ORIGINAL RESEARCH PAPER

Biological Science

BIOCHEMICAL CHARACTERIZATION AND 16S RRNA SEQUENCING OF PROBIOTIC STRAIN ISOLATED FROM COW MILK

KEY WORDS: probiotic, *Leuconostoc pseudomesenteroids*, 16S rRNA sequencing, GenBank

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ABSTRACT

Aim: To study the Biochemical characterization and molecular characterization of sequencing of probiotic organism isolated from cow milk.

16S rRNA gene sequencing approach to identify a novel species of Probiotic *Leuconostoc pseudomesenteroides*. The cow milk sample was collected from alwarkurichi, Tirunelveli District, Tamil nadu, India. Subsequently the sample was serially diluted and the aliquots were incubated for a suitable time period following which the suspected on colony was subjected to biochemical characterization, antibiotic sensitivity assay and 16S rRNA sequencing. The sequence aligned against other species were concluded to be a novel, Probiotic *Leuconostoc pseudomesenteroides* bacteria, further which were named *Leuconostoc pseudomesenteroides* strain RBSS10. After the sequence characterization, the isolate was deposited in GenBank Database, maintained by the National Centre for Biotechnology Information NCBI. The sequence can also be retrieve from EMBL and DDBJ repositories with accession number MK290325.

I.INTRODUCTION

Probiotics, are living microorganisms with low or no pathogenicity that exert beneficial effects on the health of the host. The properties of probiotic are strain-specific, it is imperative that they should be correctly identified, and their properties to be studied properly. It is essential that a potential probiotic strain is able to tolerate these stress conditions in order to survive in the Gastro intestinal tract, for this study isolate and identify *Lactobacilli* and search the potential probiotic properties of these isolates using raw cow milk as a natural source.

The guidelines proposed by FAO/WHO (2002) for evaluation of probiotics recommended that every potential probiotic strain should be correctly identified using both phenotypic and genotypic methods, followed by various tests to investigate its survival ability and functional properties. In this chapter identification of the selected probiotic isolate RBSS10 was done by phenotypic and genotypic methods for further analysis.

II. MATERIALS AND METHODS

IDENTIFICATION OF PROBIOTIC ISOLAT (S10)

The isolate number S10 was inoculated into MRS broth and incubated for 24 to 48 hrs at 37°C. This culture source was used for further identification analysis.

PRESUMPTIVE IDENTIFICATION

Lactobacilli was presumptively identified by following methods described in Bergey's Manual of Systematic Bacteriology. Macroscopic appearance of the colony, which was obtained after 48 hrs of incubation on MRS agar plates, was examined for cultural and morphological characteristics. Size, shape, colour and texture of the colonies were noted. Bacterial isolates were tested for catalase production by catalase test. Carbohydrate fermentation, Nitrate Reduction. Indole production, Voges Prauskar, Methyl Red and Citrate utilization tests were also performed. Cell morphology was examined after Gram staining,

MICROSCOPICAL EXAMINATION

A loopful of isolate was smeared on separate clean glass slides and heat fixed. Then it was subjected to Gram's staining method. Primarily, crystal violet was applied to the smear and kept for 60 seconds and washed with water. Secondly, Gram's iodine was used as mordant. Then it was washed with water. Afterwards it was washed with 95% alcohol and then treated with saffranin and kept for 45 seconds and examined under oil immersion objective microscope. The gram positive organism appears purple blue whereas Gram negative organism appeared pink.

MOTILITY TEST: HANGING DROP METHOD

Vaseline was smeared on the four edges of a cover slip and a very small drop of the culture was placed on the centre of the cover slip. Then a concave slide was inverted carefully on the cover slip. The cover slip was turned up right and examined motility of cultures under the microscope.

IDENTIFICATION BASED ON BIOCHEMICAL TEST

The following biochemical tests were performed to identify the bacteria isolate S10 described such as Catalase test, Starch hydrolysis test, Gelatin hydrolysis test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Indole production test, Carbohydrate fermentation test.

ANTIBIOTIC SENSITIVITY ANALYSIS

Antibiotic sensitivity of the isolated organism was analysed by Kirby-Bauer disc diffusion method. The most widely used antibiotics were selected for antibiotic sensitivity assay. Mueller Hinton agar was prepared and sterilized. The sterile medium was poured into the sterile petriplates, the plates were inoculated with organism broth of S10 isolate as lawn using sterile swabs. The discs of Amoxicillin (10mcg) Ampicillin (30 mcg), Gentamycin (30 mcg), ciprofloxacin (30 mcg), Streptomycin (10 mcg), Tetracycline (30 mcg), were placed on the lawn culture with a sterile forceps and gently pressed down and the plates were incubated at 37°C for 24 hrs. After incubation the diameter of the zone (mm) was measured and compared with the standard chart to find out the sensitivity of organisms against various antibiotics.

PHENOTYPIC IDENTIFICATION

Cells grown in monolayer should be lysed by suspending1-3 colonies aseptically and mixed with 450 µl of "B Cube" lysis buffer in a 2 ml micro centrifuge tube and lyse the cells by repeated pipetting. Add 4µl of RNAse A and 250µl of "B Cube" neutralization buffer. Vortex the content and incubate the tubes for 30 minutes at 65˚C in water bath. To minimize shearing the DNA molecules, mix DNA solutions by inversion. Centrifuge the tubes for 20 minutes at 14,000 rpm at 10 ˚C. Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 ml micro centrifuge tube without disturbing the pellet. Add 600 µl of "B Cube" binding buffer to the content and mix thoroughly by pipetting and incubate the content at room temperature for 5 minutes. Transfer 600 µl of the contents to a spin column placed in 2 ml collection tube. Centrifuge for 2 minutes at 14,000 rpm and discard flow-through. Reassemble the spin column and the collection tube then transfer the remaining 600µl of the lysate. Centrifuge for 2 minutes at 14,000 rpm and discard flow-through. Add 500µL "B Cube" washing buffer I to

the spin column. Centrifuge at 14,000 rpm for2 mins and discard flow-through. Reassemble the spin column and add 500 µl "B Cube" washing buffer II and Centrifuge at 14,000 rpm for 2 mins and discard flow-through. Transfer the spin column to a sterile 1.5-ml micro centrifuge tube. Add 100 µl of "B Cube" Elution buffer at the middle of spin column. Care should be taken to avoid touch with the filter. Incubate the tubes for 5 minutes at room temperature and Centrifuge at 6000 rpm for 1min.Repeat the above mentioned step 14 and 15 for complete elution. The buffer in the micro centrifuge tube contains the DNA.DNA concentrations were measured by running aliquots on 1% agarose gel. The DNA samples were stored at -20°C until further use.

PCR PROTOCOL

Denaturation: The DNA template is heated to 94°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

Annealing: The mixture is cooled to anywhere from 60℃. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

Extension: The reaction is then heated to 72° C, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

Stages temperature time: Initial Denaturation 94˚C 3 min Denaturation 94˚C 30 sec 30 cycles. Annealing 60˚C 30 sec Extension 72˚C 1 min Final extension 72˚C 10 min Hold 4˚C

Purification of PCR Production: Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

SEQUENCING PROTOCOL

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI3730xl sequencer (Applied Biosystems).

BIOINFORMATICS PROTOCOL

Sequence alignment: The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences. The resulting aligned sequences were cured using the program Gblocks 0.91b.ThisGblocks eliminates poorly aligned positions and divergent regions (removes alignment noise).

III. RESULT

S.No	Biochemical Tests	Result	
1	Catalase	-	
2	oxidase	+	
3		-	
	Indole	-	-
4	Methyl Red	-	
5	Voges Proskauer	+	
6	Citrate Utilization	-	
7		-	
	Motility		
8	Starch hydrolysis	+	
9	Gelatin Hydrolysis	-	
10	Glucose fermentation	+	
11	Lactose Fermentation	+	
12	Sucrose Fermentation	+	
13	fructose Fermentation	+	
14	maltose Fermentation	-	
15	dextrose Fermentation	+	

+ Sign indicates positive result. - Sign indicates negative result.
Table 1: Biochemical characteristeristics of the probiotic isolate S10 from raw cow milk.

CATALASE TEST

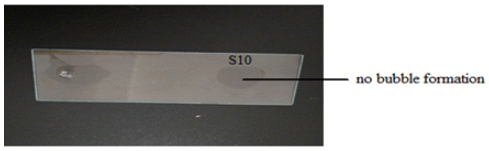
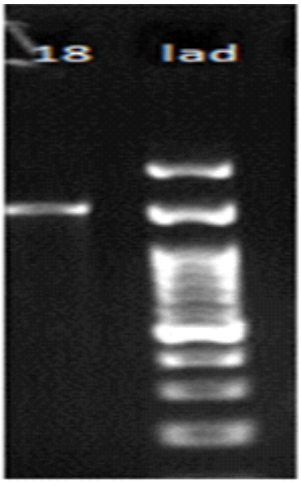


Figure 1

Table.2: Antibiotic sensitivity assay of probiotic isolate S10 against different antibiotics

Antibiotic	Zone of inhibition in Mm	Sensitivity
Amoxicillin	No zone	R
Ampicillin	No zone	R
Gentamycin	6	S
ciprofloxacilin	No zone	R
Streptomycin	No zone	R
Tetracycline	No zone	R

R- Resistant. S- Sensitive.



lad- 100bp ladder (NEB), lane 18 - sample ID -S10
Figure.2. 16S rRNA Amplification of probiotic isolate S10

IV. DISCUSSION

BIOCHEMICAL TEST

In the present study The gram staining results indicated that the isolated bacteria could be identified as Leuconostoc. Hanging-drop method showed that the isolated bacteria were nonmotile. This method conformed that the bacteria under investigation was nonmotile. Results of biochemical characteristeristics of the probiotic isolate S10. In performing catalase test, no bubble was observed indicating that the isolated bacterium was catalase negative. Catalase test was one of the most useful diagnostic test for the recognition of bacteria due to their simplicity. And observed that S10 was Indole production-negative MR test-negative, VP test- positive, citrate utilization test-negative, Gelatin hydrolysis test- negative, starch hydrolysis positive.In carbohydrate fermentation test showed that the isolated bacteria S10 could ferment glucose, lactose, sucrose, fructose, dextrose, but not maltose. Probiotic strains are could ferment sugars such as glucose, fructose and lactose.

ANTIBIOTIC SENSITIVITY ANALYSIS

Probiotic isolate S10 was found resistant to amoxyllin, ampicillin, gentamycin, ciprofloxacin, streptomycin, and tetracycline. It found sensitive to gentamycin (zone of inhibition 6mm). So this resistant strain can be used for antibiotic therapy. So unreasonable to use susceptible probiotics in combination with antibiotics but resistant probiotics can be used with antibiotics in case of bacterial infections.

16S rRNA SEQUENCING

The result of 16s rRNA sequencing showed that S10 isolate belonged to the genus Leuconostoc. The S10 probiotic isolate was 95% homology between *Leuconostoc pseudomesnteroides.* The PCR amplification of 16s rRNA of S10 potent probiotic resulted in the characteristics of single band about 969 bp sequenced by using the 27F/1492R primers. But the reference ladder DNA about 100 bp, it showed more than one band in the separated agarose gel.

BIOINFORMATICS

In bioinformatics the ssequence of *Leuconostoc pseudomesenteroides* were aligned and it deposited into genbank and maintained into NCBI. The accession number of Leuconostoc pseudomesenteroides was Mk290325.

V. CONCLUSION

The present investigation conclude that isolated and selected probiotic strain S10 was Leuconostoc pseudomesenteroides based on biochemical, antibiotic sensitivity assay and molecular characterization.

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