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Isolation, identification and characterization of *Lysinibacillus sphaericus* from fresh water fish intestinal tract

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Abstract
The main aim of this study was isolation of strains from different source like *idly* batter, *dosa* batter, raw milk and fresh water fish intestinal tract. Characterization of isolated bacterial culture was identified by morphological and 16s RNA molecular identification. Further it was selected for the evaluation of probiotic cation property using resistance to acid and bile tolerance, anti-microbial activity. It was rod-shaped, Gram-positive, catalase negative and produced ammonia from Arginine hydrolysis, which is the main characteristic feature, as observed with all lactobacilli. The culture could grow at different temperatures 30-37ºC, pH (5.8-6.2) and NaCl concentrations (2-12%) and in the presence of bile salt. The fish intestinal (FI) culture was similar to *Lys. sphaericus* strain L2 based on nucleotide homology and phylogenetic analysis. The FI sequence was producing 100% homology and significant alignments with taxa *Lys. sphaericus* strain L2. It was shown better growth even at room temperature hence this *Lysinibacillus sphaericus* was may be suggested used for probiotication of vegetable fruit juices.

Keywords: *lysinibacillus sphaericus*, antimicrobial activity, probiotication, fish intestinal tract

1. Introduction
According to the definition contained in the report by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO), probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Most probiotics consist of lactic acid bacteria, such as *Lactobacilli*, *Lactococci* and *Bifidobacteria*. These bacteria are also found in large quantities in the human intestines. However, several strains of *Streptococci*, *Enterococci*, *Pediococci*, *Bacilli* and some yeast are also regarded as probiotic strains (Antoine, 2010) [2]. The concept of probiotic (which means, "for life") was introduced in early 20th century by Elie Metchnikoff, who observed that the consumption of fermented milk could reverse putrefactive effects of the gut micro-flora (Metchnikoff, 1908) [10].

Probiotics particularly *Lactobacillus*, *Bifidobacterium* and yeast cells (*Saccharomyces boulardii*) have been associated with alleviation of lactose intolerance, prevention and cure of viral, bacterial and antibiotic or radiotherapy-induced diarrhoeas, immune-modulation, anti-mutagenic and anti-carcinogenic effects; and even blood cholesterol reduction (Blandino *et al*., 2003) [3]. The optimism associated with probiotics is counter-balanced by skepticism as many "Probiotic" products are unreliable in content and unproven clinically. There are no established or validated testing criteria and methodology to determine the safety and to assess the efficacy of a probiotic product. The regulatory mechanism differs from country to country and within a country from the way the product is labeled.

The past 5 years have witnessed a strong expansion of the probiotic market and in parallel, a rise in the number of research projects addressing fundamental and applied aspects of probiotics. They are live microbial food supplement that are consumed with the aim of providing a health benefit to the host by contributing to an improved microbial balance within the intestinal micro-biota (Blandino *et al*., 2003) [3]. These probiotics (microorganisms) residing in the human gastrointestinal tract are larger and complex microbial ecosystem that develops through infancy and childhood to form a diverse, but relatively stable community in adults (Demir *et al*., 2006) [4]. These autochronoous bacteria interact with the diet and the host, contributing to protection against intestinal pathogens through colonization resistance and providing nutritional and colonic health benefits *via* their metabolic activities.

The potential roles of probiotics as natural barrier to pathogens associated with intestinal disease are scientifically well documented. The effects on human health like alleviation of symptoms of lactose intolerance, anti-diarrheal effect, reduced risks of some cancer, improved intestinal and urogential health, enhanced immune functioning, moderation of allergic reactions, inhibition of *Helicobacter pylori* stomach infection, cholesterol lowering effect, mild anti-hypertensive effect, effect on alcohol induced liver diseases are some of the talk about the benefits of probiotics. New research technologies have supported earlier suggestions of health promoting properties of probiotic lactic acid bacteria (LAB) as reviewed by Naidu *et al*. (1999) [12] including stabilization of the intestinal micro-flora by competition against pathogens (Gibson *et al*., 1997) [8], and reduction of lactose intolerance (de Vrese and Marteau, 2007).

The ability of probiotic strains to hydrolyze bile salts has often been included among the criteria for probiotic strain selection and a number of bile salt hydrolases (BSHs) have been identified and characterized (Mishra *et al*., 2005) [11]. However, microbial BSH activity has also been mooted to be potentially detrimental to the human host, and thus it is a yet not completely clear whether BSH activity is in fact a

desirable trait in a probiotic bacterium. The ability of the probiotic organisms has been attributed to the presence of alpha- galactosidase apart from the presence of the usual beta galactosidases, which are capable of breaking the alpha-glycosidic linkage in carbohydrates such as stachyose, raffinose, verbascose, trehalose and others resulting in the formation of simple sugars that are subsequently suitably metabolized.

Lysinibacillus is commonly found in soil, plants and animals. The genome of *Lys. sphaericus* was the first strain in the genus *Lysinibacillus* which is taxonomically classified on the basis of polyphasic cell wall peptidoglycan. The antimicrobial potential of *Lysinibacillus* reported and its bacteriocins used as food preservative to combat against food borne bacterial and fungal pathogens (Ahmad *et al*., 2014). The main objective of this study was to isolate, identification, characterization and detection of probiotic and antimicrobial property of isolated culture.

2. Materials and Methods

Microorganism

Lysinibacillus sphaericus was isolated from large intestine of fresh water fish, (Catla, *Catla catla*) by using MRS (de Man, Rogosa and Sharpe) agar medium (Hi-media, Mumbai, India). The isolate was identified on the basis of morphological, physiological and molecular characteristics.

Purification of the bacterial isolates

The cultures isolated were purified by streak plate method. A loopful of appropriate culture was taken in a sterile inoculation loop and was streaked on to MRS agar plates for getting single well isolated colonies.

Morphological and biochemical characterization

To determine the ability of the isolate to grow in NaCl, MRS tubes containing 2, 4, 6, 8 and 12% NaCl were inoculated with a loopful of actively growing 24 h old culture of *Lys. sphaericus* and incubated at 37°C for 72 h. To determine the optimum growth temperature, the culture was inoculated in 10 mL of sterile MRS broth tubes and was incubated at 20, 25, 30, 35, 37, 40 and 45°C for 24 - 48 h. To optimize the pH for growth, the pH of the MRS broth was adjusted from 9.2 to 9.8, individually, and a loopful of 24 h old culture was added to each tube and incubated at 37°C for 72 h.

Gram staining

Crystal violet and ammonium oxalate were dissolved separately in ethyl alcohol and distilled water, respectively and the two solutions were then mixed. The prepared stain was filtered and stored in a clean, dry glass stopper bottle. The heat-fixed smear of individual isolates of lab cultures on a glass slide was stained with crystal violet for one minute, followed by washing off excess stain with water. Then iodine mordent was added and allowed to react for one min. After washing, Gram-positive cells appear pink in color. The above stock solutions of 10 mL mixed with 90 mL of distilled water and were used as a counter stain for gram staining.

Catalase reaction

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen that causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme. $2H_2O_2 \rightarrow 2\ H_2O + O_2$

A loopful of 24 h old broth culture was taken on a clean glass slide and a drop of 3% hydrogen peroxide was added and allowed to remain for 30 seconds. The production of effervescence in the form of gas bubbles indicates positive catalase production.

Growth at different temperatures

Three sets of tubes each having sterile MRS broth in 10 mL amounts were inoculated with a loopful of 20 h old MRS broth culture of individual isolates and incubated at 10, 37 and 45°C. Growth (turbidity) was observed for 37 and 45°C after 24 and 48 h, while at 10°C up to 5°C, there was no growth.

Salt tolerance test

MRS tubes containing 2, 4, 6.5% NaCl were inoculated with a loopful of 20 h old MRS broth culture and incubated at 37°C for 24-48 h. Growth was observed as change in turbidity.

Growth at pH 8.2 and 9.6

To MRS broth tubes adjusted to pH 8.2 and 9.6, respectively, a loopful of 20 h old MRS broth culture was added and incubated at 37°C for 24-48 h. Growth was observed as change in turbidity.

Gelatin hydrolysis

The gelatin agar stabs were inoculated with 20 h old MRS broth cultures, and incubated at 37°C for 24-48 h and observed for liquefaction and failure to solidify at 8°C.

Starch hydrolysis

A loopful of 20 h old MRS broth culture was taken and streaked on to the starch agar plates and incubated at 37°C for 24-48 h after which the utilization of starch was tested by exposing the plates to iodine vapours. Absence of blue color in the surrounding area of the streak indicated the starch hydrolysis, which was taken as positive test.

Carbohydrate fermentation

To a requisite number of test tubes, containing the desired carbohydrate fermentation medium, a loopful each of 20 h old MRS broth culture was inoculated individually and incubated for a period of 5 days at 37°C, the tubes were observed for change in color from purple to yellow, which gave the positive result for the specific carbohydrate in the medium.

Types of fermentation

MRS broth in 5 mL amounts was dispensed in test tubes and Durham's tubes were then inserted into these tubes in inverted position. These were then autoclaved, inoculated with individual cultures of LAB and incubated at 37°C for 48 h. Incubated tubes were observed for gas production in Durham's tubes, which replaced the medium in the tubes. Obligate homo-fermenters will not produce gas, obligate hetero fermenters will produce gas and facultative homo-fermenters may produce gas depending on the type of sugar

available in the growth medium was observed at O.D at 620 nm.

Selection criteria for the determination of probiotic properties of isolates

Acid and bile tolerance test

Resistance to pH 3 is often used *in vitro* assay to determine the possible resistance to stomach pH. Because the foods stay in the stomach for 3 h, this period of time limit was taken into account (14). For this purpose, active culture (grown for 18 h) was used. Cells were harvested by centrifugation for 10 min at 5000 rpm and 4°C. The cell pellets were washed once in phosphate-buffer (PBS at pH 7.2) and were resuspended in PBS (pH 3) and incubated at 37°C. Viable microorganisms were enumerated after the incubation for 0, 1, 2 and 3 h with pour plate technique. Appropriate dilutions were plated and plates were incubated at 37°C under anaerobic conditions for 48 h and the viable colony counts enumerated. Bile salt tolerance is potentially a probiotic property of LAB cultures. In this experiment, MRS broth containing 0.3% of bile salt (taurocholic acid) was inoculated with *Lys. sphaericus* and incubated at 37°C for 24 h. The control comprised of MRS broth without bile salt. Bacterial growth was monitored by measuring optical density at 600 nm after overnight incubation.

Molecular identification

DNA was isolated from actively growing culture of *Lys. sphaericus* and it was evaluated on 1.2% agarose gel as shown in the Fig 1. Fragment of 16S rDNA gene was amplified by PCR using 8F and 1492R (forward and reverse primers) from the isolated DNA. The obtained single discrete PCR amplicon band (1500 bp) was purified and further processed for sequencing. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1189 bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI GenBank Database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP Database and the phylogenetic tree was constructed using MEGA 5.

Determination of antimicrobial activity

The agar-well-diffusion method was used to determine the antimicrobial property of the isolated culture. A 24 h culture of the pathogenic strains were grown individually in Luria Broth (LB) medium and the cell suspension was spread over the surface of Muller- Hilton agar plates using a sterile spreader. The plates were allowed to dry and a sterile well borer of diameter (5 mm) was used to cut uniform wells in the agar. Each well was filled with 100 and 150 µl of isolated 24 h old active culture. After incubation at 30°C for 24 h, the plates were observed for a zone of inhibition (ZOI) around the well. Results were considered positive if the diameter (mm) of the ZOI was greater than 1 mm (Vinderola *et al*., 2000) [15].

3. Results and Discussion

The isolated *Lys. spharecius* was initially characterized and tested for primary probiotic properties. It is rod-shaped, Gram-positive, catalase negative and produced ammonia from arginine hydrolysis, which is the main characteristic feature, as observed with all lactobacilli. The culture could grow at different temperatures 30-37ºC, pH (5.8-6.2) and NaCl concentrations (2-12%) and in the presence of bile salt (Table 1).

In the isolation of probiotic strains from different source like *idly* batter, *dosa* batter, raw milk and also from fresh water fish intestinal tract. Among the sources fish intestinal tract bacteria showed better biomass, cell viability and more turbidity growth. Based on this result the isolated culture from fish intestine was characterized by 16s RNA molecular identification and identified as *Lys. sphaericus*.

Report of microbial identification

The isolated culture was also identified based on the molecular characterization of 16s RNA gene using the specified primers and conditions mentioned in the material and methods section as *Lysinibacillus spharecius* (Accession no KR140152). The fish intestinal (FI) culture was similar to *Lys. sphaericus* strain L2 (Gen Bank Accession Number: KM070813.1) based on nucleotide homology and phylogenetic analysis as in the (Fig 2). The FI sequence was producing 100% homology and significant alignments with taxa *Lys. sphaericus strain* L2 (Fig 3).

The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the boot strap test (1000 replicates) are shown next to the branches (Felsenstein, 1985) [7]. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) [9] and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1189 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011) [14]. Based on the homology and phylogenetic analysis (Fig. 2), it was concluded that the isolated FI culture as *Lys. sphaericus.* The antimicrobial activity of the lysinibacillus sphaericus culture was evaluated against *E. coli* MTCC 40, *B. cereus* MTCC 6840 and *B. cereus* MTCC7190 as compared to the Ampicillin control (Fig. 4) and their inhibition zone sizes are presented in (Table 2). Further it was selected for probiotication of vegetable and fruit juices, because this culture exhibited probiotic properties like antimicrobial activity, resistance to acid and bile tolerance and better growth even at room temperature.

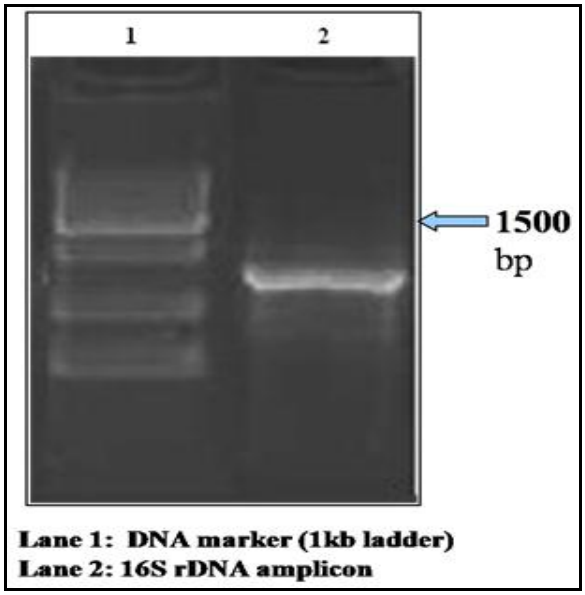

Fig 1: Agarose gel electrophoresis showing single 1.5 kb of 16S rDNA amplicon.

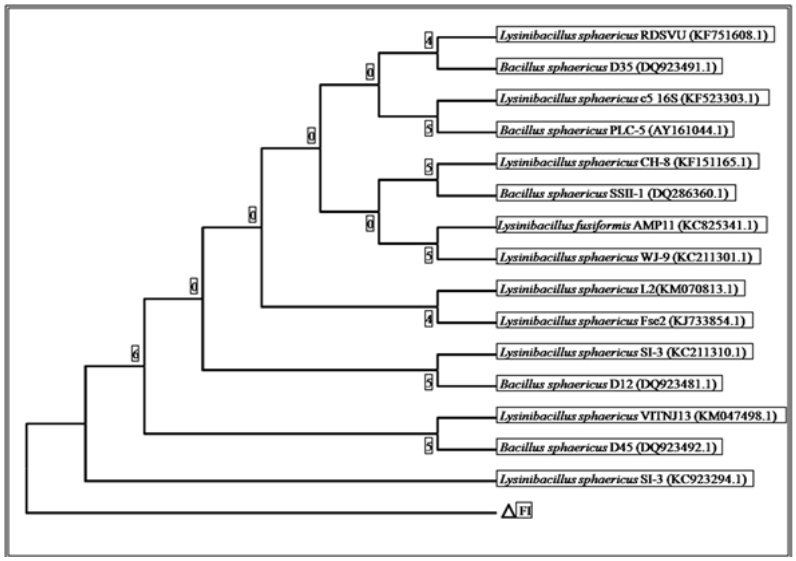

Fig 2: Phylogenetic tree of *Lysinibacillus sphaericus* isolate (FI).

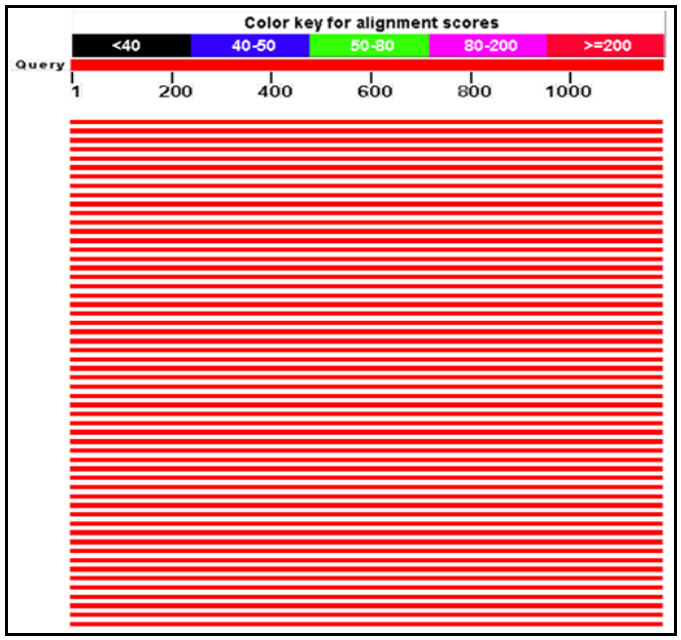

Fig 3: Distribution of 101 Blast hits on the query sequence.

Table 1: Characteristic features of *Lys. sphaericus* isolate (+ indicates = growth)

Parameter	Observation
Grams staining	+
Cell morphology	Rod shaped
Catalase	-
Arginine hydrolysis	+
Growth at temperature 10 - 45ºC	+
Growth at pH range pH 3.0 - 4.0 and pH 5.0 -9.6	+
Growth at NaCl concentration 1-10%	+
Fermentation of xylose, maltose, fructose, Dextrose, sucrose, mannose, inulin, cellobiose.	+
Acid tolerance	+
Acidifying activity	+
Bile salt tolerance	+

Table 2 Antimicrobial activity of *Lys. sphaericus.*

Sample	Zone of inhibition in (mm)		
	***E. coli* MTCC 40**	***B. cereus* MTCC 6840**	***B. cereus* MTCC 7190**
100 µl (A)	8.8±0.1	8.2±0.2	7.2±0.1
150 µl (B)	9.2±0.2	8.9±0.1	8.4±0.3
Ampicillin (C)	17.77+1.74	15.23+0.86	13.73+0.27

*Values are mean of three replicates (± standard deviation). A and B- Indicates concentration of *Lys. sphaericus*, C- indicates Ampicillin only.

4. Conclusions

Among the probiotic cultures isolated and identified from different available sources like cow milk, curd, cheese, *idly* batter, *dosa* batter and fish intestine, it found that, the strain associated with fish intestine was promising microflora, which is new and potentially safe probiotic strain. The isolated bacterium was identified based on the molecular characterization as *Lysinibacillus sphaericus* strain. Based on the results obtained, the bacterium present in fish intestine has found to be a beneficial probiotic strain that could play a main role in human therapies due to its antimicrobial activity against pathogenic organisms.

5. Acknowledgement

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