CHAPTER 4

Characterization and Molecular Screening of selected Probiotic isolates

4.1. INTRODUCTION:

Probiotics, prebiotic and synbiotics altogether gave same kind of idea that the healthy foods have the potentiality to multiple in the intestine so that they can perform different health promoting activities in the host on addition of some bacteria along with the indigestible but fermentable carbohydrates. The basic function of these probiotic bacteria is to promote health by utilization of the indigestible carbohydrates known as prebiotics [Gibson and Roberfroid, 1995].

The fact of suppressing and displacing the harmful bacteria by the beneficial bacteria on colonizing the intestinal surface improves the microbial balance and health of the host. Different beneficial organisms including bacteria may show specific abilities of the organism as well as the role of host enzymes within the same genus or even species [Ouwehand *et al.*, 1999]. These microorganisms sometime show habitat preferences for several kinds of existing species [Bernet *et al.*, 1993].

Microbes have always showed close association with humans via colonizing the gastrointestinal tract and other surfaces of the host body. The gastrointestinal tract contains a huge collection of microbial communities [Ducatelle *et al.* 2015] which is referred to as gut microbiota. [He and Shi, 2017]. Several factors such as harsh environment, antibiotic abusing, diet, aging and different pathogenic infection may lead to dysbiosis of the intestinal microbial ecosystem, and lack the immune response regulation for a low grade inflammation [Vieira et al. 2013].

The LAB group of bacteria possesses some definite characteristics such as; they are Gram positive in nature, are catalase negative and are generally no-spore former which have a long life in the history of their ancient as well as popular fermented foods [Claesson et al. 2007]. The basic criteria of a probiotic culture generally includes bile tolerance, gastric fluid tolerance,

production of anti microbial compounds, non- pathogenicity, survivality on the gastrointestinal tract as well as ability of adhering to the mucosa of the intestine etc. [Daliri and Lee, 2015]. Addition to it different taxonomic identification and molecular characterization is essential for the identification of new probiotic strains. The molecular identification may include 16S rRNA sequencing, RAPD, PCR, RFLP, genome sequencing etc. [Mohania et al. 2008].

The anti-oxidative, anti-microbial and immuno-modulative activity of the probiotics plays the key role for considering the probiotics as a health benefiting organism [Tsai et al. 2012]. Reduction of the pathogens in the gut can be controlled by replacing the Gram negative pathogenic bacteria with the probiotics which reduces the intestinal pH and also promotes exclusion by competition [Parvez *et al.* 2006].

Thus, accumulation is the preliminary criteria for effecting functional and health beneficial role of the probiotics [Kaushik *et al.* 2009]. Thus, these bacteria attaining the selective criterion help to obtain the potent probiotic cultures [O'Sullivan and Halami, 2013].

In the present study, the isolated Gram positive bacteria were taken for further phenotypic and genotypic investigation which includes its tolerance of acid, bile and gastric juice. It also includes the examination of the anti-oxidant activity as well as anti-microbial activity of the bacteria. Finally, the DNA of the isolated bacteria were also extracted to perform PCR and genome sequencing to find out the new potent probiotic strain (if atall any is present). Thus, all the bacterial isolates used here in this study can be proposed for a better therapeutic agent in order to show an antagonistic effect to different kinds of gastrointestinal disorders.

4.2. MATERIALS AND METHODS:

4.2.1. FINE CHEMICALS:

The media used in this chapter are nutrient media, MRS media, Arginine Dihydrolase Broth and Carbohydrate Fermentation Media. Other chemical compounds required are carbohydrate (Glucose, Lactose, Maltose, Cellobiose, Trehalose, Xylose and Sorbitol), antibiotic (Streptomycin and Ampicillin), dye used are (Bromocresol purple, Tetramethyl –p-phenylenediamine, Iodine,) Ox-bile, potato starch, Simulated gastric fluid media; o- thalaldehyde and phenol were procured from HiMedia, Mumbai, India.

In molecular biology experiments the chemicals required are; Proteinase K, Isopronanol and ethidium Bromide (EtBr). Ethanol, polymerase chain reaction buffer, $MgCl_2$, dNTPs, lysozyme, 10 Kb DNA ladder, were obtained from Genei Merck, Bengaluru, India.

Taurodeoxycholic acid (TDCA), 2, 2-diphenyl-1 picrylhydrazyl (DPPH), Tris Base, *Taq* DNA polymerase and primers (9F and 1492 R) were obtained from Sigma Pvt. Ltd, Bengaluru, India.

Solvents, salts and other reagents such as Hydrogen peroxide, glycerol, sodium chloride (NaCl), glucose, hydrogen chloride (HCl), urea, sodium dihydrogen phosphate, magnesium sulphate ($MgSO_4$), Disodium hydrogen phosphate, xylene, hexadecane, potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), pepsin, calcium chloride ($CaCl_2$), cholesterol (water soluble), potassium hydroxide (KOH), hexane, O-phthalaldehyde, acetic acid, sulphuric acid (H_2SO_4), chloroform, EDTA, sodium dodecyl sulphate (SDS), sodium acetate, agarose were obtained from Sisco Research Laboratory (SRL), Mumbai, India.

Software and servers used in this sets of experiment are BLASTn, MEGA –X, NCBI Server, biologicscrop.com tools, Species Finder-2.0 server. The study was designed in triplicate sets where the reports were also expressed as average value ± standard deviation. The values were statistically analyzed using SigmaPlot 10 and Microsoft Excel.

4.2.2. INSTRUMENTATION:

The equipments used for the studies were: Bacteriological incubator (Rivotek 50082001, Chennai), Centrifuge (REMI, Mumbai), Compound Microscope (Olympus CH20i, Greece), Deep freezer (Blue Star, Mumbai), Electronic balance (Shimadzu, Kyoto, Japan), Electrophoresis system for DNA (Merck, Darmstadt, Germany), Gel documentation system (Biorad, CA, USA), Laminar Air flow (Sanguine Bioinstruments, Hyderabad), Scanning electron microscope (Leo-435, Zeiss Ltd, UK), Spectrophotometer (Thermoscientific, MA, USA), Thermal cycler (Biorad, CA, USA), Waterbath (Thermoscientific, MA, USA).

4.2.3. BACTERIAL CULTURE USED:

In such experiments, human pathogens such as, *Vibrio cholerae* 0139 (MTCC No. 3906), *Pseudomonas aeruginosa* (MTCC No. 11688) was purchased from IMTECH, Chandigarh. Other than these two bacteria, laboratory control organisms are used such as; Bacillus sp.

4.2.4. PHENOTYPIC SCREENING OF THE ISOLATES (PB1 – PB10):

Phenotype is a Greek word which means, pheno – ‘showing’ and type – ‘type’. It covers the morphological characteristics, physicochemical types, its developmental process, its biochemical metabolic properties and its behavioral changes.

4.2.4.1. MORPHOLOGICAL DETAILING UNDER SEM:

The fixation of the bacterial samples was performed by the presence of 3% gluteraldehyde (in 0.1M & pH 7.2 phosphate buffer) for atleast an hour. After fixation, the cells were washed thrice with buffer and are further treated with 1% osmium tetroxide for 1 hour in the same prior buffer. Following to this process dehydration was performed in (30, 40, 50, 60, 70, 80, 90, and 100) % of ethanol and was spinned up and down five minutes each. The cells were then filtered through 0.2µ polycarbonate filter and then were placed inside a vacuum pump which was used to prepare the critical point of the samples for drying. The dry specimen was then placed into the SEM stub coated with palladium/ gold [Kimoto *et. al.*, 1999].

4.2.4.2. BIOCHEMICAL CHARACTERIZATION:

4.2.4.2.1. CATALASE TEST:

Hydrogen peroxide is a toxic byproduct of different metabolic processes that occurs in the hosts. The function of the enzyme catalase is to convert hydrogen peroxide into oxygen and water. Thereby, the oxidative damage was restricted by ROS. The experiment was performed using agar plate technique. For that isolated colonies were picked and placed onto sterile nutrient agar medium and kept for incubation. After incubation, the overnight grown colonies were treated with three drops of 3% hydrogen peroxide solution and kept for 3mins. On close observation the evolution of bubbles is evident. And this production of bubbles indicates the presence of the catalase enzyme which was recorded as positive result.

4.2.4.2.2. OXIDASE TEST:

For examining the presence of terminal enzyme cytochrome oxidase this test was performed. Isolates were streaked on MRS agar plates and one drop of reagent (tetramethyl-p-phenylenediamine dihydrochloride) was put on each culture plates. Aerobically, it acts as an artificial substrate that can donate electrons and thereby become oxidized which develops a deep purple colored compound. Plates were thus observed for 20-30 seconds after addition of the reagent. Appearance of purple color will indicate positive result.

4.2.4.2.3. STARCH HYDROLYSIS TEST:

The polysaccharide, starch is comprised of two main components – amylopectin and amylose which is too large to pass through the bacterial cell membrane. In amylose and amylopectin both the α-D-glucose molecules are bonded by 1,4-α-glycosidic linkages. Therefore, it should be first be broken into smaller fragments such as; glucose molecules and maltose. These disaccharides and monosaccharides then have the ability to enter into the the bacterial cell via a semi-permeable membrane.

Thus, this ability of the starch to breakdown the polysaccharides act as the main characteristics to evaluate the presence of amylase in the selected bacterial isolates. This test is used as an identification process to investigate the utilization property of the starch taking iodine as an indicator. Starch produces a dark blue coloration in the presence of iodine. The helical structure of starch traps iodine in it producing a clear zone indicating amylolytic activity. The diameter of the clear zone indicates the starch hydrolyzing activity of the strains.

4.2.4.2.4. ARGININE DIHYDROLASE TEST:

This experiment is performed to identify if the bacterial isolates can utilize arginine which is initiated by arginine dihydrolase enzyme. Arginine is initially converted to citrulline and then further converted to ornithine which finally follows decarboxylation to form putrescine. The pH get elevated due to the production of amine where the indicator used is bromo-cresol purple. The test shows negative result for yellow color formation or no color change. And if the medium turns purple, then the organism are designated as decarboxylase-positive.

4.2.4.2.5. CARBOHYDRATE FERMENTATION TEST:

All total 7 carbohydrates *viz.* glucose, lactose, maltose, cellobiose, trehalose, galactose and sorbitol were used to test the carbohydrate fermentation profile of selected isolates. The composition of the fermentation broth is (1 gm of Trypticase + 0.5 gm of NaCl + 0.0189 mg phenol red) dissolved in sterile water and volume makeup upto 100mL. To this fermentation broth 0.5 % of each mentioned carbohydrates were added in all tubes and was labeled respectively. The mixture was sterilized and was incubated with overnight culture of each isolates at 37^{O}C. After incubation, fermentation was confirmed by occurrence of color change to yellow which indicates the formation of acid.

4.2.4.3. ANTI-OXIDANT ASSAY BY DPPH METHOD:

The anti-oxidant activity was estimated following the protocol of Son and Lewis, 2002. Overnight culture filtrate of test cultures was obtained by centrifugation at 9000 rpm at 4°C for 10 mins. The supernatant was taken out in a fresh test tube. The culture filtrate (100 µL) and methanol (1.9 mL) was mixed thoroughly with DPPH (2 mL), whereas; DPPH (2 mL) mixed

with two mL of methanol which was considered as control and blank is taken here as methanol which was incubated in dark for 30 mins. at 37°C after thorough mixing. By the help of spectrophotometer the optical density was measured at 517 nm and % of DPPH is calculated as follows:

$$DPPH\ \% = [(A_{control} - A_{test})/ A_{control}] \times 100$$

4.2.4.4. BILE SALT TOLERANCE ASSAY:

This test was examined by Dutoit et al. (1998). Bile tolerance of isolates was evaluated by the overnight fresh growing isolates in 0.3% of bile salt (Ox- bile, Himedia Laboratories, India). The sample were inoculated on MRS medium which containing TDCA (0.5%) and 0.37 g/L $CaCl_2$. The precipitation zones around the colonies or development of white precipitate colonies after 72 hours of incubation indicate bile salt hydrolase activity.

4.2.4.5. ANTAGONISTIC EFFECT OF ISOLATES AGAINST FEW HUMAN PATHOGENS:

Antimicrobial activity of isolated bacteria (PB1-PB10) against two very well known and potent pathogenic microbes was examined by using agar cup diffusion (Ridwan et al., 2008). The principle of this method involves the antagonistic effect of selective isolated microorganism which will inhibit the growth of other pathogenic test microbes by forming clear zone of inhibition. Test microorganisms were *Vibrio cholerae 0139 (MTCC No. 3906), Pseudomonas aeruginosa (MTCC No. 11688)*. The pathogenic test microorganisms (100 µl) were added to semi-solid agar which is mixed thoroughly and solidified asceptically on MRS Agar Medium (Himedia Laboratories, India). Solidified layer of MRS agar in sterile petri-dishes was allowed to solidify. Solidifying the agar, wells of diameter 6 mm are prepared using sterile borer. Crude

culture filtrate of overnight grown cultures of selective isolates (PB1-PB10) was obtained by centrifugation at 9000 rpm at 4°C for 10 mins. 100 µl of all the selected ten isolates were poured in the respective wells on agar media which was pre inoculated with above mentioned pathogenic strains and are incubated for 24 hours at 37ºC. The inhibition zone was measured by its diameter.

4.2.4.6. ANTIBIOTIC SUSCEPTIBILITY TEST:

There are numerous methods of determining the susceptibility test of different antibiotics of isolated bacteria, which was discussed by many research workers [Klare et al., 2005; Zonenschain et al., 2009]. In this test 100 µl of fresh bacterial cultures were spread on selective media and allowed to dry. On the mediun 6 mm of wells were punched and filled up with 100µg/mL of mentioned antibiotics and were kept for incubation for 48 hours at 37°C. Antibiotics used are Ampicillin, Streptomycin, Erythromycin, Tetracycline, Chloramphenicol, Penicillin and Vancomycin of concentration 50µg/mL. Results were expressed as resistant (R) where zone of inhibition is absent and sensitive (S) if the zone of inhibition is present. The diameter of the zone of inhibition was measured and evaluated.

4.2.4.7. SIMULATED GASTRIC FLUID TOLERANCE:

For the test to occur, the formulation of the simulated gastric juice in pH 2.5 contains KCl (7 mM), $NaHCO_3$ (45 mM), NaCl (125 mM) and pepsin (3 g/L) [Zarate *et al.*, 2000]. But, the control gastric juice was fixed to neutral pH i.e., pH7 which was used for a better comparative result of both the composition and the range of pH too. Overnight grown culture of all the ten selected isolates was added (5%) in the simulated gastric fluid and was kept at

incubation for 24 hours at 37°C. The tolerance activity of PB1 to PB10 was estimated by the help of measuring turbidity at 600 nm.

4.2.4.8. CELLULAR AUTO-AGGREGATION CAPACITY OF THE ISOLATES:

The bacterial ability to auto aggregate is to maintain the microbial density in the intestine of the host [Rickard *et al.*, 2003, Nithya and Halami, 2012]. The selected cultures that were grown overnight in selective media (MRS) and were then harvested. PBS is used to was these harvested cells, and O.D. at 600 nm was adjusted to 0.5. Same suspension was then centrifuged again to harvest the samples in equal volume of MRS broth. It was further incubated for 2 to 3 hours at room temperature. After that, one ml of superficial phase was discarded to examine the optical density at λ_{600}.

$$\text{Percent auto aggregation} = \frac{\text{Initial O.D.} - \text{final O.D.}}{\text{Initial O.D.}} \text{ X } 100$$

4.2.4.9. CHOLESTEROL ASSIMILATION PROPERTY OF THE ISOLATES:

Estimation of cholesterol assimilation and reduction of the assimilated cholesterol is the key role of the potential probiotic organisms [Mathara *et al.* 2008]. The MRS broth taken in account was supplemented with 0.3% ox-bile and cholesterol (0.1 g/L) whereas; control contains MRS broth without any further supplementation. The inoculation of all the isolates in the supplemented as well as non-supplemented medium was followed by incubation at 37°C for 24 h. Post incubation, the samples were spun at 10,000 rpm at 4°C for 5min.The suspension was then collected and is used for further assessment of cholesterol reduction. Then, supernatant of

one mL was mixed with three mL of ethanol (95%) and two mL of KOH (50%) to the tubes at waterbath (60°C) for 10 mins and immediately cooled. Then, one mL distilled water was added to 5mL of hexane in each tube and was allowed to stand for ten mins for phase separation. The hexane was then evaporated and four mL of O-phthalaldehyde was added to each tube and incubated at 37°C for 12 mins. Finally, two mL of conc. H_2SO_4 was added to it and re-incubated for 10 min. The absorbance was measured at λ_{550}.

Percentage of cholesterol reduction was calculated by the following method:

$$\% \text{ cholesterol reducing rate} = [(A_0-A)/A_0] \times 100,$$

(where, A_0 is the control and A is test.)

4.2.4.10. DETERMINATION OF OPTIMUM PH AND TEMPERATURE OF ALL THE ISOLATES:

The determination of the optimum growth factors of the selected isolates is the most essential key factor for selecting the potentiality of the probiotics. The method followed to identify the optimum temperature and pH of the isolates [Al-Otaibi *et. al*. 2016]. In this method all the isolates were freshly grown on MRS liquid media and incubated overnight at room temperature. These fresh grown cultures were inoculated on MRS media which is adjusted in different pH such as, 2,3,4,5,6,7,8 by using 1(N) of HCl and 1(N) of NaOH to identify the optimum pH of all the isolates. Besides that, another experimental set-up was designed where the MRS broths adjusted to pH 7 was inoculated with all the 10 selected isolates and are subjected to overnight incubation at 37°C. Post - incubation the absorbance of both the experimental set-up was measured at 560nm where the control was taken as the un-inoculated MRS broth.

4.2.4.11. CELL SURFACE HYDROPHOBICITY BY BACTERIAL ADHESION TO HYDROCARBON (BATH)

BATH was conducted following the protocol following Rosenberg *et al.* 1980. Isolated bacterial cultures grown freshly overnight were taken for centrifugation at 9000 rpm at 4°C. The pellet was taken followed by two successive washings in phosphate urea magnesium buffer components. The initial O.D. was adjusted to 0.7. After that, 3 ml of that cell culture was mixed with 1 ml of xylene post-vortexing and the mixture was kept for 1 hour at 37°C. And thus, the final O.D. was spectrophotometerically measured at 600 nm and percentage of surface hydrophobicity was obtained by using the formula:

$$\% \text{ Surface hydrophobicity} = (A_0 - A_1)/A_0 \times 100$$

(where, A_0 - initial O.D. and A_1 - final O.D.)

4.2.5. GENOTYPIC SCREENING OF THE ISOLATES (PB1 – PB10):

The entire ten probiotic isolates were subjected initially for molecular characterization. The isolated genus of the prior mentioned putative probiotic bacteria was then selected for identification by using 16S rDNA sequencing, followed by GC analysis and finally by the construction of phylogenetic tree.

4.2.5.1. TOTAL GENOMIC DNA ISOLATION OF ALL THE ISOLATES

The total DNA was isolated by phenol: chloroform extraction [Mora et al. 2000]. The overnight grown cultures of the isolates (PB1 to PB10) were centrifuged at 9000 rpm for 10 min at 4°C and the cell pellet was taken which washed twice in TE buffer which contain (15 µl of 20 mg/mL lysozyme in 400 µL of TE buffer). Mixture was incubated at room temperature for 1 hour in a

water bath. After that, the lysate was treated with 15 µl of 10% SDS, which was mixed gently to obtain clear solution. Proteinase-K (15 µl) was added to it and was incubated for 30 mins at 55°C. Post-incubation, 250 µl of tris-saturated phenol was added to the lysate and spun at 10, 000 rpm for 15 min at 4°C. Post centrifugation, the upper aqueous layer was separated in a new tube and with equal volume (approximately, 400 µL) of chloroform was added. This tube was then re-centrifuged for 15 min at 10,000 rpm at 4°C where the top aqueous layer was evaporated and transferred into a new test tube. The clear supernatant thus obtained was precipitated with 15 µL of 3M sodium acetate and to it. Again, 400 µL of cold 100% ethanol was mixed to it was further incubated at -20°C for 24 hours and thereby precipitate the DNA. The DNA pellet was centrifuged with 70% ethanol for washing. The final pellet obtained was dried at normal condition and dissolved in TE buffer (50 µL).

4.2.5.2. PCR ASSAY OF THE PURIFIED DNA OF ALL THE ISOLATES:

Genotypic identification and amplification the 16s rDNA of selected probiotics were evaluated by gene amplification using universal primer for prokaryotes namely 9F and 1492R. The design of the primer used in the PCR was as follows; 9F – "GAGTTTGATCITIGCTCAG" and 1492R – "TACGGYTACCTTGTTACGACTT".

The reaction mixture used for the PCR amplification (approximately 25 µL) contains 10x PCR buffer (2.5 µL), 1 mM dNTP mix (5 µL), 25 mM $MgCl_2$ (2.5 µL), forward and reverse primer (0.5 µL), 50 ng conc. of DNA (5 µL) and *Taq* DNA polymerase (1.5 U). The PCR was run at adjusted temperature in a thermal cycler. After that, the finally the extension occurred at 72˚C. The PCR product after amplification was re-confirmed by agarose gel electrophoresis and obtained bands were excised and sent for sequencing.

4.2.5.3. AGAROSE GEL ELECTROPHORESIS OF THE PCR PRODUCTS

Agarose gel electrophoresis method was used to examine the purity of the amplified PCR product [Sambrook and Russell, 2001]. 0.8% agarose gel mixed with 1X TAE buffer (pH 8) containing 2 µL of ethidium bromide were used for separation. The agarose was mixed thoroughly by heating, and then cooled to 50°C, casted in a gel boat affixed with a comb and allowed to set. A 5µL of PCR product was prepared by mixing with loading dye (3 µL) which was loaded into the wells in the gel. The samples were electrophoresed at 80 V until and unless the samples reach the end. Finally, the gel was stained with EtBr and was visualized in an ultraviolet trans-illuminator.

4.2.5.4. SEQUENCE ANALYSIS AND ACCESSION NUMBERS:

The visualised gel band that was obtained prior to this experiment was excised and sent for sequencing at KPC Medical College, Kolkata, India. The obtained sequences were then analyzed by their chromatograms (Chromas Lite version 2.01). The homology was from that wasdeduced using the BLASTn algorithm search in NCBI server [Altschul et al. 1997]. The sequences were also further processed and deposited at the GenBank database of NCBI to obtain the accession numbers.

4.2.5.5. GC CONTENT ANALYSIS

GC content of the isolates is usually calculated in percentage and also called G+C ratio. And it is calculated in the form of "(G + C)/ (A + T + G + C) X 100%". Now the values have been integrated into Codon Optimization Software, which shows protein expression at species level. The study includes a special online software named as, BiologiscCrop.com.

4.2.5.6. PHYLOGENETIC ANALYSIS.

Using the 8F and 1492R primers the purified PCR product was analyzed to obtain amplified sequence to carry out BLAST at NCBI. And first 14 sequences were selected based on the maximum identity score to construct the phylogenetic tree using MEGA-4.

4.3. RESULT AND DISCUSSION:

LAB has already been proved as the most prevalent bacterial flora in different milk and milk products. In number of fermented foods their presence were recognized. They impart key flavor, improved texture and preservative qualities during fermentation process [Rina *et al.* 2009]. Probiotics imparts several health benefits which includes stably maintaining of the gut and epithelial barrier function; induce relief of gastrointestinal abnormalities such as; diarrhea, IBD, IBS and lactose intolerance etc. [Parvez et al. 2006; Nagpal et al. 2012]. So, there is always a search for a new species and strains of probiotics which are commonly considered as a potential probiotics [Batdorj et al. 2007].

4.3.1 MORPHOLOGICAL DETAILING UNDER SEM:

Surface morphology of the selected isolates was studied in detail through SEM (Scanning Electron Microscopy) and presented in **FIGURE 4.1**, where PB1, PB6 and PB7 were rods in shape, whereas, the rest of the isolates are coccus in shape. Thus, all these isolates may be considered as a putative probiotic.

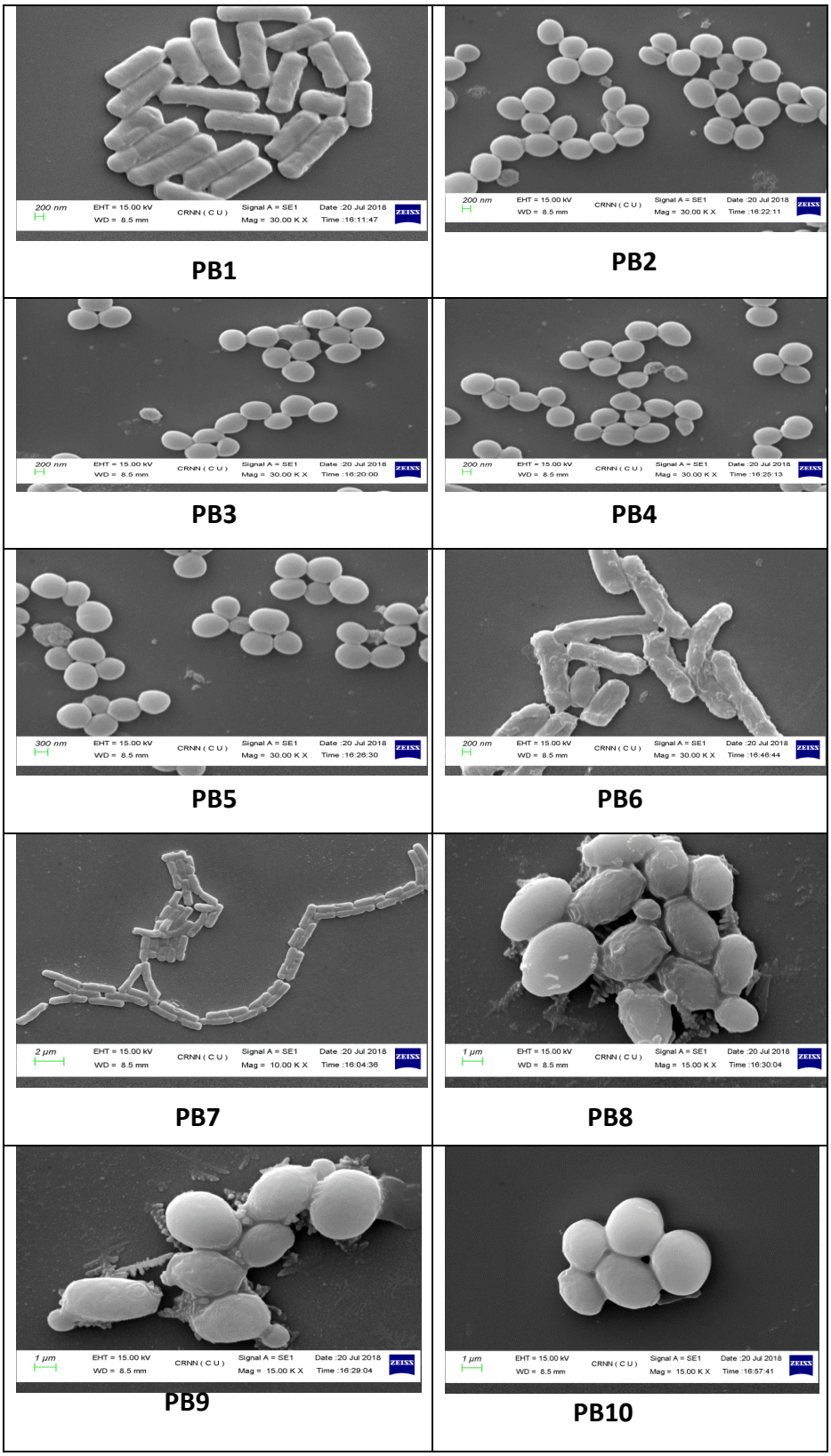


FIGURE 4.1: SEM images of the selected isolates (PB1- PB10)

4.3.2. BIOCHEMICAL CHARACTERIZATION:

All the selected ten isolates (PB1 to PB10) were projected to four different tests such as, catalase test, starch hydrolysis test, oxidase test and arginine hydrolysis test. The isolates were identified as a potential probiotic which exhibited the absence the catalase, amylase and oxidase activity, where as they were established as a putative probiotic bacteria having arginine dehydrolase enzyme. The selected isolates were shown to ferment glucose, lactose, maltose, trehalose and galactose but not cellobiose and sorbitol. The population and the biochemical characterization of the representative isolates were presented in **FIGURE 4.2. and TABLE 4.1.**

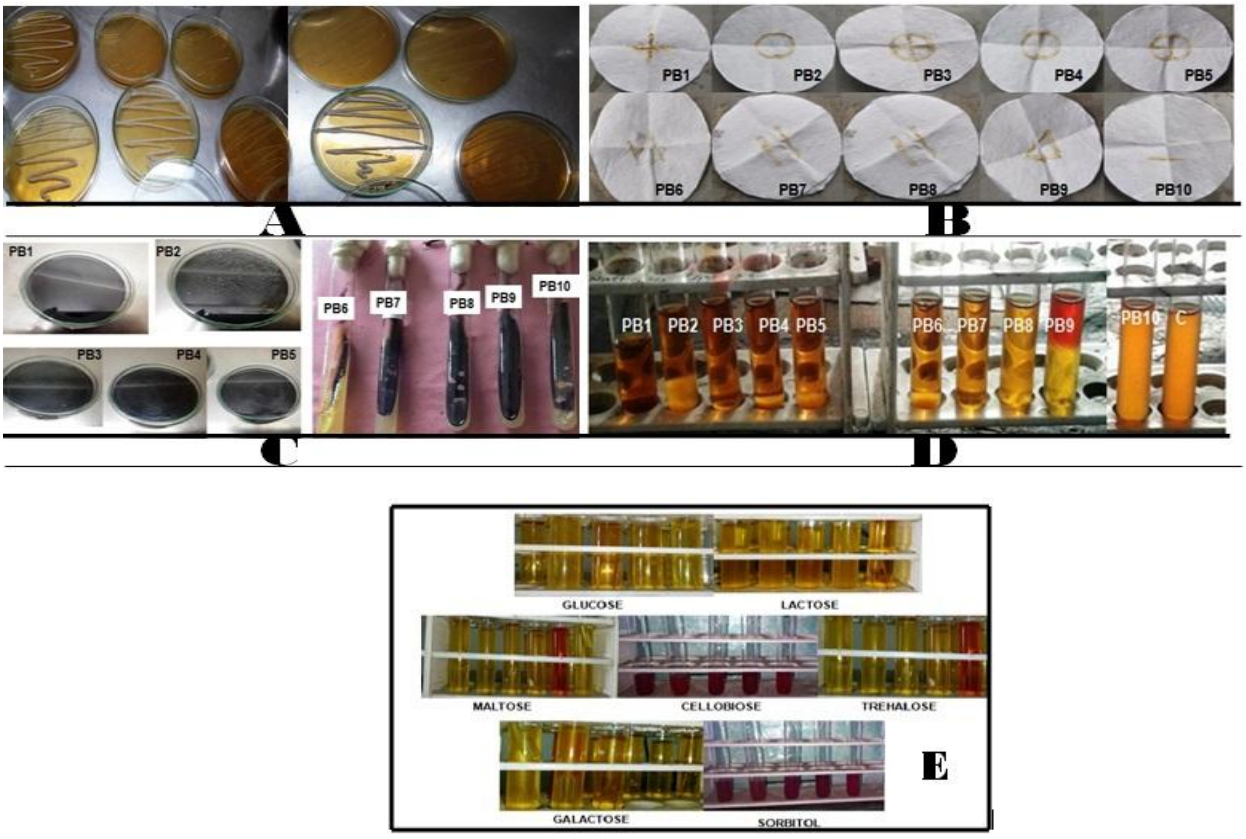


FIGURE 4.2. The biochemical characterizations of the selected isolates which includes; A: Catalase Test, B: Oxidase Test, C: Starch Hydrolysis Test, D: Arginine Hydrolysis Test, E: Carbohydrate fermentation test.

TABLE 4.1. Biochemical characterization of the isolates (PB1 – PB10).

SAMPLES	ARGININE DEHYDROLASE ANALYSIS	OXIDASE TEST	CATALASE TEST	AMYLASE TEST
PB 1	**+ ve**	**- ve**	**- ve**	**- ve**
PB 2	**+ ve**	**- ve**	**- ve**	**- ve**
PB 3	**+ ve**	**- ve**	**- ve**	**- ve**
PB 4	**+ ve**	**- ve**	**- ve**	**- ve**
PB 5	**+ ve**	**- ve**	**- ve**	**- ve**
PB 6	**+ ve**	**- ve**	**- ve**	**-/+ ve**
PB 7	**+ ve**	**- ve**	**- ve**	**- ve**
PB 8	**+ ve**	**- ve**	**- ve**	**- ve**
PB 9	**+ ve**	**- ve**	**- ve**	**-/+ ve**
PB10	**+ ve**	**- ve**	**- ve**	**-/+ ve**

SAMPLES	CARBOHYDRATES UTILIZING TEST						
	GLUCOSE	LACTOSE	MALTOSE	CELLOBIOSE	TREHALOSE	GALACTOSE	SORBITOL
PB1	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve
PB2	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve
PB3	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve
PB4	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve
PB5	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve
PB6	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve
PB7	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve
PB8	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve
PB9	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve
PB10	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	- ve

4.3.3. ANTI-OXIDANT ACTIVITY BY DPPH:

The anti-oxidative nature of isolates was evaluated by its radical scavenging ability using DPPH.

The DPPH activity was found to be between 52-64 % for all isolates. This study suggested that

these isolates were considered as high anti-oxidative potential. The result represented in the **FIGURE 4.3.** displayed high radical scavenging activity which is compared with the standard taken here as ascorbic acid (77%). The anti-oxidative activity of most of the probiotic bacteria including, LAB may help to prevent oxidative stress related destruction by the production of scavenging free radicals in the host. There are reports that suggest that LAB may be able to achieve this either by production of enzymes or by the cell surface components that supports the said activity [Wang et al. 2009].

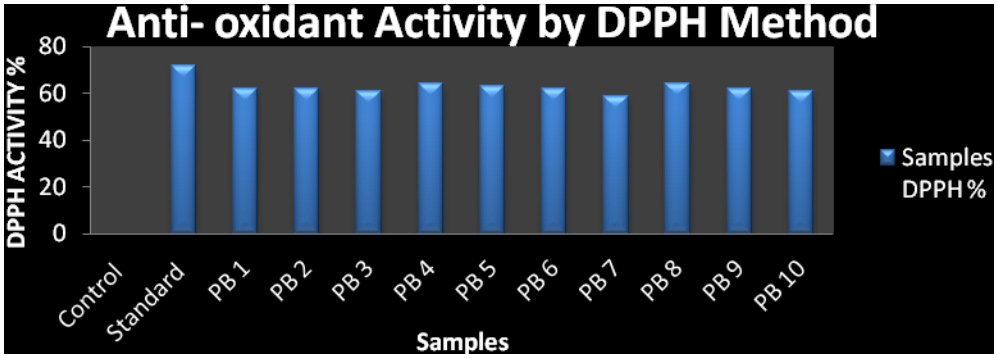


FIGURE 4.3 Anti-oxidant activity of all the isolates (PB1 – PB10) by DPPH.

4.3.4. BILE SALT TOLERANCE ASSAY:

The tolerating ability of the selected isolates towards acid and bile is one of the preliminary criteria for a functional probiotic as these isolates when consumed by the hosts can survive the passage through the harsh environment of the GI tract. The human stomach has pH of 1.5 at fasting condition and pH of about 3 to 5 at postprandial condition as hosts generally secretes 2.5 litres of gastric juice daily [Cotter and Hill, 2003]. Again, average secretion of the human liver is nearly a litre of bile (0.3-0.5%) in the small intestine [Begley et al. 2005]. The putative probiotic thus observed were required to possess tolerance abilities in order to bypass the adverse effects

and thus have the ability to colonize in the GIT to impart different health benefits. In the present study suggest that all the isolates have a very high potentiality to resist 0.2 to 0.6% of bile salt which was also reported for various LAB species [Strompfová et al. 2006].

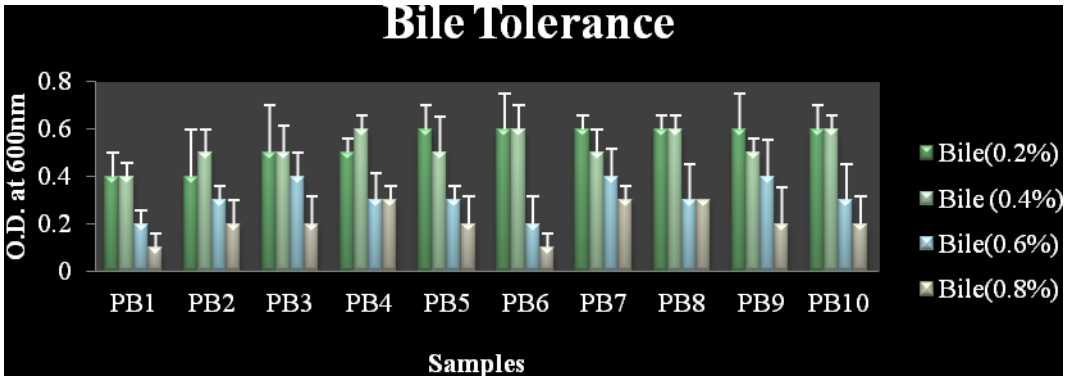


FIGURE 4.4: Tolerance of the isolates (PB1 – PB10) to bile salt.

4.3.5. ANTIMICROBIAL ACTIVITY AGAINST HUMAN PATHOGENS:

This study suggests the ability of all the selected isolates (PB1 – PB10) to inhibit human pathogenic bacteria such as, *Vibrio cholerae* 0139 (MTCC No. 3906), *Pseudomonas aeruginosa* (MTCC No. 11688) was observed. The inhibition activity of the isolates represents its antibacterial property as described in Figure 4.5 (a,b), where PB7 showed maximum zone of inhibition against both the pathogenic strains and no anti- microbial activity was observed in PB6 against *Pseudomonas aeruginosa* (MTCC No. 11688). The antimicrobial nature of LAB was confirmed by their bacteriocins production ability [Devi and Halami, 2011] and to eliminate the pathogens of the gastro-intestinal tract [Muñoz-Quezada et al. 2013].

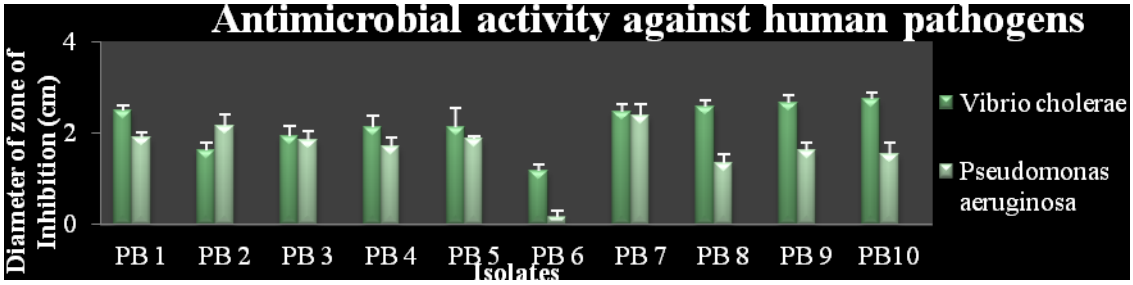


FIGURE 4.5 (a): The anti-microbial activity against human pathogens.

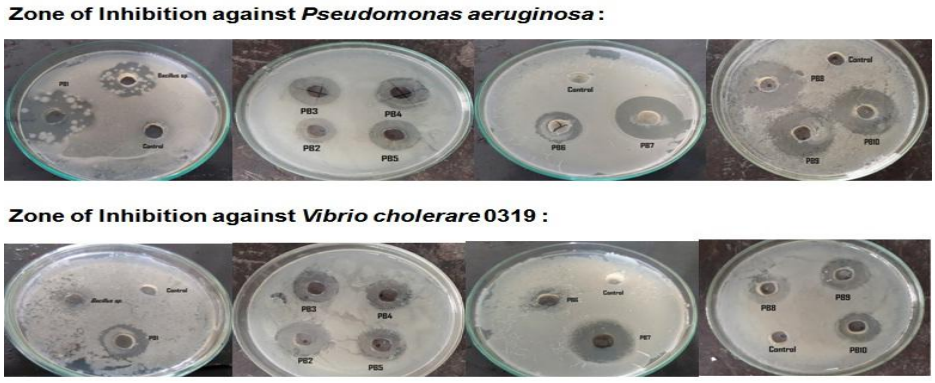


FIGURE 4.5 (b): The zone of inhibition against human pathogens.

4.3.6. ANTIBIOTIC SUSCEPTIBILITY TEST OF THE ISOLATED SAMPLES:

The antibiotic susceptibility is a property that influences the growth of probiotic bacteria in GI tract. It has been observed in many studies that the increased use of antibiotics for the treatment of GIT infections could be a potential disturbance for the balance of gut microflora [Georgieva et al., 2015]. The selected ten isolates were investigated for antibiotic susceptibility pattern by using seven commercially available antibiotics. The method followed in this case was agar cup diffusion assay, and the result obtained is represented in **TABLE 4.2.** Though, all the selected isolates showed resistance towards vancomycin but maximum antibiotics used here showed susceptibility (may be moderately in some cases)

towards all the isolates which includes ampicillin, streptomycin, erythromycin, tetracycline, chloramphenicol and penicillin.

TABLE 4.2: Antibiotic susceptibility pattern of selected isolates, where; S=Sensitive, R= Resistant, MR= Moderately Resistant and MS= Moderately Sensitive.

ISOLATES	Ampicillin	Streptomycin	Erythromycin	Tetracycline	Chloramphenicol	Vancomycin	Penicillin
PB1	MS	S	S	S	S	R	MS
PB2	S	S	S	S	S	R	S
PB3	S	S	S	S	S	R	S
PB4	S	S	S	S	S	R	S
PB5	S	S	S	S	S	R	S
PB6	S	S	S	S	S	R	MS
PB7	S	S	S	S	S	MR	S
PB8	S	S	S	S	S	R	S
PB9	S	S	S	S	S	R	S
PB10	S	S	S	S	S	R	S

4.3.7. SIMULATED GASTRIC FLUID TOLERANCE ASSAY:

The selected isolates were further investigated to identify the sustainability in simulated gastric fluid. In the study, it was observed that the isolates could survive the conditions of gastric fluid (at pH 2.5) for 24 hours. The **FIGURE 4.6** displayed that the isolate PB5 possess the maximum survival rate (about 76%) whereas, PB2 showed least survival rate (i.e., about 46%) in simulated gastric fluids. Besides that, all the ten selected isolates showed a good survival percentage in low pH of the simulated gastric juice. The gastric juice present in the stomach is the innate barrier of the humans where most of the bacterial growth got inhibited. Thus, invading this barrier itself is a criterion for selecting isoltes as a potent probiotic [Mathara *et al.*,2008].

Samples	SGF at pH 2.5	SGF at pH 7	Bacterial % Survival
PB1	0.55 ± 0.06	0.61 ± 0.04	(0.55/0.61)×100 =54.09
PB2	0.50 ± 0.04	0.66 ± 0.10	(0.50/0.66)×100 =45.45
PB3	0.50 ± 0.06	0.65 ± 0.05	(0.50/0.65)×100 = 47.61
PB4	0.50 ± 0.05	0.60 ± 0.04	(0.50/0.60)×100 = 50.00
PB5	0.50 ± 0.05	0.59 ±0.04	(0.50/0.59)×100 = 76.91
PB6	0.16 ± 0.04	0.47 ± 0.05	(0.16/0.47)×100 = 55.5
PB7	0.50 ± 0.01	0.50 ± 0.05	(0.50/0.50)×100 = 60.00
PB8	0.17 ± 0.04	0.45 ± 0.01	(0.17/0.45)×100 = 61.79
PB9	0.15 ± 0.05	0.49 ± 0.01	(0.15/0.49)×100 = 57.14
PB10	0.17 ± 0.05	0.50 ± 0.05	(0.17/0.50)×100 = 54.00

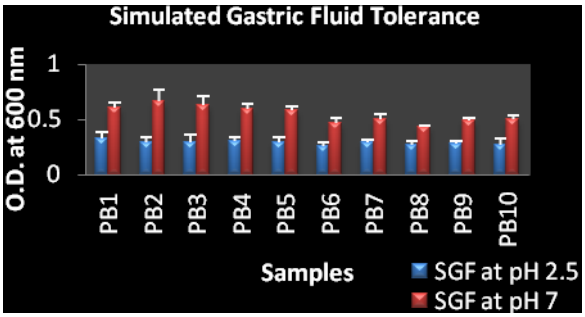


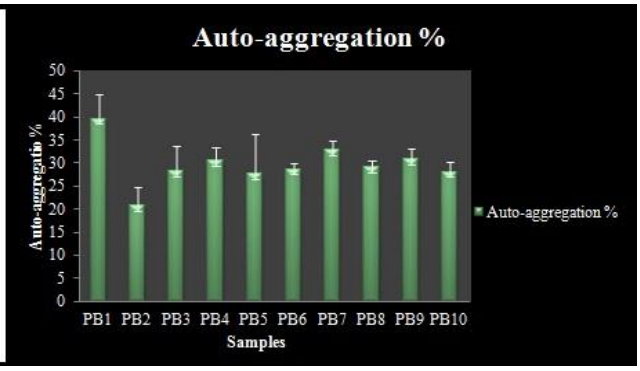
FIGURE 4.6: Simulated gastric fluid tolerance at two different pH (2 & 7).

4.3.8. CELLULAR AUTO- AGGREGATION & BATH ANALYSIS:

The adhesion property of bacteria was characterized by some preliminary evaluation which includes auto-aggregation and cell surface hydrophobicity of the cells. All the selected isolates showed a very good auto–aggregation property (i.e., about 21 to 40%) as well as cell surface hydrophobicity (i.e., about 21 to 26%), which varied from one to the other isolates based on their origin and genus they belong to.

Bacterial adhesion to hydrocarbons portrays the role of hydrophobic interactions and hence is a key method to assess probiotic nature of any bacteria [Rosenberg, 2006]. From the study of the Draksler et al. 2004, it can be concluded that adhesion is highly strain specific property and may differ depending on the expression of cell surface proteins and also may get affected by the environmental conditions [Ramiah et al. 2007]. Auto-aggregation also varies in genus level as well as species level according to Kaushik et al. 2009. It also showed a connection between hydrophobicity and auto-aggregation. [Nikolic et al. 2010]. From our study as represented in **FIGURE4.7**, it is evident that this correlating property is very useful for selection of probiotic strains *in vitro* studies.

Samples	Auto-aggregation %			MEAN ± SD
PB1	36.36	45.76	36.53	39.55 ± 5.3
PB2	22.64	16	23.52	20.72 ± 4.1
PB3	24.56	25.45	34.42	28.14 ± 5.4
PB4	27.86	33.84	29.62	30.44 ± 3.07
PB5	18.18	29.31	35.48	27.65 ± 8.7
PB6	29.31	27.11	29.5	28.64 ± 1.3
PB7	34.48	30.5	33.33	32.77 ± 2.0
PB8	29.09	27.58	30.64	29.10 ± 1.5
PB9	33.33	30.5	28.57	30.8 ± 2.4
PB10	28.81	25.8	29.68	28.09 ± 2.0



Samples	Surface Hydrophobicity %			MEAN ± SD
PB1	21.42	15.71	27.14	21.43 ± 5.7
PB2	21.42	28.57	27.14	25.71 ± 3.8
PB3	18.47	21.42	27.14	22.34 ± 4.4
PB4	27.14	21.42	22.85	23.80 ± 2.9
PB5	21.42	21.42	27.14	23.32 ± 3.3
PB6	21.42	15.71	27.14	21.42 ± 5.7
PB7	21.42	15.71	21.42	19.52 ± 3.3
PB8	24.28	21.42	27.14	24.28 ± 2.9
PB9	18.57	21.42	21.42	20.47 ± 1.6
PB10	15.71	27.14	22.85	21.90 ± 5.8

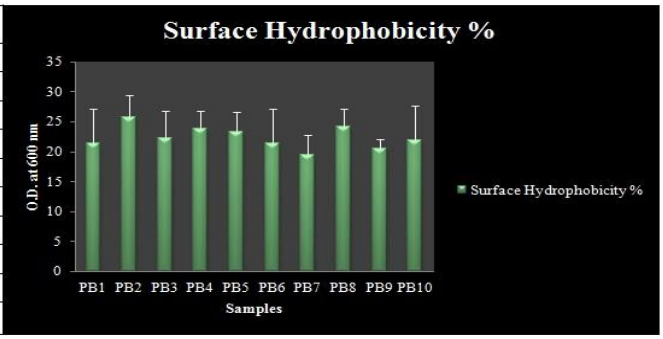


FIGURE 4.7: The auto-aggregation and the surface hydrophobicity of the selected isolates (PB1-PB10).

4.3.9. CHOLESTEROL ASSIMILATION OF THE SELECTED ISOLATES:

The breakdown of amide bond which was situated between the side chain of the amino acid and steroid moiety is initiated by the bile salt hydrolases enzyme which is intracellular in nature [Lebeer et al. 2008]. Thus, this is one of the key selection criteria for probiotic organisms that are dominantly found in inhabitants of the GIT where bile salt exists. From a study, it was evident that probiotic bacteria are known to acquire these *bsh* genes by some horizontal gene transfer [Begley et al. 2006]. Thereby, deconjugated bile salts at low pH stimulate co-precipitation of the cholesterol and which can be excreted through fecal matter [Mathara et al. 2008]. Thus, it could be the probable mechanism by which bacteria reduce cholesterol which could be an added functional health promoting property.

The capability of the strains to reduce cholesterol in a media supplemented with bile salts was evaluated in the present study. In the **FIGURE 4.8**; it is demonstrated that all the ten isolates initiates a good cholesterol reduction in presence of bile. Among them PB3 showed the best reduction rate, whereas PB10 showed the least reduction rate of cholesterol. But their range of reduction rate of the cholesterol for all the isolates lies in between 55 to 65% approximately, which is overall a significant reduction represented.

Samples	Cholesterol Reducing Rate %			Mean ± S.D.
PB1	55.57	56.07	58.96	56.9 ± 1.9
PB2	66.10	59.01	65.45	63.5 ± 3.9
PB3	68.96	67.79	57.62	64.8 ± 6.2
PB4	62.29	57.40	57.14	58.9 ± 2.9
PB5	61.10	57.14	55.73	57.9 ± 2.8
PB6	60.71	51.92	54.71	55.8 ± 4.5
PB7	60.34	54.54	61.53	58.8 ± 3.7
PB8	53.57	57.69	66.10	59.1 ± 6.3
PB9	62.71	56.21	57.40	58.8 ± 3.4
PB10	54.71	60.34	50.98	55.3 ± 4.7

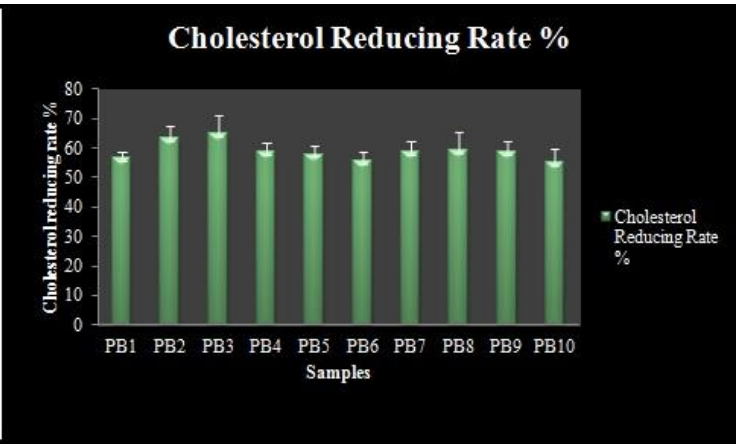


FIGURE 4.8: The cholesterol reducing rate of the isolates (PB1 – PB10).

4.3.10. OPTIMUM pH AND TEMPERATURE OF THE SELECTED ISOLATES:

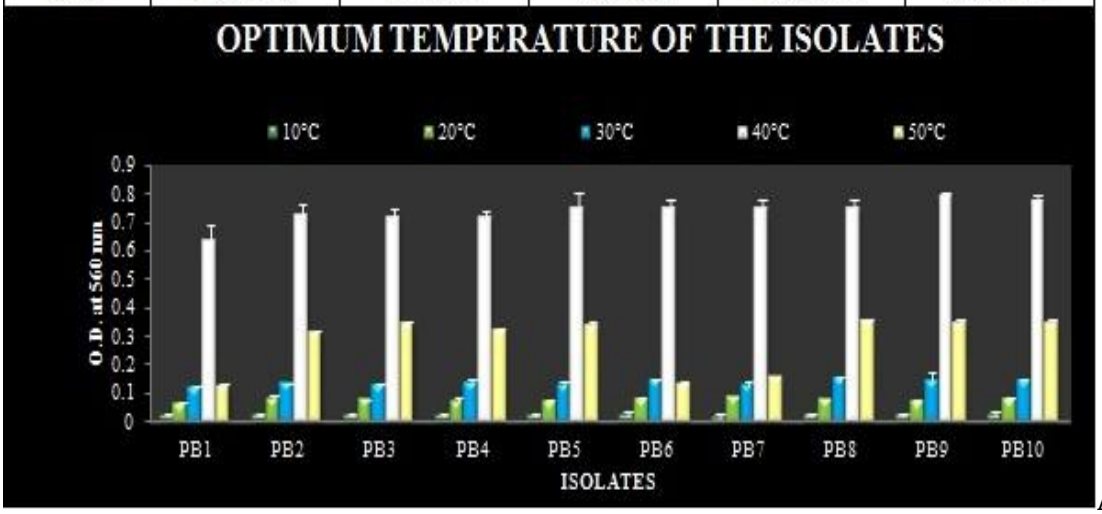
The intra-luminal pH rapidly changed in the stomach from highly acid to about pH 6 in the duodenum in most of the mammals. After that while reaching the gut the pH increases from pH 6 to 7.4, which again drops to 5.7 in the caecum, but gradually enhances while reaching the rectum at pH 6.7 [Fallingborg, 1999].

Normal human body temperature (98.6°F/37°C) generally varies. Though, different studies established that the normal body temperature varies from 97°F to 99°F. Thus, it was

evident from several researches that the growth of gut bacteria including the probiotics showed their best growth in a wide range from 36 to 40 degree Celsius [Fiorentini *et al.* 2011].

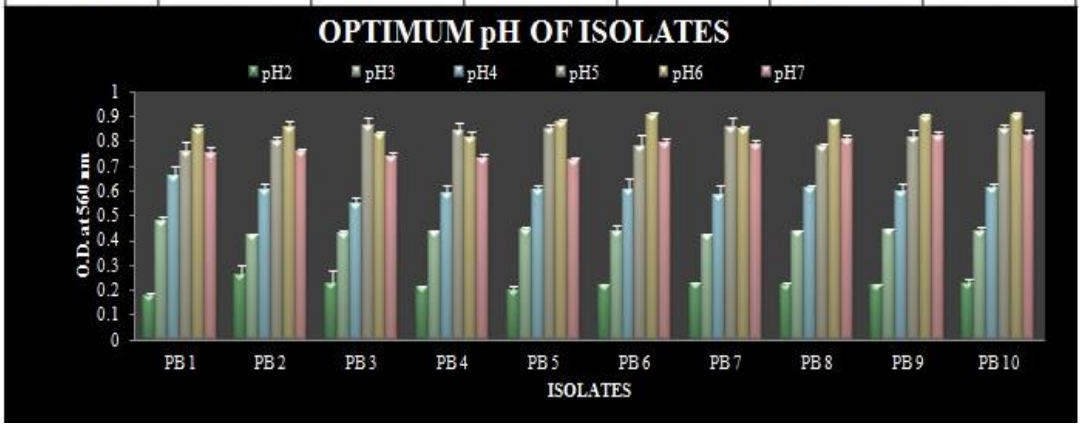
It is observed that, all the isolates exhibits optimum pH at 6 and temperature at 40°C. From the **FIGURE 4.9** it is demonstrated that, the selected isolates (PB1 – PB10) though showed best growth rate at pH 6 but grows well at a wide range pH of 4-7 and also showed growth at a very low pH such as 2. This tolerance to low pH supports the fact of being considered as a probiotic. In the same time the mentioned **FIGURE 4.9** also showed best growth rate at a temperature which is very close to normal body temperature of the humans.

ISOLATES	10°C	20°C	30°C	40°C	50°C
PB1	0.02± 0.01	0.06± 0.01	0.12± 0.01	0.63± 0.05	0.12± 0.02
PB2	0.02± 0.01	0.08± 0.02	0.13± 0.01	0.72± 0.04	0.31± 0.01
PB3	0.02± 0.01	0.07± 0.01	0.12± 0.02	0.72± 0.03	0.34± 0.01
PB4	0.02± 0.01	0.07± 0.02	0.13± 0.01	0.72± 0.02	0.32± 0.01
PB5	0.02± 0.01	0.07± 0.02	0.13± 0.01	0.74± 0.06	0.33± 0.02
PB6	0.02± 0.01	0.08± 0.02	0.14± 0.02	0.74± 0.04	0.13± 0.01
PB7	0.03± 0.01	0.08± 0.01	0.13± 0.01	0.74± 0.04	0.15± 0.01
PB8	0.02± 0.01	0.08± 0.01	0.15± 0.01	0.75± 0.04	0.35± 0.01
PB9	0.02± 0.01	0.07± 0.01	0.14± 0.01	0.79± 0.02	0.35± 0.02
PB10	0.02± 0.01	0.07± 0.01	0.14± 0.01	0.77± 0.02	0.34± 0.01



A

ISOLATES	pH2	pH3	pH4	pH5	pH6	pH7
PB 1	0.17 ± 0.02	0.48± 0.02	0.66± 0.04	0.76± 0.02	0.85± 0.03	0.75± 0.03
PB 2	0.26 ± 0.04	0.42± 0.01	0.61± 0.03	0.80± 0.01	0.86± 0.03	0.76± 0.01
PB 3	0.22±0.06	0.43± 0.01	0.55± 0.03	0.86± 0.03	0.83± 0.02	0.73± 0.03
PB 4	0.21 ± 0.01	0.43± 0.01	0.59± 0.04	0.84± 0.02	0.82± 0.03	0.73± 0.03
PB 5	0.2± 0.01	0.44± 0.02	0.61± 0.02	0.85± 0.04	0.88± 0.02	0.72± 0.02
PB 6	0.21± 0.01	0.44± 0.03	0.60± 0.04	0.78± 0.01	0.90± 0.02	0.79± 0.02
PB 7	0.23± 0.01	0.42± 0.01	0.58± 0.04	0.85± 0.04	0.85± 0.01	0.78± 0.02
PB 8	0.22± 0.01	0.44± 0.01	0.61± 0.02	0.78± 0.02	0.88± 0.01	0.81± 0.02
PB 9	0.22± 0.01	0.44± 0.01	0.59± 0.04	0.81± 0.02	0.90± 0.02	0.82± 0.02
PB 10	0.23± 0.02	0.43± 0.02	0.61± 0.04	0.85± 0.02	0.90± 0.02	0.82± 0.03



B

FIGURE 4.9: The investigation of optimum temperature (A) and pH (B) of the selected isolates.

4.3.11. MOLECULAR DETECTION AND IDENTIFICATION OF THE ISOLATES:

The purified DNA from each isolates which was further amplified with the universal primer 9F and 1492R, which universally specific primer of 16S rDNA of the entire bacterial world. The PCR product was further analyzed by following the agarose gel electrophoresis taking a ladder of 1kb. The size of the band was deduced from the **FIGURE 4.10** that is about 1500 bp in size. The presence of band of each isolated DNA predicts that the 16S rDNA of each isolates were successfully purified and amplified with the giver universal primer. Thus, the samples were ready to submit for the commercial sequencing was analyzed by BLAST.

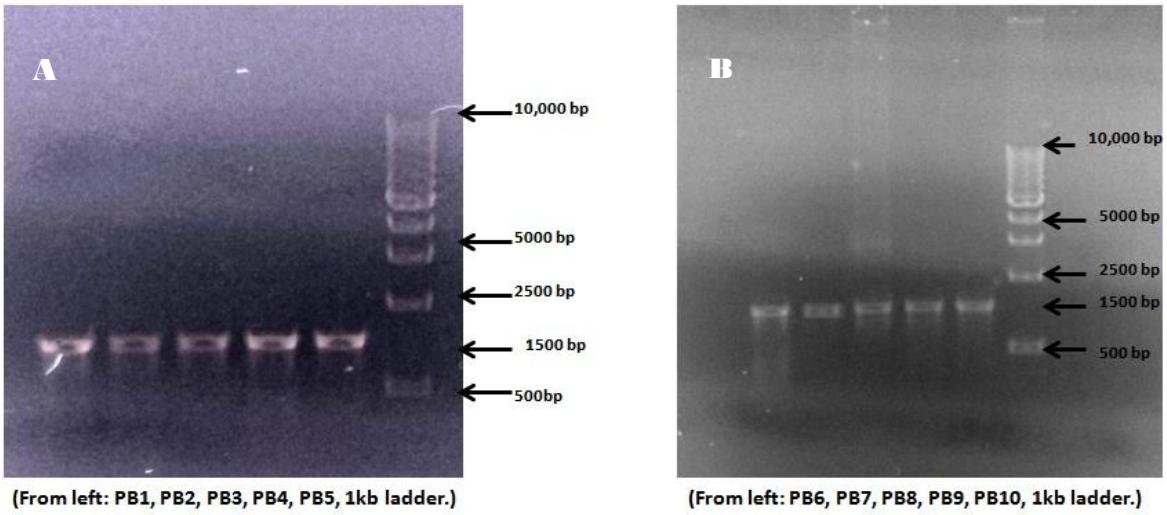


FIGURE 4.10: The agarose gel electrophoresis of amplified PCR product of all the isolates (PB1 – PB10).

The lane represents the DNA of the following isolates respectively;

In A: PB1, PB2, PB3, PB4, PB5 and 1 kb ladder

In B: PB6, PB7, PB8, PB9, PB10 and 1 kb ladder.

4.3.12. ACCESSION NUMBER OF THE OBTAINED SEQUENCES:

The sequence obtained from the KPC College, Kolkata of each isolates were first analyzed by the chromatogram obtained. Then, they are deposited at GenBank database to select few of the most homology sequences of each given isolates. The sequence we obtained for each isolates had not showed 100% homology with any of the already deposited sequences. Thus, all the isolates following BLASTn algorithm were originated to it genus and species identification which shows the maximum homology in the server. And each sequence has been assigned a new strain number. The **TABLE 4.3.** showed the genus and species identification and allotment of strain name for each of the isolates.

TABLE 4.3: The genus and species identification and allotment of the strain name to each of the isolates along with its accession ID number.

Isolates	Submission ID	Genus	Strain	Source	Accession ID
PB1	SUB5880577	*Bacillus cereus*	BURD CU1	CURD	**MN103825**
PB2	SUB5371268	*Streptococcus thermophilus*	BURD 1	CURD	**MK696580**
PB3	SUB5897956	*Streptococcus thermophilus*	BURD PB3	CURD	**MN121713**
PB4	SUB5897962	*Streptococcus thermophilus*	BURD PB4	CURD	**MN121714**
PB5	SUB5440220	*Streptococcus thermophilus*	PB5	CURD	**MK779139**
PB6	SUB5897933	*Bacillus sp.*	BURD CU7	COW MILK	**MN121712**
PB7	SUB5897890	*Lactobacillus fermentum*	BURD PB7	HUMAN MILK	**MN121704**
PB8	SUB5897970	*Streptococcus thermophilus*	BURD PB8	YOGURT	**MN121741**
PB9	SUB5897973	*Streptococcus thermophilus*	BURD PB9	YOGURT	**MN121749**
PB10	SUB5897980	*Streptococcus thermophilus*	BURD PB10	YOGURT	**MN121751**

4.3.12. PHYLOGENETIC ANALYSIS:

Based on their abundance, the majority of the isolates (i.e., PB2, PB3, PB4, PB5, PB8, PB9 and PB10) were closely related to the Genus *Streptococcus* and species *thermophilus* which is deduced by the presence of the maximum percentage of alignment of the sequence already present in the NCBI server.

The phylogenetic tree of each of the isolates with their new designated strain name is given below which describes the relation of the each isolates with their most related genus as well as species on the basis of their branch length, bootstrap value etc.

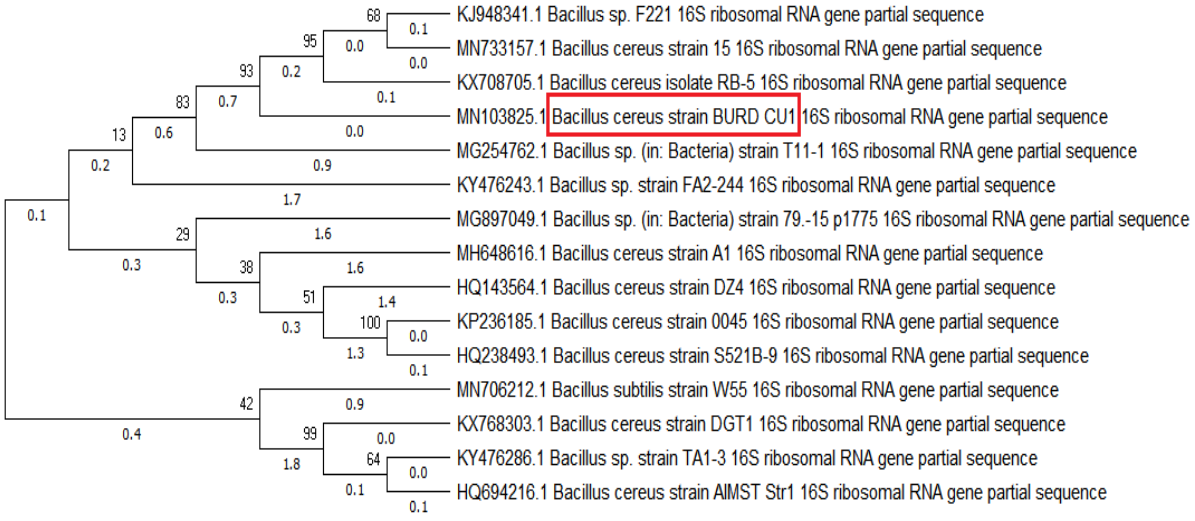


FIGURE 4.11: The phylogenetic tree of PB1 which showed maximum homology with the genus *Bacillus* and species *cereus* and the strain name designated to this isolate is BURD CU1.

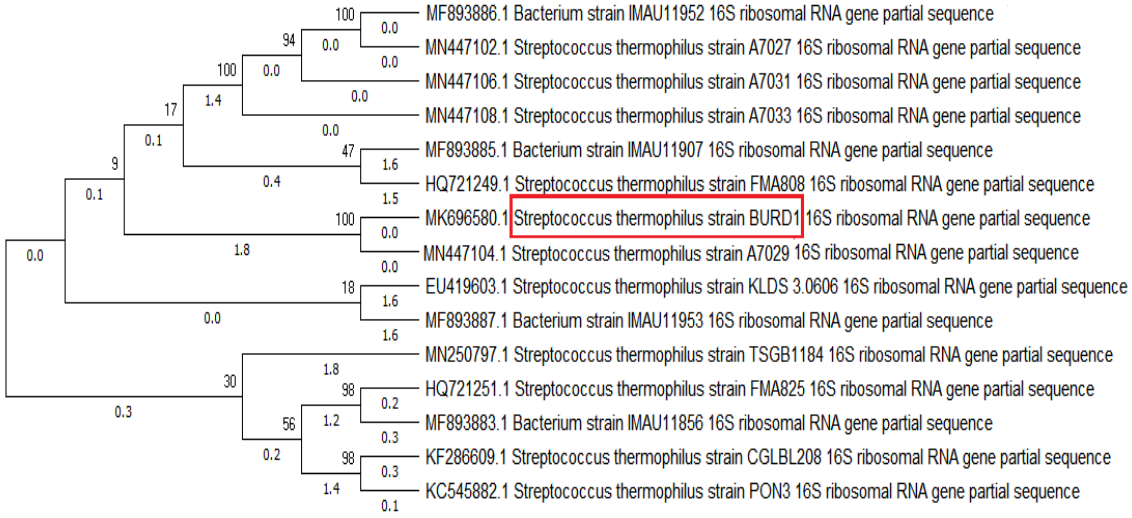


FIGURE 4.12: The phylogenetic tree of PB2 which showed maximum homology with the genus *Streptococcus* and species *thermophilus* and the strain name designated to this isolate is BURD 1.

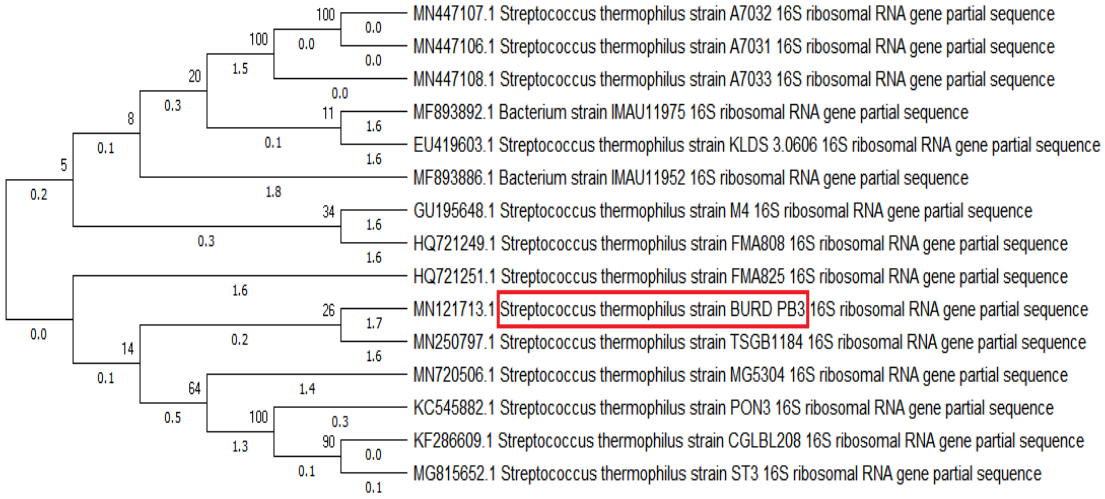


FIGURE 4.13: The phylogenetic tree of PB3 which showed maximum homology with the genus *Streptococcus* and species *thermophilus* and the strain name designated to this isolate is BURD PB3.

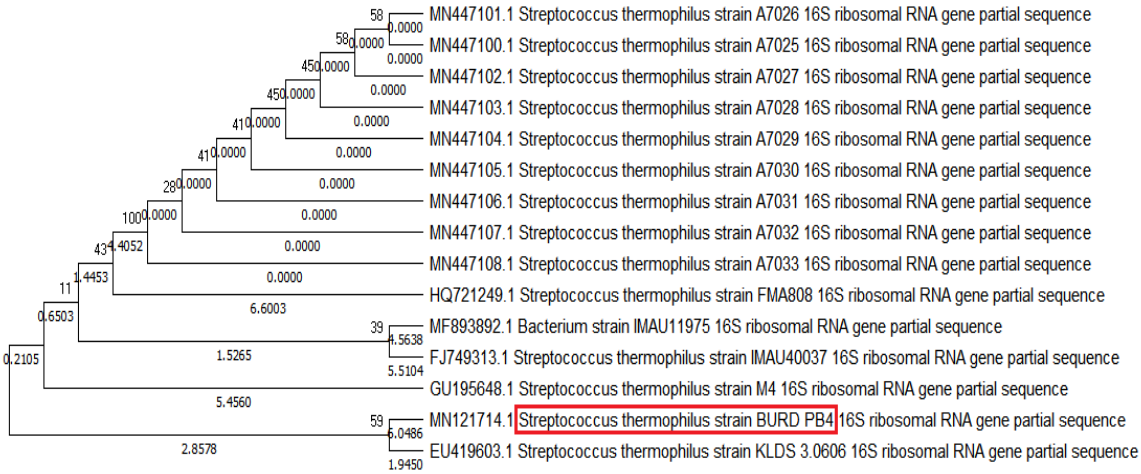


FIGURE 4.14: The phylogenetic tree of PB4 which showed maximum homology with the genus *Streptococcus* and species *thermophilus* and the strain name designated to this isolate is BURD PB4.

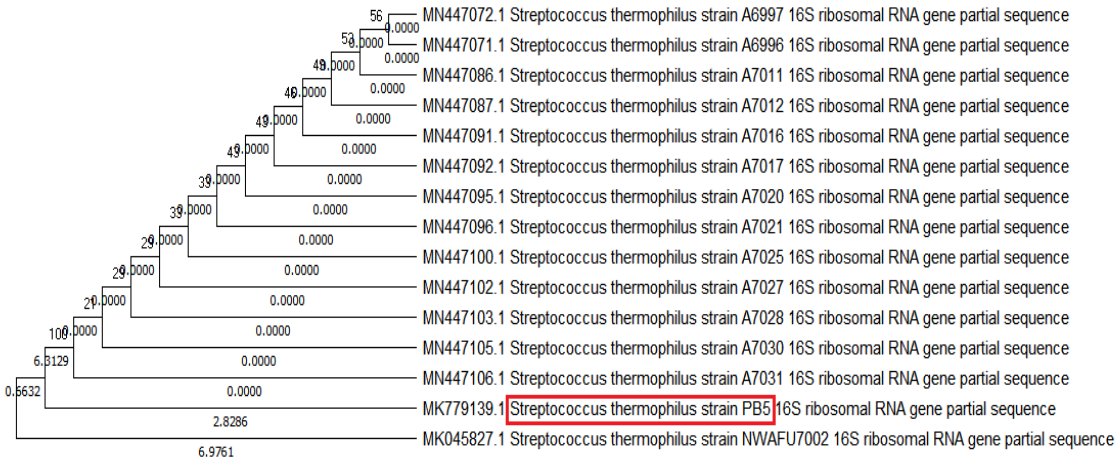


FIGURE 4.15: The phylogenetic tree of PB5 which showed maximum homology with the genus *Streptococcus* and species *thermophilus* and the strain name designated to this isolate is PB5.

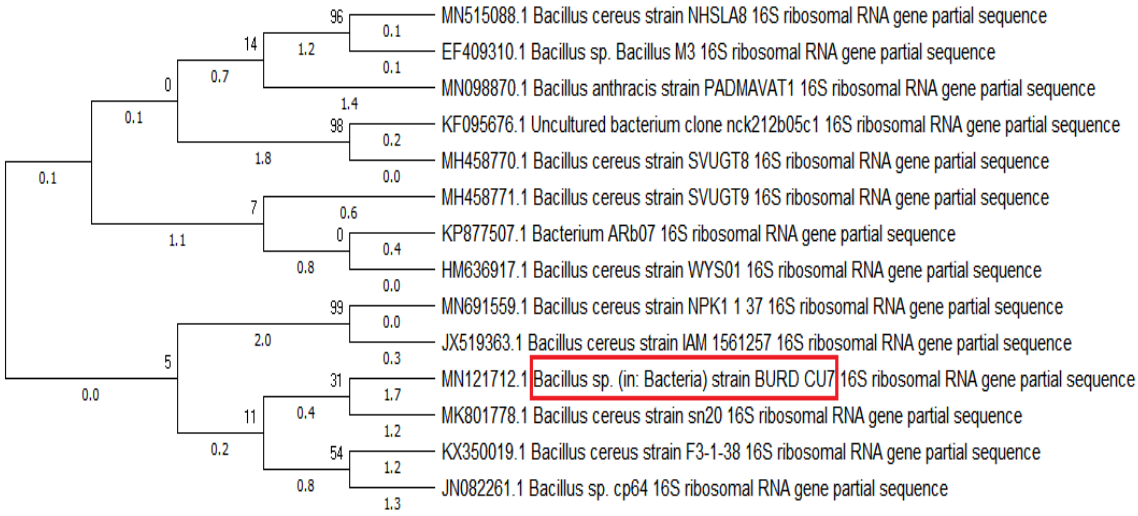


FIGURE 4.16: The phylogenetic tree of PB6 which showed maximum homology with the genus *Bacillus* and the strain name designated to this isolate is BURD CU7.

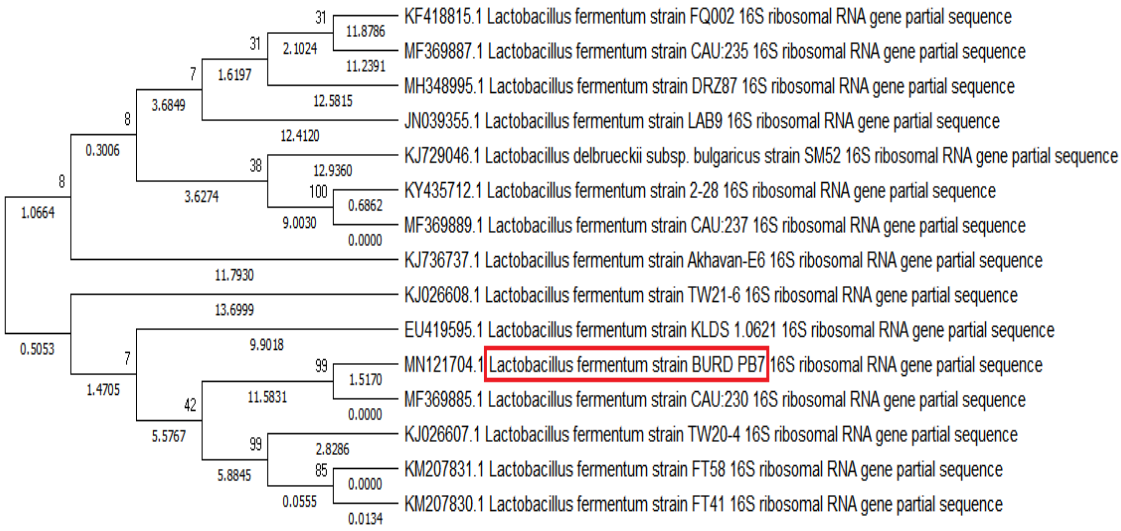


FIGURE 4.17: The phylogenetic tree of PB7 which showed maximum homology with the genus *Lactobacillus* and species *fermentum* and the strain name designated to this isolate is BURD PB7.

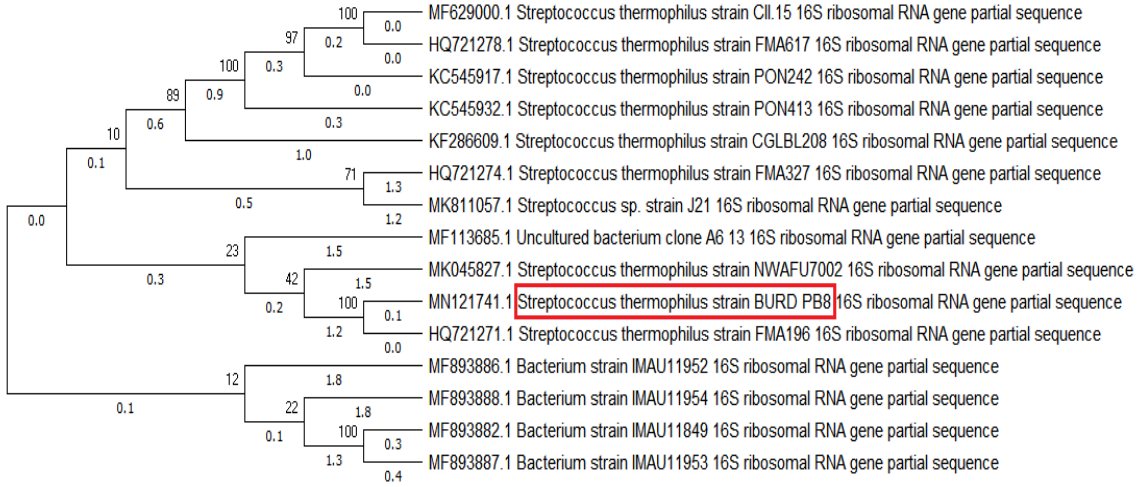


FIGURE 4.18: The phylogenetic tree of PB8 which showed maximum homology with the genus *Streptococcus* and species *thermophilus* and the strain name designated to this isolate is BURD PB8.

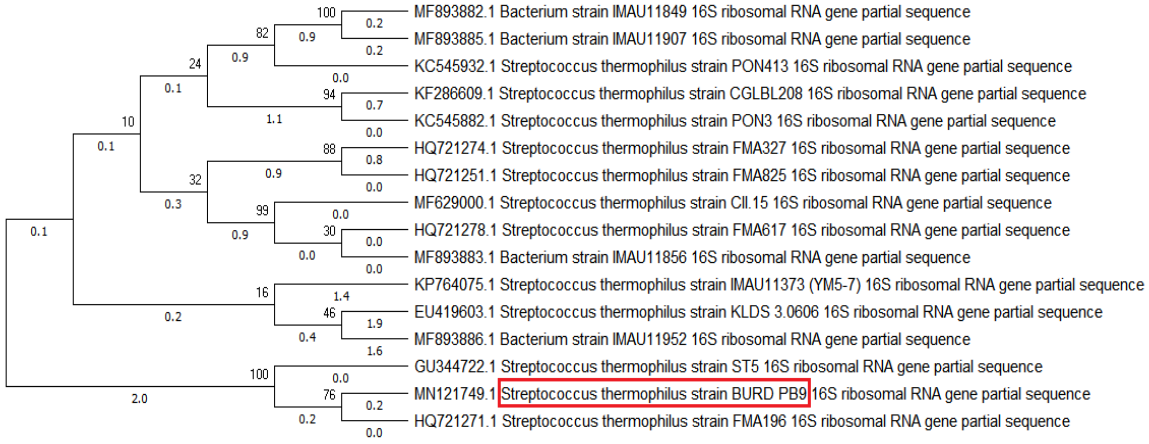


FIGURE 4.19: The phylogenetic tree of PB9 which showed maximum homology with the genus *Streptococcus* and species *thermophilus* and the strain name designated to this isolate is BURD PB9.

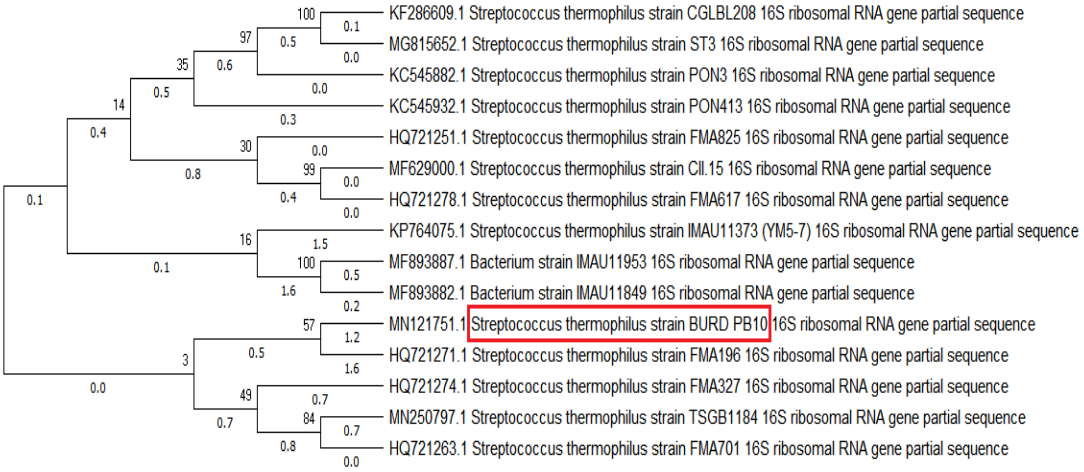


FIGURE 4.20: The phylogenetic tree of PB10 which showed maximum homology with the genus *Streptococcus* and species *thermophilus* and the strain name designated to this isolate is BURD PB10.

After the individual phylogenetic tree construction of each and every isolates, it can be depicted that all the isolates may belongs to various genus but most of them are originating from the same group of bacteria, i.e., Lactic Acid Bacteria. Among the 10 isolates only PB1 and PB6 belongs to the genus *Bacillus*, which generally did not belongs to LAB. But they have already published potentiality of being a probiotic [Duc *et al.* 2004]. Rest all the bacteria belong to the LAB group of bacteria.

Now, when it is established that all the isolates have the ability to be considered as a probiotic (based on both, physiochemical characterization and phylogenetic categorization); all the isolates were taken under one phylogenetic tree to establish the relationship between all the isolates. Such co-relation is described in the **FIGURE 4.21**.

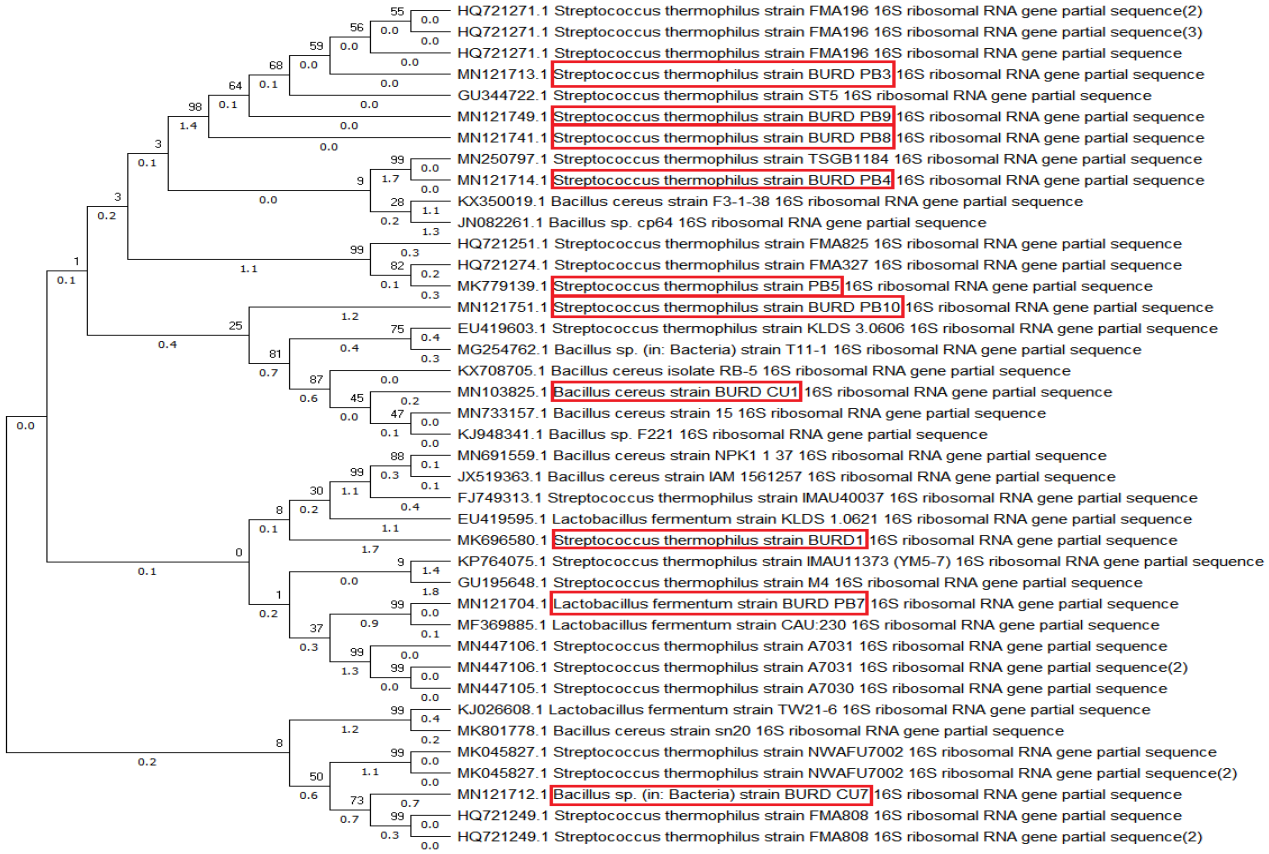
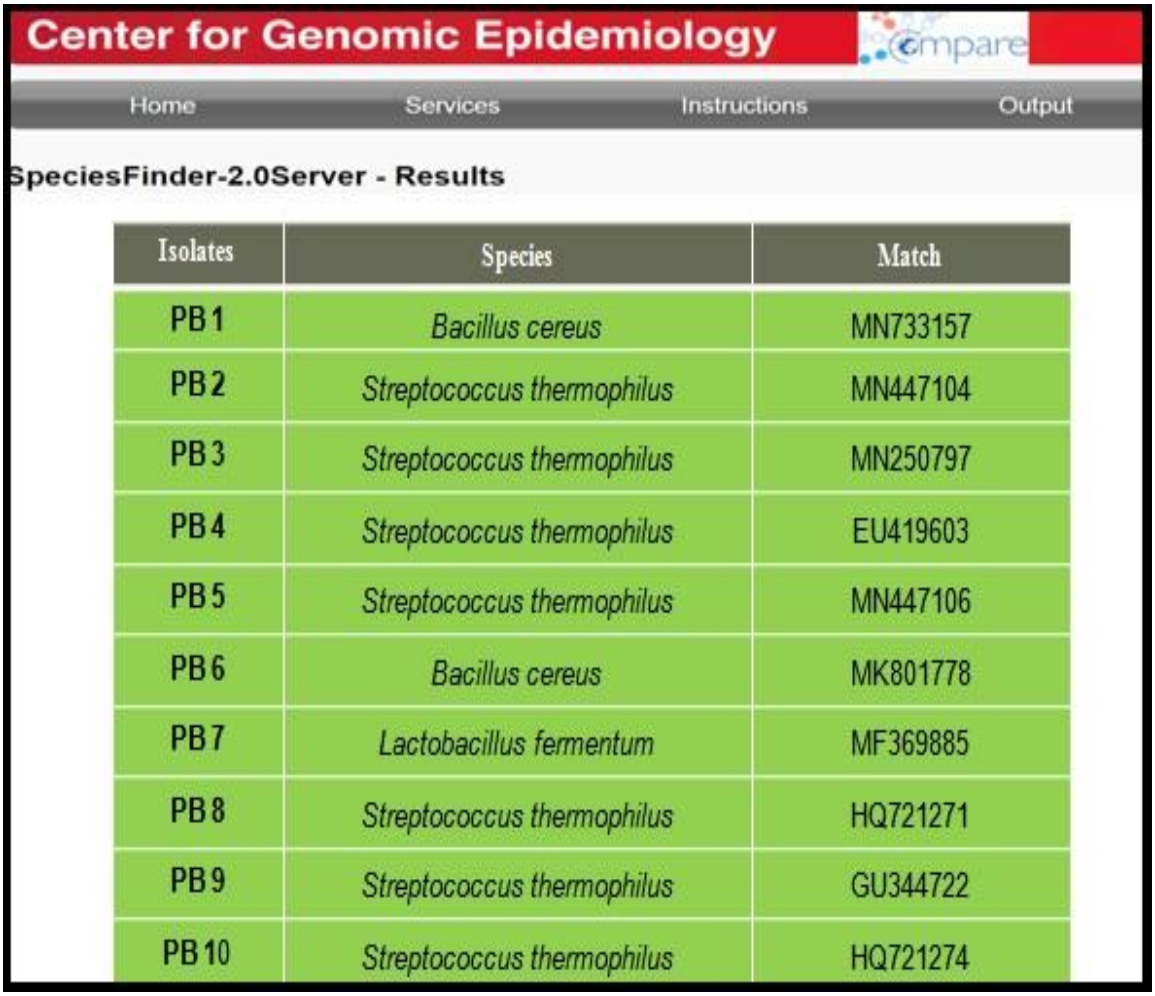


FIGURE 4.21: The overall phylogenetic tree of all the isolates (PB1 to PB10) showed maximum homology with different genus *Bacillus*, *Lactobacillus* and *Streptococcus* and which are all constructed under one tree and showed the detailed relationship between the entire isolates.

4.3.13. GC CONTENT ANALYSIS:

The GC content analysis is determined from a particular website known as, SpeciesFinder 2.0; which showed the maximum species similarity match of all the selected isolates. Therby, it helped to confirm the identification of the all these isolates based on their 16s rDNA sequence. The **FIGURE 4.22** showed the details of the homology study.


Center for Genomic Epidemiology

Home Services Instructions Output

SpeciesFinder-2.0Server - Results

Isolates	Species	Match
PB1	*Bacillus cereus*	MN733157
PB2	*Streptococcus thermophilus*	MN447104
PB3	*Streptococcus thermophilus*	MN250797
PB4	*Streptococcus thermophilus*	EU419603
PB5	*Streptococcus thermophilus*	MN447106
PB6	*Bacillus cereus*	MK801778
PB7	*Lactobacillus fermentum*	MF369885
PB8	*Streptococcus thermophilus*	HQ721271
PB9	*Streptococcus thermophilus*	GU344722
PB10	*Streptococcus thermophilus*	HQ721274

FIGURE 4.22: The GC analysis showing best match to confirm the species of the selected isolates.

4.4. CONCLUSION:

Among the all other bacteria, Lactic Acid Bacteria are known as ubiquitous microorganisms which are widely known for their probiotic properties. Selective screening helped in obtaining bile tolerant cultures. All the physiological, biochemical and safety characterization helped in the identification and selection of all the non-pathogenic isolates. All the 10 selected isolates including *Streptococcus thermophilus*, *Lactobacillus fermentum* and *Bacillus sp.* displayed a

wide range of probiotic properties which includes proliferation in simulated gastrointestinal fluids, cell surface hydrophobicity, auto-aggregation, antibacterial activity, cholesterol reduction and anti-oxidant activity. Molecular characterization differentiates between the isolates based on genomic diversity isolated from different sources. The strains are all emerging from different genus as well as species but the fact that remained similar is that the *in vitro* study of all the isolates are considered as potential probiotic. Thus, the isolates were taken for further *in vivo* characterization and the immunomodulatory effects of that.