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Molecular Typing and Probiotic Attributes of a New Strain of *Bacillus coagulans* – Unique IS-2: a Potential Biotherapeutic Agent

M Ratna Sudha, Prashant Chauhan, Kalpana Dixit, Sekhar Babu, Kaiser Jamil*
Centre for Research & Development, Unique Biotech Limited, SP Biotech Park, Phase-II, Plot-2, Shameerpet, Hyderabad- 500078, AP, India

*Correspondence to: Kaiser Jamil, kaiser.jamil@gmail.com
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Abstract

The genus *Bacillus* has been in use in the biotechnology industry for a very long time with a number of new cultures exhibiting a variety of benefits to humans. The present study focuses on phenotypic and genotypic characterization of a probiotic lactic acid bacteria, *Bacillus coagulans* (IS-2 a Unique-Biotech strain) isolated from human feces and contaminated soils. Probiotics are live microbes which when administered in adequate amounts confer a health benefit on the host and are increasingly used in nutraceuticals, functional foods or in microbial interference treatment. In addition to morphological and biochemical characterization, of this isolate, molecular characterization was done. In vitro assays performed to confirm the probiotic attributes of this isolate *B.coagulans*-Unique IS-2. The mole % G+C content of IS-2 strain was found to be 46 indicating the identity of the culture as *Bacillus coagulans*. PCR based amplification of the 16S rDNA, using universal primers, and sequencing done to study the diversity, as it allows identification of prokaryotes as well as the phylogenetic placement of the isolates. 16S rDNA sequence data was used to identify our lab isolates followed by RAPD profile of the new isolate- IS-2 of *B. coagulans.* The phylogenetic tree using Clustal W constructed by UPGMA based on the 16S rRNA gene sequence further confirmed that Unique Biotech strain IS-2 formed a coherent cluster with two other strains of *B. coagulans* namely *B. coagulans* ATCC 7050 and *Bacillus coagulans* NCIM 2030. Evaluation of its probiotic attributes revealed that *Bacillus coagulans* Unique IS-2 had promising Probiotic properties with reference to its survival in gastrointestinal conditions in the *in-vitro* assays and therefore offers excellent opportunities for its successful commercialization as a biotherapeutic agent**.**

Keywords: *Bacillus coagulans* "Unique IS-2"; Molecular Identification; RAPD; Probiotics; Phylogeny.

Introduction

Bacteria belonging to the genus *Bacillus* species are commonly associated with soil, and as such are isolated almost ubiquitously from soil, water, dust, and air [1]. *Bacillus coagulans* is a lactic acid producing spore-forming organism first isolated and characterized by Hammer [2] from evaporated milk. Since then the thermo-stability of *B. coagulans* and its probiotic attributes explored by many researchers to develop a useful and stable probiotic culture for use in a tropical country like India [3]. Currently, there are 77 recognized species of the genus *Bacillus*. This group of bacteria is quite diverse, with a range of G + C content from 44 to 50% [4]. Identification of genus *Bacillus* based on phenotypic traits, has always been difficult, but with the genetic approach, we are able to give clear idea of their identification and characterization. It may be worthwhile to mention that the name "*Lactobacillus sporogenes*" appeared in the scientific literature as early as 1932. However, it was never accepted as a species of *Lactobacillus*, which are non-spore-forming rods. In fact, it was a spore-forming bacterium like a species of *Bacillus*. It is for this precise reason that the species *Lactobacillus sporogenes* does not find any mention either in Bergey's Manual of Systematic Bacteriology (1986) or in the Approved List of Bacterial Names with Standing in Nomenclature. The ATCC catalogue contains one deposited strain named as *L. sporogenes*, but the organism classified by ATCC as "*Bacillus coagulans*" which is the accepted classification. However, for commercial convenience even though the name "*Lactobacillus sporogenes*" has no scientific validity, it continued to be used on the labels of probiotic supplements worldwide. Therefore, there is a need to establish beyond doubt that what is labeled as "*Lactobacillus sporogenes*" is indeed "*Bacillus coagulans*".

The mole % G + C content of DNA is a characteristic feature of all bacteria and strains of the same species have very similar mole % G + C content of DNA. The 16S rRNA genes [5] and DNA – DNA hybridization have also become the standard for the determination of phylogenetic relationships [6]. Now-a-days RAPD (Rapid amplified polymorphic DNA) profile of organism has also become an authorization tag. RAPD is used to characterize and trace the phylogeny of diverse living organisms.

Species such as *Bacillus coagulans* (*Lactobacillus sporogenes*) are used as probiotics and are known to replenish the normal microflora after depletion due to onset of disease or antibiotic therapy. Suvarna and Boby [7] have reported that probiotics are gaining importance because of the innumerable benefits, e.g. treating lactose intolerance, hypercholesterol problems, and managing cardiac problems like atherosclerosis and arteriosclerosis. Previously, we [8] have reviewed the role of probiotic organisms as alternative or complementary therapy in combating large number of gastrointestinal disorders and their ability to enhance immune response that attracts global attention. In addition, their therapeutic use towards cholesterol-lowering activities has further increased their applications as effective probiotics for humans as supplements in milk and yoghurt, since there are no other supplements for hypercholesterolemia, which is the crucial risk factor for cardiovascular diseases. With the current focus on disease prevention and the quest for optimal health at all ages, the probiotics market potential is enormous. Health professionals are in an ideal position to help and guide their clients toward appropriate prophylactic and therapeutic uses of probiotics that deliver the desired beneficial health effects.

With this in view, we tried to identify the isolate of *Lactobacillus sporogenes* using various phenotypic characteristics, and DNA based molecular techniques to establish the taxonomic identity and to determine the phylogenetic position of this *Bacillus* species based on its mole % G + C content of DNA, 16S rRNA gene sequence and DNA-DNA hybridization with closely related strains of *Bacillus*. The present study also tried to determine the probiotic attributes of genetically identified *Bacillus coagulans*-Unique IS-2.

Methods

All the chemicals and culture media used in this study were from Himedia, Difco and Sigma chemicals and were of analytically pure grades.

Isolation and presumptive identification: Isolation of *Bacillus* cultures from various types of samples (green marsh leaves, and human fecal soils) collected from nearby areas of Hyderabad Andhra Pradesh (India) carried out. A total number of 29 tentative isolates picked based on morphological characteristics and checked by microscopy (gram staining and spore staining) tested for motility and for presumptive identification.

Biochemical characterization: Eleven presumptive isolates subjected to biochemical characterization were based on sugar fermentation pattern in basal broth medium as per the standard method. The *Bacillus* isolates were tested using 15 carbohydrate discs (Himedia, Mumbai) for their ability to ferment different sugars. Isolates were also tested for catalase, indole, gelatin hydrolysis and lactic acid production. Among 11 presumptive isolates, only one isolate from human feces was identified as *Bacillus (sporogenes) coagulans* – (IS-2).

Molecular identification and characterization of the new isolate (IS-2)

To identify and characterize our new strain (Unique Biotech IS-2) we procured known standard cultures from ATCC, MTCC , NCIM and IICT:

(i) *Bacillus coagulans* (NCIM 2030)

(ii) *Bacillus coagulans* (MTCC 492 and ATCC 7050)

(iii) *Bacillus subtilis* (I.I.C.T.)

For molecular characterization, the following tests were carried out:

(a) Determination of the mole % G + C content of DNA of the *Bacillus* isolate.

(b) 16S rRNA gene analysis of the *B. coagulans* isolates along with the new isolate.

(c) DNA-DNA hybridization of the Unique Biotech Isolate with the other three strains.

The cultures were maintained on MRS plates at room temperature. MRS broth is an improved medium for *lactobacilli*, it supports good growth and is particularly useful for a number of fastidious strains which grow poorly on other general media. MRS broth was developed by De **M**an, **R**ogosa and **S**harpe (1960) to support luxuriant growth of all *lactobacilli* from oral, fecal, dairy and other sources.

(d) DNA prepared by inoculating 10 ml of an overnight grown culture in MRS into 500 ml of MRS medium and the cultures were kept at 37°C in a water bath shaker for growth.

Isolation of DNA for molecular characterization

The procedure of DNA isolation was essentially according to the method described by Marmur *et al* [9]. 500 ml of a bacterial culture grown to the late log phase harvested at 6000 rpm for 10 min. The cell pellet thus obtained washed with 0.9% NaCl by centrifugation and suspended in 50 ml of STE buffer (0.1 M Tris-HCl, 0.1 M NaCl and 1 mM EDTA pH 8.5). Lysozyme was then added to a concentration of 10 µg/ml and incubated at 37°C for 1 h. Protease (10 µg/ml) was added if the cells did not lyse after lysozyme treatment. Cells lysed with 5 ml of 10% SDS and incubated at 55°C for 10 min. The suspension cooled at room temperature and 5 M NaCl added such that the final concentration was 1 M. Subsequently, 70 ml of $CHCl_3$: iso-amyl alcohol mixture (24:1) added, the flasks gently mixed and the contents centrifuged at 6000 rpm for 15 min. The upper aqueous phase recovered 7.5 ml of 20% sodium acetate and 60 ml of isopropanol added and the DNA was recovered by spooling the DNA fibers on to a glass rod. The DNA was dissolved in 5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8), treated with DNAase free RNase (20 µg/ml) at 37°C for 1 h. Proteinase-K (20 µg/ml) added and the contents incubated for another 1 h. The DNA was then deproteinised thrice with Tris-saturated phenol (Phenol: $CHCl_3$: iso-amyl alcohol, 50:48:2), and then with $CHCl_3$: isoamyl alcohol (24:1). DNA was then precipitated with 2 % sodium acetate and iso-propanol and spooled on to the glass rod. The purified DNA dissolved and used for PCR and for DNA-DNA hybridization studies.

Determination of mole % G+C content of DNA

The purified DNA dissolved in 0.1X SSC was dialysed against 0.1 X SSC overnight and DNA equivalent to 25 µg in 0.1 X SSC was used for the determination of the % G + C content. For this purpose 25 µg of DNA in 3 ml of 0.1X SSC was taken in a 3 ml quartz cuvette and placed in a Hitachi 330 spectrophotometer (Model No. K-12-2055P, Hitachi Ltd, Tokyo, Japan) set at 260 nm and the contents were heated at a rate of 1°C per min using a Hitachi programmer (Model No. SPR-7, Hitachi Ltd, Tokyo, Japan) and the spectrum was recorded. The initial temperature was 25°C and the heating continued until the DNA melted as evidenced by the hyperchromic effect that plateaued after some time. The melting point (T_m) of DNA obtained by plotting the OD at 280 nm versus temperature and is equivalent to the midpoint of the transition. The T_m value used to calculate the G+C content (mole %) according to the equation of Schildkraut and Leifson [10].

The equation is as follows:

Mole % G+C = [Tm - 81.5 - 16.6 log (M)] 2.44

where, M = sodium ion concentration in 0.1 X SSC

For 0.1 X SSC mole % G + C = (T_m – 81.5 + 28.3854) 2.44

PCR amplification of the 16S rRNA gene

The small subunit rRNA gene amplified using the two primers namely:

16S1 (5'-GAG TTT GAT CCT GGC TCA-3') and

16S2 (5'-ACG GCT ACC TTG TTA CGA CTT-3')

complementary to the conserved regions at the 5' and 3' ends of the 16S rRNA gene of *E. coli* corresponding to positions 9 to 27 and 1498 to 1477 respectively [11] . The PCR amplification reaction mix of 50 μl contained bacterial DNA (≈200 ng), 1 μl (3 units) Taq-DNA polymerase, 5 μl of Taq buffer (10 mM TAPS, pH 8.8, 3 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin), 5 μl of 2 mM dNTP mix and 5 μl of each primer (10 pM/μl). Amplification carried out in a Peltier Thermocycler (Model No. PTC-200, MJ research, USA) programmed for 30 cycles. In each cycle denaturation was at 94°C for 20 sec, annealing was at 48°C for 20 sec and extension was at 72°C for 40 sec. A final extension of 5 min carried out at 72°C at the end of 30 cycles. In all the reactions, sterile water was used in place of DNA as a negative control. The amplified DNA fragment of approximately 1.5 kb separated on a 1% agarose gel and purified by using Qiagen spin columns. The purified fragment used directly for DNA sequencing.

Procedure for the use of Qiagen spin columns

The desired DNA band from agarose gel excised, weighed and transferred to a sterile microfuge tube. The gel piece dissolved in thrice the volume of Buffer QE (300 μl / 100 mg) in a water bath maintained at 65°C for 10 min. The content passed through Qiagen column spun at 8000 rpm for 2 min (Catalogue No. 28704, Qiagen Inc. USA). Then the column washed twice with 750 μl of Buffer PE and eluted with 10 μl of sterile water.

16S rRNA gene sequencing

The purified 1.5 kb DNA product was sequenced using primers namely 16S1 and 16S2 and, in addition, a set of five forward primers pB (TAA CAC ATG CAA GTC GAA CG-3'), [50-70]; pC (CTA CGG GAG GCA GCA GTG GG), [341-361]; pD (CAG CAG CCG CGG TAA TAC), [518-536]; pE (AAA CTC AAA GGA ATT GAC GG), [908-928]; pF (CAT GGC TGT CGT CAG CTC GT), [1053-1073] and one reverse primer pC* (CCC ACT GCT GCC TCC CGT AG), [341-301]. The nucleotide positions of the synthetic oligomers (as indicated in parentheses) are related to the 16S rDNA of *E. coli* [12] .

Sequencing of the purified PCR product (~ 200 ng/reaction) was carried out using 5 pmoles of a given sequencing primer and 3 μl of ready reaction mix from the Big Dye Terminator sequencing kit (Perkin Elmer) in a total volume of 5μl. Cycle sequencing carried out in a Gene Amp PCR machine (Perkin Elmer, 9600) for 30 cycles. Each cycles consisted of a denaturation step at 96°C for 10 sec, an annealing step at 50°C for 10 sec and an extension step at 60°C for 4 min. After the PCR, the products were precipitated using 1 μl of sodium acetate (3M, pH 4.6) and 50 μl of ethanol and incubated on ice for 15 min. The pellet was recovered by centrifugation at 15000 rpm for 20 min at 4°C, washed with 70% ethanol, dried under vacuum and dissolved in 10 μl of loading buffer [formamide:25 mM EDTA (4:1)]. About 2 μl of the sample

was used to analyze the sequence on an automatic DNA Sequencer (ABI Prism Model 3700, Applied Biosystems, California, USA).

DNA-DNA hybridization

DNA-DNA hybridization was performed by the membrane filter method of Tourova and Antonov [13] and essentially involves three steps:

i. Radioactive labeling of DNA to be used as a probe.

ii. Immobilization of the DNA.

iii. Hybridization between the immobilized DNA and the radioactive DNA.

a. Immobilization of DNA

About 10 µg of DNA taken in an eppendorf tube to which 20X SSC were added to a final concentration of 6X and the contents were boiled for 10 min. The tube chilled immediately on ice and the DNA immobilized on to a Hybond N^{++} membrane using a dot blot apparatus. The wells washed with 100 µl of 0.5N NaOH after which the filter removed, dried and baked in a vacuum oven at 80°C for 2 h.

b. Radioactive labeling of the DNA used as a probe

Nick translation is a procedure by which pre-existing nucleotides in a DNA molecule replaced by radioactive nucleotides thus generating ^{32}P-labelled DNA with a high specific activity. The procedure takes advantage of the fact that *E.coli* DNA polymerase I is capable of adding nucleotide residues to the 3' hydroxyl terminus to one strand, of a double stranded DNA molecule, which is nicked. Further, the same enzyme by its 5' to 3' exonucleolytic activity can remove nucleotides from the 5' side of the nick. It eliminates nucleotides from the 5' end and adds nucleotides at the 3' end thus resulting in movement of the nick.

Labeling of the DNA was carried out in an eppendorf tube in a 100 µl reaction mix consisting of the following:

DNA	5 µl (200 – 300 ng)
10 X Nick translation buffer	5 µl (Supplied by BRIT, India)
DTTP	7 µl
DCTP	7 µl
DGTP	7 µl
α-^{32}P-dATP	5 µl (50 µCi)
H_2O	64 µl

After 2 h of incubation at 15°C the reaction stopped with 8µl of 0.25 M EDTA and 56 µl of 5M ammonium acetate, 50 µl of carrier DNA and 500µl cold alcohol were added. The contents incubated at –70°C for 1 h or over night at –20°C and spun at 15000 rpm for 20 min at 4°C. The pellet washed with 70% alcohol, briefly dried under vacuum, dissolved in 6X SSC, boiled for 10 min and immediately chilled on ice and used for hybridization.

c. Hybridization

The baked filter was soaked in the prehybridization buffer (0.5 M Phosphate buffer pH 7.2 and 7% SDS) for 1 h at a temperature corresponding to 20°C less than the T_m of the DNA being used for hybridization. The prehybridization buffer discarded and the probe dissolved in fresh buffer added and hybridization done for 16 h at the same temperature as above. The filter washed with 0.5X SSC containing 0.1% SDS for 10 min at room temperature and with 0.1X SSC containing 0.5% SDS for 20 min at 50°C. Subsequently the filter was dried, exposed to an X-ray film for 24 h and developed using a Kodak X-ray film. The autoradiogram scanned and quantified using a phosphoimager. Simultaneously, the filters processed for determination of radioactive counts in the ^{14}C channel of the Tri-carb liquid scintillation counter (Model No. B1500, Zurich, Switzerland).

The percent hybridization calculated as follows:

$$\% \text{ Hybridization} = \frac{\text{Counts obtained from heterologous hybridization}}{\text{Counts obtained from homologous hybridization}} \times 100$$

Phylogenetic analysis

The reference sequences required for comparison were downloaded from the EMBL database using the site http://www.ncbi.nlm.nih.gov/Genebank. All the sequences aligned using the multiple sequence alignment program CLUSTAL W developed by Higgins et al [14]. The aligned sequences checked for gaps manually, arranged in a block of 250 bp in each row, and saved as an in-file in text format in Phylip version 3.5. The pairwise evolutionary distances computed using the DNADIST program with the Kimura 2-parameter model as developed by Kimura [15]. To obtain the confidence values the original data set re-sampled 1000 times using SEQBOOT program of phylip and subjected to bootstrap analysis. The bootstrapped data set was either used directly for constructing the phylogenetic tree using the DNAPARS program or used for calculating the multiple distance matrix. The multiple distance matrix obtained was used to construct phylogenetic trees using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). In this method, the input order of species added to the topology being constructed, were randomized via the jumble option with random seed of 7 to 10 replications. Majority rule (50%) consensus trees constructed for the topologies found by each method by using CONSENSE. All these analyses performed using the PHYLIP package, Version 3.5 of Felsenstein [16].

RAPD analysis using random primers

For RAPD analysis, genomic DNA was amplified using a set of 18 primers (Table 2). PCR reactions performed in a total volume of 10 µl containing 100 µM each of dATP, dCTP, dGTP, and dTTP, 2 picomoles (1 µl) of a single 10-base primer, 25 ng of genomic DNA, and 0.2 units of Taq DNA polymerase. Amplification was performed in a thermal cycler (MJ research., Inc., USA.) programmed for 35 cycles of 1 minute at 94°C, 1 minute at 36°C , and 2 minutes at 72°C. Amplified products analyzed by electrophoresis on 1.5% agarose gels and detected by staining with ethidium bromide.

***In vitro probiotic attributes of Bacillus coagulans* "Unique *IS-2*"**

In vitro tests were done to check the probiotic attributes of *B. coagulans* IS-2 (a Unique Biotech strain). The probiotic strains tested for their survival by simulated acid and bile tolerance tests that quantitatively reflect their level of tolerance in stomach and intestinal region. Probiotic attributes included a battery of tests like the tolerance of *B. coagulans* IS-2 strain in highly acidic and bile concentration, adherence tests by cell surface hydrophobicity, antibiotic sensitivity profiles and antibacterial activity tests.

Acid and bile tolerance test: The survival of *B. coagulans* IS-2 strain at high acidic environment carried out by the method described by Clark *et al* [17]. Low pH solutions were prepared in sterile sodium chloride solution (0.5%) adjusting the pH to 1.5, 2.0 and 3.0. The tolerance capacity of *B. coagulans* IS2 strain for high bile concentration checked as suggested by H. S. Chung et al [18]. High bile percentage solution was prepared by suspending bile salts (1%, 2% and 3%) in a sterile sodium chloride solution (0.5%). To check the tolerance level of *B. coagulans* (IS-2) strain, pour plating done at 0, 1, 2 and 3 h interval from both acid and bile solutions.

The adherence capability: Any probiotic microorganism could only be effective in the host body if it possesses good adherence ability with the inner lining of the gastro intestinal tract especially large and small intestine. The method adopted by Wiencek et al [19] and Doyle and Rosenberg [20] determined the adherence capacity of B. coagulans (IS-2) with n-hexadecane. The adherence ability of the strains was evaluated based on cell surface hydrophobicity tests with different hydrocarbons.

The antibiotic susceptibility: the disk diffusion method of Charteris et al [21] was used for determining the antibiotic sensitivity of the strains/isolates. The isolates assayed against twenty-one antibiotics and the zone of inhibition around antibiotic discs measured and sensitivity level was determined using high performance standards for antimicrobial disc susceptibility of clinical and laboratory standard institute (CLSI, 2005) [22].

Antimicrobial activity tests

The selected isolates screened for their antimicrobial activity against three pathogens namely Escherichia *coli* NCDC 135, *Shigella flexneri* MTCC 1457 and *B. cereus* NCDC 240. Using spot on lawn assay [23] the antibacterial activity was determined. The qualitative analysis of antibiotic sensitivity done with around 25 different broad spectrums and/or commonly used antibiotics for profiling the resistance behavior of the test strains towards different antibiotic agents.

Statistical analysis

Results of acid and bile tests statistically analyzed using Randomized Block Design and processed using Duncan Multiple Range Test (DMRT). Significant difference among treatment means compared by Fisher's least significant difference at 5% and 1% level of significance. Where ever required statistical analysis carried out.

Results and Discussion

Probiotic bacteria are recognized as potential therapeutic agents for over a century but only recently has medical science attempted to use probiotics for the dietary management of serious GI disorders. Among the various known probiotic organisms, two main genera of Gram-positive bacteria, *Lactobacillus* and *Bifidobacterium*, are extensively used as probiotics.

In this investigation –a search for new probiotics was attempted using a total number of 29 isolates of *Bacillus spp* from the local region selected based on colony morphology. Among the preliminary isolates, 11 isolates tentatively identified as *Bacillus* based on their microscopic observation and motility testing. All the twenty-nine isolates were biochemically tested for their identification. However, we could shortlist only one isolates based on biochemical parameters. This was further identified as *Bacillus coagulans*-IS2 (Table 1 and 2).

The biochemically identified *B. coagulans* IS2 strain was further characterized genetically (i) by mole G+C content of DNA, (ii) 16S rRNA gene sequence and Phylogenetic analysis, (iii) and DNA-DNA hybridization. The results clearly

Table 1: Sugar fermentation test for the identification of finally selected *Bacillus coagulans* (IS2) isolate.

Carbohydrates	**Acid**	**Gas**
Glucose	+	-
Lactose	+	-
Inulin	-	-
Maltose	+	-
Mannitol	+	-
Sorbital	-	-
Sucrose	+	-
Xylose	-	+
Arabinose	+	-
Rhamnose	-	-
Maltose	+	_
Dextrose	+	_
Raffinose	-	+
Fructose	+	_
Galactose	+	_
Carbohydrates	**Acid**	**Gas**
Glucose	+	-
Lactose	+	-
Inulin	-	-
Maltose	+	-
Mannitol	+	-
Sorbital	-	-
Sucrose	+	-
Xylose	-	+
Arabinose	+	-
Rhamnose	-	-
Maltose	+	_
Dextrose	+	_
Raffinose	-	+
Fructose	+	_
Galactose	+	_

Table 2: Biochemical tests for the identification of finally selected *Bacillus coagulans* (IS-2) isolate.

Catalase	+
Indole test	-
Gelatin Hydrolysis	-
Gram reaction	+
Lactic acid production	+

Table 3: The mole % G+C content of DNA of *Bacillus* strains**.**

S. No.	Bacillus Strains	Mole % G + C of DNA
1	*B. coagulans* (Unique Biotech Isolate, IS2)	46.08
2	*B. coagulans* (ATCC 7050, MTCC 492)	45.50
3	*B. coagulans* (NCIM 2030)	35.10
4	*B. subtilis* (IICT)	42.90

Table 4: Dissimilarity matrix showing the distance between *B. coagulans* (Unique Biotech Isolate, IS-2) and other related species of the genus *Bacillus* at the 16S rRNA gene level.

S. No.	Isolate	1	2	3	4	5	6	7	8	9	10	11	12
1	*B. subtilis* NCDO 1769^T												
2	*B. licheniformis* DSM 13^T	1.74											
3	***B. coagulans* (Unique Biotech Isolate, IS2**)	7.57	7.19										
4	***B. coagulans* (ATCC 7050, MTCC 492)**	7.33	7.12	0.62									
5	*B. badius* ATCC 14574^T	7.68	7.46	2.10	1.44								
6	*B. coagulans* JCM 2257^T	7.50	7.52	1.16	0.43	1.31							
7	*B. smithii* DSM 4216^T	7.25	6.82	6.15	6.08	6.71	6.60						
8	*B. lentus* IAM 12466^T	6.47	6.75	6.86	6.78	6.48	6.61	5.89					
9	*B. firmus* IAM 12464^T	5.30	5.03	6.81	6.73	6.81.	6.79	6.19	3.75				
10	***B.coagulans* (NCIM 2030)**	6.36	6.71	7.84	7.54	8.22	7.81	8.07	6.34	6.12			
11	*B. circulans* IAM 12462^T	6.36	6.0	6.96	6.89	7.0	6.97	5.66	3.66	3.90	6.21		
12	*B. azotoformans* DSM 1046^T	7.79	7.74	10.01	10.05	10.88	10.18	9.72	7.74	7.02	8.45	6.73	
13	*P.citreus* FO-074a^T	8.27	8.28	9.10	9.43	9.67	9.45	8.07	7.58	6.82	7.84	6.62	10.28

Table 5: Homology (%) of *B. coagulans* (Unique Biotech Isolate, IS-2) with other strains of *B. coagulans* and *B. subtilis* by DNA-DNA hybridization.

S. No.	Strain	Hybridization (%)
1	*B. coagulans* (Unique Biotech Isolate, IS2)	100
2	*B. coagulans* (ATCC 7050, MTCC 492)	88
3	*B. coagulans* (NCIM 2030)	5.6
4	*B. subtilis* (IICT Isolate)	5.2

indicated that the new strain of *Bacillus coagulans* IS2 had a mole % G + C content of DNA with value of 46 which is in accordance with what has been reported for *Bacillus coagulans* (44 to 50%) [4]. Further, the mole % G+C content of *Bacillus coagulans* ATCC 7050 was found to be 45.5 which further strengthened the identity of *Bacillus coagulans* IS2 strain (Table 3). The indigenous isolate *Bacillus coagulans* IS2 was confirmed at the genus and species level by specific PCR assays as well as by 16S rRNA sequencing and was subjected to a battery of tests as per FAO/WHO guidelines as documented below.

For the identity and phylogenetic position of *B. coagulans* (Unique Biotech Isolate, IS-2), the 1.5 kb 16S rRNA gene was amplified by PCR along with the genes of *B. coagulans* (NCIM 2030) and *B. coagulans* (MTCC 492) and sequenced . The 16S rDNA sequences of 1470 bp compared between the three strains of *B. coagulans* and closely related species of the genus *Bacillus*. The 16S rDNA level *B. coagulans* (Unique Biotech Isolate, IS-2) was closely related to two strains of *B. coagulans* namely *B. coagulans* (MTCC 492) and *B. coagulans* (JCM 2257^T) and the similarity was 99.4 and 98.8% respectively (Table-4). Further, *B. coagulans* IS2 differed from the other species of *Bacillus* by a minimum of 2.1 with *Bacillus badius* to a maximum of 10% with *Bacillus azotoformans* (Table-4). In fact, it appeared more closely related to *Bacillus subtilis*. The phylogenetic tree construction by UPGMA based on the 16S rRNA gene sequence (Fig. 1) using Clustal W further confirms that the Unique Biotech Isolate-IS2 is indeed *Bacillus coagulans* and it forms a coherent cluster with two other strains of *B. coagulans* namely *B. coagulans* ATCC 7050 and *Bacillus coagulans* JCM 2257^T which is a type strain of *Bacillus coagulans*. The greater than 70% bootstrap values between the three strains further confirms that the *Bacillus coagulans* IS2 is phylogenetically very close to the other *B. coagulans* strains implying that it is yet another strain of *Bacillus coagulans.*

The results of DNA-DNA hybridization studies between *Bacillus coagulans* IS-2 and other strains of *Bacillus coagulans* and *Bacillus subtilis* indicate a high level of DNA-DNA homology (88%) between *Bacillus coagulans* (Unique Biotech Isolate, IS-2) and *B. coagulans* ATCC 7050. However, it showed very little homology with *B. coagulans* NCIM 2030 and *Bacillus subtilis* IICT isolate (Table 5 and Fig. 2).

The RAPD profile evaluated with PCR amplification of genomic DNA of *Bacillus coagulans* NCIM2030, *Bacillus coagulans* IS-2, *B. coagulans* ATCC 7050, and *Bacillus subtilis.* On electrophoresis, amplified DNA bands were visible only when OPA-7, OPA-9, OPA-10, OPA-11, OPA-13, OPA-14, OPA-16, OPA-20, OPB-7 and OPB-12 were used as the primers. The number of DNA bands varied between 1 to 6 depending on the primer used and the bacterial strain (Figs. 3A and 3B) are representative RAPD profiles of the Bacillus strains. The primers that did not yield any amplified product were not utilized.

For phylogenetic analysis 75 bands generated by 8 random primers and DNA from *B. coagulans* NCIM 2030, *B. coagulans* ATCC 7050, *B. coagulans* IS-2 and *B. subtilis* were considered (Table 6). The data converted to binary digits that formed the source of data for phylogenetic analysis. The phylogenetic tree based on UPGMA analysis of the RAPD data

Table 6: Scoring of RAPD bands after assigning (+) or (–) sign based on presence or absence of band corresponding to the DNA ladder.

Primers/ Products (kb)	*B. coagulans* (NCIM 2030)	*B . subtilis*	*B. coagulans* (Unique Biotech Isolate IS2)	*B. coagulans* ATCC 7050, MTCC 492
OPA – 7				
>1.5	-	+	-	-
>1.5	-	-	+	+
>1.5	+	-	-	-
1.2	-	+	-	-
1	-	-	+	+
0.7	-	-	+	-
0.6	-	-	-	+
0.35	+	-	-	-
OPA – 9				
>1.5	-	+	-	-
>1.5	+	-	-	-
>1.5	-	+	-	+
>1.5	-	-	+	-
1.3	-	+	+	-
1.2	-	-	-	+
0.9	-	-	+	+
0.6	-	-	+	-
0.4	-	+	-	-
OPA – 10				
>1.5	-	-	+	-
>1.5	-	+	-	-
>1.5	+	+	+	+
1.4	-	-	+	+
1.2	-	-	-	-
0.8	+	-	-	+
OPA – 11				
>1.5	-	-	+	-
>1.5	-	-	-	+
>1.5	+	-	-	-
>1.5	-	+	-	-
1.2	-	-	-	+
0.9	+	+	-	+
0.8	-	+	-	-
0.7	-	-	+	+
0.6	+	+	-	-
0.5	-	+	-	-
OPA – 13				
>1.5	-	-	+	-
>1.5	+	-	-	-
>1.5	-	+	-	-
>1.5	+	-	-	+

Table 7: Antibiotic sensitivity test results for *Bacillus coagulans* (IS-2).

Name of the Antibiotic	Reference
Bacitracin	Resistant
Cefaclor	Sensitive
Cephoxitin	Sensitive
Chloramphenicol	Sensitive
Ciprofloxacin	Sensitive
Clindamycin	Intermediate
Colistin	Resistant
Doxycycline	Intermediate
Erythromycin	Intermediate
Gentamycin	Sensitive
Kanamycin	Sensitive
Methicilin	Resistant
Metronidazole	Resistant
Nalidixic Acid	Sensitive
Penicillin	Intermediate
Polymixin B	Sensitive
Rifampcin	Sensitive
Streptomycin	Resistant
Trimethoprim	Sensitive
Novobiocin	Sensitive
Tetracycline	Intermediate

comprising 75 bands indicated a similarity between all the 4 strains at the 22% level (Fig. 4). Nevertheless, it was interesting to note that *B. coagulans* IS-2 and *B. coagulans* ATCC 7050 had a higher degree of similarity (35%). These results clearly indicate that the *B. coagulans* Unique IS-2 is a distinctly different strain of *B. coagulans*. The results are in accordance with De Clerck et al [24].

The use of *Bacillus coagulans* as probiotic offers great opportunities in the area of probiotics because of their spore forming ability and enhanced survival in harsh and adverse conditions. This renewed interest in the use of probiotics is, in part, due to the recent publication of numerous clinical studies, which provide sufficient and exclusive data regarding the safety and efficacy of the probiotics in different disorders and medical indications. Evaluation of *in-vitro* probiotic test for Bacillus *coagulans* IS2 strain reveals that the strain survived well even at pH 1.5 . Only two log cycle reductions have been found for the strain at pH 1.5, which is thought to be very drastic condition for all microorganisms. Only one log cycle reduction was found at pH 2 and pH 3 (Fig 5). The two log cycle reduction were observed for bile 3% while only one log cycle reduction was observed in bile 1% and 2% (Fig 6). The high degree of survivability of *Bacillus coagulans* under gastrointestinal condition could be attributed to the spore forming ability of the bacteria that prevents the bacteria from harsh environment [25].

In the present study, N-hexadecane was used for evaluating the adherence ability of the *Bacillus coagulans* (IS-2). It showed cell surface hydrophobicity 32%, which conferred the survival and growth of *Bacillus coagulans* (IS-2) in GI tract. Results of the antibiotic sensitivity profile are shown in (Table7). This unique microorganism [8]

Fig. 1: UPGMA phenogram showing the phylogenetic relationship between *B. coagulans* (Unique Biotech Isolate, IS-2) and other related species of *Bacillus* and *P. citreus*.

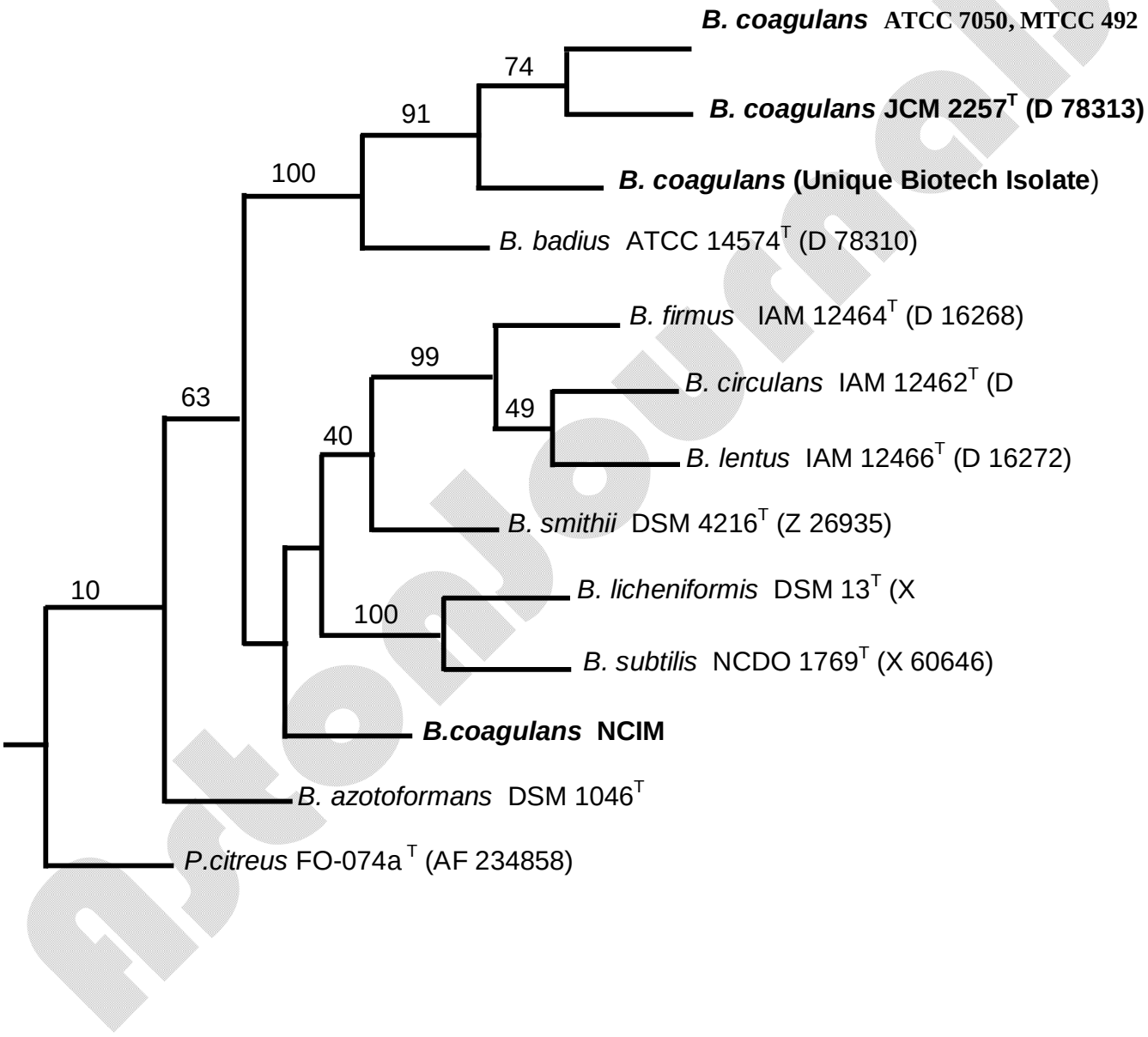
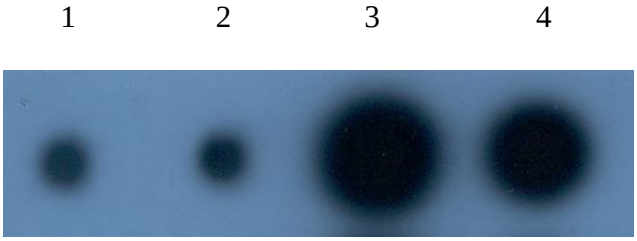


Fig. 2: DNA-DNA Hybridization of *B. coagulans* (Unique Biotech Isolate, IS-2) with (1) *B. coagulans* (NCL), (2) *B. subtilis*, (3) *B. coagulans* (Unique Biotech Isolate, IS-2) and (4) *B. coagulans* (ATCC 7050, MTCC 492).



proved effective in lowering cholesterol by 104 points in a three-month study performed at the G.B. Pant hospital in New Delhi, India. There was a highly significant reduction in the LDL cholesterol levels, and a small but significant increase in HDL cholesterol levels. Use of this microorganism is an attractive alternative to drug therapy since there are no side effects. It also provides an excellent preventative effect against various diseases of the intestine according to one researcher (www.needs.com/insights.probiotics.asp).

Probiotics have the capacity to suppress the pathogens by natural ways to maintain a normal balance in the intestine. The antimicrobial components include different organic acids like lactic acid, acetic acid along with H_2O_2 and bacteriocin. On evaluating the antibacterial activity of the strain, the maximum zone of inhibition was found against *Bacillus cereus* NCDC 240 (14mm) followed by *E. coli* NCDC 135 (12mm). However, the strain was not found to be effective in inhibiting *Shigella flexneri* MTCC 1457. Yilmaz et al [26] found the same type of results in their study.

Conclusion

The interests in probiotics also stems from the growing universal awareness among the consumers regarding the safety aspects related with chemical drugs. The emerging need of safe and natural therapeutics without any adverse effects could be one of the main reasons behind the expanding market of probiotics. Probiotics being endowed with large number of beneficial attributes offer tremendous opportunities for their extensive application in almost all segments including food, pharma and cosmetics. The present study identified a new isolate -*Bacillus coagulans* “Unique IS-2” and its diversity at genetic level from other *Bacillus* species. This strain *Bacillus coagulans* “Unique IS-2” exhibited probiotic attributes and survivability at low pH and high bile concentration, which is similar to human gastrointestinal tract. The genetically identified *Bacillus coagulans*- IS-2 therefore opens a new horizon in the field of biotherapeutic applications. The possibility of using spore formers like *Bacillus coagulans* as probiotics is of great commercial value because of their survivability in acidic, alkaline, heated/ cooked and cold preserved foods and beverages as well. Many other potential applications exist for probiotics, however, large number of controlled and clinical trials and an in-depth research is the need of the hour to further perpetuate the probiotics for a better tomorrow.

Competing Interests

The authors declare that they have no competing interests.

Fig. 3A: RAPD profile of *B. coagulans* (NCIM 2030) (1), *B. subtilis* (2), *B. coagulans* (Unique Biotech Isolate, IS-2) (3) and *B. coagulans* (ATCC 7050, MTCC 492) (4) with the random primers OPA-07, OPA-09, OPA-10 and OPA-11. In lane M, DNA size markers were loaded.

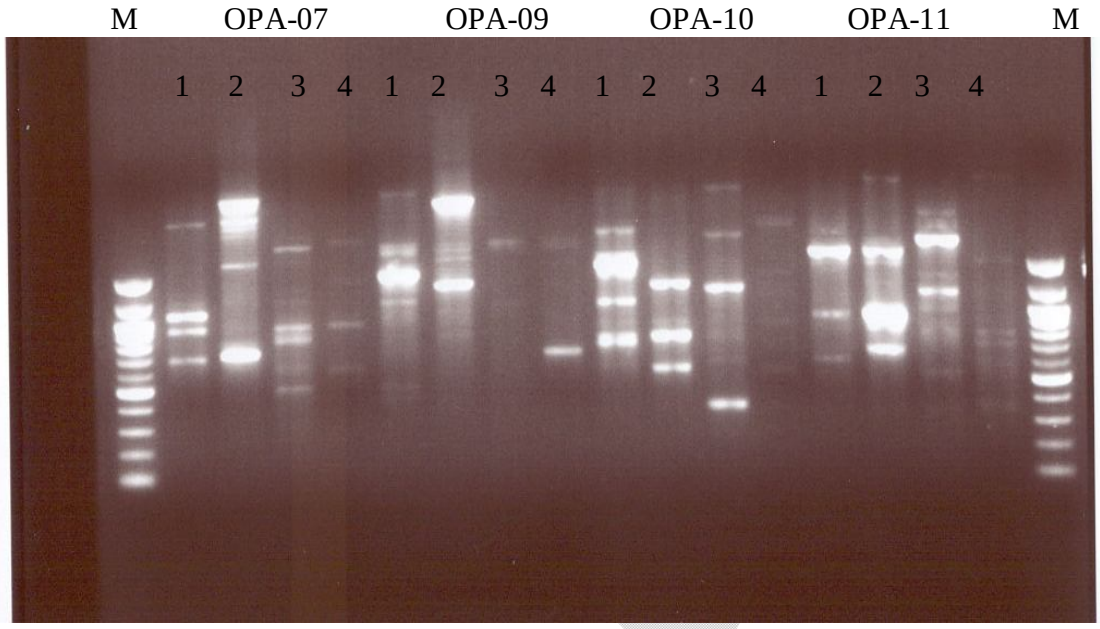


Fig. 3B: RAPD profile of *B. coagulans* (NCIM 2030) (1), *B. subtilis* (2), *B. coagulans* (Unique Biotech Isolate, IS-2) (3) and *B. coagulans* (ATCC 7050, MTCC 492) (4) with random primers OPB-7 and OPB-12. In lane M, DNA size markers were loaded.

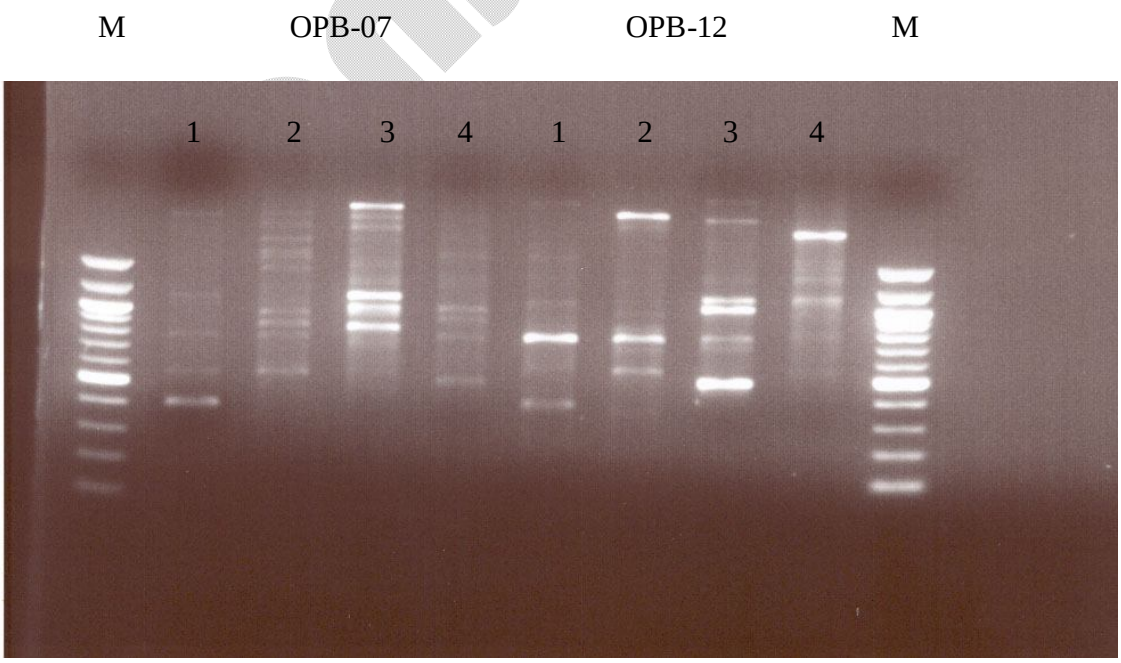


Fig. 4: Phylogenetic relationship of *B. coagulans* (Unique Biotech Isolate, IS-2) with reference to the standard strains based on RAPD analysis.

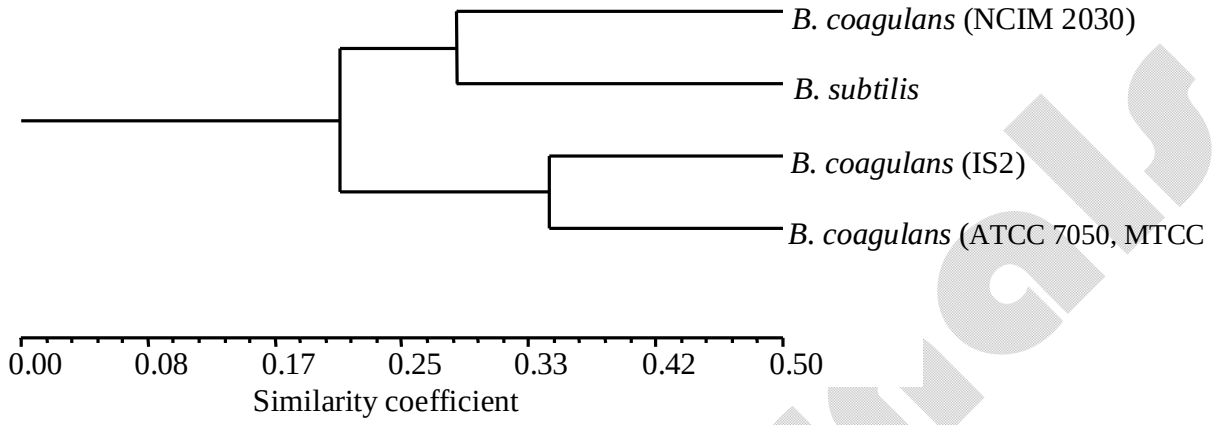


Fig. 5: Acid Tolerance ability of *Bacillus coagulans* (IS-2).

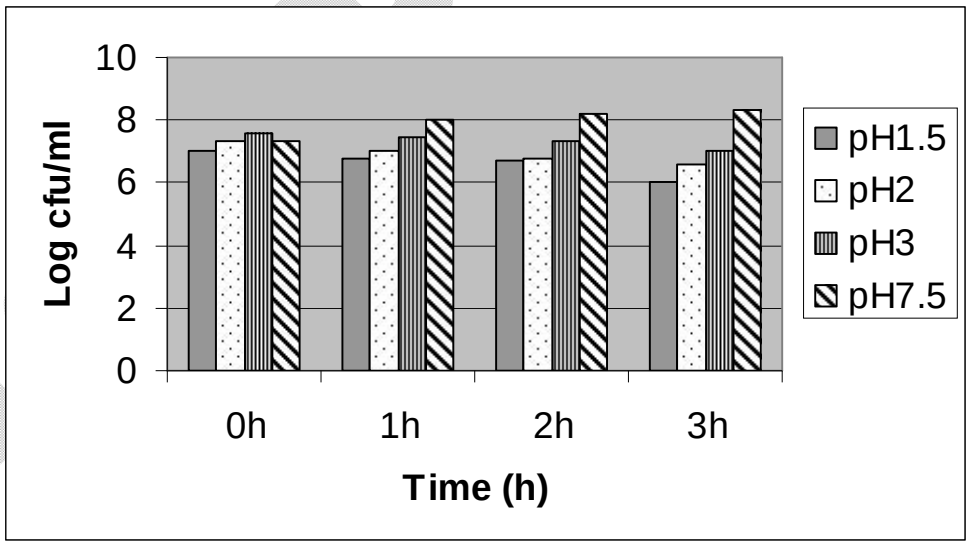
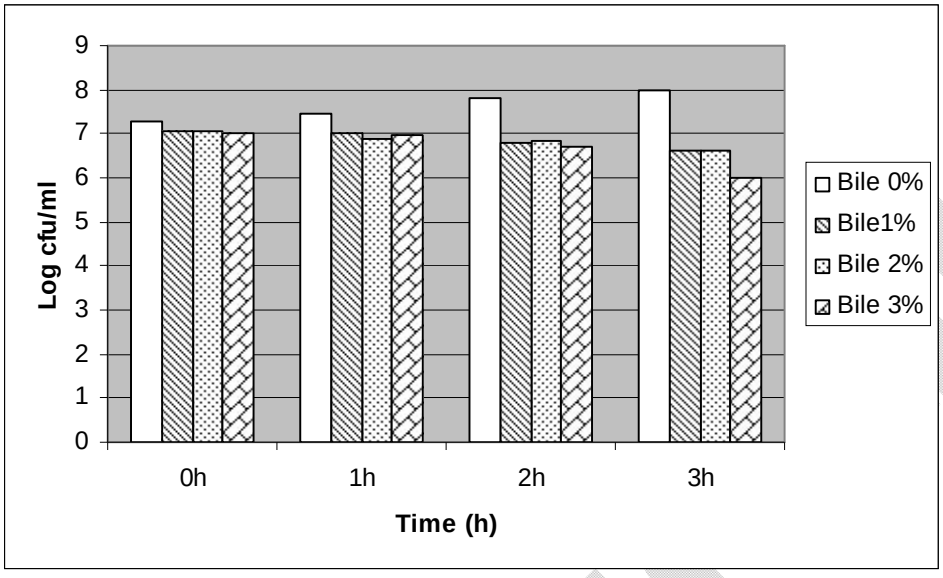


Fig. 6: Bile Tolerance ability of *Bacillus coagulans* (IS-2).



Authors' Contributions

RS and SB developed the project and maintained the cultures; PC and KD evaluated the probiotic attributes of the Unique IS-2 strain. RS and SB carried out the work with the help of CCMB scientists; KJ supervised, analyzed the data and prepared the manuscript.

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