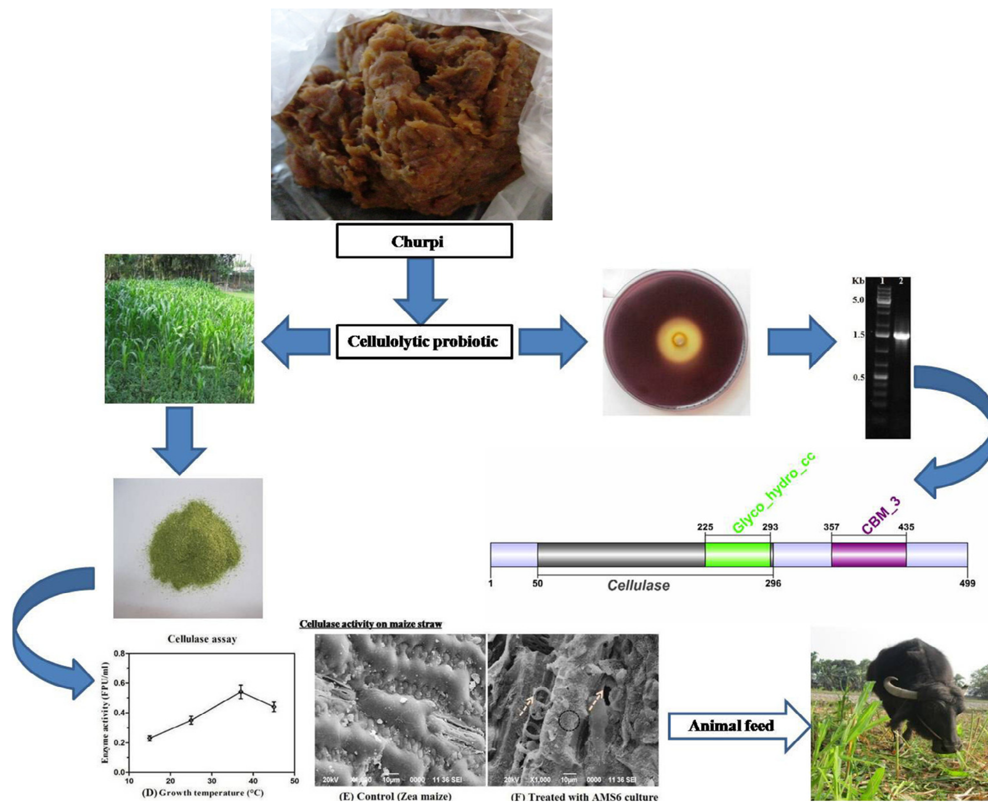
CHAPTER 7

CELLULOLYTIC POTENTIAL OF PROBIOTIC *BACILLUS SUBTILIS* AMS6 ISOLATED FROM TRADITIONAL FERMENTED SOYBEAN (CHURPI): AN *IN-VITRO* STUDY WITH REGARDS TO APPLICATION AS AN ANIMAL FEED ADDITIVE



Graphical Abstract

CELLULOLYTIC POTENTIAL OF PROBIOTIC *BACILLUS SUBTILIS* AMS6 ISOLATED FROM TRADITIONAL FERMENTED SOYBEAN (CHURPI): AN *IN-VITRO* STUDY WITH REGARDS TO APPLICATION AS AN ANIMAL FEED ADDITIVE

7.1. Abstract

The aim of the present study is to evaluate the probiotic attributes of *Bacillus subtilis* AMS6 isolated from fermented soybean (Churpi). This isolate exhibited tolerance to low pH (pH 2.0) and bile salt (0.3 %), capability to autoaggregate and coaggregate. AMS6 also showed highest antibacterial activity against the pathogenic indicator strain *Salmonella enterica typhimurium* (MTCC 1252) and susceptibility towards different antibiotics tested. The isolate was effective in inhibiting the adherence of food borne pathogens to Caco-2 epithelial cell lines, and was also found to be non-hemolytic which further strengthen the candidature of the isolate as a potential probiotic. Further studies revealed *B. subtilis* AMS6 showed cellulolytic activity (0.54 ± 0.05 filter paper units ml^{-1}) at 37 °C. The isolate was found to hydrolyze carboxymethyl cellulose, filter paper and maize (*Zea mays*) straw. The maize straw digestion was confirmed by scanning electron microscopy studies. The isolate was able to degrade filter paper within 96 hours of incubation. A full length cellulase gene of AMS6 was amplified using primers consisting of 1499 nucleotides. The ORF encoded for a protein of 499 amino acids residues with a predicted molecular mass of 55.04 kDa. The amino acids sequence consisted of a glycosyl hydrolase family 5 domain at N-terminal; Glycosyl hydrolase catalytic core and a CBM-3 cellulose binding domain at its C terminal. The study suggests potential probiotic *Bacillus subtilis* AMS6 as a promising candidate envisaging its application as an animal feed additive for enhanced fiber digestion and gut health of animal.

7.2. Introduction

Probiotics are live microbes, which when administered in adequate amounts confer a health benefit to the host[1]. The main probiotics include lactic acid bacteria, such as *Lactobacillus, Bifidobacterium* and *Enterococcus*, which are inherent members in the gastrointestinal tract of humans and animals[2]. To ensure proper functionality and promote health benefits by probiotics, the organisms must resist the harsh environment such as low pH and bile toxicity prevalent in the upper digestive

tract[3]. In addition, they should possess good surface hydrophobicity and aggregation properties for colonization in gut[4] (. Lactic acid bacteria have been extensively studied as a potential probiotic for ruminants as compared with *Bacillus* probiotics[5-9] . However, *Bacillus* sp. also has potential probiotic as well as other attributes[10]. Spore forming characteristics of *Bacillus* sp. has an advantage over other non-spore formers such as *Lactobacillus* sp. to withstand harsh environment such as low pH[11] and high temperature. So there is a need to study *Bacillus* probiotics in order to explore the untapped potential they harbor. Further, there are few reports on the cellulolytic nature of the *Bacillus* probiotics. The usage of probiotics as animal feed additives demands these attributes and thus envisages a tremendous scope. Cellulase converts the highly recalcitrant cellulose to fermentable mono- and oligo-saccharides that can be easily assimilated in the body, thus improving utilization of dietary carbohydrate and enhancing digestion. The byproducts formed after action of enzymes is utilized as a prebiotic source by probiotics and thus enhancing digestion of dietary feed rich in cellulose. The most considerable effects of probiotics have been reported after incorporation of live beneficial microbes in the animal feed during stressful periods for the gut microbiota and the animal: at weaning; at the beginning of the lactation period; and after a dietary shift from high forage to high readily fermentable carbohydrates[12]. In this context, the possibility to use cellulolytic probiotic feed supplements to attain better digestibility of the feed and productivity of animal through management of the gut microbial ecosystem has gained considerable interest.

This study describes a *Bacillus subtilis* strain that, in addition to showing potential probiotic attributes, could inhibit the adherence of food borne pathogens to Caco-2 epithelial cell lines and showed significant cellulolytic activity, thus showing the prospect of a possible use as an animal feed additive.

7.3. Materials and methods

7.3.1. Sampling and isolation of bacteria

Churpi, an indigenous fermented soybean product (Fig. 7.1), was collected from Bomdila, Arunachal Pradesh, India, located at Latitude 27.25 °N and Longitude 92.4 °E, and stored at 4 ºC until processed. The bacterial strain used in the study was

isolated from libi Churpi (prepared from yellow soybean seed) and it was tested for its probiotic attributes and cellulose degrading capability.

Fig. 7.1 Fermented soybean libi Churpi

7.3.2. Identification of the isolate

16S rRNA gene sequence analysis followed by phylogenetic studies was done for the molecular identification of selected isolate. Universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used to amplify the conserved 16S rRNA gene sequence[13]. The PCR amplification was performed in Eppendorf thermocycler in a total volume of 25 µl reaction mixture by following protocol described in our previous chapter (section 3.3.2**)**. PCR product was purified after electrophoresis in 1 % (w/v) agarose gel and used for the automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystem, Switzerland). The sequence obtained was subjected to NCBI BLAST search tool in order to retrieve the homologous sequences in Genbank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was generated by Neighbor-Joining (NJ) method using MEGA 5.05[14, 15, 16].

7.3.3. Screening of probiotic properties

7.3.3.1. In vitro gastrointestinal stress tolerance test

Overnight culture of *Bacillus subtilis* AMS6 and probiotic reference strain *L. rhamnosus* MTCC 1408, grown in Luria Bertani broth and MRS (de Man, Rogosa and Sharpe) broth respectively. The bacterial cells were harvested by centrifugation at 6000 × g for 10 min at 4 °C, followed by washing twice with phosphate buffered saline (PBS, pH 7.4) and then re-suspended (10^8cfu ml^{-1}) in different PBS solutions with various pH. Resistance to simulated gastric fluid (SGF) and small intestinal fluid (SIF) condition was tested as described in previous chapter (Section 5.3.4.1). The resistance of isolates in every condition was assessed in terms of viability on respective agar plates expressed in percentage after the treatment.

$$\text{Viability (\%)} = \left(\frac{N_t}{N_0}\right) \times 100$$

where N_0 is the initial cell count and N_t is final cell count (log cfu/ml).

7.3.3.2. Antibacterial activity

The antibacterial activity of the isolate was checked against Gram positive and Gram negative indicator strains by agar well diffusion method. The strains *Klebsiella pneumoniae* (MTCC 618), *Pseudomonas aeruginosa* (MTCC 7815), *Mycobacterium smegmatis* (ATCC 14468),*Salmonella enterica typhimurium* (MTCC 1252) and *Listeria monocytogenes* (KF894986, local isolate) were used as indicator cultures in the study. The antimicrobial activity was recorded as appearance of clear zone around the wells[17] .

7.3.3.3. Autoaggregation and Co-aggregation

Autoaggregation and co-aggregation assay was performed as mentioned in previous chapter (Section 3.3.3.4 and 3.3.3.5).

7.3.3.4. Cell surface hydrophobicity

The cell surface hydrophobicity of the selected bacterial isolate was measured using n-hexadecane and xylene by following method described in previous chapter (section 5.3.4.3).

7.3.3.5. Cell culture

The human colorectal adenocarcinoma Caco-2 cell line obtained from NCCS, India (National Centre for Cell Science, Pune) was used to study the adhesion capability and the inhibition of food borne pathogenic bacterial adhesion. The cell lines were routinely grown and maintained by following the standard procedure reported in previous chapter (Section 3.3.3.6).

7.3.3.6. Adhesion and inhibition of pathogen adhesion to Caco-2 cells by *B. subtilis* AMS6

The adhesion and inhibition study was performed by following the procedure described in previous chapter (Section 3.3.3.7). All the experiments for each of the strains were performed independently in triplicates. For visualization of adhesion, Caco-2 cell monolayers were washed thrice with PBS, dried in air and adherent bacterial isolates were observed under 100 x after fixing with 3 % paraformaldehyde and gram stained.

7.3.4. Antibiotic susceptibility test

The antibiotic susceptibility test was performed by following Bauer-Kirby method[18]. The standard antibiotic discs (Octadisc) supplied by HiMedia were used. The zones of inhibition were measured by HiMedia Antibiotic Zonescale.

7.3.5. Hemolysis assay

The pattern of hemolysis was studied following the protocol of Anand et al. [19] with some modifications. Briefly, the isolate was streaked on nutrient agar plate supplemented with 5 % goat blood and incubated for 24 h – 48 h at 37 ºC to detect patterns of hemolysis.

7.3.6. Cellulolysis and identification of cellulase gene in *B. subtilis* AMS6

The cellulolytic potential of the isolate was assessed on carboxymethyl cellulose (CMC) agar plate[20] . Wells were prepared on CMC agar plate loaded with the culture supernatant of *B. subtilis* AMS6, incubated for 18 h at 37 ºC and then flooded with Gram’s iodine to visualize the zone of hydrolysis. The gene encoding cellulase was identified using primers (CF 5’-ACAGGATCCGATGAAACGGTCAATTTCTATTTT-3’ and CR 5’-

ACTCTCGAGATTGGGTTCTGTTCCCAAAT-3') as mentioned in the previous chapter (Section 6.3.1). The sequence obtained was analyzed with EXPASY tools (http://expasy.org/). The deduced amino acid sequence obtained was further subjected to BLASTp programme (http://www.ncbi.nlm.nih.gov) search tool in order to retrieve the homologous sequences in Genbank. The obtained similar sequences were aligned using CLC sequence viewer 7.5. software and phylogenetic tree was constructed by Neighbor-Joining (NJ) method using MEGA 5.05. Domain structure was prepared using DOG 1.0[21].

7.3.7. Cellulase activity assay

Total cellulase activity was determined by measuring the amount of reducing sugar released from filter paper[22] . Briefly, 0.5 ml of culture supernatant was incubated with 1.0 ml of 0.05 M sodium citrate buffer (pH 4.8) containing Whatman no. 1 filter paper strip (1.0 × 6.0 cm). After incubation for an hour at 50 ºC, the reaction was terminated by adding 3 ml of 3, 5-dinitrosalicylic acid (DNS) reagent. The reducing sugar was estimated spectrophotometrically[23] using glucose as standard. One unit of enzyme activity was defined as the amount of enzyme that released 1 µ mol of glucose (from filter paper) in unit time under assay conditions.

7.3.8. SEM analysis of the degradation of maize (Zea mays) straw

The maize (*Zea mays*) straw used in this study was obtained locally, washed, and dried. The dried straw was chopped into small pieces and then ground into smaller homogenous particles using grinder mixer. SEM analysis of the degradation of straw by incubating them [2 % (w/v)] with crude culture of *B. subtilis* AMS6 for 6 h was done as reported in previous chapter (section 5.3.9). The specimen were platinum coated using JEOL JFC-1600 auto fine coater and observed under SEM (JEOL model JSM-6390 LV) at 15 KV.

7.3.9. Filter paper degradation

Filter paper degradation was studied by culturing the bacterial isolate in Minimal Salt Medium (MSM) supplemented with Whatman no. 1 filter paper as mentioned earlier (5.3.10).

7.4. Results and discussion

7.4.1. Isolation of the bacterial strain

Sixty-four bacterial strains were isolated from Churpi and checked for *in-vitro gastrointestinal* tolerance test (Appendix-17). On further screening, based on probiotic attributes, AS6 bacterial isolate was selected and identified based on sequencing of 16S rDNA, and subsequently named as *Bacillus subtilis* AMS6 (GenBank Accession: KP723361).

7.4.2. Identification of the isolate

The isolate was identified by sequencing 16S rRNA gene and the sequence obtained was used to construct phylogenetic tree (Fig. 7.2) using Neighbor-Joining method. The isolate showed maximum sequence similarity with *Bacillus subtilis* SBB16 (KP790031).

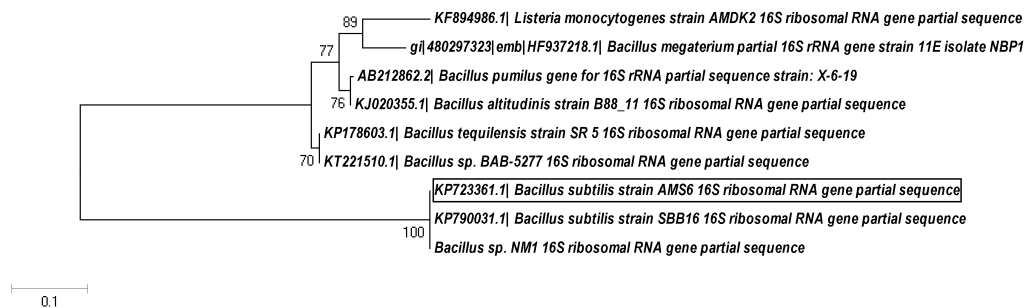


Fig. 7.2 Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain *B. subtilis* AMS6 with closely related species. Tree was constructed by the neighbour-joining method. *Listeria monocytogenes* strain AMDK2 was used as an out-group. Bootstrap values (%) based on 1000 replications are given at nodes.

7.4.3. Probiotic attributes

7.4.3.1. In vitro gastrointestinal stress tolerance test

The survival of microorganisms inhabiting the gastro-intestinal tract is dictated by their ability to withstand physical and chemical mechanisms active in the host[24]. So the viability of *B. subtilis* AMS6 in simulated gastric and small intestinal fluid as compared with *L. rhamnosus* MTCC 1408 was checked and is presented in Table 7.1. In the present study, the percentage survivability of *B. subtilis* AMS6 was

found to be >69 % after 3 h incubation at highly acidic pH 2.0. However, survivability of *B. subtilis* AMS6 increased upto 90.95 % compared to 95.2 % demonstrated by probiotic reference strain *L. rhamnosus* MTCC 1408 after 3h of exposure to simulated gastric fluid (pH 4.0).

Table 7.1 Percentage viability of *B. subtilis* (AMS6) and *L. rhamnosus* (L.r) under simulated gastric fluid (SGF) and simulated Intestinal fluid (SIF) transit

Time	Strains	pH 2.0 (SGF)	pH 3.0 (SGF)	pH 4.0 (SGF)	pH6.8.0 (SIF)	pH 8.0 (SIF)
1h	AMS6	76.74 ± 0.64[ade]	85.08±1.3[ab]	91.58±1.58[a]	95.68±1.02[abcf]	93.38±0.52[ac]
	L.r	85.59 ± 2.04[b]	95.78±3.18[b]	98.15±0.6[b]	95.12±1.55[abcf]	98.13±0.47[b]
2h	AMS6	71.13 ± 3.17[af]	82.46±8.045[ac]	91.13±0.19[a]	93.86±2.73[ac]	91±0.97[ac]
	L.r	81.33 ±1.37[beg]	91.4±7.13[bc]	95.76±0.5[c]	98.08±0.22[bcf]	94.96±2.8[a]
3h	AMS6	69.58 ± 2.33[cf]	78.57±2.68[c]	90.95±0.68[a]	91.96±1.39[ae]	91.64±3.12[ac]
	L.r	77.36 ± 2.02[dg]	97.44±0.33[b]	95.2±0.096[c]	97.58±1.49[cf]	93.84±0.31[ac]
4h	AMS6	ND	ND	ND	89.75±0.512[de]	90.177±0.36[cd]
	L.r	ND	ND	ND	97.91±0.7[f]	93.28±0.31[ad]

Data are expressed as mean ± Standard deviation. Significant differences are determined by Tukey's multiple comparison test at $p<0.05$. Values on the same column with different letters are significantly different. ND indicates not determined.

B. subtilis AMS6 recorded survivability of >89 % under simulated intestinal fluid (SIF) transit tolerance. The viability varied significantly ($p < 0.05$) at pH 2.0 and 8.0 between *B. subtilis* AMS6 and *L. rhamnosus* MTCC 1408. It has been previously reported that some strains of *B. subtilis* possess high level of viability in simulated gastric and intestinal fluids at pH 2.0 and 8.0 respectively[25]. Hence our results corroborate these findings.

7.4.3.2. Antibacterial activity

The isolate showed antibacterial activity against different indicator strains tested such as *Listeria monocytogenes* (KF894986), *K. pneumoniae* (MTCC 618), *Salmonella enterica typhimurium (*MTCC 1252*), Pseudomonas aeruginosa* (MTCC 7815), and *M. smegmatis* (ATCC 14468) (Table 2). The highest zone of inhibition (22

mm) was formed against *Salmonella enterica typhimurium* followed by 16 mm zone against *M. smegmatis* (Table 7.2). Since *S. typhimurium* is a major cause of calf morbidity and mortality[26, 27], the administration of probiotics that may specifically inhibit these pathogenic strains while conferring health benefit on the host is indispensible. *L. monocytogenes* causes invasive diseases in ruminants (cattle, sheep, and goats) including encephalitis and uterine infections. Improperly fermented silage contaminated by soil and crops can allow subsequent amplification of *L. monocytogenes* to high numbers; therefore, silage feeding with the isolate *B. subtilis* AMS6 could potentially inhibit the growth of these pathogenic bacteria, thus preventing the onslaught of eye infections and keratitis[28]. *B. subtilis* AMS6 shows significant antibacterial activity against both these pathogenic bacterial strains, thus envisaging a promising application as an animal feed additive.

Table 7.2 Antibacterial activity

Indicator strains	**Zone of inhibition (mm)**
Listeria monocytogenes (KF894986)	12
Klebsiella pneumoniae (MTCC 618)	12
Salmonella enterica typhimurium (MTCC 1252)	22
Pseudomonas aeruginosa (MTCC 7815)	12
Mycobacterium smegmatis (ATCC 14468)	16

Zone of inhibition showed are mean of three replicates.

7.4.3.3. Autoaggregation, co-aggregation and hydrophobicity of isolate

According to Pithva et al.[29], with an increase in incubation time the autoaggregation increases rapidly in case of *L. rhamnosus*. In the present study, *B. subtilis* AMS6 showed a significant autoaggregation pattern with highest degree of autoaggregation after 24 h incubation (64.7 ± 6.4 %). These values were comparable with those of the reference strain *L. rhamnosus* MTCC 1408 (Fig. 7.3A). The isolate showed co-aggregation (Fig. 7.3B) with all the tested pathogens and the highest was observed against *Salmonella enterica typhimurium* (50.6 ± 1.7 %) followed by *L. monocytogenes* (48.9 ± 6.5 %). The reference strain showed comparable co-aggregation abilities. Co-aggregation with a pathogen enables the probiotic strain to

produce antimicrobial substances in a very close vicinity of them which may inhibit the growth of pathogenic strains in the urogenital and gastrointestinal tracts[30].

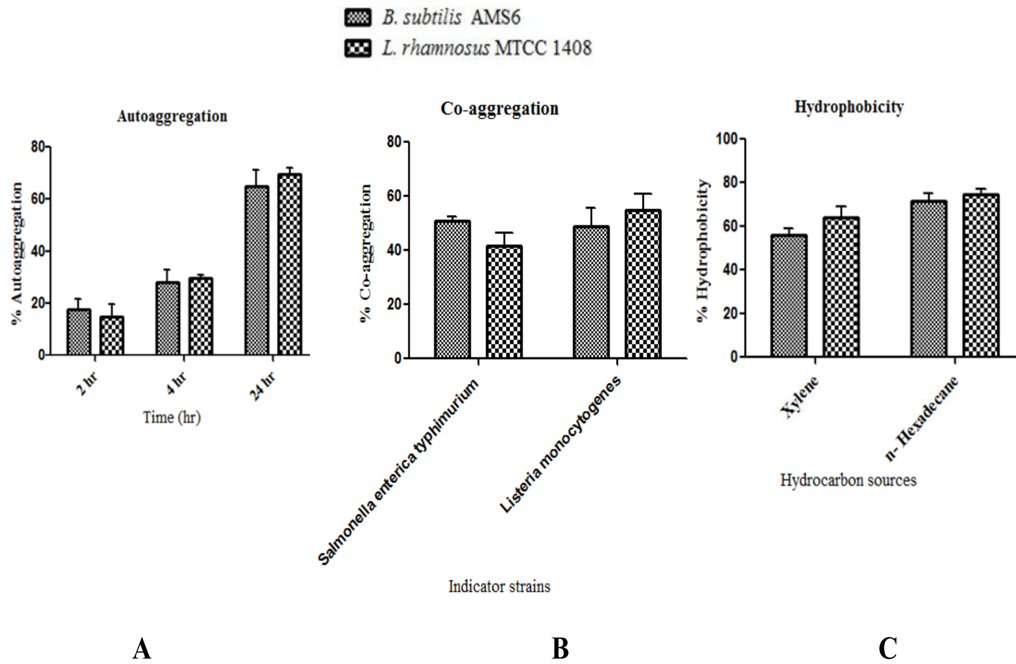


Fig. 7.3 (A) Autoaggregation; **(B)** Co-aggregation with *Salmonella enterica typhimurium* (S.t) and *Listeria monocytogenes* AMDK2 (L.m) and **(C)** Cell surface hydrophobicity of *B. subtilis* (AMS6) and *L. rhamnosus* MTCC 1407 (L.r).

Hydrophobicity measures the capacity of a bacterial strain to adhere to hydrocarbons and holds a strong relationship with ability to adhere to the epithelium of digestive tract[31]. The percent cell surface hydrophobicity of *B. subtilis* AMS6 was compared with *L. rhamnosus* and it was found that *B. subtilis* AMS6 showed maximum hydrophobicity with n-hexadecane (71.4 ± 3.8 %) followed by xylene (56.1 ± 3.1 %) (Fig. 7.3C). Thankappan et al.[32] reported 33 % cell surface hydrophobicity of *Bacillus* spp. using xylene. Microbial adhesion to n-hexadecane reveals cell surface hydrophobicity or hydrophilicity because electrostatic interactions are absent[33]. Therefore, *B. subtilis* AMS6 shows substantial hydrophobicity as compared with *L. rhamnosus* which further reflects its adhesion ability to *in-vivo* conditions on epithelial gut surfaces.

7.4.3.4. Adhesion and inhibition of pathogen adhesion to Caco-2 cells by *B. subtilis* AMS6

The adhesion of bacteria to epithelial cells such as Caco-2 cell line is considered as one of the selection criteria for probiotic strains[34]. *B. subtilis* AMS6 was examined for its capability to adhere to Caco-2 cells using *L. rhamnosus* MTCC 1408 as a reference strain. Microscopic studies showed adhesion between Caco-2 cells and probiotic *B. subtilis* AMS6 strain (Fig. 7.4A) after gram staining. The adhesion results showed that *B. subtilis* AMS6 (8.3 ± 0.52 %) was able to adhere to Caco-2 cells with no significant difference in comparison with the reference strain (10.41 ± 0.49 %) (Fig. 7.4B).

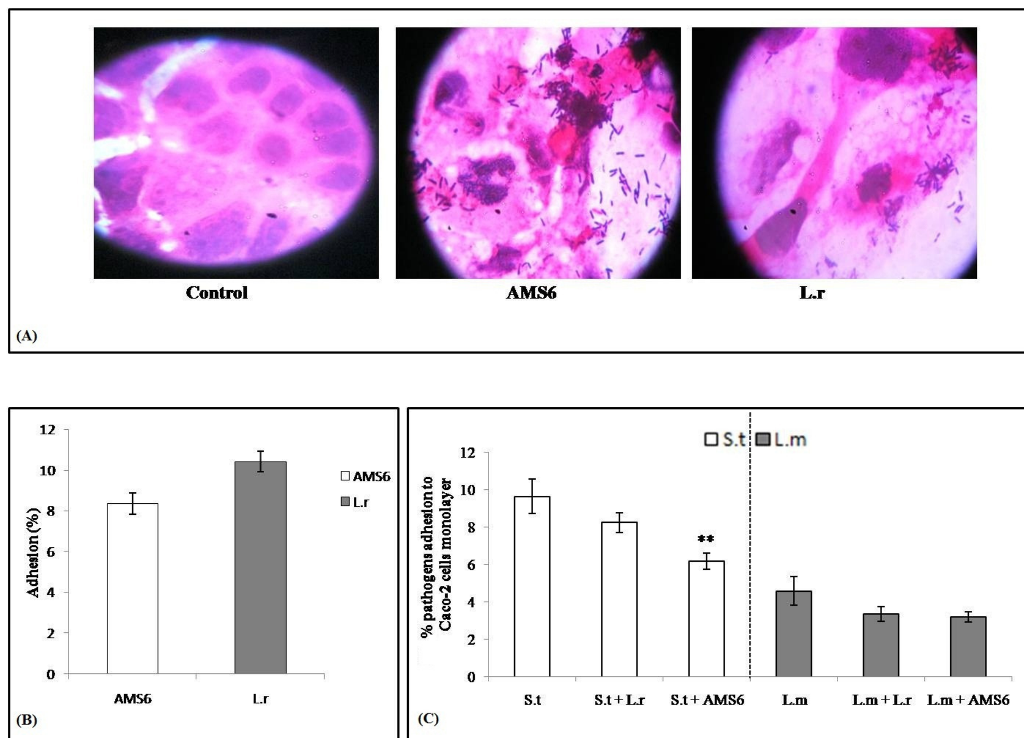


Fig. 7.4 (A) Adhesion of *B. subtilis (*AMS6) and *L. rhamnosus* MTCC 1408 (L.r) to Caco-2 cell line observed by Gram staining under bright field microscope at 100 x. Control indicates only gram stained Caco-2 cells. **(B)** Adherence (%) of *B. subtilis (*AMS6) and *L. rhamnosus* MTCC 1408 (L.r) to Caco-2 monolayer. **(C)** Percentage adhesion of *Listeria monocytogenes* AMDK2 (L.m) and *Salmonella enterica typhimurium* (S.t) to Caco-2 monolayer in the presence or absence of probiotic isolate.

The adhesion capacity observed in *B. subtilis* AMS6 was more as compared with previously reported *Bacillus subtilis var. Natto* and *B. subtilis* PY79[35]. Our study suggested that *B. subtilis* AMS6 significantly (P < 0.01) reduced the adherence of *Salmonella enterica typhimurium* (MTCC 1252) to Caco-2 cells by 35.83 ± 0.42 % whereas *Listeria monocytogenes* AMDK2 (KF894986, local isolate) adherence was reduced by 28.20 ± 0.27 %. *L. rhamnosus* MTCC 1408 also showed inhibition of *Listeria monocytogenes* AMDK2 and *Salmonella enterica typhimurium* (MTCC 1252) adherence by 24.51 ± 0.37 % and 14.47 ± 0.53 % respectively (Fig. 7.4C). Anti-adherent evaluation of novel probiotic is of significance for the inhibition of pathogen infections[36]. Therefore, *B. subtilis* AMS6 can be considered as a novel potential probiotic to prevent animal diseases and improve safety of animals.

7.4.4. Antibiotic susceptibility test

Antibiotic susceptibility test indicated that *B. subtilis* AMS6 was susceptible to each tested antibiotic included in Table 7.3 which ensures its inability to transfer antibiotic resistance, except for penicillin G (1 unit). Resistance to penicillin G may be an intrinsic characteristic of *B. subtilis* AMS6 and might be useful for the *Bacillus* taxonomy. Our results corroborate the finding of Hoa et al.[37] who reported resistant nature of probiotic *Bacillus* against penicillin and ampicillin. The resistance nature to some antibiotics could be used for both preventive and therapeutic purposes in fast restoration of normal micobiota ratio during antibiotic therapy[38].

Table 7.3 Antibiotic Susceptibility Test

Antibiotics	Zone of inhibition (mm)
Ampicillin (10 μg)	+
Chloramphenicol (25 μg)	+++
Penicillin G (1 Unit)	−
Streptomycin (10 μg)	++
Sulphatriad (300 μg)	+
Tetracycline (25 μg)	+++

Zone of inhibition showed are mean of three replicates, + indicate 10–20 mm zone of inhibition, ++ indicate 20–30 mm zone, +++ indicate >30 mm zone, − shows no zone of inhibition.

7.4.5. Hemolysis

The safety of *B. subtilis* AMS6 towards its use as potential probiotics was confirmed by its non-hemolytic activity on goat blood agar plate (Fig. 7.5). Similar results were also reported by Cao et al.[39] and Sorokulova et al.[40] on rabbit blood agar and sheep blood agar, respectively.

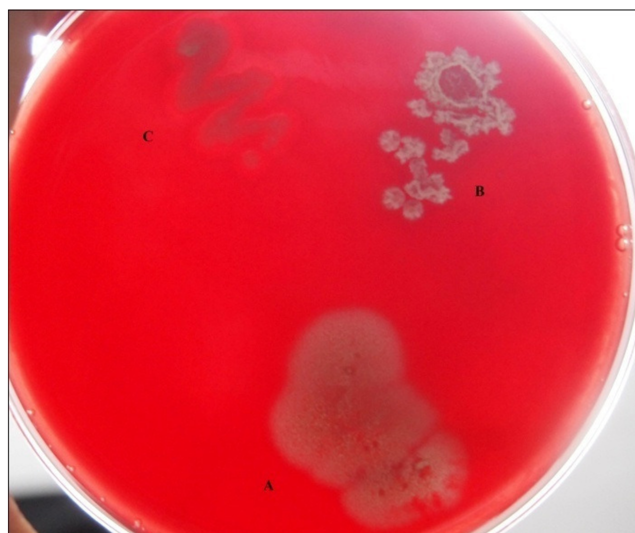


Fig. 7.5 Hemolysis assay on blood agar. No hemolysis was observed on experimental isolates A (*B. amyloliquefaciens* AMS1, a probiotic isolate studied in previous chapter) and B (*B. subtilis* AMS6, Present study). C indicates positive control, *Bacillus cereus* (MTCC 430) showing hemolysis.

7.4.6. Cellulolysis and SEM analysis of cellulolytic activity on substrate

The isolate formed a zone of hydrolysis on carboxymethyl cellulose agar plate on flooding with Gram's iodine, thus confirming its cellulolytic potential (Fig. 7.6A). Further, the maize (*Zea mays*) straw powder was treated with crude culture of *B. subtilis* AMS6. The morphological changes after the treatment were recorded by SEM. As shown in Fig. 5, the control figure (Fig. 7.6B) depicts the structure of the guard and subsidiary cells in maize (*Zea mays*) leaves whereas the treated sample (Fig. 7.6C) is found to be disordered and the peeling off of the linters is clearly visible. The entwined cellulosic skeleton is swollen as a result of imbibition of probiotic culture showing cellulase activity at 37 °C in the cellulase plate assay. The isolate showed significant cellulolytic activity at 37 °C (0.54 ± 0.05 filter paper units ml^{-1}) which was more than that at 15, 25, and 45 °C temperature (Fig. 7.6D). The

difference in cellulolytic activity at 37 °C as compared with activity at other temperatures was found to be significant (*P* <0.05). The isolate showed a remarkable cellulolytic activity as SEM revealed erosion of cell wall and different levels of degradation which is accordance with previous study. The colonized cellulose substrates showed cavity formation and hollow degradation patterns similar to that observed with efficient cellulase producing *Micromonospora* pure cultures[41]. Results of this study suggest that *B. subtilis* AMS6 very efficiently digests cellulose which makes this strain a better candidate for its industrial application in animal feed as well as food and fermentation industries.

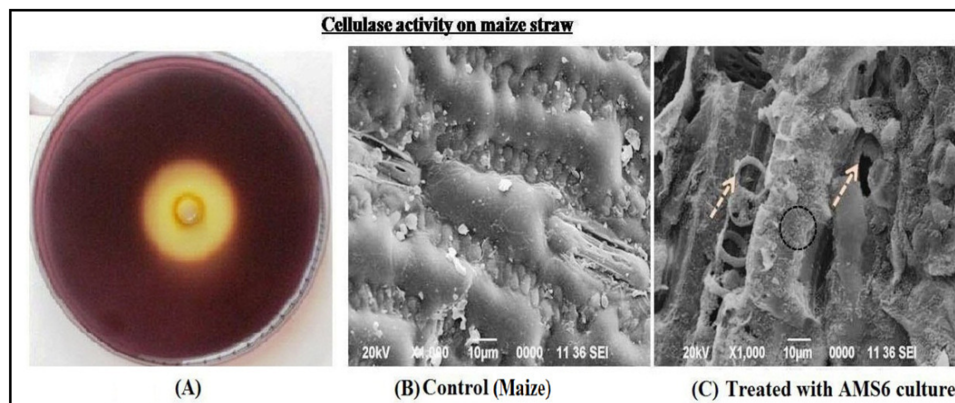
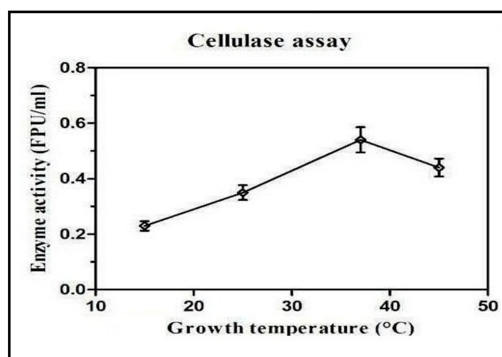


Fig. 7.6 (A) Screening of cellulase activity of *B. subtilis* AMS6 by plate assay. Scanning electron micrograph of **(B)** maize (*Zea mays*) straw without AMS6 culture and **(C)** maize (*Zea mays*) straw with AMS6 culture showing degradation of cellulose incubated at 37 °C. Arrow indicates degradation area and circle indicates adhered *B. subtilis* AMS6 cells.



(D)

Fig. 7.6 (D) Optimization of cellulase production at different tempearture.

7.4.7. Identification of cellulase gene in *B. subtilis* AMS6

The primers amplified approximately 1500 base pairs of the cellulase gene (Fig. 7.7A). The nucleotide sequence of the cellulase gene was deposited into the

NCBI GenBank database as *Bacillus subtilis* strain ASM6 cellulase gene under accession number KP724684. The phylogenetic studies of cellulase gene (Fig. 7.7B) showed close relationship to cellulase of *Bacillus subtilis* (ABS70712).

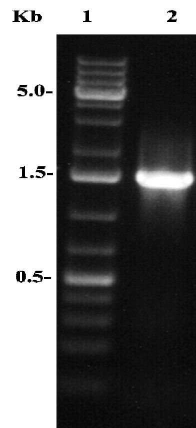


Fig. 7.7A Gel electrophoresis image showing amplification of AMS6 cellulase gene. Lane 1: marker ($1kb^+$); Lane 2: cellulase gene of 1.5 Kb size.

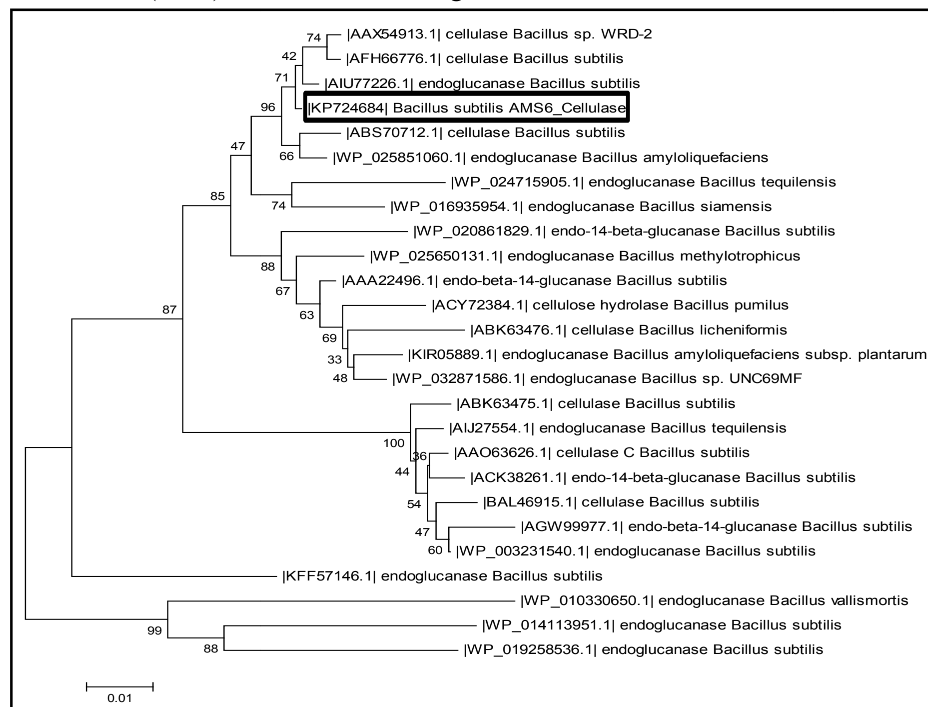


Fig. 7.7B Phylogenetic tree showing the relationship of strain *B. subtilis* AMS6 cellulase gene sequence with closely related species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

The open reading frame of the cellulase gene consists of 1499 nucleotides encoding a protein of 499 amino acids (Fig. 7.8).The isoelectric point (pI)

and molecular weight (Mw) of ASM6 cellulase gene was predicted to be 7.12 and 55.04 kDa, respectively. The SignalP 4.1 program revealed a 29 amino acid signal peptide at N-terminal region. The sequence analysis performed using Motif Finder online tool (http://www.genome.jp/tools/motif/) showed that the amino acids sequence consisted of a glycosyl hydrolase family 5 domain (12-83 bp) as reported earlier[42] ; Glycosyl hydrolase catalytic core (225-293 bp) and a CBM-3 cellulose binding domain (357-435 bp) at its C terminal (Fig. 7.9).

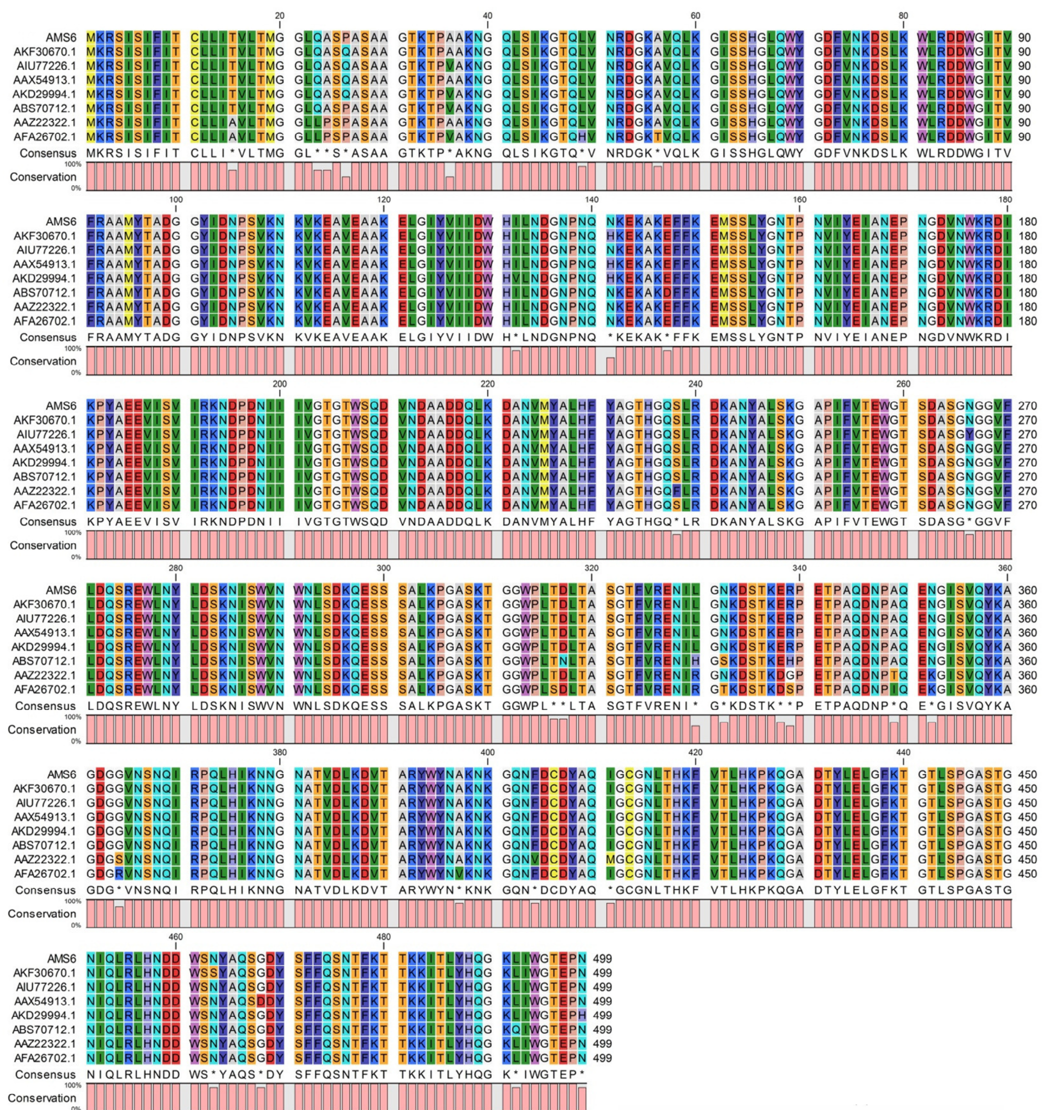


Fig. 7.8 Multiple sequence alignment of AMS6 cellulase gene with other reported cellulase gene retrieved from databases at the NCBI using the BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

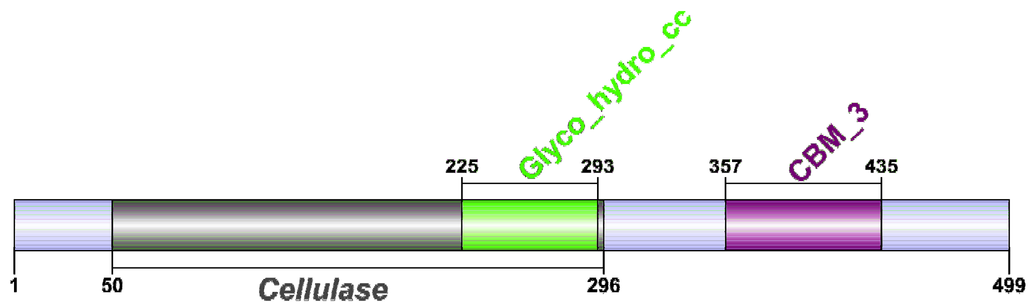


Fig. 7.9 Domain structure of AMS6 cellulase gene prepared using DOG 1.0.

7.4.8. Filter paper degradation

Filter paper degradation results showed that Whatman no.1 filterpaper (1 × 6 cm) was completely disintegrated by *B. subtilis* AMS6 within 96 h of incubation (Fig. 7.10). This shows the tremendous cellulolytic potential of the strain under investigation.

Fig.7.10 Filter paper degradation by *B. subtilis* AMS6 in basal salt medium supplemented with Whatman filter paper no.1 (1 × 6 cm strip × 2) at the end of 96 hrs of incubation represented by ‘S’. Flask marked ‘control’ is the control for this experimental set up and does not show any filter paper degradation.

7.5. Conclusion

In the present work, *B. subtilis* AMS6, isolated from a traditional fermented food Churpi, displayed potential probiotic characteristics as well as significant cellulolytic activity *in vitro*. *B. subtilis* AMS6 showed most of the probiotic functional characteristics desirable to survive harsh physico-chemical environment present in the gastrointestinal tract. The ability to inhibit the adherence of pathogens to Caco-2 epithelial cell lines emphasizes the potentiality of the isolate to prevent animal diseases and improve health of animals. The isolate was able to degrade maize (*Zea mays*) straw, CMC and filter paper conferring cellulolytic potential on the bacterium. This explores the possibility of combining cellulase producing ability of a microbe with its probiotic attributes to enhance gut health of animal and digestibility of the feed. *In vitro* studies prove the potentiality of *B. subtilis* AMS6 to be used as an animal feed additive although the work needs to be further affirmed by *in vivo* studies.

7.6. References

1. Araya, M., et al. Guidelines for the evaluation of probiotics in foods, *FAO/WHO report*, 2002.
2. Guo, L., et al. Probiotic properties of *Enterococcus* strains isolated from traditional naturally fermented cream in China, *Microb. Biotechnol*. **2015**, doi: 10.1111/1751–7915.12306.
3. Kaushik, J.K., et al. Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*, *PloS One* **4**:e8099, 2009.
4. Del Re, B., et al. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*, *Lett. Appl. Microbiol.* **31**, 438--442, 2000.
5. Brashears, M.M., et al. Prevalence of *Escherichia coli* O157:H7 and performance by beef feedlot cattle given *Lactobacillus* direct-fed microbials, J. Food Prot. **66**, 748--754, 2003.
6. Maragkoudakis, P.A., et al. Probiotic potential of *Lactobacillus* strains isolated from dairy products, *Int. Dairy J.* **16**, 189--199, 2006.
7. Peterson, R,E., et al. Effect of *Lactobacillus acidophilus* strain NP51 on *Escherichia coli* O157:H7 fecal shedding and finishing performance in beef feedlot cattle, *J. Food Prot.* **70**, 287--291, 2007.
8. Tabe, E.S., et al. Comparative effect of direct-fed microbials on fecal shedding of *Escherichia coli* O157:H7 and *Salmonella* in naturally infected feedlot cattle, *J. Food Prot.* **71**, 539--544, 2008.
9. Younts-Dahl, S.M., et al. Dietary supplementation with *Lactobacillus*- and *Propionibacterium*-based direct-fed microbials and prevalence of *Escherichia coli* O157 in beef feedlot cattle and on hides at harvest, *J. Food Prot.* **67**, 889--893, 2004.
10. Ji, J., et al. Probiotic *Bacillus amyloliquefaciens* mediate M1 macrophage polarization in mouse bone marrow-derived macrophages, *Arch. Microbiol*. **195**, 349--356, 2013.
11. Cutting, S.M. *Bacillus* probiotics, Food Microbiol. **28**, 214--220, 2011.
12. Chaucheyras-Durant, F., & Durant, H. Probiotics in animal nutrition and health, *Benef. Microbes* **1,** 3--9, 2010.

13. Guo, X.H., et al. Screening lactic acid bacteriam from swine origins for multistrain probiotics based on in vitro functional properties, *Anaerobe* **16,** 321--326, 2010.

14. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, *J. Mol. Evol.* **16**, 111--120, 1980.

15. Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783--791, 1985.

16. Tamura, K., et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, *Mol. Biol. Evol.* **28**, 2731--2739, 2011.

17. Aslim, B., et al. Determination of the bacteriocin-like substances produced by some lactic acid bacteria isolated from Turkish dairy products, *LWT – Food sci. technol.* **38**, 691--694, 2005.

18. Bauer, A.W., et al. Antibiotic susceptibility testing by a standardized single disk method, *Am. J. Clin. Pathol.* **45**, 493--496, 1966.

19. Anand, C., et al. Pig and goat blood as substitutes for sheep blood in blood-supplemented agar media, *J. Clin. Microbiol.* **38**, 591--594, 2000.

20. Kasana, R.C., et al. A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine, *Curr. Microbiol.* **57**, 503--507, 2008.

21. Ren, J., et al. DOG 1.0: illustrator of protein domain structures, *Cell Res.* **19**, 271--273, 2009.

22. Ghose, T.K. Measurement of cellulase activity, *Pure. Appl. Chem.* **59,** 257–268, 1987.

23. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.* **31**, 426--428, 1959.

24. Canganella, F., et al. A microbiological investigation on probiotic pharmaceutical products used for human health, *Microbiol. Res.* **152**, 171--179, 1997.

25. Wang, Y., et al. In vitro assessment of probiotic properties of *Bacillus* isolated from naturally fermented congee from Inner Mongolia of China, *World J. Microbiol. Biotechnol*. **26**, 1369--1377, 2010.

26. Rothenbacher, H. Mortality and morbidity in calves with salmonellosis, *J. Am. Vet. Med. Assoc.* **147**, 1211--1214, 1965.

27. Sojka, W.J., et al. Incidence of salmonella infection in animals in England and Wales 1968—1974, *J. Hyg. (Lond).* **78**, 43--56, 1977.

28. Nightingale, K.K., et al. Ecology and Transmission of *Listeria monocytogenes* Infecting Ruminants and in the Farm Environment, Appl. Environ. Microbiol. **70,** 4458--4467, 2004.

29. Pithva, S., et al. Probiotic attributes of autochthonous *Lactobacillus rhamnosus* strains of human Origin, *Appl. Biochem. Biotechnol.* **173**, 259--277, 2014.

30. Reid, G., et al. *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability of uropathogens, *Can. J. Microbiol.* **34**, 344--351, 1988.

31. Kos, B., et al. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92, *J Appl. Microbiol.* **94**, 981--987, 2003.

32. Thankappan, B., et al. Characterization of *Bacillus* spp. from the gastrointestinal tract of *Labeo rohita*--towards to identify novel probiotics against fish pathogens, *Appl. Biochem. Biotechnol.* **175**, 340--53, 2015.

33. Bhanwar, S., Singh, A., & Ganguli, A. Probiotic characterization of potential hydrolases producing *Lactococcus lactis* subsp. *lactis* isolated from pickled yam, *Int. J. Food Sci. Nutr.* **65**, 53--61, 2014.

34. Botes, M., et al. Adhesion of the probiotic strains *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells under conditions simulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments, *Arch. Microbiol*. **190**, 573--584, 2008.

35. Hong, H.A., et al. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics, *J. Appl. Microbiol.* **105**, 510–20, 2008.

36. Sribuathong, S., Saengprakai, J., & Trevanich, S. In vitro anti-adherent assessment of selected lactic acid bacteria isolates against *salmonella typhimurium* and *listeria monocytogenes* to Caco-2 cells, *J. Food Saf.* **34,** 270--282, 2014.

37. Hoa, N.T., et al. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders, *Appl. Environ. Microbiol.* **66**, 5241--5247, 2000.

38. Lata, S., et al. *In vitro* profile based selection of indigenous actinobacterial probiont *Streptomyces* sp. JD9 for enhanced broiler production, *J. Biosci. Bioeng.* **121**,124-131, 2016.

39. Cao, H., et al. *Bacillus amyloliquefaciens* G1: A potential antagonistic bacterium against Eel-pathogenic *Aeromonas hydrophila*, *Evid. Based Complement Alternat. Med.* **2011**, 824104, 2011.

40. Sorokulova, I.B., et al. The safety of two *Bacillus* probiotic strains for human use, *Dig Dis. Sci.* **53**, 954--963, 2008.

41. de Menezes, A.B., et al. Cellulose degradation by *micromonosporas* recovered from freshwater lakes and classification of these *actinomycetes* by DNA gyrase B gene sequencing, *Appl. Environ. Microbiol.* **74**, 7080--7084, 2008.

42. Pandey, S., et al. Cloning and expression of β-1,4-endoglucanase gene from *Bacillus subtilis* isolated from soil long term irrigated with effluents of paper and pulpmill, *Microbiol. Res.* **169**, 693--698, 2014.