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ORIGINAL PAPER

Hydrolysis of flatulence causing oligosaccharides by α-D-galactosidase of a probiotic *Lactobacillus plantarum* MTCC 5422 in selected legume flours and elaboration of probiotic attributes in soy-based fermented product

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Abstract In the background of increasing research interest in food fermentations involving beneficial culture, the focus present study was to evaluate the ability of a single desirable bacterial culture to impart multiple benefits to the product being prepared therein. The intracellular α-D-galactosidase elaborated by *Lactobacillus plantarum* MTCC 5422 in formulated soy whey broth for Lactobacillus on partial purification exhibited an activity of 1.2 U/mL with specific activity of 12.0 U/mg and a threefold purification. The partially purified enzyme had a molecular mass of 84 kDa, pH and temperature optima of 5.8 and 45 °C, respectively, and enzyme stability of 30 min at 55 °C. Selected legume flours treated (2 h at 45 °C) with partially purified α-D-galactosidase resulted in lowering raffinose and stachyose levels in the range of 50–74 and 66–85 %, respectively. Soy-based fermented probiotic curd prepared with *L. plantarum* MTCC 5422 from α-D-galactosidase treated soy protein isolate showed almost 99 % decrease in levels of raffinose and stachyose. In the final product, the probiotic culture of *L. plantarum* MTCC 5422 reached viable population of 9.4 $\log_{10}$ CFU/g and elaborated short chain fatty acids such as acetic, butyric and propionic acids in appreciable quantity. The product was devoid of any contaminating foodborne pathogenic bacterial species. At the same time, the product had appropriate levels of pH, acidity and antibacterial activity against *Bacillus cereus*. The innovative approach was the lowering of raffinose and stachyose by α-D-galactosidase of *L. plantarum* MTCC 5422 and elaboration of probiotic attributes by the same culture in the final product.

Keywords *Lactobacillus plantarum* · α-D-galactosidase · Probiotic soy curd · Raffinose · Stachyose · Legume flours

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Introduction

Cereal and legume-based foods are a major source of economical dietary energy and nutrients [1]. A challenging problem associated with most of the food legumes is the cause of flatulence that results from the oligosaccharides namely raffinose, stachyose and verbascose and together is commonly referred to as flatulence causing oligosaccharides (FCOs). As human system lacks α-galactosidases, these oligosaccharides are not easily digested and hence, not absorbed. As a result, anaerobic microbiota of large intestine ferments these oligosaccharides, resulting in excessive levels of rectal gas [2, 3]. Legumes are rich in FCOs to the extent of 4–5 % of the total sugars present and in general, are hydrolyzed by microbial α-D-galactosidase [4–6].

In the background of growing interest on prebiotics, the current status and significant aspects of dietary prebiotics is the topic of several reviews [7]. Studies have focused on the use of viable populations of lactic acid bacteria (LAB) in fermenting several types of beans (legumes) that are of common occurrence in different geographical regions [8, 9]. At the same time, research studies have established the beneficial attributes of LAB in terms of their antimicrobial activity, survival in simulated gastric environment, viability during extended storage and higher growth profile in presence of growth promoting substrates/factors [10–15].

Microorganisms are known to elaborate either extracellular or intracellular α-D-galactosidase (EC 3.2.1.22), which do have a major functional role in food fermentations [4, 16]. α-D-galactosidases from fungi, yeasts and bacteria have been purified to homogeneity and characterized with focus on cultures of lactobacilli and fungi [17–20]. Most of the earlier research studies have focused on isolation, purification and characterization of this enzyme, with lesser emphasis on the use of the enzyme in bringing about desirable activity profile in substrates of food uses.

Most of the traditional fermented foods are prepared from the locally available lesser known legumes, which invariably contain nutrient limiting factors [1]. An important property of desirable microbial cultures involved in fermentation has been their ability to elaborate certain beneficial and functional attributes that could contribute toward healthy and safe foods for human population [7]. Among the microbial cultures, desirable attributes associated with probiotic LAB are studied more so in soy milk-based yogurts along with additional cultures of *Bifidobacterium* spp. and *Lactobacillus* spp. [21–24]. However, the potential of desired probiotic LAB exhibiting multiple beneficial attributes in the same fermented product are less studied.

The main objective of present study was to evaluate the potential of a native probiotic culture of *Lactobacillus plantarum* MTCC 5422 to exhibit multiple benefits in the preparation of a product. The aspects of study included characterization of partially purified α-D-galactosidase elaborated by this probiotic culture and its efficacy to hydrolyze two of the FCOs, namely raffinose and stachyose in selected commercial legume flours. Finally, the probiotic attributes of L. *plantarum* was assessed in the preparation of soy-based fermented product.

Materials and methods

Materials

The dehydrated media and general chemicals used in this study were procured from HiMedia Laboratories (Mumbai, India). All fine biochemicals, dialysis tubing, protein molecular weight markers and Sephadex G-75 were from Sigma & Co., Bengaluru, India. The water used in the experimental trials was Milli Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

Ingredients/substrates

Commercial flours of legumes namely green gram (*Phaseolus aureus*), double bean (*Phaseolus lunatus*), cow pea (*Vigna sinensis*) and soy bean (*Glycine max*) in requisite quantities were procured from the local market of the City of Mysore, South India. Similarly, commercial pasteurized cow milk and prepared set curd in desired amounts were procured from the local Dairy.

Bacterial culture and inoculum preparation

The culture included was a native food isolate of *L. plantarum* MTCC 5422 with the deposition in the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India [25]. The bacterial culture was maintained in Lactobacillus MRS broth at 4 °C in the culture stock of the Department of Human Resource Development, CSIR-CFTRI, Mysore where the study was undertaken. The culture was propagated successively twice in the respective broth, prior to its use in experimental trials.

Cell suspension of the bacterial culture was prepared for use in the experimental trials. Isolate of *L. plantarum* was grown in Lactobacillus MRS broth for 18 h at 37 °C under static condition. After incubation period, the respective culture broth was centrifuged at 10,000 rpm for 20 min at 4 °C (Superspin R-V/F_M, Plasto Crafts, Mumbai, India), washed with sterile 0.85 % saline and cell pellet was resuspended in 10 mL of sterile saline taken in sterile screw-capped tubes and stored at 4 °C until further use. This suspension was serially diluted and appropriate aliquots of the dilutions were used to get an initial level of 2.3 $\log_{10}$ CFU/mL.

Preparation of soy protein isolate

Soy bean seeds in 50 g quantities were soaked along with 1.6 g of sodium bicarbonate in 400 mL potable water at ambient temperature for 12 h. The swollen soy bean seeds were then blended in a blender by adding 400 mL Milli Q water. The resultant slurry was filtered through muslin cloth. Soy milk was collected in a clean container; pH was adjusted to 4.5 with 6 M citric acid and boiled for 5 min. This was then filtered, and resultant protein coagulate was thoroughly washed till it comes to neutral pH. Later, water was drained off from the coagulate and frozen. The soy protein so obtained was lyophilized and prepared into a fine powder and stored at 6 °C until further use in the experiment as soy protein isolate.

Preparation of pre-set curd

Commercial pasteurized cow milk in 100 mL quantities was taken in pre-sterilized glass beaker of 250 mL capacity, covered with aluminum foil, steamed for 20 min and cooled to ambient temperature. Steamed milk aliquots were inoculated with individual viable cell population of 2.3 $\log_{10}$ CFU/mL of *L. plantarum* MTCC 5422 and incubated for 20 h at 37 °C. Set curd at 2 % level was used in the preparation of soy-based probiotic curd.

Medium for α-D-galactosidase of *L. plantarum*

The intracellular α-D-galactosidase from the culture of *L. plantarum* MTCC 5422 was prepared as described in our earlier paper [26]. The culture was grown in formulated culture medium, namely soy whey broth for Lactobacillus (SWBL). Soy whey broth was prepared from 50 g quantity of overnight soaked soy beans, which was then made into a slurry, filtered, pH adjusted and boiled to obtain soy milk. The soy milk was placed for 12 h at 6 °C, following which clear supernatant was collected and membrane filtered for use in the final medium. The basal medium of pH 6.5 ± 0.2 consisting of requisite quantities of peptone, yeast extract, galactose, desired salts of Mg and Mn, triammonium citrate was autoclaved at 121 °C for 15 min. The basal medium was added with 6 % of membrane filtered soy whey, inoculated with *L. plantarum* at a level of 2.3 $\log_{10}$ CFU/mL and incubated for 36 h at 37 °C under static condition. The intracellular α-D-galactosidase from the culture broth was obtained by centrifugation, sonication of resultant cells in 50 mM McIlvaine buffer of pH 5.8 and final cell-free extract was stored at 6 °C for further use in the study.

Assay for α-D-galactosidase activity and determination of total protein

In the sample aliquots of *L. plantarum* grown SWBL medium, intracellular α-D-galactosidase activity in cell-free extract resulting from sonicated cells was assayed in McIlvaine buffer following the method of Church et al. [27] as described in our earlier paper [25]. The total protein in sample aliquots was determined by the Coomassie Blue G-250 binding method [28]. The protein content was calculated using the standard curve prepared from 10 to 100 μg/mL of bovine serum albumin.

Partial purification of α-D-galactosidase

The culture of *L. plantarum* MTCC 5422 was inoculated into 50 mL aliquot of pre-sterilized SWBL at a level of 2.3 $\log_{10}$ CFU/mL and incubated for 36 h at 37 °C under static condition. The culture broth was centrifuged (10,000 rpm, 15 min, 4 °C), and the resulting cell pellet was washed twice with 10 mM McIlvaine buffer of pH 5.8. The cell pellet was subjected to sonication for the release of intracellular α-D-galactosidase. The cell debris was removed by centrifugation (10,000 rpm, 15 min, 4 °C), and the resultant supernatant was lyophilized in a Laboratory Model Lyophilizer (Heto Dry Winner, Jouan Nordic, Allerod, Denmark) to obtain a completely dried sample. The lyophilized enzyme preparation was resuspended in appropriate aliquot of 10 mM McIlvaine buffer and stored in sterile screw cap tubes at −20 °C until further use. Prior to lyophilization, aliquot of culture supernatant was assayed for α-D-galactosidase activity and total protein content as described previously.

The lyophilized sample containing α-D-galactosidase was fractionated with requisite quantities of well powdered ammonium sulfate by uniform mixing on a low-speed magnetic stirrer for 15 h at 4 °C. The protein precipitated with 65 % ammonium sulfate was collected by centrifugation (10,000 rpm, 20 min, 4 °C). The resultant precipitate was then resuspended in aliquots of 10 mM McIlvaine buffer of pH 5.8 and dialyzed thoroughly against 500 mL of 10 mM McIlvaine buffer of pH 5.8 for 24 h at 4 °C on a low-speed magnetic stirrer using a dialysis tubing of MWCO 12.4 kDa. The dialysate was lyophilized, and the same in 5 mL aliquot of 10 mM McIlvaine buffer was applied to a Sephadex G-75 column (30 cm length × 2.2 cm i.d.) that had been equilibrated with 10 mM McIlvaine buffer of pH 5.8, which later served as the eluant. The flow rate was maintained at 1 mL/min using a Minipuls 3 peristaltic pump (Gilson, Villiers Le Bel, France), and 5 mL fractions each were collected at 4 °C. The absorbance values of the individual fractions were read at 280 nm in the UV–VIS Spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) and also assayed for α-D-galactosidase activity as described previously. The eluted fractions exhibiting enzyme activity were pooled, lyophilized and stored at −20 °C until further use.

The sample aliquots of 65 % ammonium sulfate precipitate, lyophilized dialysate and lyophilized pooled fractions from Sephadex G-75 were assayed for α-D-galactosidase activity and total protein content as described previously.

The lyophilized pooled active fractions from Sephadex G-75 column was separated under reducing condition in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10 % polyacrylamide gel by the method of Laemmli [29]. The electrophoretic run was at a constant current of 60 V for 2 h in a Mini Slab Gel Electrophoresis System (GeNei, Bengaluru, India). After completing the run, the protein bands in the gel were visualized by staining with Coomassie brilliant blue R-250. The molecular mass of the partially purified α-D-galactosidase was calculated against the high range (36–200 kDa) molecular weight markers (S8320, Sigma-Aldrich, Bengaluru, India) consisting of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), chicken egg ovalbumin (45 kDa), bovine liver glutamic dehydrogenase (55 kDa), bovine serum albumin (66 kDa), rabbit muscle phosphorylase B (97 kDa), *Escherichia coli* β-galactosidase (116 kDa) and rabbit muscle myosin (200 kDa).

Characterization of α-D-galactosidase

The pH stability of partially purified lyophilized preparation (pooled fractions from Sephadex G-75) of α-D-galactosidase at a level of 30 U/mL was determined in 10 mM McIlvaine

buffer at pH levels of 2.8–7.8 by incubation for 12 h at 4 °C. Similarly, temperature stability of the partially purified α-D-galactosidase at a level of 30 U/mL was determined over the range of 30–60 °C at regular intervals of 10 °C in 10 mM McIlvaine buffer of pH 5.8 for 30 min. Further, stability of α-D-galactosidase to higher temperatures was assessed at individual temperatures of 55, 65 and 75 °C for 0–30 min with aliquots of 30 U/mL. At the respective intervals of treatment, sample aliquots were tested for residual activity. The treated (experimental) buffer sample aliquots were assayed for α-D-galactosidase activity as described previously. Untreated buffer aliquots served as control.

The effect of selected metal ions such as Ba^{2+}, Ca^{2+}, Cd^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{2+} and Zn^{2+} at a concentration of 5 mM and Hg^{2+} at a concentration of 1 mM as well as inhibitors such as ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), β-mercaptoethanol and phenyl methyl sulfonyl fluoride (PMSF) at a level of 1 mM was individually treated for 60 min at 4 °C with known concentration of the partially purified lyophilized α-D-galactosidase in aliquots of 10 mM McIlvaine buffer of pH 5.8. The treated buffer sample aliquots were assayed for α-D-galactosidase as described previously. The activity recorded in terms of U/mL was expressed as relative activity (%) of the enzyme. Aliquots of buffer devoid of metals/inhibitors served as control.

Action of α-D-galactosidase on raffinose and stachyose in legume flours

Commercial flours of green gram, double bean, cow pea and soy bean in quantities of 2 g each were mixed with 4 mL of 10 mM McIlvaine buffer (pH 5.8) containing 10.0 U/mL lyophilized pooled fractions (from Sephadex G-75) α-D-galactosidase. As the probable use of α-D-galactosidase is in the initial step of preparing legume-based foods, the mixed samples (experimental) were incubated for 2 h at 45 °C in an orbital shaker maintained at 120 rpm. Immediately after incubation period, the reaction was terminated by keeping the reaction mixture in a boiling water bath for 5 min. The individual reaction mixtures were lyophilized and then soluble sugars were extracted with 80 % (v/v) aqueous ethanol [4, 30]. The solvent was evaporated at 45–50 °C, and the sugars were resuspended in 1 mL of 80 % aqueous ethanol and analyzed by HPLC.

The HPLC analysis was performed in a Hewlett-Packard HPLC model HP 1100 Series (Hewlett-Packard, CA, USA) equipped with a quaternary pump, 30 °C oven and fitted with aminopropyl (NH_2) Inertsil (GL Sciences Inc., Tokyo, Japan) analytical column (4.6 mm × 250 mm, 5 μm particle size). The mobile phase was acetonitrile:water isocratic mixture (70:30 v/v), which was membrane filtered through 0.45 μm membrane filter (Millipore, Bengaluru, India) under vacuum and degassed by sonication for 15 min. Injections were made with a 20 μL loop. The flow rate was 1.0 mL/min. The detection was carried out using a HP 1100 Series Refractive Index Detector. The individual sugars were identified and quantified by HP Chemstation software with a comparison with the retention times of standard sugar concentrations.

In addition to the above, experiment trial was undertaken to find out the hydrolyzing efficiency of crude enzyme extract of α-D-galactosidase on standard sugars like stachyose and raffinose. For this purpose, a mixture of 5 mL of lyophilized crude enzyme extract containing 50 U of α-D-galactosidase activity and 0.1 mL of 10 mg/mL of stachyose and raffinose, individually in 10 mM McIlavaine buffer (pH 5.8). This was incubated at 45 °C for various periods of 20, 40 and 60 min. The hydrolyzed sugars were analyzed quantitatively by HPLC as described previously. Besides, thin layer chromatography (TLC) was also performed on pre-coated silica gel plates. Plates were developed at ambient temperature in a saturated chamber containing 1:1:0.1 (v/v/v) of *n*-propanol:acetic acid:water as a solvent system. After final development was completed, the TLC plates were sprayed with 1 % α-naphthol in absolute ethanol containing 10 % of *ortho*-phosphoric acid. The plates were then kept in an oven at 140 °C for 5 min [31].

Probiotic attributes of *L. plantarum* in soy-based curd

As a means of overcoming the beany flavor of soy beans and also impart probiotic attributes as well as lowering levels of raffinose and stachyose, in this experimental trial, probiotic soy curd was prepared with the probiotic culture of *L. plantarum* MTCC 5422 from a mixture of α-D-galactosidase-treated soy protein isolate and cow milk. On the basis of preliminary trials, soy protein isolate was used at 4 % for obtaining a good set curd in 10–12 h at 37 °C. The protocol of preparation of probiotic soy curd is presented in Fig. 1. In this experimental study, two types of samples were included:

Sample 1 (Experimental): 4 % soy protein treated with 10 U/mL of α-D-galactosidase + commercial dairy milk + 2 % pre-set curd of *L. plantarum.*
Sample 2 (Control): 4 % of soy protein treated with 10 U/mL of α-D-galactosidase + commercial dairy milk + 2 % of commercial dairy curd (no identity of lactic culture used).
The final set curd was analyzed for viable population of *L. plantarum*, acidity, pH, raffinose, stachyose and hydrolyzed sugars and short chain fatty acids (SCFAs).

Viable population of *L. plantarum* in probiotic soy-based curd

As the probiotic soy-based curd (experimental sample) was prepared with probiotic culture of *L. plantarum* MTCC

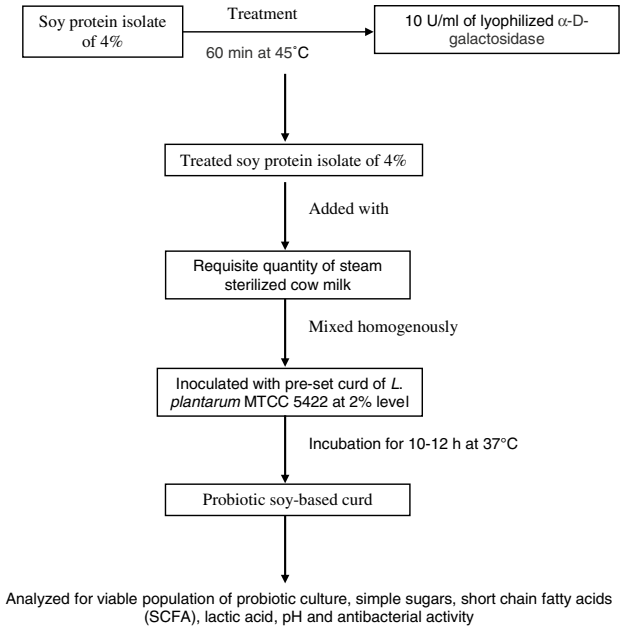

Fig. 1 Flow sheet for the preparation of probiotic soy curd. In this experimental study, two types of samples were included: Sample 1 (Experimental): 4 % of soy protein + 10 U/mL of α-D-galactosidase + commercial dairy milk + 2 % pre-set curd of *L. plantarum* MTCC 5422. Sample 2 (Control): 4 % of soy protein + 10 U/mL of α-D-galactosidase + commercial dairy milk + 2 % of commercial dairy curd (no mention of lactic culture name)

5422, it was desired to enumerate the viable population of this culture in the prepared product. At the same time, the control sample was also enumerated for viable population of LAB, as the culture inoculum was commercial curd with no identity of culture used. Requisite aliquots of appropriate serial dilutions of experimental and control samples of soy-based curd were pour plated with Lactobacillus MRS agar and incubated for 24–48 h at 37 °C. The number of colonies appearing in the incubated plates of respective dilutions was counted, averaged and expressed as $\log_{10}$ CFU/g. The colonies appearing in the incubated plates of probiotic soy-based curd (experimental) were selected at random and subjected to selected morphological, cultural and biochemical characteristics [25]. The colonies resulting from control samples were also tested for Gram staining and catalase production, two of the main identifying characters of LAB. The isolated culture of *L. plantarum* MTCC 5422 and the lactic culture of control samples were assayed for α-D-galactosidase activity as described previously. Further, the presence of α-galactosidase gene in these two lactic cultures was confirmed by performing PCR with melA primers of 398 bp (F 5′-CGGAAGTCTATTCGCCACAT-3′ and R 5′-CTGCTTGTCCGCATCACTTA-3′) with about 5 % of randomly selected individual colonies appearing in the incubated MRS agar plates. The preparation of total genomic DNA and PCR detection was performed as described in our earlier paper [25].

Profile of raffinose and stachyose

This was evaluated in the prepared product to know the extent of functional efficiency of α-D-galactosidase in the preparation of soy-based curd. The profile of FCOs was determined by HPLC as described previously.

Profile of short chain fatty acids

Analysis of SCFAs in the soy curd was performed by slight modification of the method of Kihara and Sakata [32]. Aliquot of 2 mL of homogenized soy curd sample was taken, and 7 mL of 10 mM NaOH containing 0.1 mM crotonic acid was added. The mixture was kept in a shaker incubator for 6 h at 30 °C. To remove fat soluble substances, 1 mL chloroform was then mixed with the sample and centrifuged at 10,000 rpm for 15 min at 4 °C in a refrigerated centrifuge. The supernatant was filtered through 0.22 μm membrane filter. The HPLC analysis was performed in a Hewlett-Packard HPLC model HP 1100 Series (Hewlett-Packard, CA, USA). An analytical column [C 18] was used for this purpose and eluted with 0.1 % (v/v) phosphoric acid isocratic mixture at 30 °C at a flow rate of 0.7 mL/min, using an UV detector at 210 nm. The individual SCFAs were identified and quantified by comparison with the retention times and standard SCFA concentrations.

Antibacterial activity

The ability of probiotic cultures to elaborate antibacterial activity in soy-based curd was assessed in supernatant of the prepared product by performing the assay against one of the foodborne pathogenic bacterial species namely *Bacillus cereus* F 4810 (courtesy Dr. J.H. Kramer, Central Public Health Laboratory, UK) by agar well-diffusion method as described earlier [25, 33, 34]. Antibacterial activity was expressed as activity unit per millimeter (AU/mL).

Analysis of acidity and pH

A requisite amount of prepared soy-based curd sample was taken and then diluted with known amount of Milli Q water. One to two drops of phenolphthalein indicator was added. It was titrated against 0.1 N NaOH till the appearance of permanent pale pink color as the end point appeared [35]. The pH value of each curd sample was measured in a digital pH meter (Eutech, pH tutor, Singapore). The pH meter was standardized using pH 4 and 7 buffer solutions. The curd samples were stirred with a small amount of Milli Q water prior to pH measurement.

Evaluation of prepared product for microbial safety

Experimental samples of prepared soy-based curd were enumerated for viable counts of mesophilic aerobes, yeasts and molds, *E. coli*, *B. cereus* and *Staphylococcus aureus* using plate count agar, potato dextrose agar, violet red bile agar, *B. cereus* agar and Baird-Parker agar, respectively. Appropriate dilutions of the samples in 0.1 mL aliquots prepared in 0.85 % saline were surface plated on the pre-poured plates of respective agar media except for *E. coli*, which was pour plated with violet red bile agar. For bacterial species, inoculated plates were incubated for 24–48 h at 37 °C, while for yeasts and molds, the incubation was 48–72 h at 30 °C. The counts of characteristic colonies appearing in the respective incubated plates were recorded and expressed as $\log_{10}$ CFU/g.

Statistical analysis

All experimental trials were conducted in triplicates, and the average values with standard errors (SE) were calculated using Microsoft Excel Software Programme 2010 (Microsoft Corporation, Redmond, WA, USA). The significance of results from data analysis was considered by $P < 0.05$ for presenting the final results.

Results

Characterization of α-D-galactosidase of *L. plantarum*

The associated activities during partial purification of intracellular α-D-galactosidase of *L. plantarum* MTCC 5422 elaborated in SWBL are presented in Table 1. The purification revealed an increasing activity from 0.69 U/mL in 65 % ammonium sulfate precipitation to 1.2 U/mL in lyophilized pooled active fractions from Sephadex G-75 column. The specific activity was in the range of 5.7–12.0 U/mg, protein. The elution profile (Fig. 2) revealed the highest activity in fraction 21 with an eluant volume of 105 mL. The resulting purification was threefold. The molecular weight of the partially purified α-D-galactosidase as determined in SDS-PAGE was 84 kDa (data not shown). The maximum activity of α-D-galactosidase was at pH 5.8, with the activity being appreciable between pH 4.8 and 6.2 (Fig. 3a). The optimum temperature profile for higher enzyme activity was 40–45 °C (Fig. 3b). It was observed that the enzyme activity was stable for 30 min at 55 °C, while it decreased to 80 % in 30 min at 65 °C (Fig. 4).

The effect of metal ions and inhibitors on α-D-galactosidase activity revealed that Hg^{2+} at concentration of 1 mM almost completely inhibited the enzyme, while 5 mM of Zn^{2+} caused a decrease in enzymatic activity (Table 2). The addition of other divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+} and K^{+} at concentration of 5 mM to the reaction buffer did not alter α-D-galactosidase activity, while Ba^{2+} and Cd^{2+} ions moderately inhibited enzyme activity. The inhibitors like EDTA, DTT and β-mercaptoethanol at a concentration of 1 mM had no effect on α-D-galactosidase activity, whereas PMSF slightly inhibited the activity.

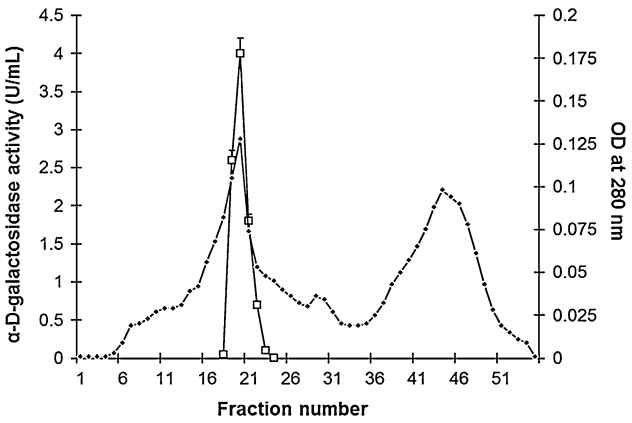

Fig. 2 Elution profile of α-D-galactosidase activity of *L. plantarum* MTCC 5422 obtained through separation in Sephadex G-75 column. *Open square* represents activity, *filled square* OD at 280 nm

Table 1 Partial purification of α-D-galactosidase of *L. plantarum* MTCC 5422 elaborated in soy whey broth for *Lactobacilllus*

Purification parameters	Purification steps			
	Culture supernatant (values ± SD)	65 % ammonium sulfate pptn.	Lyophilized dialyzate	Lyophilized pooled fractions (Sephadex G-75)
Volume (mL)	50	20	10	05
Activity (U/mL)	0.60 ± 0.2	0.69 ± 0.18	0.76 ± 0.22	1.2 ± 0.16
Protein conc. (mg/mL)	0.15 ± 0.06	0.12 ± 0.04	0.11 ± 0.03	0.1 ± 0.0
Specific activity (U/mg, protein)	4.0 ± 0.8	5.7 ± 1.2	6.9 ± 0.8	12.0 ± 1.4
Purification (fold)	1.0	1.4	1.7	3.0

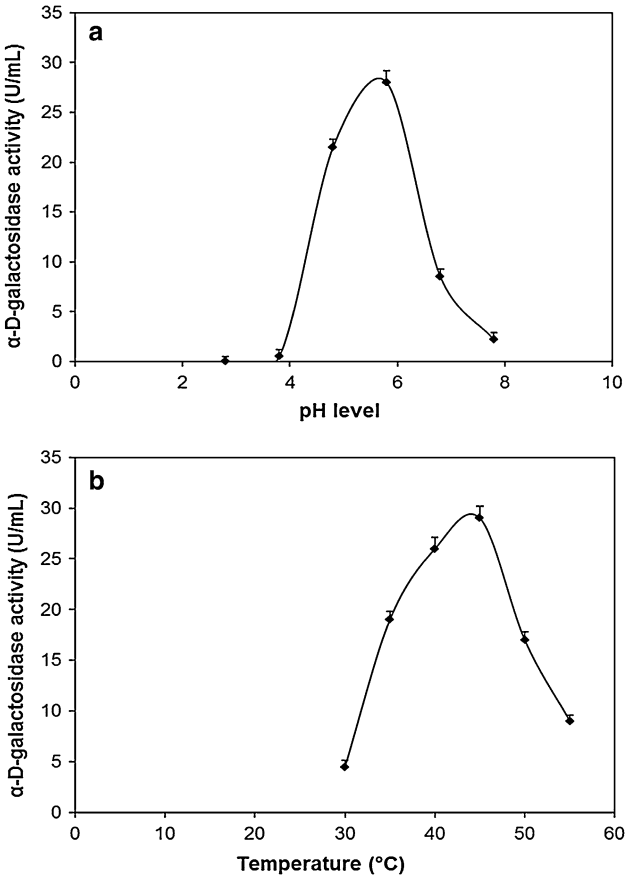

Fig. 3 Activity of α-D-galactosidase in *L. plantarum* MTCC 5422 under optimal pH (**a**) and temperature (**b**)

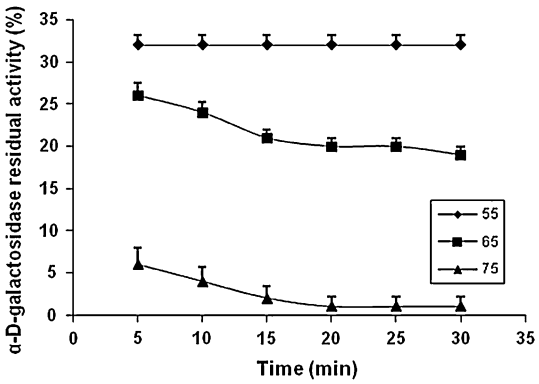

Fig. 4 Temperature stability of α-D-galactosidase activity in *L. plantarum* MTCC 5422

Hydrolysis of FCOs in legume flours by α-D-galactosidase of *L. plantarum*

In the present study, the partially purified α-D-galactosidase of *L. plantarum* MTCC 5422 was very much effective to hydrolyse FCOs such as raffinose and stachyose in commercial legume flours. In terms of quantitative evaluation of action of α-D-galactosidase in legume flours, the HPLC chromatograms (Figs. 5, 6) did indicate the reduction in levels of raffinose and stachyose in 2-h-treated flour samples of selected legumes as against their levels in untreated (control) samples. Quantitatively, α-D-galactosidase treated flours of green gram, cow pea, double bean and soy bean revealed reduction in the levels of raffinose as against their initial levels in untreated flours was 64, 50, 74 and 63 %, respectively, and that of stachyose was 76, 66, 72 and 85 %, respectively (Table 3). Simultaneously, there was also an increase or decrease in levels of other sugars such as sucrose, galactose, fructose and glucose. At the same time, the potential of α-D-galactosidase of *L. plantarum* MTCC 5422 to hydrolyze the oligosaccharides present in selected legume flours were also visualized by TLC (Fig. 7). The sugar profile of α-D-galactosidase treated standard sugars namely stachyose and raffinose was determined by HPLC (Figs. 8, 9). Stachyose was hydrolyzed to galactose and sucrose with raffinose as the intermediate, whereas raffinose was hydrolyzed to galactose and sucrose. During the treatment period of 20, 40 and 60 min, the amount of stachyose and raffinose got reduced, and at the same time trace amounts of sucrose and galactose were detected (Table 4). In control samples (untreated), stachyose was quantified as 0.64 mg/mL and raffinose as 0.84 mg/mL. The sample incubated for 60 min showed complete absence of both standard sugars.

Table 2 Effect of metal ions and inhibitors on α-D-galactosidase of *L. plantarum* MTCC 5422

Metal ion[a]/inhibitor[b] parameters	Relative activity (%)
Positive control	100
Ba^{2+}	62
Ca^{2+}	98
Cd^{2+}	74
Fe^{2+}	86
K^{+}	92
Mg^{2+}	96
Mn^{2+}	96
Zn^{2+}	62
Dithiothreitol	90
Ethylenediaminetetraacetate	94
β-mercaptoethanol	78
Phenyl methyl sulfonyl fluoride	ND

Hg^{2+} at a concentration of 1 mM inhibited α-D-galactosidase activity

ND not detected

[a] Metal ions were used at a concentration of 5 mM

[b] Inhibitors at 1 mM

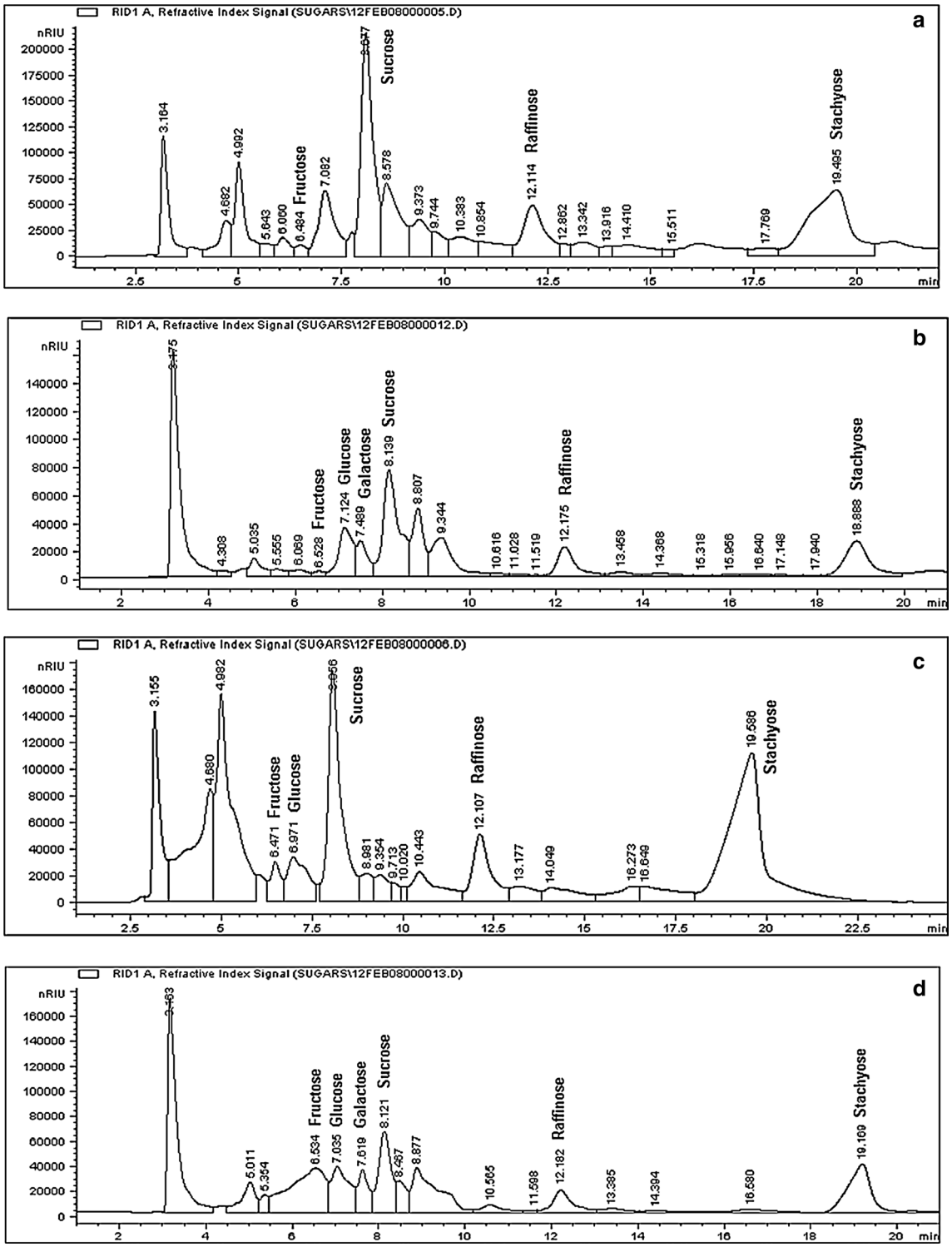

Fig. 5 HPLC chromatogram of sugar profile in flours of green gram and cow pea due to the action of α-D-galactosidase of *L. plantarum* MTCC 5422. Untreated (**a**) and 2 h at 45 °C treated (**b**) flour of green gram; and untreated (**c**) and 2 h at 45 °C treated (**d**) flour of cow pea

Probiotic attributes in soy-based curd

In the present study, an attempt was made to optimize the preparation of a probiotic curd from a combination of soy protein isolate and cow milk using the characterized native probiotic isolate of *L. plantarum* MTCC 5422. Both the experimental and control products exhibited characteristic setting pattern of curd as the lactic cultures were able to grow

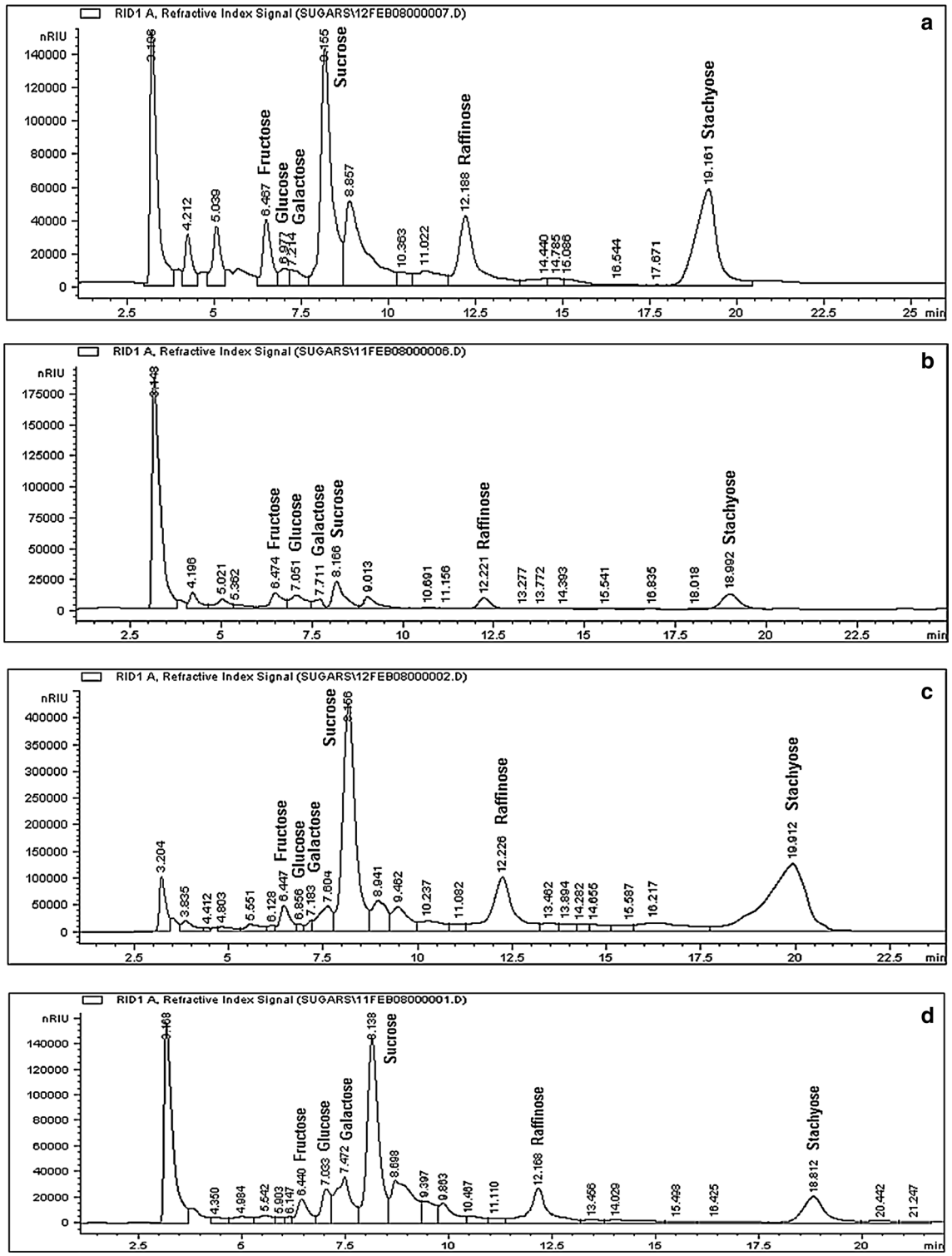

Fig. 6 HPLC chromatogram of sugar profile in flours of double beans and soy beans due to the action of α-D-galactosidase of *L. plantarum* MTCC 5422. Untreated (**a**) and 2 h at 45 °C treated (**b**) flour of double beans; and untreated (**c**) and 2 h at 45 °C treated (**d**) flour of soy beans

well and produce acidity. In the prepared product (experimental Sample 1), this probiotic culture was able to grow exceedingly well reaching viable population of 9.4 ± 1.8 $\log_{10}$ CFU/g. The colonies appearing in the incubated plates of this product confirmed them to be *L. planatrum* MTCC 5422 based on Gram positive and rod-shaped cells, negative for

Table 3 Quantitative profile of sugars in commercial legume flours treated with partially purified α-D-galactosidase of *L. plantarum* MTCC 5422

Sugar profile	Green gram		Cow pea		Double beans		Soy bean	
	0 h	2 h[a]	0 h	2 h[a]	0 h	2 h[a]	0 h	2 h[a]
	(Conc. g/kg, flour)[b]							
Raffinose	22.4 ± 0.3	8.1 ± 0.2	21.7 ± 0.4	11.0 ± 0.2	18.5 ± 0.2	4.8 ± 0.1	51.2 ± 0.3	19.2 ± 0.3
Stachyose	63.1 ± 0.5	15.1 ± 0.3	10.3 ± 0.2	35.7 ± 0.4	35.7 ± 0.2	10.0 ± 0.2	129.7 ± 0.4	20.1 ± 0.4
Sucrose	33.7 ± 0.3	14.7 ± 0.2	33.2 ± 0.3	10.0 ± 0.2	24.5 ± 0.2	6.5 ± 0.2	76.5 ± 0.4	28.5 ± 0.4
Galactose	ND	5.6 ± 0.1	ND	7.8 ± 0.2	3.3 ± 0.2	3.2 ± 0.1	3.1 ± 0.1	6.4 ± 0.2
Fructose	4.4 ± 0.1	1.6 ± 0.0	12.0 ± 0.1	41.5 ± 0.4	14.1 ± 0.2	12.0 ± 0.3	19.2 ± 0.3	8.9 ± 0.2
Glucose	ND	15.3 ± 0.2	24.0 ± 0.3	18.3 ± 0.3	18.3 ± 0.2	10.0 ± 0.2	3.1 ± 0.1	9.2 ± 0.3

ND not detected

[a] Substrate treated with α-D-galactosidase for 2 h at 45 °C

[b] Values are the mean ± SD

catalase production, positive growth at 15 and 37 °C as well as 2, 4 and 6.5 % NaCl and acid from few individual sugars. The control (Sample 2) product showed a viable lactic count of 6.7 ± 1.3 $\log_{10}$ CFU/g. Herein, the lactic culture used in commercial curd tentatively appeared to be that of *Lactococcus* sp. based on Gram positive, cocci in single or in pairs and negative for catalase production. Qualitatively, culture of *L. plantarum* MTCC 5422 was positive for α-D-galactosidase activity and also showed positive amplification in PCR with melA primers of α-galactosidase (Data not shown). However, the culture of *Lactococcus* sp. from the commercial curd was devoid of α-D-galactosidase activity in qualitative assay and in PCR, there was no positive amplification with melA primers.

The ability of α-D-galactosidase of *L. plantarum* to reduce FCOs was also included in terms of incorporation of partially purified α-D-galactosidase during the preparation of this product. The profile of FCOs and other sugars in prepared probiotic soy curd is shown in Fig. 10, and quantification data are presented in Table 5. It was observed that levels of FCOs in soy protein isolate could be lowered through the use of 10 U/mL of α-D-galactosidase. It was of interest to record that in the experimental product (Sample 1), with the probiotic culture of *L. plantarum* MTCC 5422, the decrease in the levels of FCOs, lactose and sucrose was near to 99 %. In the control product (Sample 2), wherein commercial dairy curd (with no probiotic culture) was used in the preparation, there were the appreciable levels of lactose, sucrose, raffinose and stachyose. At the same time, there was an increase in concentration of fructose, glucose, galactose and melibiose in both the Samples 1 and 2, as could be evidenced in Fig. 10.

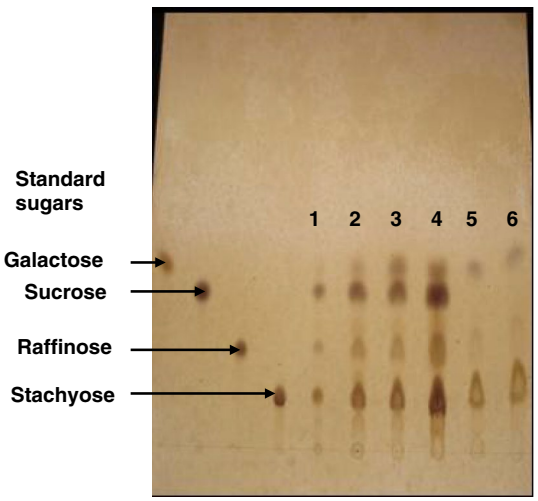

Fig. 7 Chromatogram (TLC) of sugar profile in untreated and α-D-galactosidase of *L. plantarum* MTCC 5422 treated legume flours. *Lane 1* double beans, *lane 2* cow pea, *lane 3* green gram, *lane 4* soy bean, *lane 5* 2-h-treated soy flour, *lane 6* 4-h-treated soy flour

The experimental probiotic soy curd (Sample 1) did show the presence of SCFAs namely acetic, propionic and butyric acids (Fig. 11; Table 6), while in the control product (Sample 2), the amounts were comparatively less. The titratable acidity (0.8–1.2 %) was slightly higher in probiotic soy curd, and accordingly lower pH levels were recorded (4.3–4.1). The experimental probiotic soy curd (Sample 1) exhibited 12 AU/mL of antibacterial activity against the culture of *B. cereus* and at the same time was devoid of any viable populations of foodborne pathogenic bacterial species such as *E. coli*, *B. cereus* and *S. aureus* and thus ensuring microbial safety of the prepared product. The control product (Sample 2) with the use of commercial curd did not exhibit any antibacterial activity. However, the product was devoid of any viable population of foodborne pathogenic bacterial species included in this study.

Discussion

Most of the food legumes are known to cause flatulence due to the presence of oligosaccharides namely raffinose,

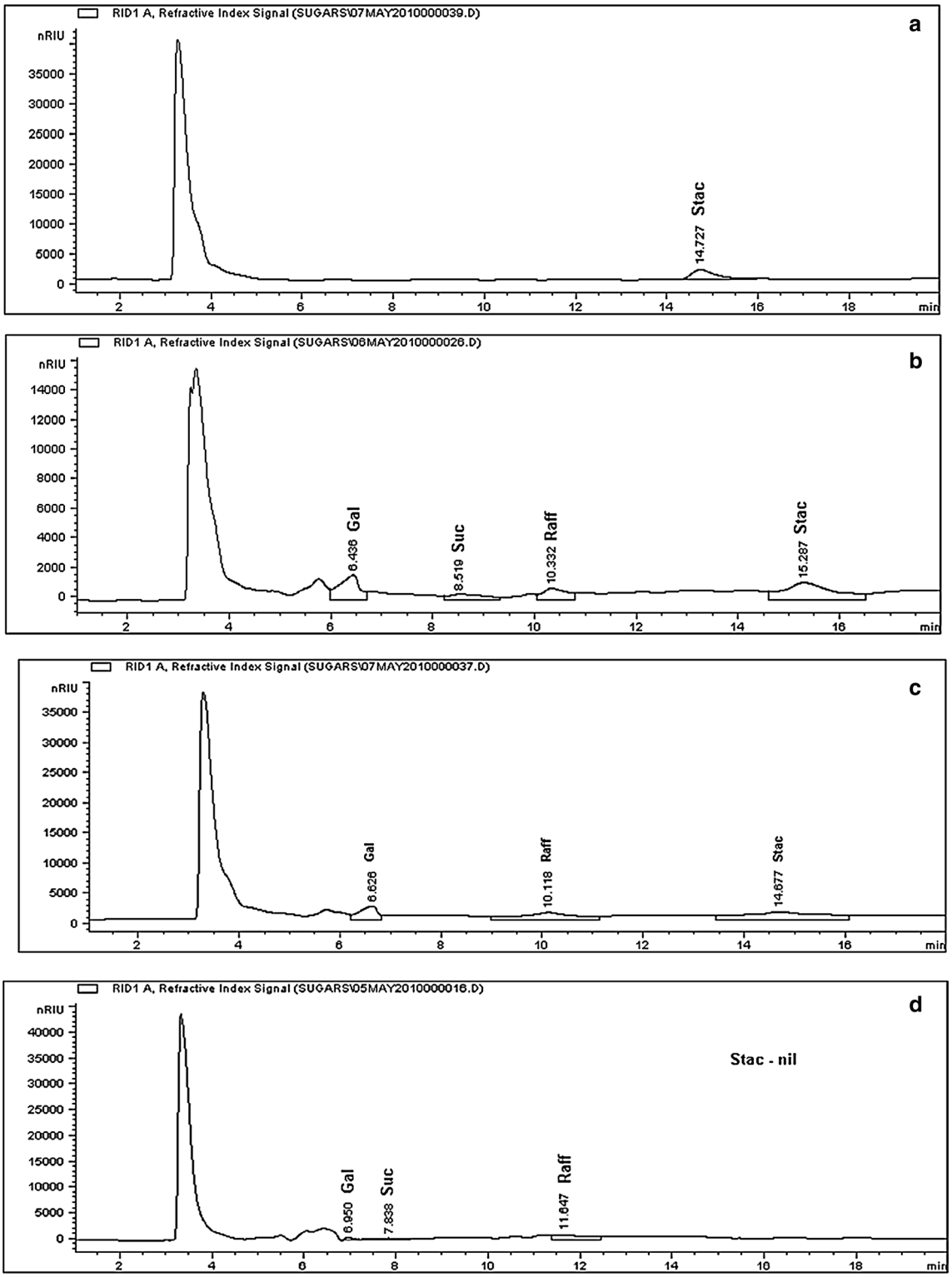

Fig. 8 HPLC chromatogram of untreated standard stachyose (**a**) and stachyose treated with α-D-galactosidase of *L. plantarum* MTCC 5422 for 20 min (**b**), 40 min (**c**) and 60 min (**d**). *Gal* galactose, *Raff* raffinose, *Stac* stachyose, *Suc* sucrose

stachyose and verbascose and microbial fermentation could break down these FCOs to easily absorbable sugars [2, 36]. Further, it is well established that fermentation of legumes by LAB can effectively reduce FCOs, which in turn improves the nutritive value [8, 9]. Several microbial sources of this enzyme are reported in literature, but the

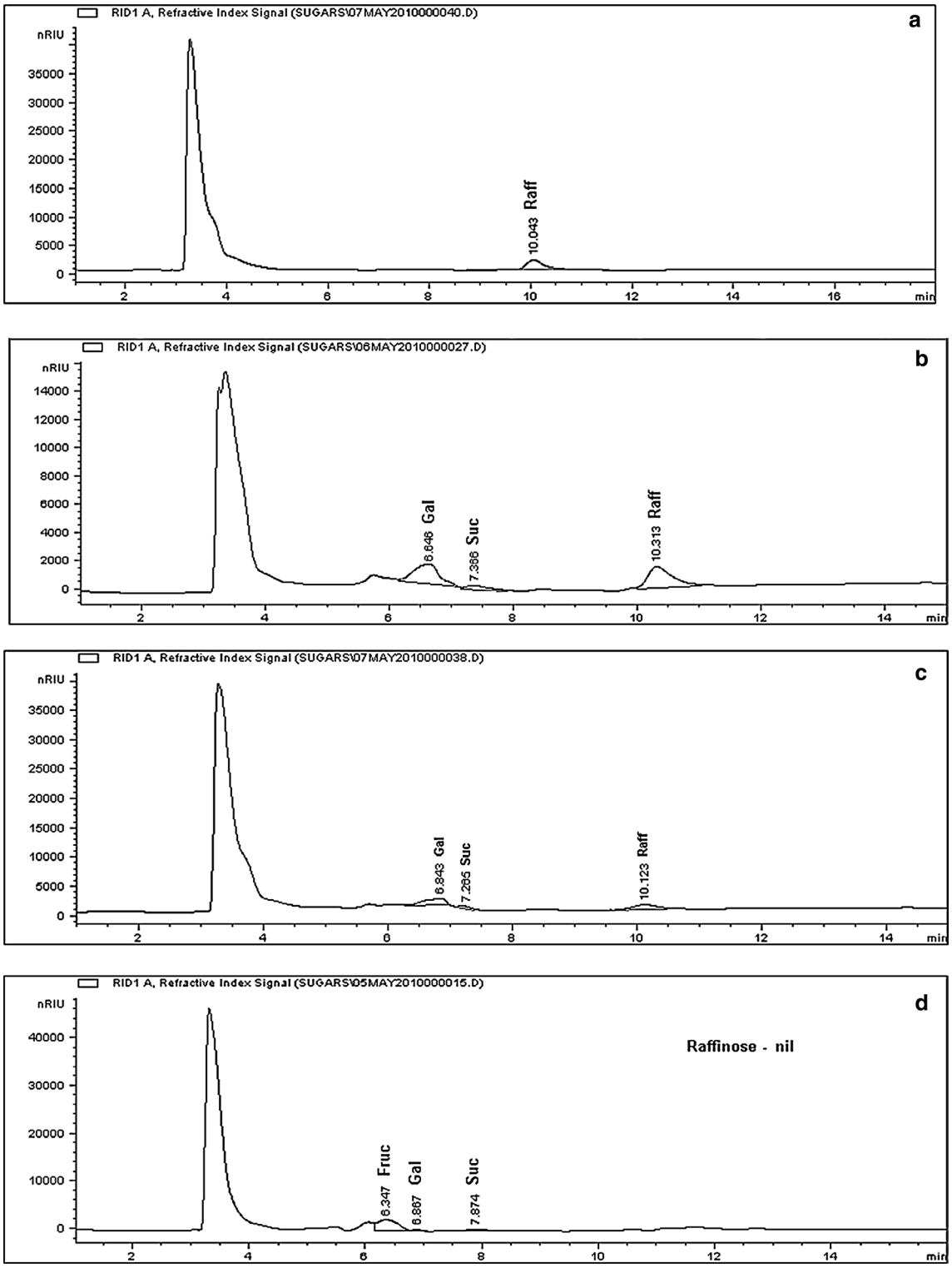

Fig. 9 HPLC chromatogram of untreated standard raffinose (**a**) and raffinose treated with α-D-galactosidase of *L. plantarum* MTCC 5422 for 20 min (**b**), 40 min (**c**) and 60 min (**d**). *Fruc* fructose, *Gal* galactose, *Raff* raffinose, *Suc* sucrose

research interest in this area continues with a focus toward isolating and characterizing α-D-galactosidase from desirable microbes for its specificity of action in a wide variety of legumes. It is quite likely that the presence of raffinose and stachyose in the formulated SWBL could have enabled a better enzyme activity of the native isolate of *L. plantarum*

Table 4 Sugar profile of standard stachyose and raffinose treated with partially purified α-D-galactosidase of *L. plantarum* MTCC 5422 as determined by HPLC

Standard sugar (10 mg/mL)	Incubation period (min)	Stachyose Conc. (mg/mL)[a]	Raffinose	Sucrose	Galactose
Stachyose	20	0.17 ± 0.03	0.12 ± 0.02	0.45 ± 0.08	0.73 ± 0.11
	40	0.13 ± 0.01	0.23 ± 0.04	0.61 ± 0.09	0.87 ± 0.09
	60	ND	0.07 ± 0.00	0.59 ± 0.03	0.14 ± 0.01
Raffinose	20	ND	0.54 ± 0.05	0.16 ± 0.02	0.73 ± 0.08
	40	ND	0.33 ± 0.07	0.18 ± 0.04	0.87 ± 0.11
	60	ND	ND	0.31 ± 0.05	0.68 ± 0.07

ND not detected

[a] Values are the mean ± SD

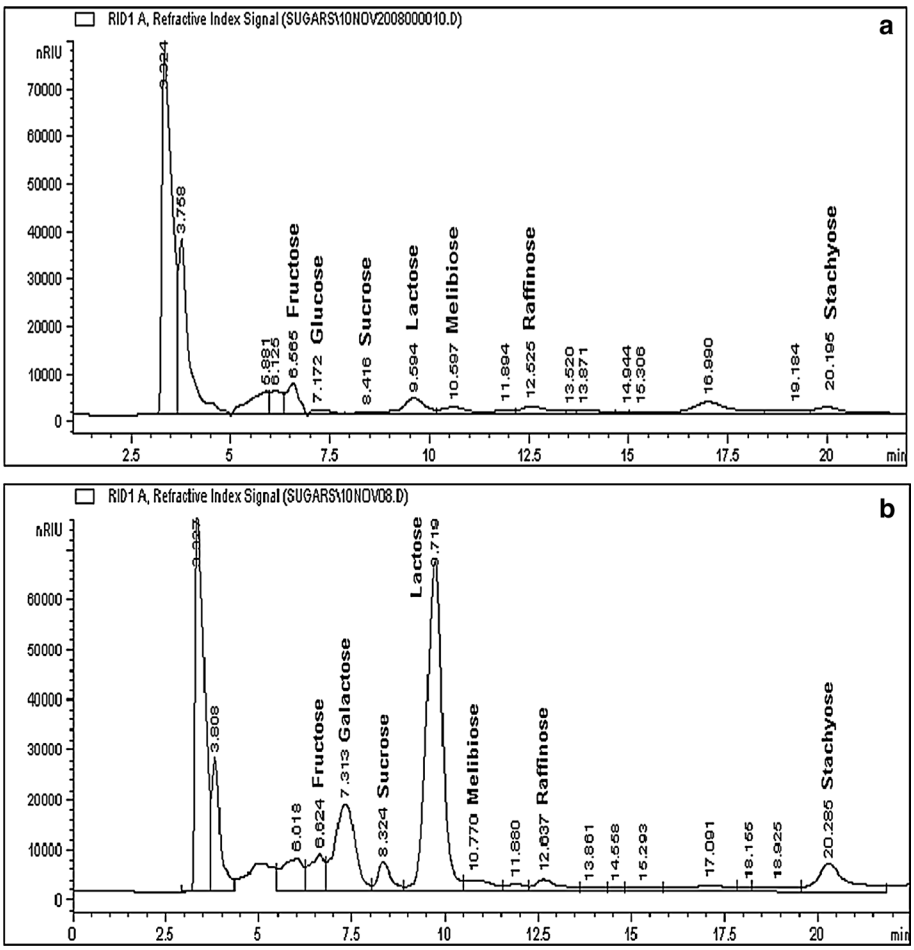

Fig. 10 HPLC chromatogram profile of FCOs and other sugars in experimental sample of probiotic soy curd (**a**) and control sample of soy curd prepared with commercial curd (**b**)

MTCC 5422, as a similar observation was documented in one of the earlier studies relating to optimization of growth of *L. acidophilus* FTCC 0291 for certain desired functional attributes [37]. The molecular mass of α-D-galactosidase appears to be very diverse with a range of 45 kDa to 300 kDa. The pH and temperature optima of the partially purified enzyme from *L. plantarum* MTCC 5422 was more or less same as those reported for other lactobacilli, wherein α-galactosidase activity of *L. plantarum* was active between pH 4.5 and 5.2 [38]. Further, slight variations in terms of activity of α-galactosidases were observed due to the effect of metal ions and inhibitors, which could be related with cultural parameters and degree of purification achieved [17–20, 39, 40]. It could be visualized that the native culture of

Table 5 Quantitative profile of sugars in soy-based curd samples

Sugar identified	Soy-based curd samples Probiotic soy-based curd (Experimental Sample 1) Conc. (g/100 g)[a]	Non-probiotic soy-based curd (Control Sample 2)
Stachyose	0.002 ± 0.00	0.07 ± 0.01
Raffinose	0.007 ± 0.01	0.06 ± 0.01
Melibiose	0.023 ± 0.00	0.07 ± 0.00
Lactose	0.07 ± 0.01	1.72 ± 0.09
Sucrose	0.01 ± 0.00	0.09 ± 0.01
Galactose	0.012 ± 0.01	0.37 ± 0.08
Glucose	ND	ND
Fructose	0.04 ± 0.00	0.10 ± 0.00

ND not detected

[a] Values are the mean ± SD

L. plantarum used in this study does exhibit the attributes that very much desirable in food fermentations.

Research investigations have revealed the ability of α-galactosidases from microbial and plant sources to reduce the levels of FCOs in legumes, particularly soy bean has been the focus of many of these studies [5, 20]. Besides soy bean, there have been a large number of other less utilized legumes, which constitute the base material of a diverse range of traditional fermented and non-fermented foods including those of Indian subcontinent [41]. The use of legume sprouts and other healthy and nutritional foods necessitates having these legumes with reduced levels of FCOs. Almost in line with the present study, several earlier research investigations have shown the ability of purified and crude preparation of α-galactosidase from microbial and plant sources to reduce FCOs with varying reduction levels being achieved [20, 30, 42]. Few of the studies with legumes revealed that fermentation using individual cultures of LAB could effectively reduce raffinose and stachyose through higher activity of α-galactosidase of probiotic LAB [43, 44]. In comparison with these earlier investigations, the efficacy of from *L. plantarum* MTCC 5422 was quite significant, wherein reduction in raffinose and stachyose achieved was higher (50–85 %), considering the treatment time being just 2 h at 45 °C as against the reduction levels (56–77 %) achieved in fermentation period of 24–72 h with individual 72 h of fermentation with pure cultures in

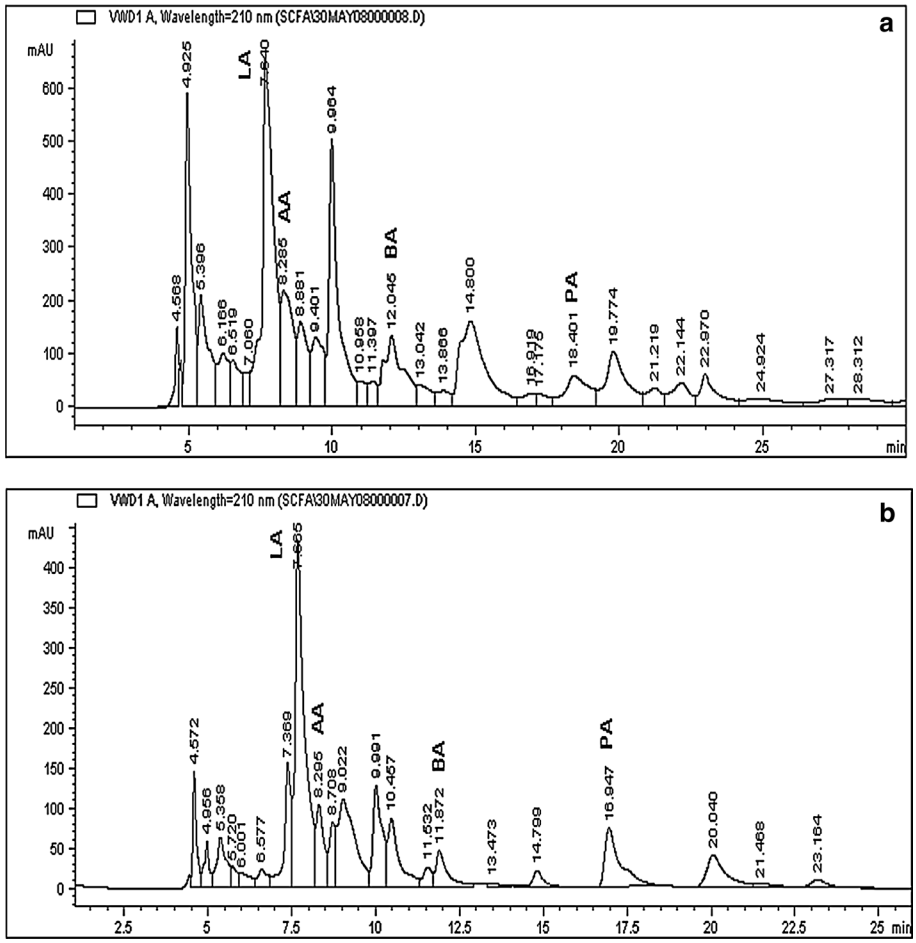

Fig. 11 HPLC chromatogram profile of short chain fatty acids and lactic acid in experimental sample of probiotic soy curd (**a**) and control sample of soy curd prepared with commercial curd (**b**). *AA* acetic acid, *BA* butyric acid, *PA* propionic acid, *LA* lactic acid

Table 6 Quantitative profile of short chain fatty acids (SCFA) and lactic acid in soy-based curd samples

SCFA identified	Soy-based curd samples Probiotic soy-based curd (experimental Sample 1) Conc. (g/100 g)[a]	Non-probiotic soy-based curd (control Sample 2)
Acetic acid	0.63 ± 0.08	0.21 ± 0.02
Butyric acid	0.52 ± 0.06	0.18 ± 0.01
Propionic acid	0.61 ± 0.06	0.14 ± 0.03
Lactic acid	1.22 ± 0.07	0.92 ± 0.08

ND not detected

[a] Values are the mean ± SD

those earlier studies [43–45]. Further, as documented in our earlier paper, the molecular identity of this activity in *L. plantarum* MTCC 5422 was established with primers (*melA*) designed for α-D-galactosidase [25].

A number of cereal/legume-based and milk-based foods are fermented by the growth of cultures of LAB, which imparts specific characteristics for the product. Often, these benefits are studied more so in soy-based products such as soy milk and soy yogurts. Research studies have contributed toward the development of soy yogurt as a product with desirable health benefits [46]. Few studies reported the presence of high percentage of FCOs in soy yogurt, because of the non-hydrolysis of these sugars by yogurt bacteria [47]. However, a good number of research studies have focused on methods for the reduction in FCOs in soy milk and soy bean products [21, 48–50]. The variations in the levels of FCOs may be due to the activity of the enzyme that could utilize some of these sugars as substrates for oligosaccharide formation [51].

Research studies have focused on viability, confirmed identity and few of the desirable attributes of selected probiotic cultures of *Lactobacillus* and *Bifidobacterium*, either in commercial fermented milks and/or in fermented soy milk. Often, studies in soy milk with probiotics have been more of optimizing cultural parameters for higher α-galactosidase activity, organic acids production and one or two associated metabolic activities [38, 43, 44, 52–54]. In the market, several soy-based products are available, but it has a healthy competition with dairy products. Probiotic cultures of *L. delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus* and *Bifidobacterium* spp. were able to exhibit increased viable populations in soy yogurt formulations [22–24]. The advantage or a step of innovation achieved in present study during preparation of probiotic soy-based curd is the pre-treatment of soy protein isolate with partially purified α-D-galactosidase of *L. plantarum* MTCC 5422 to effectively reduce the level of FCOs (raffinose and stachyose) in resultant probiotic soy curd and gain the probiotic attributes in the product in the form of SCFAs (Fig. 11). An observation similar to the present study was observed with cultures of *L. bulgaricus* and *S. thermophilus* used in soy yogurt preparation that were able to hydrolyze the FCOs [55]. It is also more likely that in the present study, the use of α-D-galactosidase treated soy protein isolate could eliminate the beany flavor, a finding that was reported due to metabolization of aldehyde by probiotic bacteria in soy yogurt [22]. A point of interest was that both the types of curd samples were free from beany flavor, which may be due to the use of only soy protein isolate and also the addition of α-D-galactosidase in the initial stages of preparation. The active proliferation of probiotic LAB in the product is reflected by the desirable attributes being present in the product for which the desired cultures were used in product preparation. One of the important attributes associated with active LAB is the production of SCFAs in food products that mainly depends upon the growth of these microbes and their ability to ferment available carbohydrates. It was reported that SCFAs were produced from oligosaccharides due to the metabolism by the microbes in hind gut [56].

The levels of acidity (0.8–1.2 %) and pH (4.3–4.1) observed in probiotic soy-based curd of present study was in line with the commercial fermented milks that revealed acidity of 0.79–1.16 % and pH of 3.9–4.2 [53]. Besides, the product also revealed slightly higher lactic acid content, which may be due to the active proliferation of *L. plantarum* MTCC 5422, resulting in complete utilization of lactose. On the other hand, in the sample 2, the unknown lactic culture used in commercial dairy curd was not so potent such as *L. plantarum* and, thus resulting in less lactic acid due to slower fermentation. Few of the earlier studies did report higher pH in probiotic yogurt than in natural yogurt due to low viable populations of probiotic cultures [57, 58]. However, increased titratable acidity can occur during storage period [59]. In general, acidification and decrease in pH is dependent upon strains used and time–temperature combination of storage [60, 61].

Conclusion

The findings of our research study reveal more than one benefits of native culture of *L. plantarum* MTCC 5422, exhibiting the efficacy of α-D-galactosidase activity in reducing FCOs in the base material/ingredient and later impart probiotic attributes in the product prepared therein. This gives an opportunity to use a single desirable culture to achieve quality consistent fermented product with benefits of health and microbial safety.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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