

Molecular characterization of native isolates of lactic acid bacteria, bifidobacteria and yeasts for beneficial attributes

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Received: 23 February 2009 / Accepted: 25 March 2009 / Published online: 1 May 2009
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Abstract In a changing scenario of food habits being associated with wellness factors through the concepts of probiotics and prebiotics, an attempt has been made to characterize on molecular basis, the desirable benefits associated with natural isolates of lactic acid bacteria, bifidobacteria, and yeasts. From a diverse range of foods and related samples, based on conventional microbiological protocols, three well-characterized natural isolates of *Lactobacillus plantarum* MTCC 5422, *Bifidobacterium adolescentis* MTCC 5423 and *Saccharomyces cerevisiae* MTCC 5421 were selected. The cultures of *L. plantarum* and *B. adolescentis* showed positive polymerase chain reaction (PCR) amplification with oligonucleotide primers targeting genus-specific 16 S rRNA for *Lactobacillus* and fructose-6-phosphate phosphoketolase for *Bifidobacterium*. Similarly, species-specific positive amplification in PCR was observed with primers of phytase (acid phosphatase) in *S. cerevisiae* and α -D-galactosidase and bile salt hydrolase in *L. plantarum* and *B. adolescentis*. The cultures of *L. plantarum* and *B. adolescentis* exhibited a broad spectrum antibacterial activity against selected foodborne pathogenic bacterial species and tolerance to acid and bile. Gene sequence of respective PCR-amplified products confirmed the genetic identity of the isolated cultures as *L. plantarum* and *B. adolescentis* showing 99% homology with the documented sequence of established gene bank.

Keywords *Lactobacillus plantarum* · *Bifidobacterium adolescentis* · *Saccharomyces cerevisiae* · Probiotic · Prebiotic · Molecular characterization · Phylogeny

Introduction

Use of microorganisms in preparing foods based on milk, cereals, and legumes have been a traditional practice since prehistoric times. In a majority of fermented foods, particularly those of traditional foods of India, which are based on cereals and legumes, the nature of fermentation has been a mixed type with the involvement of lactic acid bacteria (LAB) and yeasts. With the growing interest in health consciousness and idea of health promotion without associated health risks, the concept of probiotic foods has attracted much attention. In this context, probiotics, prebiotics, and synbiotics all have a significant role. Probiotics are live microbial additions to the diet; prebiotics are food components that have a selective metabolism in the hind gut, while synbiotics are combinations of the two approaches. A number of *Lactobacillus* spp., *Bifidobacteria* spp., and *Saccharomyces* spp. have been in use as desirable cultures, so as to act as beneficial food supplements and contribute to a balanced intestinal microflora for human wellness (Ljungh and Wadstrom 2006; Masco et al. 2007; Woodmansey 2007). These beneficial effects have been very much diversified ranging from suppression of harmful bacteria in large intestine to immune system activation effects (Fooks et al. 1999). Considering the potential of beneficial cultures, food industry has been looking toward well-characterized cultures with defined probiotic and other desirable attributes (Masco et al. 2007).

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Cereals and legumes are considered as effective substrates for the production of probiotic-incorporated functional food, as they can be used as a source of nondigestible carbohydrates which stimulate the growth of lactobacilli and bifidobacteria, both endogenous and supplemented, and contain water soluble fibres like β -glucan, arabinoxylan, galacto-oligosaccharides, and fructo-oligosaccharides, which are prebiotics (Swennen et al. 2006). They are fermented by several groups of bacteria in the large intestine, yielding a variety of fermentation products, particularly short-chain fatty acids (SCFA). The resulting SCFA are known to provide an acidic environment in the large intestine, which stimulates the proliferation of probiotic cultures (Roberfroid 1997; Macfarlane et al. 2006).

In general, the conventional protocols of morphological, cultural, and biochemical characterization of microbial cultures are in use to identify probiotic microbial cultures. Often, these attributes do not clearly express in the targeted microorganisms due to several causative factors. In such circumstances, focus has been on molecular characterization, particularly by polymerase chain reaction (PCR) methods using primers specific to 16 S rRNA and targeted genes (Matsuki et al. 2003; Mullie et al. 2003; Berthoud et al. 2005; Kwon et al. 2005). At the same time, phylogenetic relatedness among probiotic cultures are being deduced through sequencing of PCR amplifications of target genes of interest (Coeuret et al. 2003; Rekha et al. 2006).

In the background of a predominant mixed-type microbial fermentation, the objective of present study was to assess the occurrence of native isolates of yeasts, LAB, and bifidobacteria in foods and feeds and characterize them on molecular basis for associated beneficial attributes with a focus on phylogenetic relatedness.

Materials and methods

Samples

In all, a total of 65 samples were analyzed in this study, which included juices of grapes, pineapple, apple and tomato, cow milk, curds, cereal/legume-based fermented batters and doughs of traditional foods, concentrated milk sweets, mother's milk, feces of infants and rabbits. Samples were collected locally under sterile conditions and brought to the laboratory within 60 min of collection and subjected to analysis.

Reference cultures

These included (1) reference cultures of *Staphylococcus aureus* FRI 722, *Bacillus cereus* F 4810, *Listeria monocytogenes*

Scott A obtained through courtesy from Dr. S. Notermans, Public Health Laboratory, The Netherlands, Dr. J.H. Kramer, Central Public Health Laboratory, United Kingdom, and Dr. Arun K. Bhunia, Purdue University, USA and (2) strain of *Yersinia enterocolitica* MTCC 859 procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The individual bacterial strains were maintained at 4°C on BHI agar (HiMedia Dehydrated Laboratories, Mumbai, India) slants and subcultured at regular intervals of 15 days. The cultures were propagated in BHI broth at 37°C, prior to use in experiments.

Isolation and characterization of native microbial cultures

Requisite aliquots of appropriate serial dilutions of samples were plated for the respective types of microbial cultures as desired in the present study. For obtaining yeast isolates, dilutions were surface plated on sterile pre-poured plates of potato dextrose agar (HiMedia), and inoculated plates were incubated for 48–72 h at 25°C. For lactic acid bacteria (LAB) isolates, dilutions were pour plated with sterile deMan, Rogosa, and Sharpe (MRS) *Lactobacillus* agar (HiMedia) and incubated for 24–48 h at 37°C. Similarly, bifidobacteria isolates were obtained by pour plating of sample dilutions with MRS *Lactobacillus* agar supplemented with 0.05% cysteine hydrochloride and incubated for 48–72 h at 37°C under anaerobic condition in anaerobic jar (Don Whitley, UK) using anaerobic gas pack (HiMedia). For isolation of bifidobacteria, the diluent, 0.85% saline was also supplemented with 0.05% cysteine hydrochloride.

The presumptive isolates thus obtained were purified by established protocols using the respective isolation media. Isolated cultures were maintained at 6°C on the slants or broth of respective media with subculturing at regular intervals of 30 days for use in further studies. As a means to select all the presumptive isolates under the scope of present study, initially, conventional method of identification based on morphological, cultural, and biochemical characteristics was followed (Cappuccino and Sherman 2004). This would avoid the probable chances of losing isolates which may fail to exhibit amplification of specific 16 S rRNA PCR products.

Fructose-6-phosphate phosphoketolase (F-6-ppk) is the key enzyme of the so-called “bifidobacteria shunt,” a unique hexose metabolism that occur via the phosphoketolase pathway in *Bifidobacterium* is used as a key biochemical character in the identification protocols. Sonicated cells of overnight-grown presumptive native isolates of *Bifidobacterium* were subjected to the qualitative assay of F-6-ppk following the documented method (Orban and Patterson 2000). Presumptive isolates of LAB and *Bifidobacteria* were identified to their respective genus and species level according to the Bergey's Manual

of Systematic Bacteriology (Kandler and Weiss 1986). Native isolates of yeasts were identified to their respective genus and species following morphological, cultural, and biochemical characteristics and the identification key described earlier (Deak and Beuchat 1996).

Alpha-D-galactosidase activity

Activity of intracellular α -D-galactosidase in native characterized isolates of LAB and *Bifidobacterium* spp. was determined in cell-free extract obtained from sonicated cells of 36-h-old MRS *Lactobacillus* culture broth. α -D-galactosidase activity was assayed in MCILvaine buffer by measuring the absorbance at 400 nm of the *p*-nitrophenol (PNP) released by the action of the enzyme upon its specific substrate, *P*-nitrophenyl- α -D-galactopyranoside (PNPG). One unit of enzyme was defined as the amount of enzyme that released 1 μ mol of PNP from the substrate PNPG per milliliter per minute under the assay conditions (Church et al. 1980).

Antibacterial activity

Extracellular culture supernatants of isolates of LAB and bifidobacteria were assayed for antibacterial activity against foodborne pathogenic bacterial cultures of *B. cereus*, *S. aureus*, *L. monocytogenes*, and *Y. enterocolitica*, individually, by agar well diffusion method as described earlier (Schillinger and Lucke 1989; Varadaraj et al. 1993). Antibacterial activity in the culture supernatants was defined as the reciprocal of the highest dilution showing definite inhibition of indicator bacterial strains and expressed as activity units per millimetre (AU/ml).

Bile salt and acid tolerance

Native isolates of *L. plantarum* MTCC 5422 and *B. adolescentis* MTCC 5423 were assessed for their ability to grow in the presence of bile salt concentrations of 0.15%, 0.3%, and 0.45% (*w/v*) added in MRS *Lactobacillus* broth (HiMedia) and at pH levels ranging from 2.0 to 5.5 with a difference of 0.5 U using 1 N HCl or 1 N NaOH in MRS broth, according to the method described earlier (Gotcheva et al. 2002). Prepared broth tubes were inoculated with 1% (*v/v*) cell suspension of 18 h old culture broth, incubated at 37°C for 48 h and absorbance read at 560 nm in a UV-VIS Spectrophotometer (Genesys 5, Milton Roy, USA) at intervals of 12 h. MRS broth without added bile salt and of pH 6.5, respectively, served as controls.

Bile salt hydrolase activity

Bile salt hydrolase activity in the native isolates of *L. plantarum* MTCC 5422 and *B. adolescentis* MTCC 5423

was determined in two steps assay (Tanaka et al. 2000), wherein the amount of amino acids liberated from conjugated bile salts used as a substrate. One unit of BSH activity was defined as the amount of enzyme which liberates 1 μ mol of amino acids from the substrate per minute.

Phytase activity

Extracellular phytase activity in native identified isolates of yeasts was determined in 48-h-old potato dextrose culture broth supernatants. In the activity assay, inorganic phosphate released from sodium phytate used as a substrate was measured following the earlier described established procedure (Heinonen and Lahti 1981). One unit of the enzyme activity was defined as the amount of phytase required to liberate 1 μ mol of inorganic phosphate per minute under defined assay conditions.

PCR detection for species specificity and probiotic and prebiotic attributes

In the present study, the target genes included were those of phytase (*pho*) for yeasts; 16 S rRNA, α -D-galactosidase (*melA*), and bile salt hydrolase (*bsh*) for *Lactobacillus* spp.; fructose-6-phosphate phosphoketolase (F-6-ppk), bile salt hydrolase (*bsh*), and α -D-galactosidase (α -D-gal) for *Bifidobacterium* spp. The nucleotide sequences and amplification conditions of these PCR primers are presented in Table 1. The synthesized primers were obtained from a commercial company (Sigma Aldrich, Bangalore, India). The total genomic DNA of individual isolates was extracted from 1.5-ml aliquots of overnight culture broth by boiling in Triton-X (Wang et al. 1997). The dried DNA was then dissolved in TE buffer (pH 8.0) and used as template DNA for amplifying the target genes using the respective primers. PCR was performed with the individual characterized isolates of yeasts, *Lactobacillus* and *Bifidobacterium* according to the procedure described earlier (Klocke and Mundt 2004). PCR amplification was performed in an automated DNA thermal Cycler (Eppendorf, Master Cycler, Cedex, France) following the conditions as detailed in Table 1. The PCR products were run in 1.2% agarose gels in Tris acetate buffer for 1.5 h at 100 V and stained in 0.5 μ g ml⁻¹ ethidium bromide solution (Sambrook and Russel 2001) and documented in Gel Documentation System (Vilber Lourmat, France).

Nucleotide sequence analysis of amplified PCR product and alignment

Considering the positive results with PCR primers and probiotic and prebiotic activities, one isolate each of *L. plantarum* MTCC 5422 and *B. adolescentis* MTCC

Table 1 Nucleotide sequence of specific PCR primers and conditions for targeted genes among microbial cultures

Microbial culture	Target gene	Sequence / PCR conditions	Amplicon size (bp)
<i>Lactobacillus</i> sp	16S rRNA	F 5' GGAAGCTCAGACACGGTCCAT 3' R 5' TACGGATTCCACCGCTAAAC 3' 95°C 3'; 35 cycles of 94°C 40 s; 46°C 40 s; 72°C 2'; final one cycle of 72°C 15'	385
<i>L. plantarum</i>	Bile salt hydrolase <i>bsh</i>	F 5' GGATTGTGTATTGCGGGATT 3' R 5' AGTCCGCCCATCTCTACT 3' 95°C 4'; 35 cycles of 94°C 1'; 55°C 40 s; 72°C 2'; final one cycle of 72°C 10'	422
<i>L. plantarum</i>	α -D-galactosidase <i>mela</i>	F 5' CGGAAGTCTATTGCCACAT 3' R 5' CTGCTTGTCGCATCACTTA 3' 95°C 4'; 35 cycles of 94°C 1'; 55°C 40 s; 72°C 2'; final one cycle of 72°C 10'	398
<i>Bifidobacterium</i> sp.	Fructose-6-phosphate phosphoketolase F-6- <i>ppk</i>	F 5' GCAGTCCAACAAGCTCATCA 3' R 5' AACAGTCTTCCGGTTGTA 3' 94°C 4'; 35 cycles of 94°C 30 s; 60°C 30 s; 72°C 1'; final one cycle of 72°C 10'	468
<i>B. adolescentis</i>	Bile salt hydrolase <i>bsh</i>	F 5' ACCTCGACTGGAGCTTCTCA 3' R 5' CTGGTTGGTCAGCACGTCTA 3' 94°C 4'; 35 cycles of 94°C 30 s; 60°C 30 s; 72°C 1'; final one cycle of 72°C 10'	467
<i>B. adolescentis</i>	α -D-galactosidase <i>aga</i>	F 5' TCAAAGCGTCATACCAACCA 3' R 5' GATGTCGGTGACCTGGAAC 3' 94°C 4'; 35 cycles of 94°C 30 s; 60°C 30 s; 72°C 1'; final one cycle of 72°C 10'	381
<i>Saccharomyces cerevisiae</i>	Acid phosphatase <i>pho</i>	F 5' CAAACCAGTTTTGCCGTTTT 3' R 5' TGAAGCGTTGAACAAGTTGG 3' 95°C 5'; 35 cycles of 95°C 30 s; 61.5°C 30 s; 72°C 3'; final one cycle of 72°C 5'	465

5423 were selected for this experimental trial. The resultant PCR amplified products with the three respective primers, 16 S rRNA, α -D-gal, and *bsh* were purified using commercially available PCR purification Spin Kit (HiPur A, HiMedia Laboratories, Mumbai, India) and subjected to sequence analysis by a commercial company (Sigma Aldrich, Bangalore, India). The resulting nucleotide sequences were subjected to BLAST programme of NCBI (online access) to assess the percent homology with closely related strains/variants documented in Gene Bank databases. A similarity network tree was constructed based on force type of neighbour-joining method from the same program.

Results

Native cultures of *Lactobacillus* sp. and *Bifidobacterium* sp. with probiotic attributes

Characterization and identification of native isolates

Native isolates of LAB and bifidobacteria were obtained from food and related samples analyzed in this study. In all, from a total of 70 presumptive isolates of LAB and bifidobacteria, 12 isolates were identified to the species of *Lactobacillus*, *Leuconotoc*, and *Pediococcus* and two isolates to those of *Bifidobacterium* based on conventional characterization protocols. The remaining native isolates were characterized

to their respective genus level. The occurrence of completely identified microbial cultures in different food and related samples is presented in Table 2. Three cultures of LAB were isolated from samples of curd, which is a natural lactic fermented homemade milk product, followed by another three cultures of LAB from naturally fermented batters based on cereal and legumes, which constitute a common base material in the preparation of a variety of traditional foods. Similarly, fruit juices did harbor LAB.

In the background of rare occurrence of bifidobacteria in foods, in the present study, only two well-characterized isolates of *Bifidobacterium* based on the qualitative assay for fructose-6-phosphate phosphoketolase activity were obtained from feces of breast-fed infant and rabbit. The characterized isolates of LAB and bifidobacteria were designated with specific numbers for convenience of presentation, wherein CFR refers to the host Institute's name. The native isolates which exhibited potent beneficial attributes were selected and deposited in a publicly accessible culture collection center of India, namely, the Microbial Type Culture Collection at the Institute of Microbial Technology, Chandigarh, India. Among the native isolates obtained in this study, only two isolates, one each of LAB and bifidobacteria were identified as the most potent and used in further experimental trials. These two cultures had the deposition accession details of *L. plantarum* MTCC 5422 and *B. adolescentis* MTCC 5423.

Table 2 Distribution pattern of native isolates of yeasts, lactic acid bacteria, and bifidobacteria from foods and related sources

Sample	Identified cultures		
	Yeasts	Lactic acid bacteria	Bifidobacteria
Curd	<i>Schizosaccharomyces japonicus</i> CFR 522 <i>Candida magnoliae</i> CFR 526	<i>Lactobacillus plantarum</i> CFR 2254 <i>L. fermentum</i> CFR 2196 <i>L. casei</i> subsp. <i>tolerans</i> CFR 2197	
Cow milk		<i>L. plantarum</i> CFR 2255	
Cereal/legume	<i>S. malidevorans</i> CFR 523	<i>L. plantarum</i> MTCC 5422	
Fermented batter and dough (traditional foods)	<i>S. octosporus</i> CFR 524 <i>C. foliorum</i> CFR 525 <i>Pichia norvegensis</i> CFR 528 <i>Rhodospiridium minuta</i> CFR 529	<i>Pediococcus pentosaceus</i> CFR 2253 <i>L. reuteri</i> CFR 2200	
Fruit juices (grapes, pineapple, apple tomato)	<i>Saccharomyces cerevisiae</i> MTCC 5421 <i>C. agrestis</i> CFR 527	<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i> CFR 2251 <i>Leu. oenos</i> CFR 2252 <i>L. plantarum</i> CFR 2256	
Infant feces			<i>Bifidobacterium adolescentis</i> MTCC 5423
Rabbit feces			<i>B. longum</i> CFR 2202
Concentrated milk		<i>L. casei</i> subsp. <i>pseudopantarum</i> CFR 2198	
Sweet (traditional food)		<i>L. casei</i> subsp. <i>rhamnosus</i> CFR 2199	

As confirmatory evidence and for further selection of isolates, PCR analysis based on 16 S rRNA primers designed for the specificity of genus *Lactobacillus* resulted in positive amplification with all conventionally characterized *Lactobacillus* spp. in PCR (data not shown). The same primers failed to show any positive amplification with identified isolates of *Leuconostoc mesenteroides* subsp. *dextranicum* CFR 2251, *Leuconostoc oenos* CFR 2252, and *Pediococcus pentosaceus* CFR 2253, indicating the high specificity of designed PCR primers for the genus *Lactobacillus*.

α-D-galactosidase and bile salt hydrolase activities

Among the native isolates of LAB assayed for this activity, the only potent culture was that of *L. plantarum* MTCC 5422, which exhibited an intracellular activity of 6.6 U ml⁻¹, followed by 1.5 U ml⁻¹ by *L. fermentum* CFR 2196 (Fig. 1). Similarly, another important probiotic property defined for LAB and bifidobacteria has been that of bile salt hydrolase activity. Native isolates of LAB failed to exhibit visible activity in the assay. In order to establish the molecular identity of these two important attributes, all the native well-characterized isolates of LAB were subjected to PCR with primers designed for *α*-D-galactosidase (*melA*) and bile salt hydrolase (*bsh*). The isolate of *L. plantarum* MTCC 5422 showed positive amplification with *melA* and *bsh* in PCR. Similarly, isolates of *B. adolescentis* MTCC 5423 and *B. longum* CFR 2202 showed very negligible *α*-D-galactosidase activity, while bile

salt hydrolase activity was to the extent of 0.48 U ml⁻¹. In PCR, the isolate of *B. adolescentis* MTCC 5423 showed positive amplification with primers designed (Table 1) for fructose-6-phosphate phosphoketolase (*F-6-ppk*), bile salt hydrolase (*bsh*), and *α*-D-galactosidase (*aga*) in PCR (Fig. 2). In a few of the native isolates of LAB and bifidobacteria, the gel band in PCR was faint for *α*-D-galactosidase.

Antibacterial activity against foodborne pathogenic bacteria

Another important probiotic attribute associated with cultures of LAB and bifidobacteria is that of antibacterial activity toward foodborne pathogenic bacterial species. In the present study, the native isolates exhibited a diversified pattern with respect to antibacterial activity against the four indicator bacterial species (Table 3). The antibacterial activity toward *Staphylococcus aureus* FRI 722 was very low, and most of the isolates failed to have any positive effect. In terms of activity (AU ml⁻¹), most of the native isolates could inhibit strains of *Bacillus cereus* F 4810, *Listeria monocytogenes* Scott A, and *Yersinia enterocolitica* MTCC 859. Among the native isolates of LAB, *L. plantarum* MTCC 5422 did exhibit potential antibacterial activity against all the four indicator bacterial strains and could thus be a promising probiotic culture. Similarly, among the two native isolates of *Bifidobacterium*, *B. adolescentis* MTCC 5423 appeared to be a potential probiotic culture (Table 3).

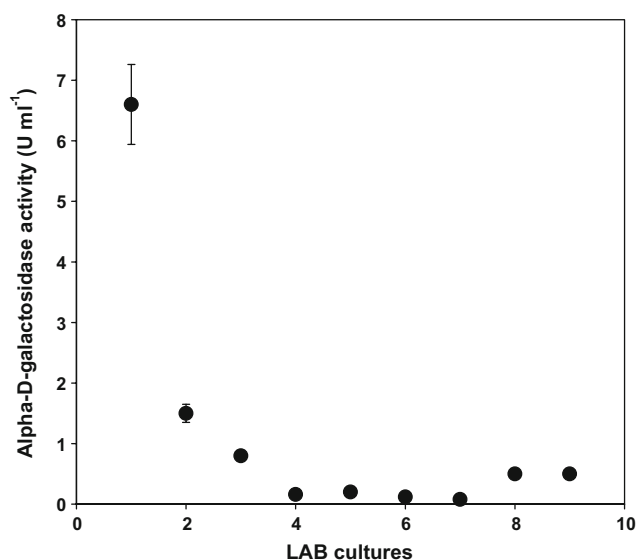


Fig. 1 Alpha-D-galactosidase activity exhibited by natural isolates of lactic acid bacteria and bifidobacteria 1, *L. plantarum* MTCC 5422; 2, *L. fermentum* CFR 2196; 3, *L. casei* subsp. *tolerans* CFR 2197; 4, *L. casei* subsp. *pseudoplantarum* CFR 2198; 5, *L. casei* subsp. *rhamnosus* CFR 2199; 6, *L. reuteri* CFR 2200; 7, *Leu. mesenteroides* subsp. *dextranicum* CFR 2251; 8, *Leu. oenos* CFR 2252; 9, *P. pentosaceus* CFR 2253

Survival/growth in presence of bile salt and acidic conditions

As there is a requirement for probiotic LAB to survive conditions of gastrointestinal tract, simultaneously, the isolates of *L. plantarum* MTCC 5422 and *B. adolescentis* MTCC 5423 were being assessed for their ability to survive/grow in presence of bile salts (0.15–0.45%) and

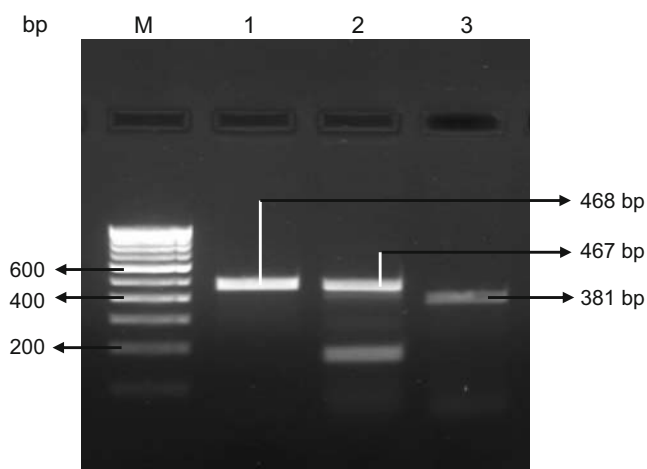


Fig. 2 Agarose gel electrophoretic pattern of PCR products showing amplicons with F-6-ppk, bsh and aga primers for fructose-6-phosphate phosphoketolase, bile salt hydrolase and α -D-galactosidase gene in a native isolate of *B. adolescentis* MTCC 5423. lane M, 100 bp marker, lanes 1 F-6-ppk, 2 bsh, 3 α -D-gal

under acidic conditions (pH 2.5–4.0). The growth performance of both the native isolates was quite appreciable in the presence of bile salts at concentration levels of 0.15%, 0.3%, and 0.45%, respectively. However, in the presence of 0.45% bile salt, the growth was slightly reduced as against that of control broth medium. Under pH levels of less than 3.5, both the cultures failed to grow, while growth was quite appreciable at pH levels of 4.5 and 5.5 compared with growth at pH 6.5 (Fig. 3 and 4).

Molecular basis for relatedness/diversity of food isolates of *L. plantarum* and *B. adolescentis*

In the present study, resultant partial nucleotide sequences of respective PCR amplicons of 16 S rRNA (385 bases), melA (398 bases), and bsh (422 bases) of *L. plantarum* MTCC 5422 and F-6-ppk (468 bases) of *B. adolescentis* MTCC 5423 were screened against BLAST algorithm of NCBI (online access). Based on the resultant outputs, force type neighbour-joining phylogram were generated for partial gene sequences of 16 S rRNA, melA, bsh, and F-6-ppk, respectively (Fig. 5). The phylogenetic tree generated for 16 S rRNA showed 98% homology with other cultures appearing in the generated tree (Fig. 5a). Similarly, the phylogenetic tree generated for the gene of α -D-galactosidase revealed that the native isolate of *L. plantarum* MTCC 5422 showed 99% homology with a strain of *L. plantarum*, while it was 78–81% with *L. reuteri* and *L. salivarius* (Fig. 5b). However, in case of the tree constructed for bile salt hydrolase revealed 97% homology with three strains of *L. plantarum* (Fig. 5c). Similarly, the phylogenetic tree of F-6-ppk in *Bifidobacterium* spp. revealed that the native isolate of *B. adolescentis* MTCC 5423 had 97% homology with another strain of *B. adolescentis* and almost 90% with other species of *Bifidobacterium* (Fig. 5d).

Native yeast cultures with phytase activity

In the case of native cultures of yeasts, 22 isolates were obtained on primary isolation and as in case of LAB, conventional characterization by physiological and biochemical attributes resulted in identification of nine cultures to their genus and species level. The remaining isolates were identified to their genus level. Two yeast cultures were isolated from samples of curd and five isolates from naturally fermented batters based on cereal and legumes. Fruit juices did harbor yeasts (Table 2). As the study was aimed at assessing beneficial attributes associated with the microbial cultures, the identified yeast cultures when assayed for phytase activity revealed that an isolate of *Saccharomyces cerevisiae* MTCC 5421 exhibited 184 U ml⁻¹ of extracellular phytase activity, while isolates of *Schizosaccharomyces malidevorans* CFR

Table 3 Antibacterial activity associated with native isolates of lactic acid bacteria and bifidobacteria

LAB cultures	Foodborne bacterial cultures			
	<i>B. cereus</i> (Antibacterial activity, AU ml ⁻¹)	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>Y. enterocolitica</i>
<i>L. plantarum</i> MTCC 5422	14	2.0	8.5	12.8
<i>L. fermentum</i> CFR 2196	4.0	0.9	7.1	11.4
<i>L. casei</i> subsp. <i>tolerans</i> CFR 2197	4.3	Nil	4.3	11.4
<i>L. casei</i> subsp. <i>pseudoplantarum</i> CFR 2198	5.7	3.0	11.4	11.4
<i>L. casei</i> subsp. <i>rhamnosus</i> CFR 2199	6.4	1.5	11.4	12.1
<i>Leu. mesenteroides</i> subsp. <i>dextranicum</i> CFR 2251	5.3	Nil	4.7	6.0
<i>Leu. oenos</i> CFR 2252	8.3	Nil	9.4	8.3
<i>L. reuteri</i> CFR 2200	4.3	Nil	8.6	11.4
<i>P. pentosaceus</i> CFR 2253	6.6	1.8	7.8	10.0
<i>Bifidobacterium adolescentis</i> MTCC 5423	5.0	Nil	1.8	0.2
<i>B. longum</i> CFR 2202	3.1	Nil	0.8	0.1

523 and *Sch. octosporus* CFR 524 could elaborate 162 and 118 U/ml, respectively. Through the use of acid phosphatase (*pho*) primers (Table 1), of the nine identified yeast cultures, only six of them gave a positive amplification in PCR. The native cultures of *Candida agrestis* CFR 527 and *Rhododsporidium minuta* CFR 529 could not give any positive amplification, although these cultures could elaborate very less quantity of phytase to an extent of 25–28 U ml⁻¹ in assay, as the primers designed was for species-specific phytase gene in *S. cerevisiae*.

Discussion

Probiotic and prebiotic attributes among LAB and bifidobacteria

Microbial cultures are being increasingly evaluated for their desirable attributes, so as to incorporate in foods to derive benefits and wellness to human health. Mixed fermentation with the involvement of LAB and yeasts are known to occur more commonly in a majority of traditional foods. In the present study, a diverse range of foods and related matter were assessed for the prevalence of lactic acid bacteria and bifidobacteria with probiotic attributes and also for native cultures of yeasts with attributes of reducing nonfunctional food constituents. Further, the native cultures were characterized for the selected attributes by PCR methods and based on these inputs, attempts were made to outline phylogenetic patterns with related genera/species. In the case of identification of bifidobacteria, the assay for F-6-ppk has been a key taxonomic identifying character, so it could be distinguished from other closely related bacterial groups such as lactobacilli, actinomycetes, and coryne-

bacteria (Scardovi 1986; Vlkova et al. 2002). Among the native isolates of LAB and bifidobacteria, based on conventional identification protocols, isolates were characterized to their respective genus and species. Two isolates were selected, which had the deposition details of *L. plantarum* MTCC 5422 and *B. adolescentis* MTCC 5423 (Table 2).

Although isolates of LAB were characterized by conventional protocols of cultural and biochemical attributes, the use of molecular-based approach in targeting the specific gene of importance gives an impetus to the study which focuses on obtaining cultures with defined characteristics. Earlier studies have focused on isolating specific cultures of *Lactobacillus*, wherein genomic DNA of test cultures was amplified using 16 S rRNA genus specific primers. In recent years, 16 S rRNA-targeted PCR primers enable rapid and specific detection of bacterial genera (Kaufmann et al. 1997). In a study with fermentative process, isolates of *L. plantarum* and *L. fermentum* were found to occur as predominant cultures based on PCR amplification of the intergenic segment between 16 S and 23 S rDNA subunits, followed by restriction enzyme digestion specific for *Lactobacillus* (Tannock et al. 1999). In a broader perspective, research studies have shown the usefulness of 16 S rRNA targeted primers to identify the genera/species of *Lactobacillus* and *Bifidobacterium* through PCR (Klocke and Mundt 2004; Mangin et al. 2006; Mignard and Flandrois 2006; Tamang et al. 2008). Molecular characterization using RAPD-PCR with M13 primers revealed the predominance of populations of *L. curvatus*, *L. plantarum*, and *L. sakei* during natural fermentation of sausages (Rantsiou et al. 2006). In a similar approach, PCR using 16 S rDNA specific primers confirmed six bacteriocinogenic strains as cultures of *L. plantarum* (Omar et al. 2006). In our study,

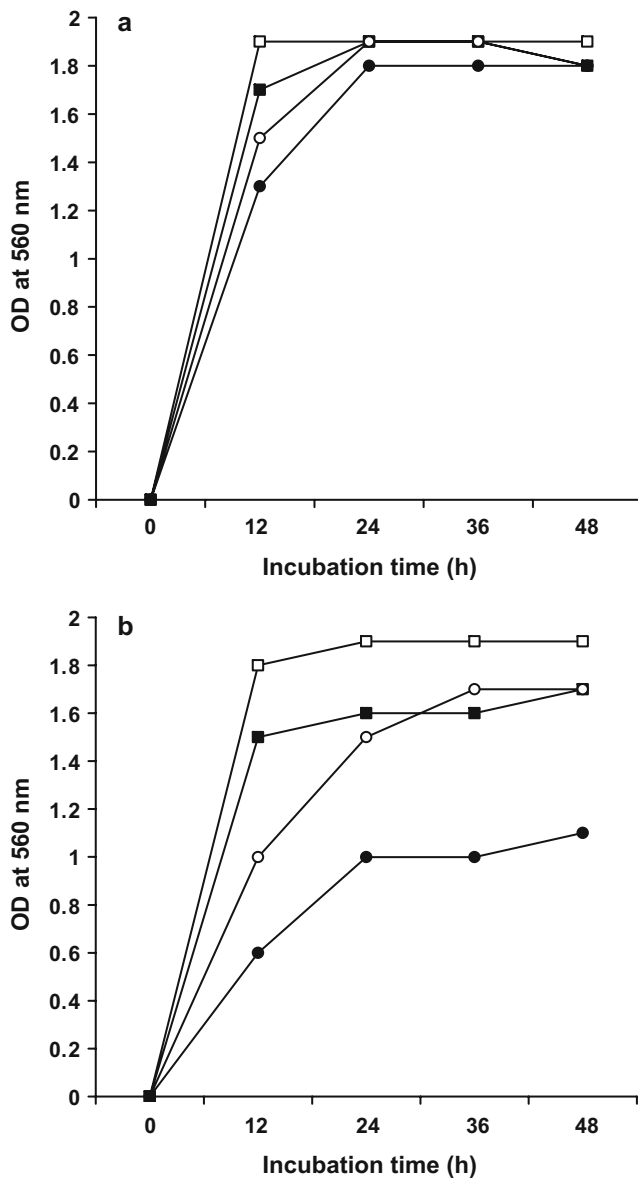


Fig. 3 Growth response of *L. plantarum* MTCC 5422 to bile salt concentrations (a) and acid pH levels (b): empty squares MRS broth without bile salt, filled squares with 0.15% bile salt, empty circles with 0.30% bile salt, filled circles with 0.45% bile salt in (a); empty squares MRS broth of pH 6.5; filled squares of pH 5.5; empty circles of pH 4.5; filled circles of pH 3.5 in (b)

the samples assessed for LAB showed the predominance of *L. plantarum*, followed by *L. casei*. The use of 16 S rRNA primers is more useful in characterization of genus and not to distinguish very closely related species and/or subspecies of a single species. This was evident in case of *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*, wherein 16 S rRNA gene sequences of these two subspecies had significant similarities (Maruo et al. 2006). A similar observation did exist in our study, wherein the 16 S rRNA primers specifically designed to identify *Lactobacillus* genus, failed to show any amplification in

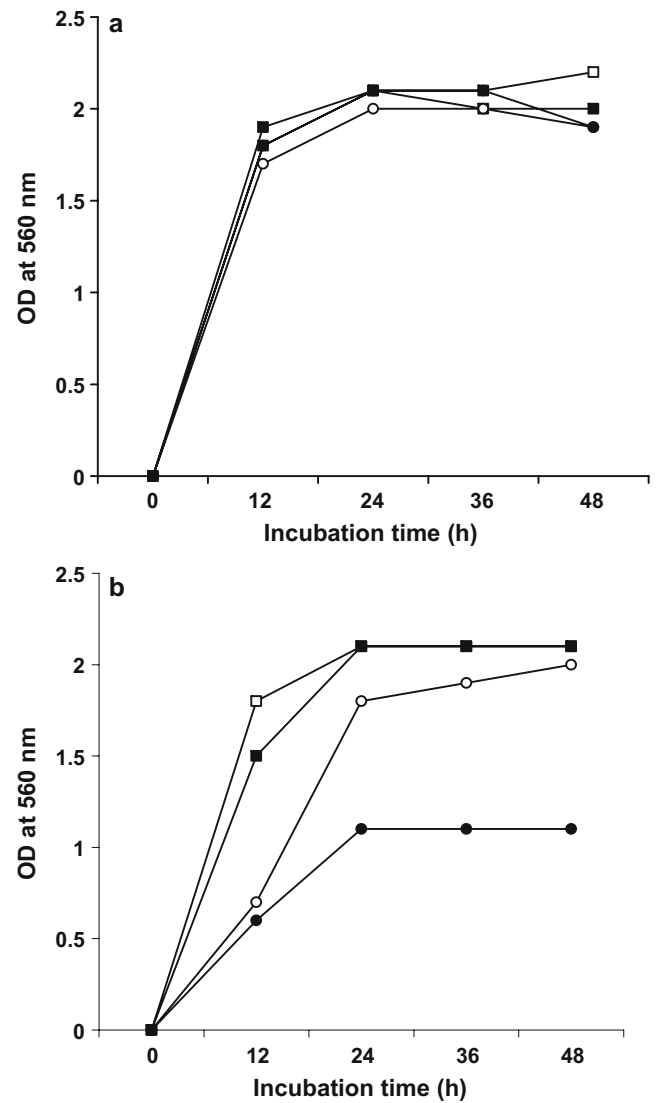


Fig. 4 Growth response of *B. adolescentis* MTCC 5423 to bile salt concentrations (a) and acid pH levels (b): empty squares MRS broth without bile salt; filled squares with 0.15% bile salt; empty circles with 0.30% bile salt; filled circles with 0.45% bile salt in (a); empty squares MRS broth of pH 6.5; filled squares of pH 5.5; empty circles of pH 4.5; filled circles of pH 3.5 in (b)

PCR with isolates conventionally identified as *Leuconostoc* and *Pediococcus*.

The cultures of LAB and bifidobacteria are known more for their probiotic attributes. However, these cultures can also function in a manner to make available prebiotic substrate through α -D-galactosidase activity. Although cultures of *Lactobacillus* spp. are known to exhibit α -D-galactosidase activity, most of the research investigations have focused on *L. fermentum* and *L. plantarum*, wherein quantification assays have resulted in diversified units of activity. The first genetic characterization of α -D-galactosidase was established by cloning and expressing *mela* gene from *L. plantarum* in *Escherichia coli* yielding an active α -Gal (Silvestroni et al.

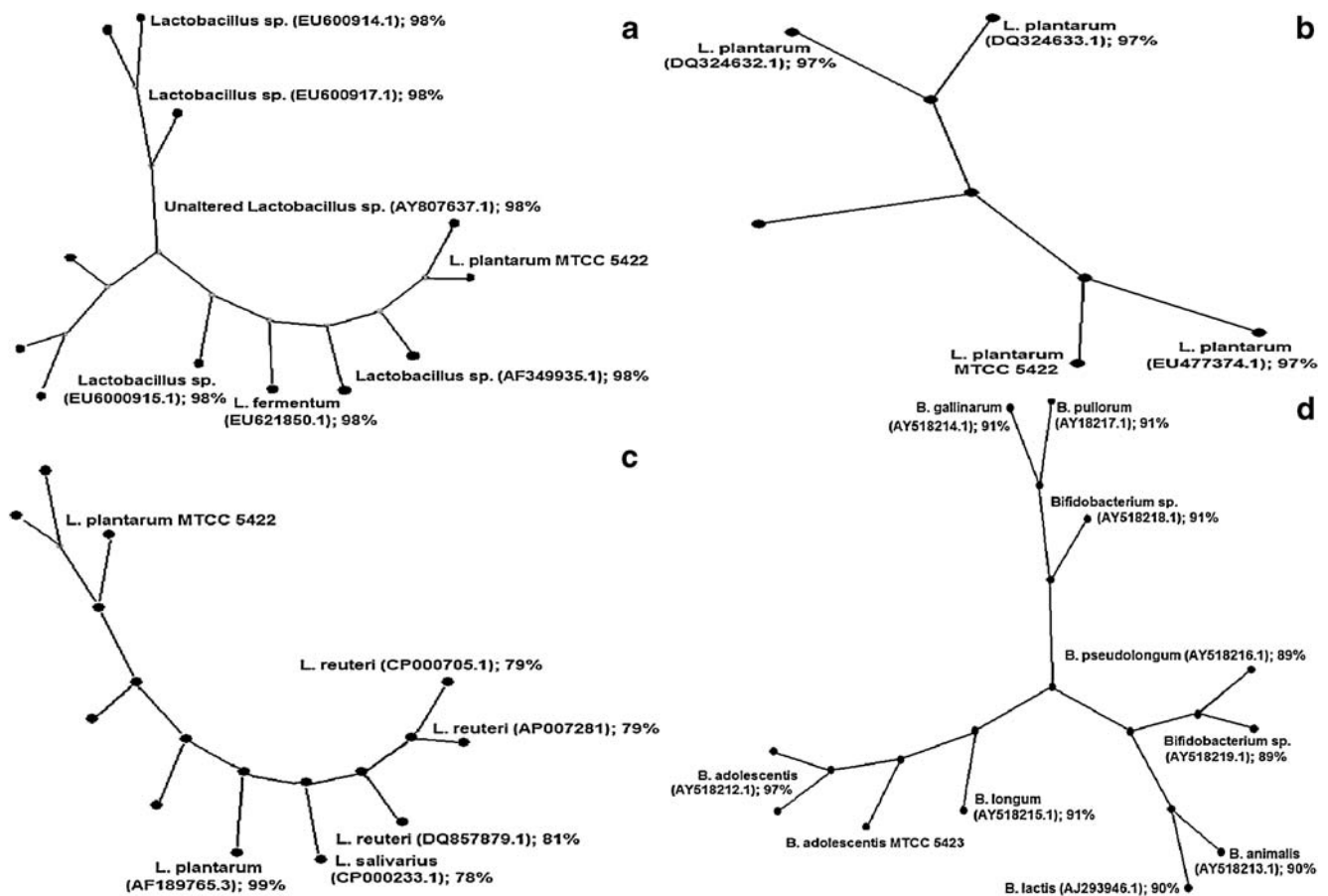


Fig. 5 Neighbour-joining phylogenetic tree of representatives of *L. plantarum* MTCC 5422 based on nucleotide sequence analysis of PCR amplified products with 16 S rRNA primers (a), *melA* primers

(b), and *bsh* primers (c) and for *B. adolescentis* MTCC 5423 based on F-6-*ppk* primers (d). The percent homology expressed is in relation with the two respective native food isolates

2002). The behavior of *L. fermentum* CRL 722 and CRL 251 evaluated under different pH conditions of 6.0 to 4.5 revealed an activity of 5 U ml^{-1} at pH 5.5 (Le Blanc et al. 2004). In our study, the native isolate of *L. plantarum* MTCC 5422 could elaborate a slightly higher activity of 6.6 U ml^{-1} . In general, bile salt hydrolase activity is being included as one of the probiotic attributes of desirable microbial cultures, which are nonpathogenic/toxigenic/virulent.

Initially, *Bifidobacterium* sp. was identified by the presence of F-6-*ppk* in cellular extracts, more so as a qualitative approach (Scardovi 1986). This was later confirmed in PCR by amplification of *Bifidobacterium* targeted gene sequence for xylulose-6-phosphate phospho-ketolase (*xfp*) primers (Matsuki et al. 2003; Berthoud et al. 2005). Besides, researchers have identified bifidobacteria of human origin as well as those exhibiting probiotic properties using 16 S rRNA-targeted primers (Mullie et al. 2003; Masco et al. 2007) and species-specific primers based on region extending from 16 S rRNA through 23 S rRNA (Shuhaimi et al. 2004; Kwon et al. 2005). In the present study, initially, we could classify the native isolate as

B. adolescentis based on carbohydrate fermentation profile and later by confirmation in PCR (Fig. 2). The identified culture exhibited almost all the major phenotypic characteristics reported for this species. Interestingly, strains of *Bifidobacterium* have shown higher *bsh* activity as against those reported for other bacterial groups. Research investigations have focused on biochemical and genetic characterization of *bsh* of *B. longum* (Tanaka et al. 2000; Shuhaimi et al. 2001). Similar characterization studies were established with *B. bifidum* strains and *B. adolescentis* (Kim et al. 2004, 2005). In a similar manner, in the present study, only the presence of *bsh* gene in a native isolate of *B. adolescentis* MTCC 5423 was established in PCR (Fig. 2). Although no definite relationship can be attributed to activity in assay system and PCR amplification for target genes in the microbial cultures, it is appropriate to mention that PCR primers designed in the present study were species specific, as it was the case in α -D-galactosidase activity and PCR amplification for the same.

Another important probiotic attribute associated with cultures of LAB and bifidobacteria is that of antibacterial activity toward foodborne pathogenic bacterial species.

Besides, this probiotic property becomes more evident with the cultures exhibiting appreciable growth performance in the presence bile salts and at low pH levels. In the present study, both the isolates of *L. plantarum* MTCC 5422 and *B. adolescentis* MTCC 5423 revealed appreciable antibacterial activity (Table 3) and survival/growth in the presence of bile salts and low pH conditions (Fig. 3 and 4). These potent beneficial attributes of LAB and bifidobacteria have found applications in food fermentation processes, a well established fact over the centuries, more particularly with that of intestinal species like *L. acidophilus* and/or *B. bifidum* in preparation of fermented products (Driessen and Boer 1989). In this context, an increasing interest exists for milk-based foods with a focus on those bacterial species providing health-improving properties (Portier et al. 1993). The interest in application of these microorganisms and their metabolites toward wellness factor for human health has taken a major leap over the last decade (Ljungh and Wadstrom 2006; Masco et al. 2007; Woodmansey 2007). The species of *Lactobacillus* and *Bifidobacterium* are well known to have health promoting effects in human and animal intestinal tract, for which they are termed as probiotics. Though a matter of debate, it is considered that these beneficial microbial cultures are to be present as live cell populations of 10^6 CFU ml^{-1} or g^{-1} of product to have the exact meaning of a probiotic (Kailasapathy and Chin 2000). As the culture-dependent methods have limitations of reliability and sensitivity in terms of specific characterization for probiotic attributes, PCR-based methods are being established as determinants of probiotic attributes. In the present study, probiotic attributes associated with selected cultures of *L. plantarum* and *B. adolescentis* were evaluated by culture-dependent methods. Further, the same attributes were supported on a molecular basis with the positive amplification for specific and targeted genes being evidenced in PCR.

Often, conventional methods of identification of microbial species based on phenotypic characteristics lead to uncertain conclusions (Bernardeau et al. 2006). The use of molecular-based approaches has added newer dimensions to characterize the desirable species of *Lactobacillus* and *Bifidobacterium* with focus on targeted attributes (Dellaglio et al. 1991). These methods mostly target rRNA genes as a primary means to identify species of *Lactobacillus*. Although intergenic 16 S-23S rRNA spacer region has been commonly targeted in a majority of the investigations (Hayashi et al. 2002; Vizoso Pinto et al. 2006), there appears to be certain inconclusive and nondistinguishable similarities among closely related species. As a means to achieve a clear understanding of the interrelationship, phylogenetic determinations have been generated based on sequence analysis data for PCR amplified product with specific target genes of interest (Chavagnat et al. 2002; Ventura et al. 2003; Blaiotta et al. 2008).

Lactobacillus with nearly 80 recognized species is a highly diverse group as could be evidenced from a complex phylogeny. This diversity of the genus *Lactobacillus* was evidenced in a study relating to comparative genomics of five species of *Lactobacillus*, namely, *L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. johnsonii*, and *L. sakei*. Phylogeny based on whole genome alignment suggested that *L. salivarius* was closer to *L. plantarum* than to *L. sakei* (Canchaya et al. 2006). Although, it is believed that during evolution, the major genes are conserved between related bacterial species, the phylogenetic trees reconstructed for individual genes may differ from this general viewpoint, and they may give an indication of horizontal transfer. Individual trees are often not resolved with clarity to bring in meaningful presentation of analysis (Nicolas et al. 2007).

Studies focusing on evolutionary trends in microbial species are gaining importance in the broad area of “microbial diversity,” which becomes evident by molecular biology approaches. In the present study, attempts were made to generate phylogeny tree for *L. plantarum* based on genus specific 16 S rRNA primers as well as individual genetic characters such as α -D-galactosidase and bile salt hydrolase, wherein *bsh*-based tree exhibited almost complete homology with the same species and in *melA*-based tree, a lower percent homology existed with *L. salivarius* and *L. reuteri* (Fig. 5b and 5c). The appearance of few strains of *L. plantarum* in the tree may be attributed to the low prevalence of this gene among species of *Lactobacillus*. In our study, it was very much evident that gene for bile salt hydrolase though detected in native isolates of *Lactobacillus* spp. by PCR, quantification of activity was not very much appreciable, which may be due to certain parameters in PCR analysis. Similarly, based on 16 S rRNA generated tree, the native isolate of *L. plantarum* MTCC 5422 did exhibit 98% homology with other cultures of *Lactobacillus* sp. (Fig. 5a). On the other hand, phylogenetic tree generated for *B. adolescentis* based on F-6-*ppk* showed the appearance of several species of *Bifidobacterium*, as this attribute being the major character of all *Bifidobacterium* spp. would definitely enable most of the species to be interrelated (Fig. 5d).

Native yeast cultures with phytase activity

Research studies have focused on biochemical and molecular characterization of acid phosphatases of yeasts (Kaur et al. 2007). These enzymes reside on the cell surface and catalyze the release of inorganic phosphate from different phosphorylated organic compounds. In *S. cerevisiae*, the acid phosphatase encoding genes *pho3*, *pho5*, *pho11*, and *pho12* have been isolated and characterized (Vogel and Hinnen 1990). Genetic studies with some of the yeast phosphatase

genes have indicated their role as phytases. These genes were *pho1* and *pho4* from *Schizosaccharomyces pombe* and *pho3* from *S. cerevisiae* (Wodzinski and Ullah 1996). As *S. cerevisiae* and its by-products have long been known to be safe for human and animal consumption, the phytases elaborated by this culture have found commercial applications to overcome the problems of antinutritional factor in cereal/legume-based foods. Studies with different yeast cultures have revealed a diverse range of phytase activity ranging from 77 to 176 U g⁻¹ of dry biomass (Bindu et al. 1998; Vohra and Satyanarayana 2004). A similar situation prevailed in the present study, wherein isolates of *Saccharomyces* spp., *Candida* spp., *Schizosaccharomyces* spp., and *Rhodospiridium* sp. elaborated phytase activity ranging from a lowest of 25 to a highest of 228 U ml⁻¹. As the PCR primers designed were species specific to *S. cerevisiae*, a few of the yeast cultures failed to show positive amplification in PCR with these primers. Further, it is likely that the targeted phytase gene in our study may not be either present in other yeast cultures or not related genetically.

The findings of this study have conclusively established that microbial cultures with genetically stable desirable attributes are present in diversified habitats, and they need to be well characterized and exploited commercially toward enriching wellness factor in human health mediated through traditional fermented and/or nonfermented foods.

Acknowledgements The authors are thankful to Dr. V. Prakash, Director, CFTRI, Mysore, India for providing the facilities and interest in present work. The first author is grateful to Council of Scientific and Industrial Research, New Delhi, India for awarding the Senior Research Fellowship.

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