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Full Length Research Paper

Pediococcus spp. – A potential probiotic isolated from *Khadi* (an Indian fermented food) and identified by 16S rDNA sequence analysis

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The probiotic potential of the lactic acid bacteria (LAB) isolated from fermented *Khadi* was investigated. The pure culture of the isolate GS4, was subjected to microbiological, biochemical characterization and 16S rDNA gene sequence analysis for species confirmation. Among other probiotic properties, GS4 exhibited antibacterial ability against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 25619), with an average zone of inhibition of 25.03 ± 2.13 mm, 18.83 ± 0.98 mm and 17.33 ± 0.58 mm respectively. The GS4 showed also significant bile tolerance and survivability at pH 3.5. Amplification of DNA was carried out by the consensus 16S rDNA primers. Sequencing was performed with the aid of big dye terminator version 3.1 cycle sequencing kit. The data was analysed using software seq scape version 5.2 and was compared by alignment against the 16S rDNA sequences of LAB. Phylogenetic tree was created based on Jukes- cantor corrected distance model and the tree was created using weighbor. The statistical tool used for estimating sample distribution by resampling with replacement from original sample was carried out using Bootstrap method. The sequence of the isolate has been deposited in Genbank repository holding an NCBI accession number HM044322. The sequence reveals the LAB isolate as *Pediococcus* sp. GS4, a closest homologue to *Pediococcus* sp. Pom 4 (EF107608) with a similarity score of 0.997. The work looks forward the future use of this probiotic strain in food biotechnology.

Key words: *Pediococcus* sp. GS4, probiotics, 16S rDNA, antibacterial, survivability.

INTRODUCTION

Khadi is a delicacy of Gujarat (India). This fermented food is prepared from sour dahi (curd) or buttermilk mixed with spices and dhal and warmed. The sour curd is made from cow's milk inoculated with a culture of curd and allowed to ferment for a day or even longer at room temperature. The sour curd is either used directly for further processing or churned into butter milk and then processed.

This food is often served with rice or the Indian bread (Naan). No study has yet been carried out on the microbial diversity of this fermented Indian delicacy and evaluated the possible probiotic potentials.

Probiotics is defined as "living organisms, which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" (Guarner et al., 1998). Lactic acid bacteria (LAB) have been of great importance as starter cultures and LAB with probiotic potential have been reported to have many beneficial effects to the human host on inhabiting the gut mucosa (Casas et al., 2000). A potential probiotic bacterium must qualify certain selection criteria as described by Gibson et al. (2000), like, acid and bile stability, antimicrobial production and antagonistic activity, ß-Galactosidase enzyme activity and should be preferably of human origin. Many studies have documented the potential of the LAB from indigenous food of different countries (Choi et al., 1998; Kim et al., 2001).

The present study aimed at studying the key probiotic properties of isolated LAB from the fermented food *Khadi*

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and to identify the bacterial strain using 16S rDNA sequence analysis.

MATERIALS AND METHODS

Bacterial isolation, enumeration and identification

The sample of *Khadi* was aseptically collected in cold condition from southern India and microbiologically processed within two hours of collection by following standard microbiological procedure. De Mann Rogosa Sharpe (MRS) broth and agar medium (Himedia, India) pH 6.5 ± 0.2 (at 25°C) was used for the isolation and culturing of the LAB. Inoculated cultures were incubated at 37°C for 24 - 48 h. Based on the colony characteristics, a single colony was selected and sub cultured onto MRS broth to obtain pure culture. The strain was preserved in stab culture in triplicates and was periodically sub cultured every two months. Standard Biochemical tests were performed following standard procedures (Cappuccino and Sherman, 1999; Sharpe, 1979; Harrigan and McCance, 1976).

Staphylococcus aureus (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 25619) were used as reference strains for this study. Stab cultures of American type culture collection (ATCC) of Gram positive bacteria *S. aureus* (ATCC 25923) and Gram negative bacterial cultures of *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 25619) were used for the study of antibacterial activity. The stab cultures were subcultured on sterile Luria Bertani broth (Himedia, India) pH 7.5 ± 0.2 (at 25°C) and incubated at 37°C for 18 - 24 h.

Their identity has been confirmed by Gram staining and morphology examination. Cells in the log phase of growth were used in the study of antibacterial activity. The reference strains were maintained in Luria Bertani agar (Himedia, India) slant and stab at 4°C.

Determination of antibacterial activity

Antibacterial activity was determined by Agar well diffusion method (Lasta et al. 2008). The petridishes containing Muller Hilton agar (pH 6.5 ± 0.2 at 25°C) was seeded with the reference strain in the log phase using sterile swab. Wells were created on the seeded agar using incerinated Agar Boarer. A 100 µl of 18h grown cell free extract (CFE) of GS 4 strain was instilled into the wells, with Ampicillin (10µg/100 µl) as positive control and sterile (un-inoculated) MRS broth as negative control. Antibiotic activity was determined by the measurement of zone of inhibition (ZOI)) around the wells after 24 h of incubation at 37°C.The diameter of the zone was measured (in mm) and compared with that of positive and negative control. The experiment was performed in triplicates and each of the readings was taken by two observers and the average was calculated. The same procedure was repeated with the pH of the supernatant being neutralized to 6.5 with 1N NaOH to nullify the lactic acid activity.

Resistance to antibiotics

Antibiotic sensitivity test of the isolate was performed by standard disc diffusion method (NCCLS 1999). The LAB culture suspension containing 3×10^8 cfu/ml of the 18 h culture at 37°C were spread on the MRS agar plates to form a uniform smear. Selected antibiotic discs (Erythromycin 10 µg, Gentamycin 30 µg, Vancomycin 30 µg, Tetracycline 30 µg, Chloramphenicol 30 µg and Nalidixic acid 30 µg) (Himedia, India) were aseptically transferred on to the seeded plates. The diameters of the zone of inhibition were measured in mm under the colony counter after 24 h of incubation. The experiment was repeated thrice and the average inhibitory zone diameters were compared with the standards provided by the National Committee for Clinical Laboratory Standards (NCCLS, 1999).

β–Galactosidase activity

The method described by Karasova et al. (2002) was followed to test the presence of β-galactosidase activity. GS4 was streaked onto MRS agar plate containing 0.01% X-gal (5 Bromo, 4 Chloro,3-indolyl-D-Galactopyranoside)(Hi Media, India) and 0.1 mM IPTG (Hi Media, India) dissolved in Dimethyl sulphoxide(DMSO) as an inducer .The plate was incubated for 24 - 48 h at 37°C.

Determination of lactic acid production

A 24 h culture of the isolate GS4 was used for the estimation of the organic acid produced. Supernatant was collected by centrifuging at 10,000 rpm for 15 min at 4°C. 20 ml of supernatant was taken in a conical flask; few drops of phenolphthalein (1%) were added as an indicator. Titrimetrical estimation was performed against 0.1 M NaOH. As per standards provided by A.O.A.C. 1990, one ml of 0.1 M NaOH for neutralization of acid is equivalent to 90.08 mg of lactic acid. Amount of lactic acid produced was calculated accordingly. The titration for each supernatant was carried out in triplicates and the average value was calculated.

Acid tolerance test

Acid tolerant capabilities were confirmed by viable count (Gilliland et al., 1984). MRS broth for tolerance test and MRS agar for LAB enumeration were used .One ml of the isolate grown in the MRS broth for three generations having an optical density of 0.280 at 600 nm were inoculated in 9 ml of sterile MRS broth whose pH was adjusted to 3.5 with 0.5 N HCl. The inoculated broth was incubated at 37°C for 4 h after inoculation. At 0th hour and 4th h, one ml of sample was taken and serially diluted with sterile normal saline (0.84% sodium chloride) in order to neutralize the acidity of the medium.100 µl of the specific dilution was inoculated onto MRS agar plates. The agar plates were incubated at 37˚C for 24 - 48 h. The colonies were counted using colony counter. The reduction in log cycle after exposure to low pH for 4 h as compared to control was considered as the criteria for acid tolerance (Wright et al., 1983).

Percentage survivability of the strain to acidic pH was calculated using the formula thus given:

% survivability = (log cfu 4th hour / log cfu 0th hour) ×100

Bile salt tolerance test

Bile acid tolerance capability of GS4 was confirmed by viable count method (Gilliland et al., 1984). MRS broth for the tolerance test and MRS agar for the LAB enumeration were used. From MRS broth culture having an optical density of 0.280 at 600 nm, 1 ml of the inoculum was inoculated in 9ml of sterile MRS broth enriched with 0.3% (w/v) of sodium thioglycollate (Hi Media, India) and incubated at 37°C for 24 h. At 0th h and 24th h, 1 ml of culture was taken and serially diluted with sterile saline solution (0.84%) and 100 µl of the specific dilution was inoculated on MRS agar. The plates were incubated for 24 - 48 h at 37°C and the colonies were counted using a colony counter. The reduction in the log values of survival after exposure to 0.3% bile salts for 24 h was compared with the values at 0 h (as control) was considered as criteria for bile salt tolerance.

Percentage survivability of the strains to 0.3% sodium thioglycollate was calculated using the formula thus given:

Table 1. Microbiological and biochemical characteristics of GS4[a].

Characteristics	Strain name
	***Pediococcus* sp. GS4**
Morphology and	+
Gram stain reaction	Pediococci
Spore formation	-
Cultural characteristics	
Size	>0.1 mm
Shape	Circular
Colour	Milky white
Elevation	Concave
Density	Mucoid and glistening
Biochemical characteristics	
Catalase	-
Oxidase	-
Indole	-
MR	-
Citrate	-
TSI	A/A
Physiological characteristics	
Growth in MRS broth:	
- with 0.5% NaCl	+
- with 4.0% NaCl	+
- with 6.5% NaCl	+
Growth at 10˚C	-
Growth at 37˚C	+
Growth at 45˚C	+
Gas production from glucose	-
Fermentation of sugars	
Glucose	-
Adonitol	-
Arabinose	+
Lactose	+
Sorbitol	+
Mannitol	-
Rhamnose	+
Sucrose	+

[a]+, - indicates positive and negative results, respectively.

% survivability = (log cfu 24th h / log cfu 0th h) × 100

Salt aggregation test (SAT)

Salting out method described by Lindahl et al. (1981) was performed to determine the hydrophobic characteristics of the bacterial cell surface to reveal the adherence property of GS4 in gut system. Highly hydrophobic bacteria aggregate according to characteristic patterns in the presence of ammonium sulfate. The strain cultured in MRS broth was harvested, washed, and resuspended in phosphate buffer saline (PBS) (0.02 M, pH 6.8) to get a bacterial concentration of 10^9 cfu/ml. Ammonium sulfate in PBS was prepared at different concentrations (2 - 0.01 M). Aliquots of bacterial suspensions and salt solutions were mixed for 2 min on slides. The bacterial aggregates/ clumps were observed against black background. The procedure was repeated thrice.

Amplification and sequence analysis (16S rDNA)

16S rDNA analysis was performed using big dye terminator version 3.1 cycles sequencing kit and ABI 3130 Genetic Analyser (By Chromous biotech PVT. Ltd., India). The unknown sequence of the isolate was compared by alignment against 16S rDNA sequences of LAB available in the GenBank Database. The ~1.4 kb rDNA fragment was amplified using high-fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward, reverse and internal primer. The sequence data was aligned and analyzed to identify the bacterium and its closest neighbors.

In brief, culture of LAB was used for the isolation of genomic DNA (Frederick et al., 2002). Amplification of DNA was carried out by the use of Taq DNA polymerase enzyme (3 U/ µl) in PCR Thermal Cylcer ABI2720. The reaction cocktail consisted of DNA: 1 µl, 16S forward primer 400 ng, 16S reverse primer 400 ng, dNTPs (2.5 mM each): 4 µl, 10X Taq DNA polymerase assay buffer:10 µl, Taq DNA polymerase enzyme (3U/ µl) 1 µl, MilliQWater 84 µl (total reaction volume 100 µl).The consensus 16S rDNA primers used in the reaction include 16S forward primer -5'-AGAGTRTGATCMTYGCTWAC-3'and 16S reverse primer-5'-CGYTAMCCTWTTACGRCT-3'. According to the International Union of Biochemistry convention, R represents an A/G nucleotide degeneracy, Y a C/T nucleotide degeneracy, M A/C nucleotide degeneracy and W an A/T nucleotide degeneracy, respectively (http://prowl.rockefeller.edu/aainfo/nuctrans.html). The denaturation step was carried out for conditions subjected for 5 min at 94°C , PCR was repeated for 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 2 min and 72°C for 5 min and the reaction mixture was cooled and subjected for Agarose gel electrophoresis. Sequencing was performed using ABI3130 Genetic analyzer with the aid of Big dye terminator version 3.1 cycle sequencing kit. The reaction mixture consisted of big dye terminator ready reaction mix 4 µl, Template (100 ng/ µl): 1 µl, Primer (10 pmol/λ): 2 µl, Milli Q water: 3 µl. The PCR cycles consisted of 25 cycles with initial denaturation for 96°C for 1 min, Denaturation: 96°C for 10 s, Hybridization: 50°C for 5 s and Elongation: 60°C for 4 min. The data was analysed using software seq scape version 5.2. The sequence was compared by alignment against the 16S rDNA sequences of lactic acid Bacteria. Phylogenetic tree was created based on Jukes-cantor corrected distance model and the tree was created using weighbor. The statistical tool used for estimating sample distribution by resampling with replacement from original sample was carried out using Bootstrap method. The sequence of the isolate has been deposited in Genbank repository holding an NCBI accession number HM044322.

RESULTS

Bacterial isolation, enumeration and identification

The selected isolate LAB (GS4) from *Khadi* was gram positive cocci arranged in tetrads, catalase negative, oxidase negative and non spore-forming. Other characteristics like its ability to grow at different temperatures; different salt concentration was investigated and tabulated as shown in Table 1.

Table 2. Alignment view and distance matrix table (With Sample GS4 sequence taken as reference sequence).

S_ab score	Organism name	NCBI accession No.
0.991	*Pediococcus* sp. Rrv3	EF107607
0.997	*Pediococcus* sp. Pom4	EF107608
0.994	*Pediococcus* sp. Rrt8	EF107615
0.987	*Pediococcus pentosaceus*; BMG 60	EU080990
0.988	*Pediococcus pentosaceus*; BMG 74	EU080993
0.986	*Pediococcus* sp. MMZ60A	EU157914
0.984	*Pediococcus pentosaceus*; MY-800	EU483113
0.987	*Pediococcus pentosaceus*; NS75	EU180605
0.984	*Pediococcus pentosaceus*; KC007	EU569832
0.988	*Pediococcus pentosaceus*; SH 740	EU878171

Antibacterial activity

Strain GS4 showed maximum ZOI against *S.aureus* and *P. aeruginosa* with the average zones of 25.03 ± 2.13 and 18.83 ± 0.98 (mm) respectively. The average ZOI observed with the positive control include 43.00 ± 2.65, 19.67 ± 1.04, 30.67 ± 0.29 for *S. aureus*, *P. aeruginosa* and *E. coli*, respectively.GS4 showed minimum inhibition against *E. coli* with an average zone of 17.33 ± 0.58 mm. Sterile broth as negative control did not show any zone of inhibition. In general the zone of inhibition against gram-positive bacteria was observed to be higher than that of the Gram-negative bacteria. Upon neutralization the average ZOI was 17.53 ± 0.76, 14.06 ± 0.55 (mm) and 13.20 ± 0.66 (mm) for *S. aureus, P. aeruginosa* and *E.coli*, respectively. The positive control showed zones with average of 41.00 ± 0.65, 19.00 ± 1.00, 23.08 ± 0.00 (mm) for *S.aureus*, *P. aeruginosa* and *E. coli*, respectively. Negative control exhibited no zones of inhibition.

Antibiotic resistance

GS4 stain was resistant to vancomycin, gentamycin and nalidixic acid. It was more sensititive to Chloramphenicol (34.25 ± 0.96) in comparison to erythromycin (30 ± 0.82) and tetracycline (26.25 ± 0.96) mm, respectively.

β-Galactosidase activity

β- Galactosidase activity reduces lactose intolerance and is an important probiotic property. GS4 isolate showed blue/green colonies on MRS agar supplemented with X-GAL and IPTG and thus it confirmed β-galactosidase activity.

Lactic acid production, acid and bile tolerance and SAT

GS4 produced lactic acid with an average of 2.60 ± 0.01 (g/20 ml of supernatant). The survival rate was calculated to be 87.74% at pH 3.5 and thus it qualifies one of the key properties of a potent probiotic isolate. The survivability in the bile environment (0.3%) showed an increase in the log value upon bile exposure giving rise to 114.01% survivability. As described by Olejnik et al. (2005) that the survival of bacterial cultures in bile environment is more crucial than the effect of low pH. GS4 isolate formed prominent clumps, “salt aggregation” at a low concentration of ammonium sulphate (0.01 M). This confirms the hydrophobic nature of bacterial cell surface. This property enables the bacterium to adhere to gut mucosa and exhibit the beneficial property.

Amplification and sequence analysis (16S rDNA)

The isolated genomic DNA amplified by PCR when analysed by Agarose gel electrophoresis shows a molecular weight of 1.5 k base pairs when compared with the standard markers. The phylogenetic tree was determined by comparing the homology with the existing GenBank Database as shown in Figure 1. The comparative analysis revealed their closest neighbors. The Microbe was found to be most similar to *Pediococcus* sp. Pom4; (GenBank entry: EF107608). Information about other close homologue for the microbe is shown in Table 2.

DISCUSSION

India is a land of early civilization and the most diversified democracy in the world with different delicacies and fermented foods. *Idli*, *Dosa*, *Curd*, *Khadi*, *Bodi*, and many more fermented food and food items are used regularly in Indian meals. Many foods were not examined thoroughly for establishing the health-benefit like probiotic properties. *Khadi* is one among such fermented food which has never been evaluated for the microbial ecology and beneficial effect to the consumers. However, this study enabled the isolation of a Lactic acid bacterium with ability to exhibit profound probiotic characteristics such as β-galactosidase activity, acid tolerance, bile acid tolerance

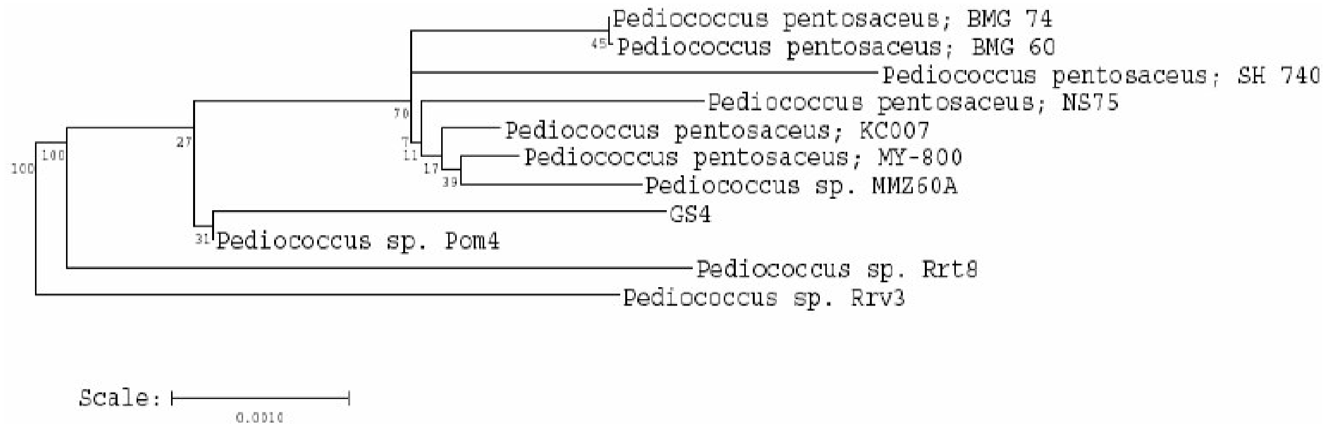

Figure 1. Phylogenetic tree of sequence analysed GS4 showing the LAB is *Pediococcus* sp. and it was determined by comparing the homology with the existing GenBank Database.

tolerance, adherence ability as well as antibacterial property. GS4 is a vancomycin resistant. Resistance to vancomycin remains one of the key characteristic of probiotic bacteria (Salminen et al., 1998). The survivability in condition of 0.3% bile concentration, low pH of 3.5 and salt aggregation have been observed and those ensure their probiotic potentials with adherence property to the gut mucosa-in turn leading to other physiological effects. β-galactosidase is also being considered, the other key property of probiotic category; this enzyme plays a major role in digestion of lactose in humans.

The antibiotic activity exhibited by the strain GS4 may be partly due to bacteriocin like peptides (pediocin) and other metabolites such as lactic acid, hydrogen peroxide and organic acids (El-Ziney and Debevere, 1998).The production of acids lowers the pH of the digestive tract and inhibits the growth of the pathogenic microorganisms. Antimicrobial activity was observed even after neutralization of pH to 6.5 using 1N NaOH. This extends the investigation further into the isolation of the protein or protein like antimicrobial compound from this isolate. Numerable peptide containing or protenaceous compounds having antagonistic activity have been isolated from different fermented food (Kim et al., 1991) To date, Nisin is the only bacteriocin that has found practical application in some industrially processed foods. It is anticipated that advances in bacteriocin research and combination treatment for food preservation will benefit both the producer and the consumer of the food industry. The 16S rDNA sequence analysis and comparative analysis revealed that our strain was found to be most similar to *Pediococcus* sp. Pom4; (GenBank entry: EF107608). *Pediococcus* sp. GS 4 is catalase negative and hydrogen peroxide is unstable at room temperature. Therefore it is less likely that either acid or hydrogen peroxide was responsible for the antimicrobial potential of *Pediococcus* sp. GS4.

The predominant microbial member in *Kimchi*- a Korean pickle has been reported to be *Pediococcus* sp. (Chin et al., 2006) in the early phase of fermentation. *Pediococcus* sp. has been also reported to be the producer of bacteriocin which may be of use as preservative in food industry. In our study, the strain produces antibacterial(s) in early stage of death phase and showed the ability to inhibit the growth of *S. aureus*, *P. aeruginosa* and *E. coli*, respectively. This antibacterial could be pediocin. *Pediococcus* sp. GS4 exhibits the probiotic potentials which may be in use in healthcare and in food industry in near future.

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