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Isolation and identification of phytate-degrading lactobacilli from indian cereal-based fermented milk product – "*Raabadi"*

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Abstract

The present study on the isolation and characterization of potential phytate degrading lactobacilli from Raabadi was carried out. Twenty five samples of country made *Raabadi* collected from different villages of Rajasthan and Haryana in India were analyzed. Out of 40 lactobacilli isolates, nine were identified as phytate degrader, one was identified as *Lactobacillus rhamnosus*, five were identified as *Lactobacillus fermentum* and remaining three were identified as *Lactobacillus brevis* through phenotypic and genotypic characterization. These isolates are very much potential to degrade the phytate in cereal based fermented products.

Keywords: Phytic acid, Phytase, Raabadi, *Lactobacillus rhamnosus, Lactobacillus fermentum*

Phytic acid [*myo*-inositol hexakis (dihydrogen phosphate)], is the predominant form of total phosphate found in cereal grains (Reddy *et al*. 1982). Phytic acid chelates the dietary minerals (Fe, Zn, Mg, Ca), protein or vitamins and is considered to be one of the primary factors limiting the nutritional values of cereals. Therefore, by decreasing the phytate content, nutritional values of cereal can be improved. This led to the increasing interests in phytate-degrading enzymes and microorganisms. Phytase is produced by bacteria, yeast and fungi. Phytase production by bacteria - *Klebsiella aerogenes* (Tambe *et al.* 1994), *Bacillus subtilis* (Shimizu, 1992; Kerovuo *et al*. 1992), *Escherichia coli* (Greiner *et al*. 1993) and some ruminal bacteria (D'Silva *et al*. 2000), by fungi - *Saccharomyces cerevisiae* and *Schwanniomyces castellii* (Segueilha *et al*. 1993), *Aspergillus ficuum* (Liu *et al*. 1999), *Aspergillus carbonarius* (Al-Asheh and Duvnjak, 1994), *Aspergillus oryzae* (Shimizu, 1993) and *Aspergillus niger* (Volfova *et al*. 1994) have been reported.

It was evident that lactic acid bacteria (LAB) used in milk and milk-based products such as strains of *L. acidophilus*, *L casei*, *Lactococcus lactis*, *L. delbrueckii* and *Streptococcus* spp. are very poor producers of phytase, whereas *L. amylovorus* and *L. plantarum*, which are present in a variety of microbial systems of plant origin, resulted in the maximum yield of enzyme.

Thus, cereal fermented products and milk-cereal based fermented products will be a good source of phytate degrading LAB. Therefore an effort was made to isolate and identify the phytate degrading lactic acid bacteria from an Indian cereal based fermented milk product – "*Raabadi*". In present study, nine Lactobacillus spp. were identified as good phytate degrader. Among them one was identified as *Lactobacillus rhamnosus*, five were identified as *Lactobacillus fermentum* and rest three were identified as *Lactobacillus brevis* by applying both phenotypic and genotypic techniques.

Materials and Methods

Isolation of LAB from raabadi

Total twenty five *Raabadi* samples were collected from different villages of Haryana and Rajasthan in India during summer (May-June). A loopfull of homogenized sample was inoculated in 4ml of MRS broth tubes (5 ml) and were incubated at 37°C for 18-24 h. Streaking was done on MRS agar plates from the broth culture and were incubated at 37°C for 24-48 h. 3 - 4 well isolated colonies were picked randomly from each plate and inoculated in MRS broth and incubated, at 37°C for 18-24 h. The isolates were purified by restreaking on MRS agar plates for three times. Gram's positive and catalase negative rod shaped cultures were preserved in 20% glycerol stock at -20^{o}C until used.

Phytase activity

This method for detecting phytase activity would rely on the disappearance of precipitated calcium or sodium phytate as an indication of enzyme activity. Microorganisms expressing phytase produce zones of clearing on agar media containing sodium or calcium phytate (Shieh and Ware, 1968; Howson and Davis, 1983). The glycerol stock cultures were reactivated twice in MRS broth by incubating at 37°C for 16-18 h before use. Activated lactobacilli cultures were screened for phytase activity on MRS agar containing 1% (w/v) sodium phytate (HiMedia, Mumbai, India) by spotting method. Active MRS broth cultures were aseptically spotted on MRS agar plates containing 1% (w/v) sodium phytate. The plates were incubated at 37°C for 48 h and plates were flooded with 2% aqueous cobalt chloride solution and kept at room temperature. After 5 min., the cobalt chloride solution was replaced with a freshly prepared solution containing equal

volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium vandate solution. Phytase activity was observed as zones of clearance in opaque background. Isolates showing positive for phytase activity were further evaluated for comparative phytate degrading ability. Sterilized circular blank discs (10 mm diameter) were dipped into the active MRS broth cultures and aseptically placed on MRS agar plates containing 1% (w/v) sodium phytate. Comparison of phytase activity was done by measuring the diameter of clear zone around the disc (Figure 1).

Phytase assay

Quantitative assay for phosphate released by phytase was done following Engelen *et al*. (1944). Briefly, the reaction mixture contained 2 ml of enzyme extract and 4 ml of substrate solution containing 0.84 gm sodium phytate in 90 ml acetate buffer, pH 5.5 [0.18 gm acetic acid (100%), 3.0 gm sodium acetate trihydrate, 0.15 gm calcium chloride dihydrate, 90 ml distilled water, adjusted to pH 5.5 with acetic acid and diluted to 100 ml]. The reaction was carried out at 37^0C for 90 min. and was stopped by adding 4 ml freshly prepared color reagent. The color reagent consisted of 25 ml ammonium molybdate solution [10 gm ammonium molybdate. $4H_2O$ distilled water, 1 ml ammonia (25%), adjusted to 100 ml with distilled water] mixed with 25 ml ammonium vanadate solution [0.235 gm ammonium vanadate added to 40 ml distilled water at 60^0C followed by slow addition of 2 ml nitric acid and adjustment to 100 ml with distilled water] and with slow stirring, 16.5 ml nitric acid (65%). After cooling to room temperature, the volume was adjusted to 100 ml with distilled water. The color developed was measured at 450 nm. One unit of phytase activity was defined as the amount of enzyme that released 1 µmole of inorganic phosphate in 1 min.

$$\text{Units/ml enzyme} = \frac{(\mu\text{moles of Phosphate released})(\text{df})}{(90)(2)}$$

df = Dilution factor

90 = Time (in minutes) of assay per the Unit Definition

2 ml = volume (in milliliter) of enzyme used

Phenotypic characterization

Bacteria were subjected to biochemical tests like arginine hydrolysis, esculine hydrolysis test and sugar fermentation using galactose, lactose, maltose, mannitol, mellibiose, sorbitol, sucrose, trehalose, cellobiose, raffinose, xylose, sorbitol, rhamnose.

Identification by API CHL50 system (bioMerieux, France)

Pured culture was streaked on solidified MRS agar. It was incubated for 24 h. Then well grown colonies were picked up by sterile loop and inoculated into API CHL medium aseptically. Inoculated CHL medium was poured into API CHL sugar strips. The sugar strips were incubated at 37°C for 24 h and observed for color change. The results were recorded after 48 h and interpreted using the APILAB Plus computer-aided identification program (bioMerieux). A percentage correct identification (% Id) value was obtained for each strain.

Genotypic characterization

After probable identification by these biochemical and sugar fermentation tests, rod shaped bacteria were further subjected to molecular identification technique of PCR by using Lactobacillus genus and species specific primer as follows.

Genomic DNA was extracted from 2 ml of overnight grown culture in MRS broth at 37°C as described (Pospiech and Neumann, 1995).

Identification of *Lactobacillus*

The forward primer used was AGA GTT TGA T(C/T) (A/C) TGG CTC AG of 21 bp (Kane et al., 1993) and reverse primer used was CAC CGC TAC ACA TGG AG of 17 bp (Heilig et al., 2002) (Table 1). PCR was carried out in 25 µl reaction mixture containing 2.5 µl PCR buffer (containing 15 mmol l^{-1} $MgCl_2$), 0.5 µl dNTP mix (10 mmol l^{-1} each dNTP), 0.5 µl of each primer (10 pmol µl−1), 18.7 µl of purified water, 0.3 µl of Taq DNA polymerase(Bangalore Genei, Bangalore, India), 2 µl genomic DNA. Reactions were performed in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). Temperature profile consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of DNA denaturation at 94°C for 0.5 min, primer annealing for 0.5 min. at 52°C and an elongation step at 72°C for 0.5 min. The final extension step was carried out at 72°C for 10 min. The estimated band length was 250 base pairs (Fig 2).

PCR for *Lactobacillus fermentum* identification

According to Dickson *et al*. (2005) The forward primer used was *L. fermentum*-F AAT ACC GCA TTA CAA CTT TG of 20 bp and reverse primer used was *L. fermentum*-R GGT TAA ATA CCG TCA ACG TA of 20 bp (Table 1). PCR mixture quantity and thermocycler were same as mentioned above. Temperature profile consisted of an initial denaturation at 95°C for 5 min., followed by 30 cycles of DNA denaturation at 94°C for 0.5 min., primer annealing for 0.5 min. at 50°C and an elongation step at 72°C for 0.5 min. The final extension step was carried out at 72°C for 10 min. The estimated band length was 337 base pairs(bp) (Figure 3 & 4).

1.5% agarose gel was used for gel electrophoresis. The agarose gels were stained with a 0.002 mg ml^{-1} ethidium bromide solution. Gel was run at 100 mV for 30 min. 100 base pairs ladder (Fermentas) was used for PCR product identification.

Partial sequencing of 16S rRNA gene

16S rRNA universal primer was used for PCR amplification. The forward primer was designated as S-G-Lab-0159-a-S-20 (Lab-0159f) 20-mer GGA AAC AG (A/G) TGC TAA TAC CG (Table 1) and reverse primer was designated as S-G-Lab-0677-a-A-17 (Lab-0677r) 17-mer CAC CGC TAC ACA TGG AG (Heilig *et al*., 2002) (Table 1). Temperature profile consisted of an initial denaturation at 94°C for 5 min., followed by 30 cycles of DNA denaturation at 94°C for 30 sec, primer annealing for 30 sec at 57°C and an elongation step at 72°C for 45 sec. The final extension step was carried out at 72°C for 7 min. The estimated band length was 750 base pairs. The obtained sequences (done by Xcleris Labs Limited, India) were compared with other bacteria by NCBI BLAST.

Table 1: List of primers used in this study for *Lactobacillus* identification

Target	Primer Sequence 5'-3'	Anneling Temp. (ºC)	Product size (bp)	Reference
Lactobacillus genus	CTCAAAACTAAACAAAGTTTC CTTGTACACACCGCCCGTCA	55	250	Dubernet *et al*. 2002
7F	AGAGTTTGAT(C/T)(A/C) TGGCTCAG	57	750	Kane *et al.* 1993, Heilig *et al.* 2002
S-G-Lab-0677-R	CACCGCTACACATGGAG			
L fermentum	AAT ACC GCA TTA CAA CTT TG GGT TAA ATA CCG TCA ACG TA	50	337	Dickson *et al*. 2005

Table 2: Zone of clearance for phytate degradation

Name of Isolate	Cell morphology	Zone of clearance (diameter in mm)
RSI3	Bacilli	33
JL2	Bacilli	31
JL3	Bacilli	28
RS6	Bacilli	23
JL7	Bacilli	23
JL6	Bacilli	21
RS2	Bacilli	18
JL1	Bacilli	16
JL5	Bacilli	16

Table 3: Phytase Assay of screened isolates

Isolate	Absorbance at 450 nm	Enzyme activity/min.
RSI3	0.475	0.011187
JL2	0.428	0.008797
JL3	0.415	0.008136
RS6	0.404	0.007577
JL7	0.387	0.006712
JL6	0.373	0.006
RS2	0.315	0.003051
JL1	0.295	0.002034
JL5	0.283	0.001424

Table 4: Biochemical and sugar fermentation pattern of isolates

	Sugar fermentation pattern																
	Arginine	Esculine	Arabinose	Xylose	Ribose	Galactose	Lactose	Maltose	Mannitol	Mannose	Mellibiose	Sorbitol	Sucrose	Trehalose	Cellibiose	Raffinose	Rhamnose
RSI3	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	+
JL2	+	-	+	-	ND	+	+	ND	-	+	+	+	+	+	-	+	ND
JL5	+	-	+	-	ND	+	+	ND	-	-	+	-	+	-	-	+	ND
JL6	+	-	+	-	ND	+	+	ND	-	-	+	-	+	-	-	+	ND
RS2	+	-	+	-	ND	-	+	+	-	+	+	-	+	-	-	+	ND
RS6	+	-	+	-	ND	+	+	+	-	-	+	-	+	-	-	+	ND
JL1	+	-	ND	-	ND	+	+	+	-	-	+	-	+	-	-	-	ND
JL3	+	-	ND	-	ND	+	+	+	-	-	+	-	+	-	-	-	ND
JL7	+	-	ND	-	ND	+	+	+	-	-	+	-	+	-	-	-	ND

ND = Not Done

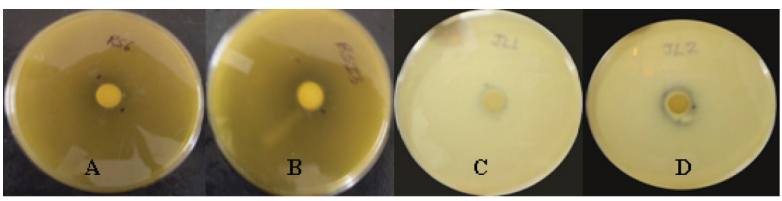

Fig. 1: Zone of clearance of phytate degradation for A = Isolate RS6, B = Isolate RSI3, C = Isolate JL1, D = Isolate JL2

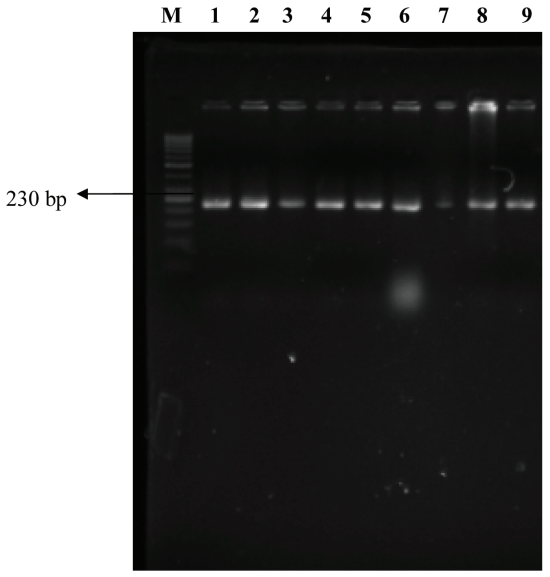

Fig. 2: Gel electrophoresis for *Lactobacillus* genus specific PCR product, LaneM= Marker (50 bp), Lane1= JL2, Lane2= JL5, Lane3= JL6, Lane4= RSI3, Lane5 =RS2, Lane6= RS6, Lane7= JL1, Lane8= JL3, Lane9= JL7

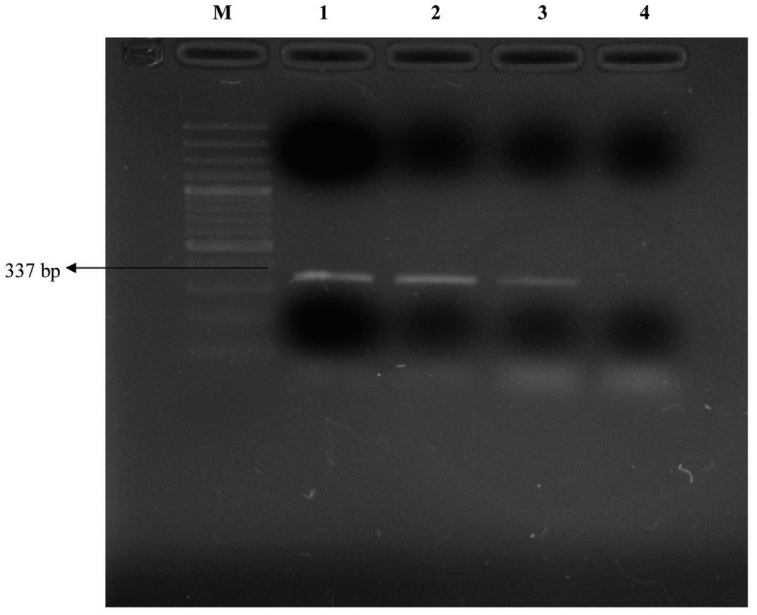

Fig. 3: Gel electrophoresis for *L. fermentum* species specific PCR product, Lane M= Marker (100 bp), Lane1= Isolate JL2, Lane2= Isolate JL5, Lane3= Isolate JL6

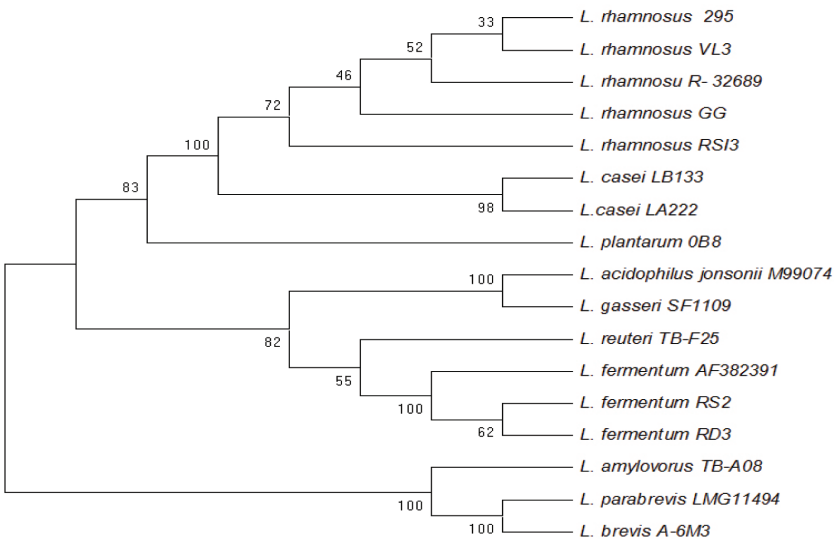

Fig. 4: Phylogenetic Dendrogram for *L. rhamnosus*RSI3, *L. fermentum*RS2 and Related Strains Based on the 16S rRNA Partial Sequence

Results and Discussion

Nine Lactobacillus bacteria have shown zone of clearance in case of phytate degradation, (Table 2 & Figure 1). Among them, isolates RSI3, JL2 and JL3 had shown significant zone of clearance (33 mm, 31 mm and 28 mm respectively) and phytase enzyme activity (0.011187, 0.008797, 0.008136 enzyme unit min^{-1} ml^{-1} respectively) as shown in Table 3. Considering the fact, only nine Lactobacillus bacteria were selected for further identification study by biochemical as well as molecular technique. Preliminary identification of nine Lactobacillus bacteria was done by biochemical tests (Table 4).

Comparing these results with differentiating characteristics of species of Streptobacterium (group IIB in Bergey's Manual, 1974) and species of Betabacterium IIA (group II of Bergey's Manual, 1974), RSI3 was tentatively determined as *Lactobacillus casei* subsp *rhamnosus,* JL2, JL5, JL6, RS2, RS6 were tentatively determined as *Lactobacillus fermentum* and JL1, JL3, JL7 were tentatively determined as *Lactobacillus brevis*.

Isolates were further subjected to API CHL50 identification system. RSI3 showed 99.8% (Very Good identification) identification similarity with *Lactobacillus rhamnosus,* RS2 showed 99.6% identification similarity with *Lactobacillus fermentum*, and JL1, JL3 and JL7 were proved as *Lactobacillus brevis*.

Partial sequencing of 16S rDNA of RSI3 and RS2 was performed by Xcleris Labs Limited, India. These sequences were analyzed using Chromas software (version 1.45, http://www.technelysium.com.au /chromas. html) and were compared with other sequences by NCBI BLAST in the database for species identification and it confirmed that RSI3 had over 99% similarity with *Lactobacillus rhamnosus* and RS2 had over 99% similarity with *Lactobacillus fermentum*. These sequence data have been submitted to NCBI GenBank database under accession numbers HQ008217 and HQ008219 for RSI3 and RS2 respectively.

In process of genotypic identification, preliminary all nine screened isolates were confirmed as Lactobacillus by PCR using genus specific primer (Figure 2.). RS2, RS6, JL2, JL5 and JL6 were further characterized as *Lb. fermentum* by PCR using species specific primer (Figure 3.). From our study we have detected that each technique has some limitation hence a polyphasic study comprising biochemical as well as molecular technique are better for identification of microbes. Phylogenetic dendrogram (Figure 4.) for *L. rhamnosus* RSI3, *L. fermentum* RS2 and related strains based on the 16S rRNA partial sequence was prepared by using mega4 software (Tamura *et al*. 2007).

In a similar study, *Lactobacilli* species were isolated from nine Nigerian fermented foods and screened for amylase, phytase, linamarase and bacteriocin productions. Isolates of *Lactobacillus* which expressed useful characters and potentials for use as starter cultures in cassava or cereal fermentations were identified as *L. plantarum, L. pentosum and L. fermentum* (Olukoya, 1995).

Twenty-six strains of Lactic Acid Bacteria (LAB) isolated from French and Bulgarian sourdoughs were screened for their enzymatic activities (phytase and phosphatase) to elucidate their possible roles during the fermentation process. Several strains have shown an interesting combination of proteolytic and phytase activities suggesting their possible roles during dough fermentation. (Valcheva *et al.*, 2009). It was reported that *Leuconostoc mesenteroides* KC51 isolated from *Kimchi*, one of the Korean traditional fermented foods can also produce phytase enzyme (Oh and In, 2009). In a study, *Lactobacillus* sp. KV1 was isolated from fermented Chinese cabbage and was found to produce phytase (Luechai and Dharmsthiti, 2010). Potential probiotic of *Lactobacillus johnsonii* LT171 for chicken nutrition showed high clear zone in culture medium containing calcium phytate (Taheri *et al.* 2009).

Conclusion

This was significant in this study that most of the phytate degrading *Lactobacillus* spp. isolated from "raabadi" sample were either *L. fermentum* or *L. brevis*. Among them one isolate RSI3 was identified as *L. rhamnosus*, whether the main source

of *L. rhamnosus* was regarded as human origin. In this regard conventionally country made "raabadi" sample gave us opportunity to isolate *L. rhamnosus*, whether *L. rhamnosus* was well recognized as potential probiotic. Our isolated *L. rhamnosus* strain may be proved as probiotic in future. That will require further study of the isolate. In our present study, we were able to isolate some potential phytate degrading *Lactobacillus* spp. which could be used as potential starter for preparation of cereal based fermented milk product as taking it into consideration that antinutritional factor phytate is present in cereal.

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