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Original Research Paper

Identification of Lactic Acid Bacteria Isolated From Natural Fermented Sources

Biological Science

KEYWORDS : Fermented vegetables, lactic acid bacteria, identification.

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ABSTRACT

Non- dairy fermented foods are known to have Lactic acid bacteria which ferment the food and increase the nutritional value. In the current study, five lactic acid bacteria were isolated from four different fermented natural sources. The isolated strains were identified using biochemical tests like Gram's test, Catalase test, Indole tests, Methyl Red test, Vogues Proskauer's test, Citrate utilization test, TSI and Hugh Liefson's test. Techniques like DNA extraction, PCR amplification, sequencing and BLAST were performed to confirm the isolates at species level. The distribution of isolated strains was as follows; Lactobacillus plantarum (C1 and C2), Pediococcus pentosaceus (G and BG) and Enterococcus faecium (M). The isolated bacteria grew well at 37ºC and showed fermentative nature when tested biochemically.

INTRODUCTION

The concept of 'Probiotics' (which means, 'for life') gains its roots in early twentieth century when the Nobel Prize winner, Eli Metchnikoff proposed that micro-organisms may have beneficial effects on human health (Metchnikoff, 1907). However, it gained momentum only recently with significant advances in selection and characterization of probiotics and their beneficial effects over human and animal health. Probiotics are live microbial feed supplement, which beneficially affects the host by improving its intestinal microbial balance (Fuller, 1991 and O'Sullivan *et al.*, 2005).

Probiotic bacteria belong to lactic acid bacteria group, among them Lactobacilli and Bifidobacteria play a reportedly significant role in intestinal health. Presence of Lactic acid bacteria (LAB) in fermented foods and dairy product and their unique existence in the intestinal region of gastrointestinal tract prove their safety. LAB are considered generally recognized as safe (GRAS) organisms and are indexed as part of human microbiota. In food industry, LAB are extensively used as starter cultures in fermented foods production (Sanders, 2000).

Probiotic microorganisms have peculiar characteristics *viz.*, acid tolerance, survival in intestine, adhesion to intestinal mucosa, antibiotic resistance and bacteriocin production. (Fuller, 1991). They show antibacterial activity against pathogenic bacteria (Schillinger and Lucke, 1989) and exert antimutagenic and anticarcinogenic activities (O'Mahony *et al.*, 2001). Lactic acid bacteria stimulate the host immune system by enhancing the natural and acquired immunity (phagocytic activity) in healthy as well as immuno compromised subjects (Gill and Rutherfurd, 2000).

Fermentation was used since ancient times as an easy method for preservation of vegetables, which also improves the nutritional properties of vegetables. The spontaneous fermentation of vegetables is caused mainly by proliferation of LAB. Fermentation also enhances digestibility of vegetables, reduces antinutrients and increases mineral bioavailability with increased Vitamin B complex (Rekha and Vijayalakshmi, 2010). The consumer awareness about effects of healthy gut microbiota is resulting in consumption of products that maintain health and prevent disease.

The identification and characterization of LAB can be performed in two steps namely biochemical and molecular identification. The biochemical identification tests include: Gram's staining, Catalase test, Oxidase test, Indole test, Methyl red test, Vogues Prokauer's test, Citrate utilization test, Nitrate reduction test and ability to ferment carbohydrates (sugar) and produce gas (Leisner *et al.*, 1999).The molecular identification is done by isolation of DNA, amplification of DNA using Polymerase Chain Reaction and sequencing. Molecular techniques have become inevitable tools in microbiology and allow evaluation and identification of isolates at species level (Jayalalitha *et al.*, 2012).

The characterization of LAB associated with indigenous fermented products in different regions or countries has been reported. These include fermented milk made by nomads, fermented dried milk cake made by Kazak, fermented vegetable juices made by farmers from China (Yi *et al.*, 2011), from fermented vegetables of Romania (Grosu-Tudor and Zamfir, 2011), from Chili Bo, a Malyasian food ingredient (Leisner *et al.*, 1999) from Kule Naoto- the Maasai traditional fermented milk in Kenya (Mathara *et al.*, 2004), from cheeses of Serbia and Montenegro (Topisirovic *et al.*, 2006) and from some fermented dairy ("Nono", "Fura", Yogurt, "Wara") and non-dairy foods ("Ogi" and "Fufu") of Nigeria (Bukola *et al.*, 2008).

The aim of the study was to isolate the lactic acid bacteria from fermented vegetable. The vegetables selected were cabbage (leaves), Ginger (tuber roots), Black gram and Green gram. Moreover, the isolated bacteria were identified using biochemical tests and also molecular techniques like PCR and sequencing.

MATERIALS AND METHODS

Sources for isolation

The samples for the isolation of LAB included the fermented cabbage, ginger (rhizome), black gram and green gram. The vegetables were finely grated and were allowed to ferment in 2% sea salt solution at ambient temperature (32°C±2), for a period of 5-6 days. The vegetables were packed tightly and pressed so as to allow a layer of brine on the top. While, the washed pulses were soaked in water for 7 hours and ground into fine paste using a home grinder. The ground paste was allowed to ferment for 8 hours at ambient temperature (32°C±2).

Isolation and Culturing

Isolation as well as culturing of isolated bacteria was done using the De Man Rogosa Sharpe (MRS) media (Hi media, Mumbai) (De Man, Rogosa and Sharpe, 1960). Isolation from the selected sample was carried out by directly plating a loopfull brine of the fermented vegetables on MRS agar plates. The plates were then incubated at 37°C for a

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period of 48 hrs. Isolated colonies with different morphology and phenotypic appearance were picked up and inoculated on MRS agar slants. The presumptive *Lactobacilli* were purified by re-streaking on MRS agar. The pure cultures obtained were characterized for their colony morphology and subjected to Gram staining. The cultures were stored at 8°C for further studies.

Identification of Isolates:
Biochemical identification
The phenotypic identification was done by microscopic examination after performing Gram's test, Catalase test and Oxidase test. The identification work was done according to The Bergey's manual of determinative bacteriology. Indole tests, Methyl Red test, Vogues Proskauer's test, Citrate utilaization test, TSI test and Hugh- Liefson's test were carried out according to standard protocol. Carbohydrate (sugar) fermentation tests of dextrose, maltose, fructose, sucrose, lactose and D- xylose were performed (Matharaa *et al*., 2004). Carbohydrate fermentation profile were determined using peptone water (2% peptone and 0.5% NaCl) with 1% sugar and phenol red as indicator. The microorganisms were grown in peptone water containing different sugars for 24 hours at 37ºC and were checked for colour change due to decrease in pH.

Molecular identification
For molecular identification, isolation of DNA from all the isolates was performed. The DNA samples were stored at -20ºC until further experiments. The isolated DNA samples were then amplified using PCR technique. The sequences of PCR products obtained by DNA sequencing were further analyzed using Basic Local Alignment Search Tool (BLAST).

Isolation of genomic DNA- Frozen microorganisms were revived by inoculating in 2ml of sterile MRS broth and were incubated 37°C for 18 hours. The log stage bacteria were harvested by centrifugation at 10000 rpm for 10min at 4°C. The supernatant media was decanted and the pellet wash washed with 500µl washing buffer (50mM Tris). After discarding the supernatant, the pellet was resuspended in 200µl Lysis buffer (50mM tris and 50mM EDTA) and 100µl lysozyme (2m g/ml) and incubated at 37°C for 1 hour with intermittent shaking. Post incubation, 20µl SDS (20%) and 10 µl protienase K (10mg/ml) were added followed by incubation at 60°C for 15minutes (Alander *et al*., 1999). After incubation, equal volume of chloroform: isoamyl alcohol (24:1) was added to the vial and the contents were mixed thoroughly. The mixture was centrifuged at 10000 rpm for 10min at 4°C and the upper aqueous layer was carefully transferred to a fresh vial without disturbing the interphase containing the cell debris. DNA was precipitated out by adding 2 volumes of chilled absolute ethanol and 1/10 volumes of 3M sodium acetate. For precipitation, DNA sample was centrifuged at 10000 rpm for 10min at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was air dried completely and resuspended in 50µl Tris EDTA buffer overnight at room temperature. The DNA was stored at -20°C till further analysis.

All the DNA samples isolated were analysed by spectrophotometry. The DNA was quantified by determining the $A_{260/280}$ ratio. Agarose (1%) gel electrophoresis was also used to evaluate the quality of DNA.

PCR analysis**-** Polymerase Chain Reaction was done to analyze the quality of isolated DNA samples. Universal 16s RNA primers 16s Forward (AGAGTTTGATCCTGGCTCAG) and 16s Reverse (TACGGCTACCTTGTTACGAC) at a concentration of 20 pmol μl^{-1} were used to perform PCR (Ulrich and Hughes, 2001 and Alander *et al*., 1999). The template DNA, extracted from isolated bacterial samples, were amplified using thermalcycler as follows: one cycle at 95ºC for 4 minutes, 35 cycles at 95ºC for 30seconds, 58ºC for 45 seconds and 72ºC for 1minute 30 seconds, followed by a final extension of 7 minutes at 72ºC. The samples (5µl) were analyzed on 2% agarose gel (100V for 40- 50minutes) containing 0.5µg/ml ethidium bromide. The PCR products were further sequenced at Eurofins Ltd.. The sequences of PCR products were analyzed using BLAST.

RESULTS AND DISCUSSION
In this study, a total of 8 colonies were picked from 4 different sources. Preliminary screening of Gram positive and catalase negative isolates was done by microscopy and 5 isolates were selected and were used for further characterization. *L. plantarum* (ATCC No.:8014), *L. bulgaricus* (ATCC No.:8001), *L. acidophilus* (ATCC No.:4963) and *L. lactis* (ATCC No.:8000) were procured from National Collection of Industrial Microorganisms (NCIM), Pune and were used for reference purpose during identification of isolates.

All the 5 isolates showed moderate growth at 37ºC, attaining an Absorbance of about 1.2 at 615nm after 24 hour incubation. The cell morphology of the isolates was examined microscopically. Out of 5, 2 isolates were rod shaped, 2 were cocci and 1 was coccobacillar.

All the isolates, except GOS, tested negative for oxidase, Indole production, methyl red, Vogues Proskauer's test and citrate utilization test. One of the isolate from fermented ginger tested to be catalase positive. The test results of the isolates were same as those to the reference *Lactobacilli* sps. When tested for the fermentative nature, the isolates showed positive results in TSI and Hugh Liefson's test.

All the micro organisms were able to ferment Dextrose, Sucrose, Lactose, Maltose and fructose. Gas production by any of the LAB was not observed. D- xylose was not fermented by any of the lactic acid bacteria.

The DNA was successfully extracted from all the isolated bacteria. The presence and quality of DNA was checked by measuring the absorbance at 280nm using spectrophotometer and by agarose gel (1%) electrophoresis using Ethidium Bromide as indicator dye. The DNA bands on the agarose gel were observed under U.V Trans-illuminator (Figure 1). The isolated DNA samples were amplified using Polymerase Chain Reaction. The amplification products were analysed by Agarose Gel (3%) Electrophoresis. Amplified PCR product was observed as a Single band corresponding to 600bp band of DNA ladder (100bp to 1000bp) (Figure 2). The PCR product was sequenced using the PCR universal 16s primers. The sequences obtained were analysed using BLAST tool. The isolates from fermented cabbage (C1 and C2) showed *Lactobacillus plantarum* as immediate hits. GOS sample isolated from fermented ginger and UB isolated from fermented Black Gram were identified as *Pediococcus pentosaceus.* The isolate (M) obtained from fermented Green Gram was identified as *Enterococcus faecium*.

Micro organism	Source	Gram's test	Colony character
L. plantarum	NCIM	Positive rods	White , elevated, smooth margin
L. bulgaricus	NCIM	Positive rods	White , elevated, smooth margin
L. acidophilus'	NCIM	Positive rods	White , elevated, smooth margin

L. lactis	NCIM	Positive rods	White , elevated, smooth margin
C1	Fermented Cabbage	Positive rods	White , elevated, smooth margin, small
C2	Fermented Cabbage	Positive rods	White , elevated, smooth margin, big
G	Fermented Ginger	Positive coccobacillar	White , elevated, smooth margin, small
BG	Fermented Black Gram	Positive cocci	White , elevated, smooth margin
GG	Fermented Green Gram	Positive cocci	White , elevated, smooth margin, small

Table 1: Preliminary analysis of isolates (Strains isolated from Cabbage- C1 and C2, Ginger- G, Black Gram- BG and Green Gram- GG)

Tests → / Micro organism ↓	Catalase	Oxidase	Indole	Methyl red	Vogues Proskauer's	Citrate utilization	TSI test	Hugh- Liefson's test
L. plantarum	-	-	-	-	-	-	K/A,no H_2S	Fermentive
L. bulgaricus	-	-	-	-	-	-	K/A, no H_2S	Fermentive
'L. acidophilus	-	-	-	-	-	-	K/A, no H_2S	Fermentive
L. lactis	-	-	-	-	-	-	K/A, no H_2S	Fermentive
C1	-	-	-	-	-	-	K/A, no H_2S	Fermentive
C2	-	-	-	-	-	-	K/A, no H_2S	Fermentive
G	+	-	-	-	-	-	K/A, no H_2S	Fermentive
BG	-	-	-	-	-	-	K/A, no H_2S	Fermentive
GG	-	-	-	-	-	-	K/A, no H_2S	Fermentive

Table 2: Biochemical tests for phenotypic identification (Strains isolated from Cabbage- C1 and C2, Ginger- G, Black Gram- BG and Green Gram-GG) (For TSI- K/A, no H_2S= red alkaline slant and yellow acidic butt due to glucose utilization)

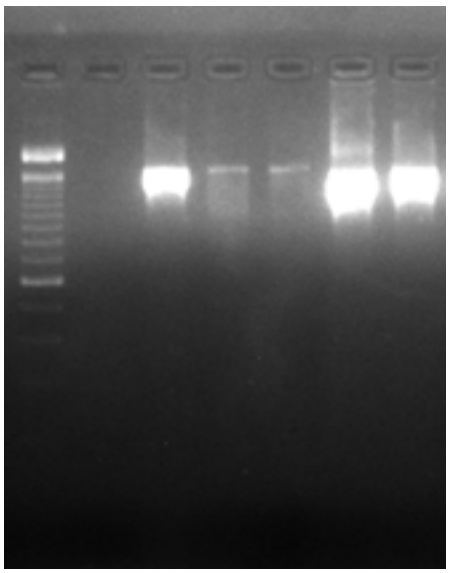


Figure 1: Agarose gel electrophoresis of chromosomal DNA of the isolates.

Lane 1: DNA ladder (100bp to 2200bp), 3- C1, 4- C2, 5- G, 6-BG, 7- GG
(Strains isolated from Cabbage- C1 and C2, Ginger- G, Black Gram- BG and Green Gram- GG)

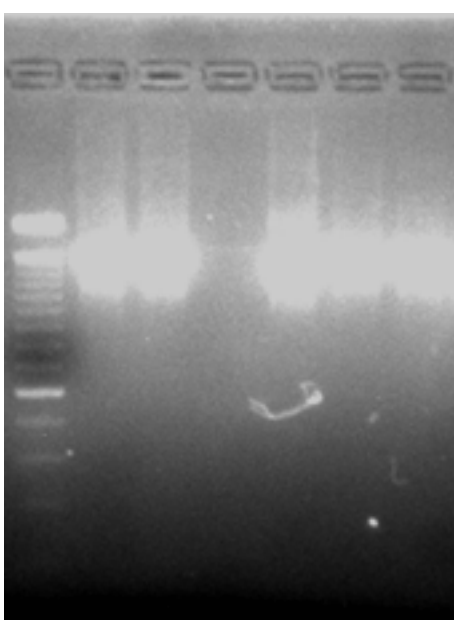


Figure 2: Agarose (2%) electrophoresis gel of PCR amplification products Lane 1- DNA ladder (100bp to 2200bp), 2- C1, 3- C2, 5- G, 6- BG, 7-GG.
(Strains isolated from Cabbage- C1 and C2, Ginger- G, Black Gram- BG and Green Gram- GG)

CONCLUSION

In current study, we have explored a range of potential non-dairy fermented sources for isolation of Lactic acid bacteria which were identified on biochemical as well as molecular level. The protocol for efficient DNA extraction and PCR amplification was standardized. The molecular analysis thus allowed the complete identification and confirmation of the potent probiotic microbial isolates. Molecular identification and characterization of the bacteria isolat-

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ed from natural sources can be eventually used as database for construction of starter cultures for production of novel functional foods. In this study, the results have confirmed the diversity of Lactic acid bacteria present in various fermented sources. The traditional belief of presence of 'good' bacteria in Fermented vegetables was found to be factual.

The innovations with respect to the isolation, characterization and utilization of new species of the microorganisms with probiotic potential and the exploration of the various natural sources for the same has been a broad research area in the today's ever-expanding food industry. In the same context, the current research provided methods for isolation of probiotics from fermented vegetable sources and their characterization. The strains isolated can be used as starter cultures with known characteristics in development of small scale fermented foods. This research also emphasizes that the fermented vegetables can provide potential probiotic strains in addition to the dairy product sources; thus introducing a new notion for the investigation of the probiotic resources. Hence, we can conclude that, apart from widely used dairy products, fermented vegetables can also prove to be potential source of probiotics.

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