Synbiotics, Immobilization, *L. casei*, *E. coli*, Antimicrobial activity, Growth kinetics

INTRODUCTION

The synbiotics, i.e., the combination of probiotics and prebiotics are rapidly gaining interest. "Probiotics are live microorganisms (bacteria or yeasts), which when ingested or locally applied in sufficient numbers confer one or more specified demonstrated health benefits for the host" [1]. Among the probiotic genera, lactic acid bacteria (LAB), typically associated with the human gastrointestinal tract, are the most widely used probiotic microorganisms till date. On the other hand, prebiotics is defined by Gibson and Roberfroid [2] as "non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." Intake of such fibers may act both in the prevention as well as in the treatment of chronic diseases [3-6]. Among different prebiotics available in natural vegetable sources, inulin extracted from chicory roots is extensively studied. The present research group has also investigated the characteristics of inulin isolated from various Indian cereals, e. g. wheat, garlic, dalia, oat etc. [7]. In a recent article, the influence of inulin on the growth kinetics of *Pediococcus acidilactici* has also been reported by the present group [8]. Among different natural sources of fibrous polysaccharide, arabinoxylan present in *Psyllium* husk has been reported to be popular in India for its medicinal activity against constipation, diarrhoea, irritable bowel syndrome, inflammatory bowel disease, ulcerative colitis, colon cancer, diabetes, and hypercholesterolemia [9-15]. *Psyllium*, a soluble fibre, has a potential to stimulate bacterial growth in the digestive system, and, in some reports, it has been termed as prebiotic [11-14]. Actually, *Psyllium*, the common name used for several members of the plant genus *Plantago,* is gel-forming mucilage composed of a highly branched arabinoxylan. While the backbone consists of xylose units, arabinose and xylose form the side chains [12, 16]. Although *Psyllium* has been in use as a laxative for decades, its prebiotic activity has not been studied systematically. As *Psyllium* has been in use as a laxative for decades, it is expected that it might have a prebiotic activity which enhances the activity of probiotic gut bacteria. However, no research study has so far been reported on the prebiotic activity of the polysaccharide namely arabinoxylan extracted from psyllium husk. Since probiotics in immobilized form are currently in demand, the effect of prebiotics like arabinoxylan on encapsulated probiotic may create an opportunity for application of a synergistic mixture of arabinoxylan and immobilized probiotics. There is a dearth of data in this area also. The present research study focuses for the first time on the arabinoxylan and its synergistic application with free and immobilized forms of probiotic bacteria, namely, *Lactobacillus casei.* The growth kinetics of *Lactobacillus casei* using glucose and arabinoxylan separately as substrate have been determined in different growth media, viz., and basal medium and modified MRS medium. The antimicrobial activity of *Lactobacillus casei* grown on arabinoxylan against *E. coli* has been compared with that grown on glucose. Since the encapsulation of probiotics is an efficient technique [17, 18], for the improvement of their viability and stability and efficient delivery of the cells to their active sites, the immobilization of *L. casei* using external gelation technique has also been studied in the present research work. The microencapsulation has been characterized in terms of encapsulation size distribution and stability. The stability of microcapsules has been determined varying different parameters viz., the speed of centrifugation (200–5000 * g), deep freezing (-20°C) and heat shock by the exposure to high temperature up to 50 °C. The effect of fortification with arabinoxylan has also been determined during the deep freezing of milk with probiotic microcapsules.

MATERIALS AND METHODS

Organism

The experimental bacterial strain, *Lactobacillus casei* (NCIM-2125) has been procured from National Collection of Industrial Microorganisms, Pune, India.

Chemicals used

Peptone, Beef extract, Yeast extract, Peptone water were purchased from Himedia, India. Dipotassium hydrogen phosphates, Sodium acetate, Tri-ammonium citrate, Manganese sulphate, Magnesium sulphate, Glucose, Tryptone, Tween 80, Sodium chloride, Potassium chloride, Magnesium chloride, Potassium dihydrogen phosphate, Ammonium chloride, 3,5-dinitro salicylic acid, Sodium hydroxide, Sodium potassium tartrate, Calcium chloride (0.27M), Sodium alginate (3%), agar-agar, de-ionized water used in the present study, were purchased from Merck, India. Cysteine HCl was purchased from Merck, Germany. TLC Silica gel G 60 Aluminium sheets 20 x 20 cm (Merck HX 816976, Germany), benzene (Ranbaxy, India), acetic acid(Merck, India), methanol(Merck, India), resorcinol(Merck, India), ethanol (Merck, India), sulphuric acid(Merck, India), HCL (Merck, India), *Psyllium* husk, Rice bran oil and Amul milk tetra-pack (200 ml) were purchased from local market.

Analytical instrument

Cold Centrifuge (C-24, Remi), Laminar air flow bench, Double Beam UV-VIS spectrophotometer (Perkin Elmer), Shimadzu FTIR Spectroscope 8400, HPTLC-CAMAD; Applicator–LINOMAT 5; Scanner 3; Reprostar 3 were used.

Media preparation

In order to prepare specific media for growth of *Lactobacillus casei* with and without the prebiotic arabinoxylan, modified MRS media containing glucose and arabinoxylan respectively in the range of 1-3 g/l were prepared. In both the media, concentrations of all other components were maintained same as in MRS medium. On the basis of 1L distilled water, the amounts of components were as follows: pH 7, 10g Peptone, 5g Yeast extract, 10g Beef extract, 2g Dipotassium hydrogen phosphate, 5g sodium acetate, 2g Tri-ammonium citrate, 0.05g Manganese sulphate, 0.05g Magnesium sulphate and 10-40 g D-glucose in 1 L distilled water (MRS medium)

In order to prepare basal medium [19], 5.0g Peptone Water, 10g Tryptone, 2.5g Yeast extract, 1g Tween 80, 4.5g Sodium chloride, 0.25g Potassium chloride, 0.1g Magnesium chloride, 0.40g Potassium dihydrogen phosphate, 0.50g Cysteine. HCl, 0.40g ammonium chloride, 0.20g Dipotassium hydrogen phosphate were dissolved in 1 L distilled water, pH set at 7. Both the media were subsequently sterilized under 2 bar pressure at 121°C for 15 min in an autoclave.

Methods

Extraction of the prebiotic from *Psyllium* husk

Isolation of arabinoxylan

Arabinoxylan was extracted from *Psyllium* husk following the protocol reported by [20]. 50 g *Psyllium* seed husk was soaked in sterilize distilled water overnight (seed husk: water 1:50, w/v) to form the mucilage. Aqueous NaOH solution (2.5%) was added to the mixture to adjust the pH to 12 for the hydrolysis of the fibres. The husk fibres were separated from the gel by applying vacuum filtration after stirring for 2–3 min. Concentrated acetic acid was added to the filtrate mixture to coagulate the sample at pH 3. The gel was then washed for several times over a period of 3–4 d with distilled water until the pH became constant and was freeze dried.

Solubilisation of arabinoxylan

Solubilisation of arabinoxylan extract was performed using the protocol suggested by [21]. 0.5 g of the freeze dried arabinoxylan was dissolved in 40 ml of distilled water and kept in a water bath for 2 h. 60 ml of colonic juice was added to it. This mixture was again heated in water bath at 100°C for half an hour. The mixture obtained after heating was cooled down and used directly as a prebiotic source for further experiments.

Assay of arabinoxylan

Arabinoxylan extract and its hydrolysate obtained after hydrolysis following the protocol recommended by [22] were analyzed using, HPTLC and TLC respectively. The chromatograms were compared with those of standard arabinose and xylose. FTIR analysis of arabinoxylan extract was also performed to identify the chemical bonds present in it.

Cell growth study

Preadaptation of lactobacillus casei culture

Adaptation of the *Lactobacillus casei* strain to a medium containing high concentrations of glucose (5 g/l) and arabinoxylan (5 g/l) was performed by repetitive subculturing for thrice. The pre-adaptation runs were conducted in an incubator at 37 °C using 250 ml Erlenmeyer flasks for 1 d, until sufficient growth (2.94x10[11]cfu/l) was observed. The cells from the last adaptation experiment were stored for use in further experiments.

Batch experiments

To study the growth kinetics of *Lactobacillus casei* on substrates, glucose and arabinoxylan, two separate series of batch experiments in 500 ml Erlenmeyer flasks containing 250 ml MMRS media, one containing glucose and the other containing arabinoxylan as the carbohydrate source, were conducted for 56 h. In the case of glucose, the concentration was varied from 1-3 g/l and for arabinoxylan, the concentration range was 1-5 g/l. These two series were also repeated with the basal medium. The pH of the medium was set at 7. For all experiments, 1% (v/v) inoculum was added. 10 ml paraffin oil was used immediately after the inoculation of the Lactobacillus casei to maintain the anaerobic condition for both sets of experiments with and without arabinoxylan. Each flask was closed with a cork through which a narrow glass tube was inserted into the solution. A 25 ml injection vial was fitted to the other end of the tube for the purpose of withdrawing the sample under anaerobic and aseptic condition at 2 h interval. The incubation temperature was 37 °C.

Determination of microbial biomass concentration

The concentration of biomass was determined using spectrophotometric method [8]. For dry cell weight technique [8] 15 ml each of samples were centrifuged at 10000 gyrations, at 4 °C for 15 min. The cell precipitate of each sample so formed, was separated, washed and transferred into a pre weighed aluminum cup. This was dried at 50 °C overnight in the hot air oven, the cup was then cooled and weighed and weight of cellular mass was determined by difference. The concentration of biomass was determined using the volume of sample and weight of cell precipitate. The samples were also analyzed by spectrophotometer at 600 nm and the ODs have been plotted against the concentration determined through dry cell weight method. For all other samples ODs were directly measured by spectrophotometer at 600 nm and dry cell weight concentrations were determined using the standard plot.

Studies on cell dynamics

Investigation on cell growth dynamics has been carried out by monitoring concentration time history of biomass and substrate. The experimental data on cell growth and the depletion of substrate concentration would qualitatively aid in understanding the cell growth dynamics. At this stage, suitable model equation for growth would be selected to get the quantitative picture of the cell dynamics in the presence of limiting substrate, i.e., either only glucose or arabinoxylan.

Determination of kinetic parameters

Calculation for specific cell growth rate μ

The growth of a bacteria growing in a batch reactor is given by,

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad \ldots\ldots\ldots (1)$$

The Monod model applicable for uninhibited growth is attempted under the present investigation. This is as follows:

$$\mu = \frac{\mu_{max} S}{Ks+S} \quad \ldots\ldots\ldots\ldots (2)$$

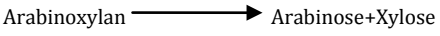
Where, μ_{max} = maximum specific growth rate for growth on glucose,/h

K_s = half saturation constant

The biomass yield coefficient is defined by, $Y_{X/S}$ = amount of biomass produced (in grams)/amount of substrate consumed (in grams)

DNS method for the evaluation of carbohydrate concentration

To calculate the carbohydrate concentration in any liquid sample DNS method [7] is used. It is a chromogenic reaction by which the amount of carbohydrate present in any liquid sample may be quantified by measuring the OD values at 540 nm. In case of experiments using glucose as the limiting substrate, the concentration of reducing sugar in the supernatant of culture samples obtained at different time interval was determined by DNS method directly. Since arabinoxylan possesses complex oligosaccharide structure, the cleavage of its glycosidic linkages through hydrolysis was done for the determination of its concentration in the batch culture sample using DNS method. According to this experimental protocol, the concentration of reducing sugar in the supernatant of a culture sample was first determined using DNS method. The same supernatant was then hydrolyzed using standard experimental method [7]. The concentration of reducing sugar in the hydrolyzed supernatant was again determined using DNS method. By subtracting the concentration of reducing sugar in the original supernatant from this reading, the concentration of reducing sugar formed through hydrolysis of unconverted arabinoxylan was determined. Hydrolysis reaction of arabinoxylan is as follows:



From this value the concentration of arabinoxylan in the supernatant of culture sample was calculated.

Antimicrobial activity of *L. casei*

Sterile MRS and Basal media were taken in two different conical flasks, one, containing 2 g/l UV-sterilized arabinoxylan and the other 2 g/l glucose. 1% inoculum of *L. casei* was added to each flask and batch culture was run for 48h maintaining the temperature at 37°C in the incubator. 1 ml sample was taken out in an Eppendorf tube every 6 h interval and centrifuged at 10000 rpm for 10 min. After centrifugation the supernatant was used for the antimicrobial study. Small discs (diameter 0.5 cm) of filter paper was prepared and loaded with 0.1 ml of the supernatant obtained after the centrifugation of the respective samples. Each disc prepared with supernatant of the sample withdrawn at every 6 h was impregnated onto the MRS agar plate spread with *E. coli* culture. These plates were then kept for incubation at 37 °C for 24 h and the zone of inhibition was measured in each plate.

Determination of cell counts by spread plate method

A known amount of freshly prepared seed culture was directly loaded on a Neubauer Haemocytometer counting chamber and covered with a coverslip, followed by direct count under a microscope and total numbers of cells were calculated from the data.

Preparation of microcapsules

One part of seed culture concentrate (5 ml) is mixed with 4 parts of 3% sodium alginate solution (20 ml) and vortexed properly for adequate mixing. On the other hand, vegetable oil (rice bran oil) was mixed with 0.2% tween 80 and 0.25% sodium lauryl sulphate. One part of the aqueous mixture was then added dropwise to 5 parts i.e. 125 ml oil mixture. Later, the whole suspension was magnetically stirred at 200 rpm by magnetic stirring for 45 min. Within 45 min, a uniformly turbid emulsion was obtained with no evidence of a free aqueous phase. Calcium chloride (0.27M) was added quickly but gently (20 ml/sec) down the edge of the beaker until the water/oil emulsion was broken. After that Calcium alginate microcapsules were allowed to form in next 30 min by leaving the beaker undisturbed. Microcapsules, thus formed at the bottom of the beaker were then filtered through fine muslin cloth. Subsequently, microcapsules were washed with 0.9% saline (0.9 g NaCl/100 ml) containing 5% glycerol and stored at 4°C [23]

Determination of size distribution of microcapsules

1 g of microcapsules which is equivalent to 24 numbers of microcapsules was selected randomly in order to determine the range of their size and to find out the size of the maximum populated microcapsules. 1 cm x1 cm area was selected on a microscopic slide and was divided into 100 equal squares. On this selected area, 1 g of microcapsules was uniformly distributed and was viewed under a microscope to determine the size distribution.

Determination of size distribution of microcapsules under centrifugal force

To check the stability of calcium alginate microcapsules, centrifugation process was selected. For this process, 24 numbers of microcapsules were centrifuged on an average each time at a range of 200* g to 5000* g centrifugation speed. The size distribution of microcapsules was determined for each case following the same protocol followed for freshly prepared microcapsules.

Determination of the effect of centrifugation on size distribution and stability of microcapsules

1 gram of microcapsules were weighed and suspended in 20 ml of distilled water. Then 8 autoclaved Eppendorf tubes each containing 1 ml of suspended microcapsules was taken. Each one of these Eppendorf tubes was centrifuged at different r. p. m at 200*g, 1000*g, 1800*g, 2600*g, 3400*g, 4200*g, 5000*g respectively for 10 min. The size distributions of the microcapsules were determined under a microscope. 10 µl of supernatant from each Eppendorf tube was taken in sterile test tube serially diluted up to 10^{-5}. 0.1 ml supernatant from the 5^{th} dilution was spread using a glass spreader on the MRS agar plate and incubated for 48 h at 37 °C. The Same procedure was followed for other samples which were obtained at different rpm. After 48 h the colonies were counted on the plates of different r. p. m.

Determination of stability of microcapsules under deep freezing condition

200 ml of Amul toned milk (Tetrapack) was taken in a beaker and was sterilized. Milk was then allowed to cool and was inoculated with *L. casei* up to 10[15] cfu level. 10 ml of milk was taken in each of 6 different small sized sterile plastic containers. Among these 6 milk samples one was kept as control and in the rest of the 5 milk samples were added 1% *L. casei* seed culture; 0.2% *Psyllium* husk; 1% *L. casei* seed culture and 0.2% *Psyllium* husk; 1% encapsulated *L. casei* microcapsules; 0.2% *Psyllium* husk and 1% encapsulated *L. casei* microcapsules respectively. All these 6 containers were tightly sealed with paraffin film and kept in deep freezer at-20 °C for 10 d. After 10 d all the containers were taken out from deep freezer and thawing was done to melt the milk solution and later each sample was serially diluted till 10^{-}[20] with double distilled water. 0.1 ml of each sample from the first dilution till 10^{-}[18] was spread evenly on the MRS agar plate using glass spreader. All the plates were incubated for 48 h at 37°C for the growth of the bacterial colony. After 48 h colony forming units (cfu) were counted for all the plates and final results were obtained by multiplying the dilution factors.

Determination of stability of microcapsules in response to thermal shock

One gram of entrapped microorganism was weighed and suspended in 20 ml of double distilled water in a beaker. Suspension of microcapsules was heated aseptically in between 45-50°C for 30 min using a heating mantle. This suspension was aseptically diluted till 10^{-9} using double distilled water. After dilution 0.1 ml of sample was taken out from the 10^{-9} dilution test tube and spread evenly on the MRS agar plate with the help of glass spreader and incubated at 37°C for 48 h. After 48 h colony forming units were counted and final results were obtained multiplying the dilution factors. All experiments were conducted in triplicate.

RESULTS AND DISCUSSION

FTIR test was done to identify the functional groups present in the *Psyllium* husk. The functional groups and the responsible wavelengths for these groups were given in fig. 1 and table 1.

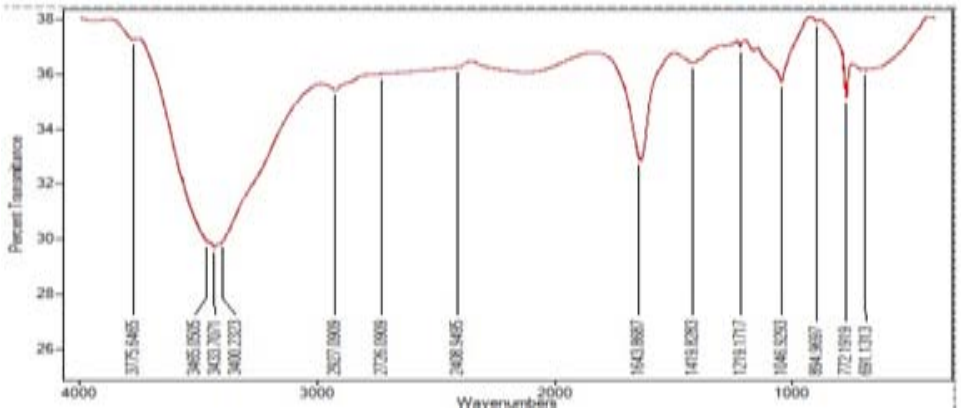


Fig. 1: FTIR of *Psyllium* husk

Table 1: Wavelength numbers for the functional groups present in *Psyllium* husk

Peak point	Functional group
3404	(m OH)
2927	(m CH)
1640	(Absorbed H_2O)
1462	(In-plane d OH)
1419	(d CH_2)
1375	(d CH)
1249.1162	(antisym, bridge oxygen d)
1046	(d C-O)
894	antisym out-of-plan d
617,534	(Polymer backbone)cm

The peaks of FTIR chromatogram (table 1) were found almost similar with small variation when compared with those obtained by other workers [24, 25]. Fig. 2 shows HPTLC fig. of arabinose, xylose and other oligosaccharides.

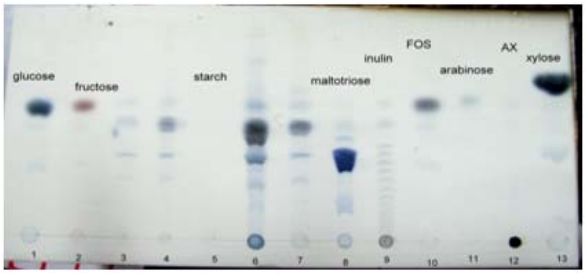


Fig. 2: HPTLC of arabinoxylan, arabinose and Xylose

Fig. 3 showed the TLC picture and the R_f values of standard arabinose (4.6 cm) and xylose (5.2 cm) and hydrolysed arabinoxylan (isolated) where two bands were formed with R_f values for arabinose and xylose 4.7 cm and 5.3 cm respectively.

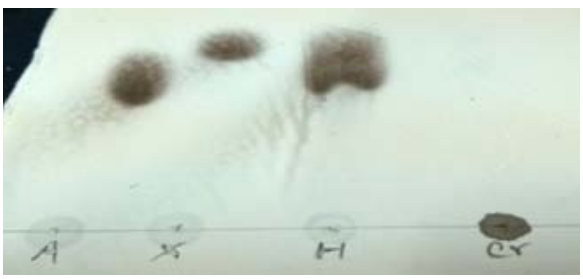
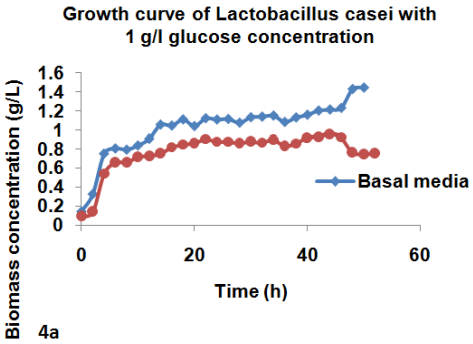
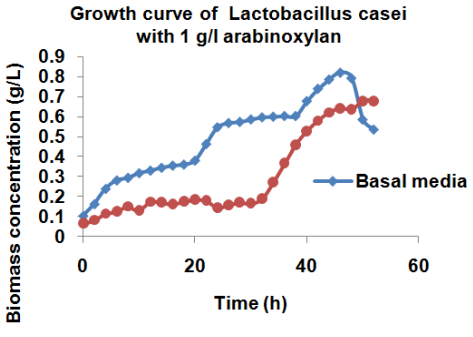


Fig. 3: TLC of standard arabinose, xylose; hydrolysed isolated arabinoxylan; isolated arabinoxylan (crude)

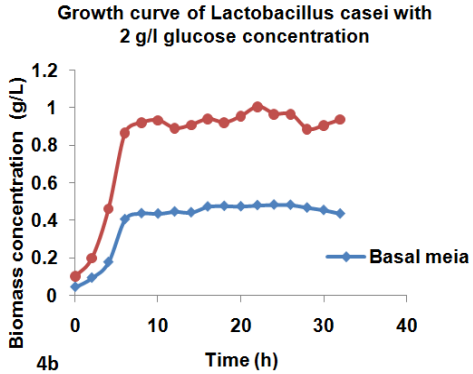
HPTLC (fig. 2) shows that arabinose, xylose and other oligosaccharides moved forward where as in case of arabinoxylan no movement was followed due to its high molecular weight. On the other hand, fig. 3 for TLC shows that, the R_f values of standard arabinose and xylose are almost same with that of hydrolysed arabinoxylan (isolated) showing two bands with almost same R_f values for arabinose and xylose.



4a



4d



4b

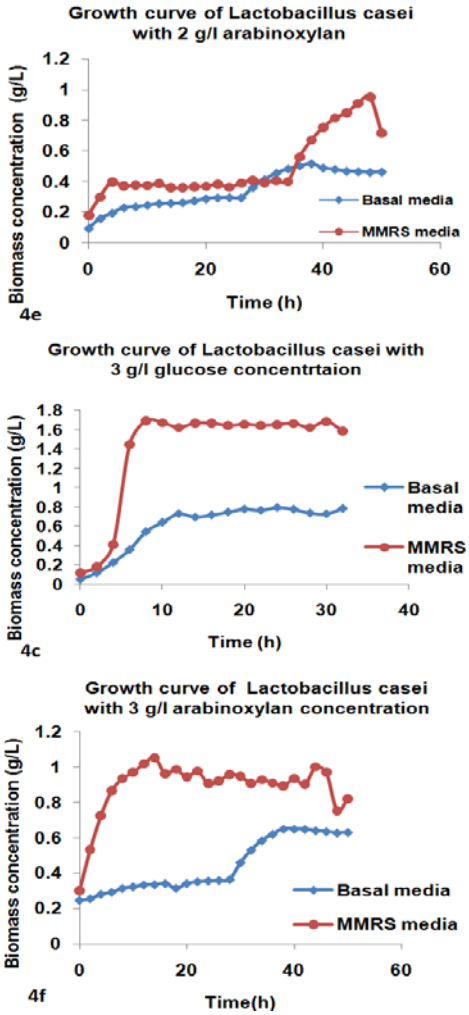


Fig. 4: Determination of maximum specific growth rate (μ_{max}) and substrate saturation constant (k_s)

Growth kinetics

Fig. 4a through 4c and 4d through 4f depict the time trajectories of biomass concentration in MMRS and Basal media over the culture period of 56 h have been plotted in fig. 4a-4c and 4d-4f respectively for glucose and arabinoxylan varying their initial concentrations in the range of 1-3 g/l.

From the analysis of the fig. (fig. 4a-4c and 4d-4f) it is clear that higher growth is always obtained in MMRS medium in comparison to Basal medium. This may be due to the favourable composition of nutrients in MMRS medium. From the plots, it is also noticed that at the same level of concentration, the growth rate of the microorganism is higher with glucose than with arabinoxylan. This may be due to the complexity of arabinoxylan molecule in comparison to glucose. In all cases, almost no lag phase is observed both in the case of glucose and arabinoxylan. This indicates excellent adaptability of the bacteria with both the carbohydrates. Another interesting fact revealed from the plots is that although the trajectory of biomass concentration in the presence of glucose follows usual exponential nature, the trajectory obtained during growth on arabinoxylan shows increasing tendency with respect to the normal exponential path at later periods. This may be caused due to the formation of simpler sugars, namely arabinose and xylose through hydrolysis of arabinoxylan after longer culture periods. In fig. 5a and 5b the values of the inverse of initial specific growth rate in MMRS and Basal media have been respectively plotted against the reciprocal of initial glucose concentrations. Similar plots were made with arabinoxylan in fig. 5c and 5d.

The linearity of plots in all cases indicates the validity of Monod model of growth kinetics. The values of kinetic parameters, μ_{max} and k_s have been determined to be 0.379/h, 0.3942 g/l and 0.08127/h, 0.3094 g/l for glucose in MMRS and Basal media respectively. Corresponding values of arabinoxylan in MMRS and Basal media are 0.0905/h, 0.071 g/l and 0.0416/h, 0.870 g/l respectively. The values of μ_{max} and k_s in each case have been determined graphically as well as through statistical non-linear regression analysis [26]. The yield coefficients of biomass with respect to arabinoxylan and glucose are-0.419 g/g and-0.25 g/g respectively.

Zone of inhibition

Antimicrobial activity of *L. casei* grown separately on glucose and arabinoxylan, against *E. coli* has been determined, measuring the zone of inhibition (fig. 6).

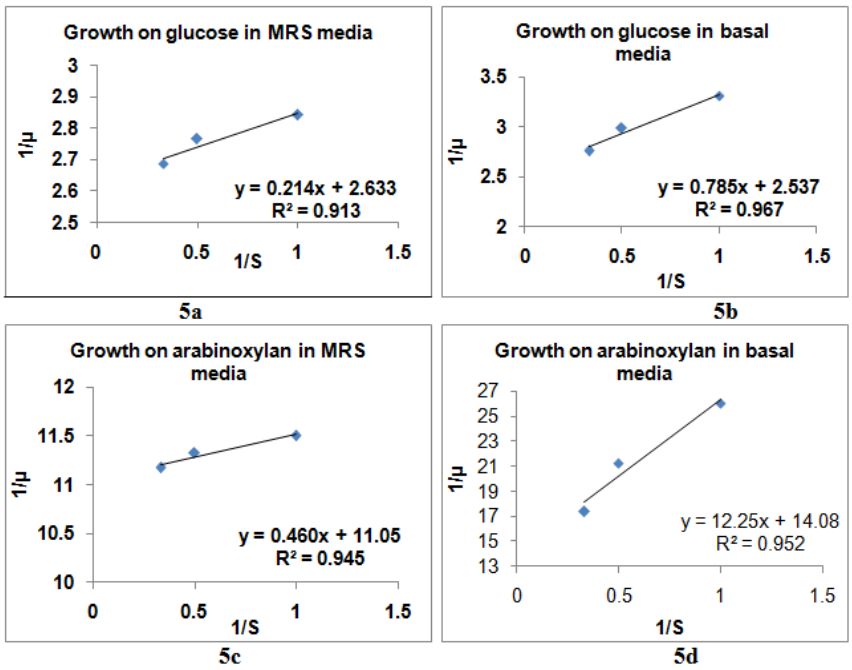


Fig. 5: μ_{max} and K_S calculation in MMRS and basal media

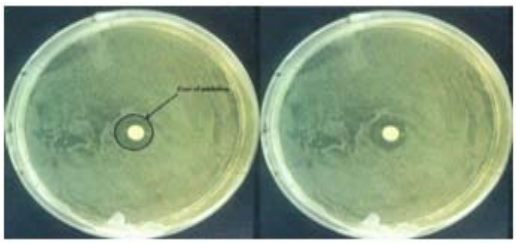


Fig. 6: Zone of inhibition against *E. coli*

While the maximum inhibitory effect of the organism against *E. coli* has been observed after 24 h of incubation with glucose, in the case of arabinoxylan it was observed after 36 h of incubation. The zone of inhibition is 14.5 mm in the case of arabinoxylan and 13 mm due to glucose.

Maximum inhibitory effect against *E. coli* was achieved in the later part of the growth (fig. 6). This may be due to the structural complexity and heavier molecular weight of arabinoxylan than that of glucose. Its uptake by the bacterial cells is expected to start at a later period as evident from the growth pattern of the bacteria. The zone of inhibition is larger in the case of arabinoxylan in comparison to that due to glucose. This indicates higher antimicrobial activity in presence of arabinoxylan. The prebiotic activity of arabinoxylan may be the cause of these findings.

Immobilization

Immobilization of *L. casei* was done using external gelation technique and the fig. 7 shows the freshly prepared microcapsules, microcapsules size determination and microscopic views of microcapsules containing microencapsulated *L. casei* under 20 X.

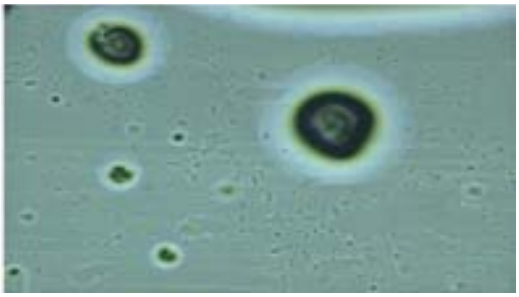


Fig. 7: Microscopic view of *L. casei* alginate microcapsules (at 20 X)

From the results of the experiments conducted using the protocol described in material and methods section, it has been observed that from 5 ml of cellular broth, 15 g of microcapsules are obtained. The calculations have been detailed below.

Cell count of *L. casei*

For free cells:

Colony forming units varying dilution factors

Order of dilutions	Colony forming unit (cfu)
10^{-10}	67
10^{-12}	64
10^{-14}	26

Calculations

Total CFU count = $(67*10^{10}+64*10^{12}+26*10^{14})/3$

= $8.8 * 10^{14}$

Since, 0.1 ml of inoculum contains $8.8 * 10^{14}$

Therefore, 1000 ml of inoculum = $(8.8 * 10^{14}/0.1) * 1000$

= $8.8 * 10^{18}$cfu/l

= $8.8 * 10^{15}$cfu/ml

1 ml of *L. casei* inoculum yields 0.04745g of dried biomass.

Beads formation:-

Seed culture+sodium alginate+rice bran oil+tween 80+SDS+$CaCl_2$

= 5 ml+20 ml+125 ml+0.25 ml+0.3125g

$2C_6H_7O_6Na+CaCl_2 \longrightarrow Ca(C_6H_7O_6)+2NaCl$

2 moles of Na-alginate $\longrightarrow$ 1 mole of Ca-alginate

Molecular weight of $Ca(C_6H_7O_6)$ = 390

Thus, 5*0.047579+((20*0.03)/198)*0.5*390+(125* 0.9)+0.25+0.3125

= 0.237895+0.590+112.5+0.25+0.3125

= 113.890 g

Since 5 ml of inoculum gives 15 g of beads

Thus, it is clear that other 98.890 g of chemicals were not used up at the time of bead formation.

Examination of bead size

24 freshly prepared microcapsules were selected randomly and their sizes were examined under a microscope. From the microscopic analysis, it has been observed that the size of the microcapsules varies from 0.1 mm to 0.55 mm in which 0.25 mm of diameter size microcapsules were maximum i.e., 38% of total microcapsules. The bead size distribution has been presented in fig. 8.

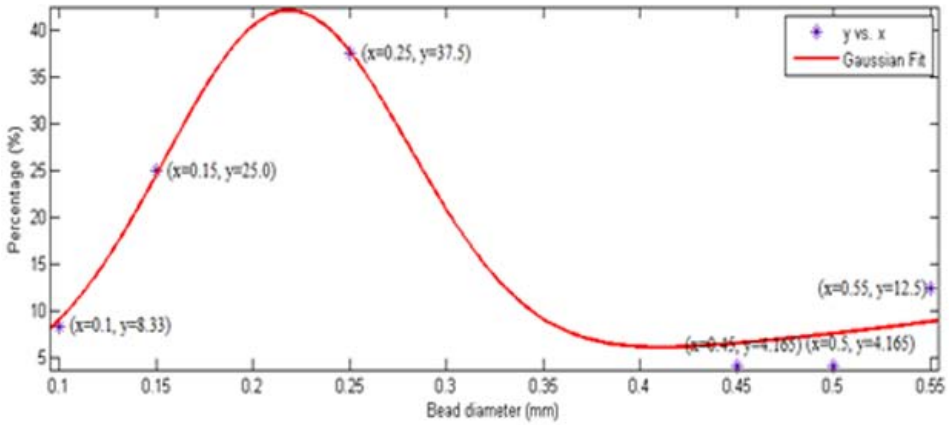


Fig. 8: Distribution of bead size

Table 2: Percentage of beads according to bead size

Bead size (mm)	Percentage
0.1	8.33
0.15	25
0.25	37.5
0.4	8.33
0.45	4.165
0.5	4.165
0.55	12.5

From the results of microscopic analysis and the bead size distribution, presented in fig. 8 it is clear that the microcapsules follow Gaussian size distribution.

Effect of centrifugal force on size distribution of microcapsules

After centrifugation at different centrifugal speed, new sets of the bead size were found and the results are shown in fig. 9. The data are provided in table 3.

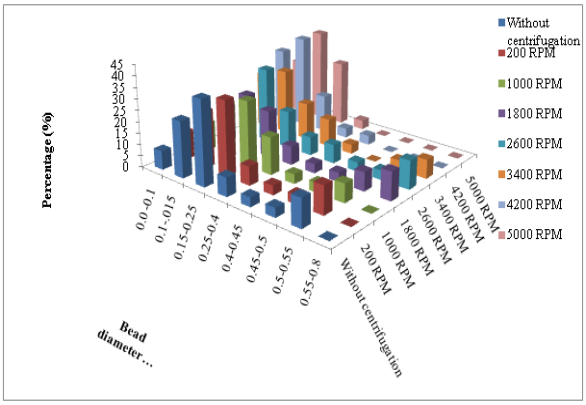


Fig. 9: Microscopic view of *L. casei* entrapped alginate microcapsules (at 20 x)

Table 3: Effect of centrifugation speed on the bead size

Bead size (mm)	Without centrifugation		200 rpm		1000 rpm		1800 rpm	
	Number of microcapsules	Percentage	Number of microcapsules	Percentage	Number of microcapsules	Percentage	Number of microcapsules	Percentage
0.0-0.1	2	8	3	13	3	13	4	17
0.1-.015	6	25	6	25	6	25	6	25
0.15-0.25	9	38	8	33	7	29	5	21
0.25-0.4	2	8	2	8	4	17	2	8
0.4-0.45	1	4	1	4	1	4	1	4
0.45-0.5	1	4	1	4	1	4	1	4
0.5-0.55	3	13	3	13	2	8	2	8
0.55-0.8	0	0	0	0	0	0	3	13
Bead size (mm)	**2600 rpm**		**3400 rpm**		**4200 rpm**		**5000 rpm**	
	Number of microcapsules	Percentage	Number of microcapsules	Percentage	Number of microcapsules	Percentage	Number of microcapsules	Percentage
0.0-0.1	3	13	6	25	8	33	6	25
0.1-.015	8	33	7	29	10	42	10	42
0.15-0.25	4	17	4	17	4	17	7	29
0.25-0.4	2	8	3	13	1	4	1	4
0.4-0.45	2	8	1	4	1	4	0	0
0.45-0.5	1	4	0	0	0	0	0	0
0.5-0.55	1	4	1	4	0	0	0	0
0.55-0.8	3	13	2	8	0	0	0	0

From the analysis of the results, it is evident that the size (0.15-0.25) of mostly populated bead remains unchanged as the centrifugation force is increased from 0 to 1000*g, beyond which there is a decrease in the size (0.1-0.15) of mostly populated microcapsules.

Effect of centrifugal force on stability of microcapsules

In fig. 10 the cell concentrations (cfu/ml) in the 48 h old MRS agar plate using the supernatant of centrifuged microcapsules have been plotted against the centrifugal speed. Values are given in table 4.

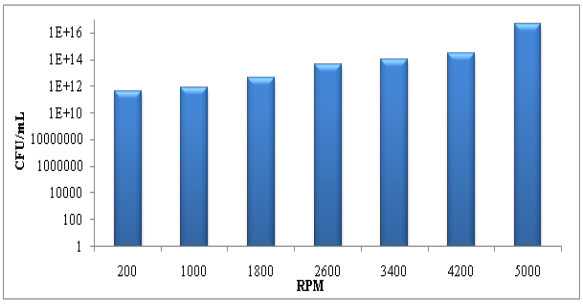


Fig. 10: Bar plot of different CFU/ml versus different centrifugal force (200rpm–5000rpm)

Table 4: Determination of beads stability varying centrifugal force

Centrifugation speed (RPM)	CFU/ml after 48 h of incubation
200	4X10[11]
1000	7.2X10[11]
1800	4.1X10[11]
2600	4X10[13]
3400	1.1X10[14]
4200	3.110[14]
5000	5X10[16]

It has been observed from fig. 10, that as the centrifugation speed is increased the corresponding supernatant cell concentration increases signifying the rate of disruption of microcapsules. This also signifies the increase of leakage of entrapped probiotic cells with the increase of centrifugal force.

Stability against freezing

The images of deep frozen entrapped *L. casei* cells captured under the microscope are shown in fig. 11 at different magnifications (20X

and 40X). It is evident that the cells are present in the bead even under freezing condition.

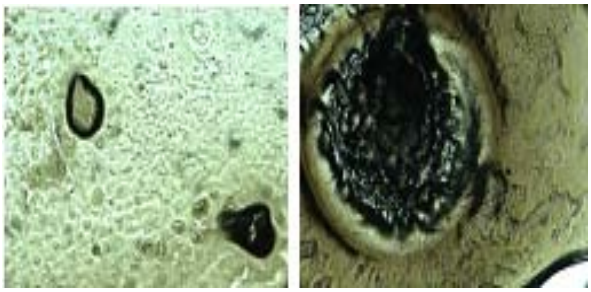


Fig. 11: Images of entrapped cells captured under (20X and 40X) microscope after deep freezing at-20°C for 10 d

Fig. 12 shows cell counts of 48 h old MRS plate of 10 d old deeply frozen milk samples with and without fortification. Data on stability against freezing with and without fortification are given in table 5.

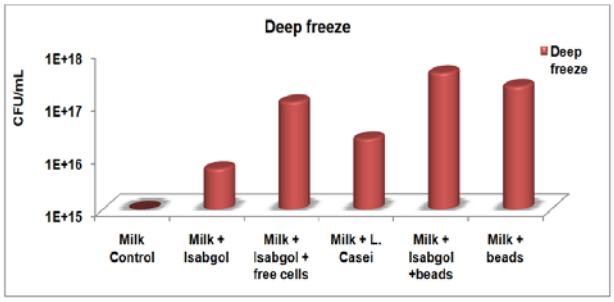


Fig. 12: Bar plot of stability of entrapped cells with and without fortification

Table 5: Effect of freezing on stability of beads fortified with milk and *Psyllium* husk (Isabgol)

Medium	Deep freeze (CFU)
Milk Control	1E+15
Milk+Isabgol	5.9E+15
Milk+Isabgol+free cells	1.30E+17
Milk+L. Casei	2.2E+16
Milk+Isabgol+microcapsules	3.9E+17
Milk+microcapsules	2.15E+17

From the analysis of fig., it appears that cell growth is enhanced by the fortification either with probiotic alone or with a combination of probiotic, *L casei* and prebiotic, arabinoxylan. It has been observed that when *L. casei* is added to milk and kept in deep freeze at-20°C, the number of viable cells decreases and more death rates occurs immediately and after a few days, it is saturated (not shown). Probably the damage to cells inside the freezer is caused by the formation of ice crystals. Effect of fortification increases when both the probiotic and its combination with prebiotics are used in encapsulated form, rather than in free form. The cell count of entrapped cell is approximately 34% more than the free cells. Thus, it is justified that entrapped cells survived better than non-entrapped cells.

Stability against thermal shock

Fig. 13 shows the size distribution of original microcapsules and that under thermally stressed condition. Data are given in table 6.

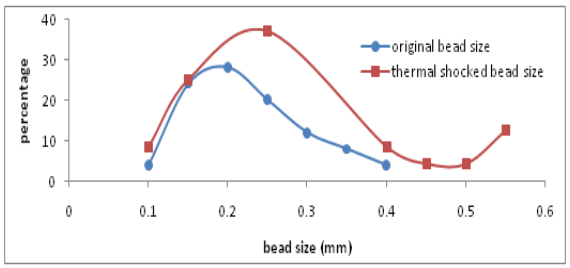


Fig. 13: Graph of reduction in bead size after thermal shock

Table 6: Stability of beads against thermal shock

Original microcapsules		Thermal shock at 47°C	
Size(mm)	Percentage	Size(mm)	Percentage
0.1	4.0	0.1	8.33
0.15	24.0	0.15	25.0
0.2	28.0	0.25	37.5
0.25	20.0	0.4	8.33
0.3	12.0	0.45	4.165
0.35	8.0	0.5	4.165
0.4	4.0	0.55	12.5

From fig. 13, it is observed that the percentage of mostly populated bead size (0.2) changes from 24% to 37.5% as the microcapsules encounter high temperature shock. This may be due to coalescence of microcapsules at high temperature.

CONCLUSION

From the above studies, it may be concluded that in the presence of arabinoxylan the growth of *L. casei* has been increased. The growth kinetics of *L. casei* followed Monod's model. The yield coefficients of biomass with respect to arabinoxylan and glucose are-0.419 g/g and-0.25 g/g respectively. The antimicrobial activity of *L. casei* against *Escherichia coli* as zone of inhibition in the presence of arabinoxylan has been observed to be larger (14.5 mm) in comparison to that obtained with glucose (13 mm). The size of microcapsules follows a normal distribution in the range of 0.1 mm-0.55 mm. The results obtained under the present investigation could not be compared with other findings due to unavailability of reported articles in this field in the literature. The retention of probiotic cells in fortified milk under freezing condition increases in the presence of arabinoxylan.

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CONFLICT OF INTERESTS

Authors have no conflict of interest to declare

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