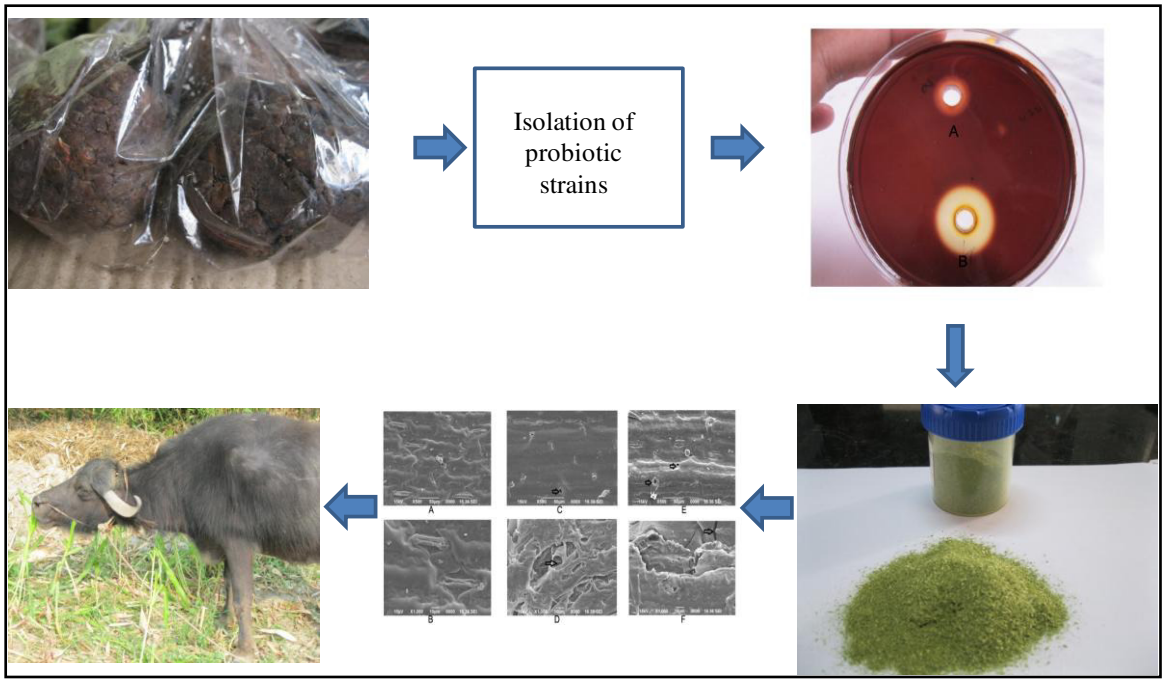
Chapter 5

IN VITRO EVALUATION OF CELLULOYTIC *BACILLUS AMYLOLIQUEFACIENS* AMS1 ISOLATED FROM TRADITIONAL FERMENTED SOYBEAN (CHURPI) AS AN ANIMAL PROBIOTIC



Graphical Abstract

IN VITRO EVALUATION OF CELLULOYTIC *BACILLUS AMYLOLIQUEFACIENS* AMS1 ISOLATED FROM TRADITIONAL FERMENTED SOYBEAN (CHURPI) AS AN ANIMAL PROBIOTIC

5.1. Abstract

A microorganism showing probiotic attributes and hydrolyzing carboxymethylcellulose was isolated from traditional fermented soybean (Churpi) and identified as *Bacillus amyloliquefaciens* by analysis of 16S rRNA gene sequence and named as *B. amyloliquefaciens* AMS1. The potentiality of this isolate as probiotic was investigated *in vitro* and it showed gastrointestinal transit tolerance, cell surface hydrophobicity, cell aggregation and antimicrobial activity. The isolate was found to be non-haemolytic which further strengthens its candidature as a potential probiotic.The maize straw digestion was confirmed by scanning electron microscopy studies. The isolate was able to degrade filter paper within 96 hours of incubation. The study opens up new avenues of combining the cellulase degrading capability of a microbe with its probiotic attributes to be used as animal feed additives for better digestibility of the feed and enhanced productivity of the animal.

5.2. Introduction

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host[1]. Probiotics exert beneficial effects on the host by providing nutrients and enzymatic contribution to digestion, improving water quality, enhancing growth, inhibiting pathogenic microorganisms and enhancing immune responses[2, 3]. The supplementation of Lactic Acid Bacteria (LAB) as probiotics in ruminants has been extensively studied as compared with *Bacillus* probiotics[4-8]. However there are very few reports on cellulolytic activities of LAB. Since *Bacillus* is spore forming, it has an advantage over other non-spore formers such as *Lactobacillus* spp., of surviving the low pH of the stomach. *Bacillus amyloliquefaciens* is a potential probiotic strain[9, 10]. *B. amyloliquefaciens* have also been found to have cellulolytic activity[11]. The enzymes produced by *B. amyloliquefaciens* are expected to be able to transform complex molecules particularly lignocelluloses, which become the limiting factor in animal feed, into

simpler molecules[12]. There is a demand for enhanced digestibility of animal feeds and it needs efficient cellulolytic microbes. It has been found that only a small subset of rumen microorganisms that include cellulolytic bacteria, fungi and protozoa, have the capacity to initiate degradation of plant cell walls. However, the most numerous group of rumen microorganisms are non-cellulolytic bacteria, and only actively cellulolytic rumen species have been found to cause extensive solubilization of plant cell wall material in pure culture[13]. Cellulolytic bacteria play an important role in energy supply for forage animals. According to Varga and Kovler[14], feed fibres were not completely converted to animal product in intensive animal farming and 20-70% undigested cellulose was carried out with feces. Keeping this in view, there is a possibility of combining the probiotic attributes of a *Bacillus* strain and its cellulose degrading capability to enhance the digestibility of animal feed and the productivity of animals.

This study discusses the potential probiotic attributes of a novel cellulolytic *B. amyloliquefaciens* strain. In the present study fermented soybean (Churpi) was collected from Bomdila, Arunachal Pradesh, India. Churpi is a fermented product that is prepared from local varieties of soybean seeds by *Monpa* tribe of Arunachal Pradesh, India. The topography is mostly mountainous and covered with the Himalayas which favor different habitats and microflora of beneficial use. In recent times, extensive research has been carried out on isolation and screening of microbes from traditional fermented foods due to their eco-friendly and genetically sturdy nature[15]. In addition, soybean meal is an important protein source in animal feed[16].

Here we report cellulolytic *B. amyloliquefaciens* AMS1 isolated from fermented soybean as a potential animal probiotic that could enhance feed digestibility.

5.3. Materials and methods

5.3.1. Sampling and isolation of bacteria

An indigenous fermented soybean product 'Churpi' prepared from brown local varieties of soybean seeds was sampled from Arunachal Pradesh, India. The

sample was collected and stored at 4 ºC until the study was performed. 1 gm of sample was appropriately diluted using 0.9% NaCl up to 10^{-8} dilutions. An aliquot of 0.1 ml of each dilution was plated on Nutrient agar plate and MRS agar plate. The isolate was first tested for its potential probiotic attributes and further examined for its cellulose degrading capability.

Fig. 5.1 Fermented soybean (libi Churpi)

5.3.2. Molecular identification

Selected isolate based upon potential probiotic attributes was identified by 16S rRNA gene sequence analysis followed by phylogenetic studies. Universal primers 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-GGTTACCTTGTTACGACTT-3´) were used for the amplification of 16S rRNA gene sequence[17]. The PCR amplification was performed in Eppendorf thermocycler in a total volume of 25 µl reaction mixture according to protocol described previously (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, Rotkreuz, 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[18, 19, 20].

5.3.4. Screening of probiotics properties

5.3.4.1. In vitro gastrointestinal stress tolerance test

The bacterial cells were harvested from overnight grown culture of isolated strains and probiotic reference strain *L. rhamnosus* MTCC 1408, grown in Luria Bertani broth and MRS (de Man, Rogosa and Sharpe) broth respectively, were used to harvest the bacterial cells 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) was tested as reported earlier[21]. SGF was prepared by suspending pepsin (0.3 mg/ml; HiMedia) and NaCl (0.5 %, w/v) in sterilized PBS adjusted to pH 2, 3 and 4. Tolerance to small intestinal fluid (SIF) condition was tested in PBS solution adjusted to pH 6.8 and 8.0, containing pancreatin[22] (0.1 mg/ml, Sigma) and 0.3 % (w/v) Oxgall (HiMedia). Bacterial cells in SGF were incubated at 37 ºC for 0, 1, 2 and 3 h, and those in SIF were incubated at 37 ºC for 0, 1, 2, 3 and 4 h. 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).

5.3.4.2. Antibacterial activity

The antimicrobial effects of the bacterial isolate against Gram positive and Gram negative indicator strains were examined by agar well diffusion method. The strains *Bacillus cereus* (MTCC 430), *K. pneumoniae* (MTCC 618), *Yersinia enterocolitica* (MTCC 859), *Salmonella enterica typhimurium* (MTCC 1252) and *Listeria monocytogenes* (KF894986, laboratory isolate) were used as indicator cultures in the study. The antimicrobial activity was recorded as appearance of clear zone around the wells[23].

5.3.4.3. Cell surface hydrophobicity

The cell surface hydrophobicity of the selected isolate was measured by the method described by Rosenberg et al.[24], with slight modifications. The overnight grown culture at 37 ºC was pelleted (6,000 x *g*, 5 min) and the cell pellet was washed twice with phosphate buffer saline and re-suspended in phosphate buffer saline. The absorbance of suspended pellet of the isolate was measured at 600 nm. The cell suspension was thoroughly mixed separately with equal volume of n-hexadecane, chloroform and ethyl acetate by vortexing for 2 min. The two phases were allowed to separate for 1 h and aqueous phase was gently taken out to measure its absorbance at 600 nm. The surface hydrophobicity was calculated as decrease in the absorbance of the aqueous phase after mixing and phase separation relative to that of original suspension ($Abs_{initial}$) as:

$$\text{Hydrophobicity}(\%) = 100 \times \frac{\text{Abs}_{\text{initial}} - \text{Abs}_{\text{final}}}{\text{Abs}_{\text{initial}}}$$

where $Abs_{initial}$ represents initial absorption before mixing with hydrocarbon sources and Abs_{final} represents final absorption after mixing with hydrocarbons.

5.3.4.4. Autoaggregation and co-aggregation

Autoaggregation assay was performed according to Del Re et al[25] as described in previous chapter (Section 3.3.3.4 and 3.3.3.5).

Autoaggregation percentage was expressed as:

$$\text{Autoaggregation}(\%) = 1 - {}^{A_t}/_{A_0} \times 100$$

where A_t = absorbance at different time interval 2, 4, 24 h and A_0 = absorbance at 0 h.

The percentage of co-aggregation was calculated using the equation of Handley et al.[26]:

$$\text{Co-aggregation}\ (\%) = \frac{(\,(Ax + Ay)/2) - A(x + y)}{Ax + Ay/2} \times 100$$

where x and y represent each of the two strains in the control tubes, and (x + y) the mixture.

5.3.4.5. Adhesion and inhibition of pathogen adhesion to Caco-2 cells by *B. amyloliquefaciens* AMS1

The adhesion and inhibition of pathogen adhesion to Caco-2 cells study was performed by using the procedure described by García-Cayuela et al.[27] with slight modification as described previously (Section 3.3.3.7). *L. rhamnosus* MTCC 1408 was used as a reference strain to study adhesion and inhibition of pathogens to Caco-2 cells.

5.3.5. Antibiotic susceptibility test

The antibiotic susceptibility test was performed by following Bauer–Kirby method[28]. The standard antibiotic discs (Octa disc) supplied by HiMedia were used. The zones of inhibition were measured by HiMedia Antibiotic Zonescale.

5.3.6. Cellulolysis

The assessment of cellulolytic potential of the isolate was routinely done on carboxymethylcellulose sodium salt, medium viscosity (CMC) agar plate[29]. CMC agar was prepared by Mineral Salt Medium (MSM) supplemented with 1% CMC (HIMEDIA). Wells were prepared on CMC plate containing culture supernatant and incubated for 18 h at different temperatures and were flooded with Gram's iodine.

5.3.7. Optimization of cellulase production at different temperatures and pH

Production medium (0.5 % CMC, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1g KCl, 0.05 g $MgSO_4$, 0.05 g peptone and 0.1 g glucose for 100 ml) at pH 7.0 was inoculated with overnight grown selected bacterial isolate. The broth was incubated at different temperatures viz. 15, 30, 37, 40, 50, and 60 ˚C for 24 h. At the end of incubation period, the cell-free culture filtrate was obtained, dialyzed (ammonium sulfate precipitation at 80% saturation followed by dialysis using HiMedia Dialysis membrane-110) and used as enzyme source.

Erlenmeyer flasks containing production medium was adjusted to 3.5, 4.5, 5.5 pH (using sodium acetate buffer), 6.5, 7.5 pH (using sodium phosphate buffer) and 8.5 pH (using Tris-HCl) respectively. The cultures were inoculated with the selected

isolate and incubated at 37 ˚C. At the end of incubation period, the cell-free culture filtrate was obtained and dialyzed and used as enzyme source.

5.3.8. Cellulase activity assay

Total cellulase activity was determined by measuring the amount of reducing sugar formed from filter paper[30]. 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 to 1 ml of reaction mixture. The reducing sugars were estimated spectrophotometrically with DNS reagent (1 g DNS, 1.6 g of NaOH and 30 g of potassium sodium tartrate for 100 ml) using glucose as standard[31]. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose per minute.

5.3.9. SEM analysis of the degradation of maize straw

The maize straw used in this study was obtained locally, washed, and dried. The dried maize straw was chopped into small pieces and then ground into smaller homogenous particles using grinder mixer. SEM analysis of the degradation of maize straw by incubating the maize straw powder [2% (w/v)] with dialyzed enzyme for 6 h was done. Samples incubated at 37 and 60 °C were centrifuged for 2500 × *g* for 10 min and supernatant was collected to estimate the amount of reducing sugars released from treated sample. The tested maize straw was fixed with 2.5% glutaraldehyde for 6 h and washed twice with 1X PBS, pH 7.4. Further samples were dehydrated in graded concentration of ethanol. Then specimen were platinum coated using JEOL JFC-1600 auto fine coater and observed under SEM (JEOL model JSM-6390 LV) at 15 kV.

5.3.10. Filter paper degradation

Filter paper degradation was studied by culturing the bacterial isolate in MSM supplemented with Whatman no. 1 filter paper (1 × 6 cm strip × 2, 0.05 g per 20 ml) at the end of 96 h of incubation.

5.4. Results and discussion

5.4.1. Isolation of the bacterial strain

Fifty-two bacterial strains were isolated from Churpi based on different colony morphology (Appendix- 16). On further screening, only one strain (AMS1) showed probiotic attributes. This isolate also showed cellulolytic activity on CMC agar plate and was selected for further studies.

5.4.2. Molecular identification

The 16S rRNA gene sequence of the isolate was used for constructing phylogenetic tree (Fig. 5.2) with the neighbor-joining method[32]. The isolate showed maximum sequence similarity (99%) with *Bacillus amyloliquefaciens* M69 (GQ340480.1). The 16S rRNA gene sequence was submitted to NCBI gene bank with GenBank Accession: KJ162392 and the isolate was named as *B. amyloliquefaciens* AMS1.

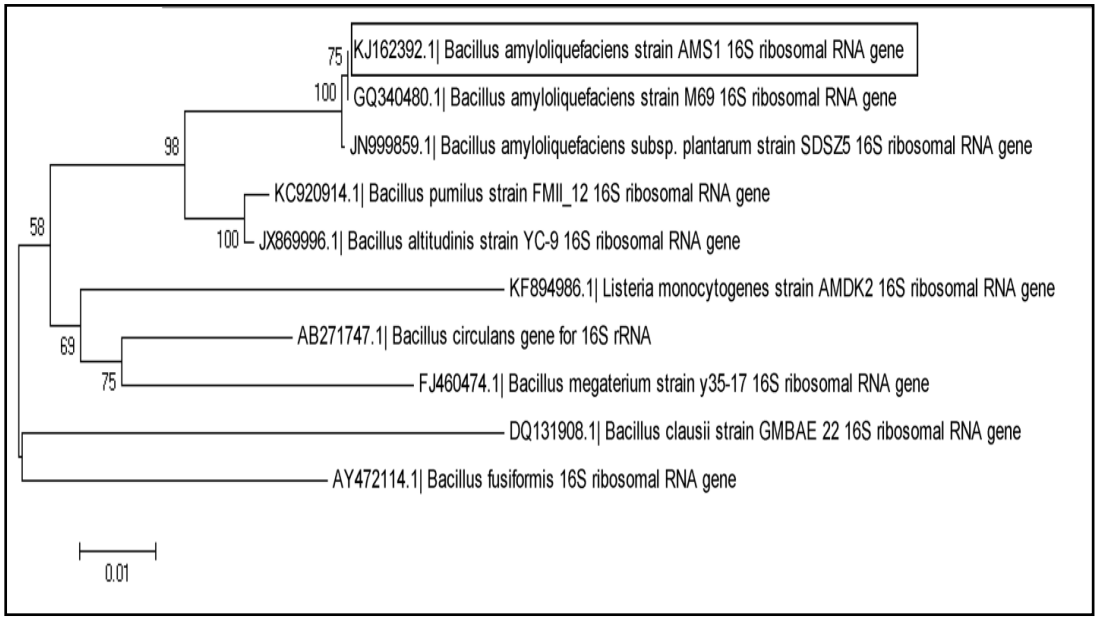


Fig. 5.2 Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain *B. amyloliquefaciens* AMS1 with closely related species. Tree was constructed by the neighbor-joining method. *Listeria monocytogenes* strain AMDK2 was used as an out-group. Bootstrap values (%) based on 1000 replications are given at nodes.

5.4.3. Screening of probiotic properties

5.4.3.1. In vitro gastrointestinal stress tolerance test

The effect of simulated gastric and small intestinal transit tolerance on the viability of the *B. amyloliquefaciens* AMS1 as compared with *L. rhamnosus* MTCC 1408 is presented in Table 5.1A and 5.1B. It has been previously reported that some strains of *B. amyloliquefaciens* possess high level of survival in simulated gastric and intestinal fluids at pH 2.0 and 8.0 respectively[33]. In the present study, the percentage survivability of *B. amyloliquefaciens* AMS1 was found to be >70% after 3h incubation period in the simulated gastric and intestinal fluid which is comparable with that of *L. rhamnosus* MTCC 1408. However, the viability varied significantly ($p<0.05$) at pH 2.0 and 8.0 between the two strains. It has been previously observed that *Bacillus* spp. is weakly tolerant or sensitive to bile salt concentration[33]. Hence our results are in agreement with those findings.

Table 5.1A Percentage viability of *B. amyloliquefaciens* AMS1 and *L. rhamnosus* under simulated gastric fluid (SGF) transit

	Strains	**pH 2.0 (SGF)**	**pH 3.0 (SGF)**	**pH 4.0 (SGF)**
1 h	*B. amyloliquefaciens* AMS1	76.91 ± 2.47^{a}	89.75 ± 0.41^{a}	90.60 ± 3.23^{a}
	L. rhamnosus MTCC 1408	83.30 ± 3.09^{bd}	89.53 ± 4.18^{a}	88.59 ± 2.54^{a}
2 h	*B. amyloliquefaciens* AMS1	71.03 ± 1.69^{a}	82.01 ± 3.86^{ade}	90.70 ± 1.64^{a}
	L. rhamnosus MTCC 1408	85.23 ± 2.04^{cd}	78.83 ± 2.39^{afg}	88.83 ± 3.81^{a}
3 h	*B. amyloliquefaciens* AMS1	70.07 ± 3.20^{a}	80.21 ± 1.14^{bdfh}	91.58 ± 0.88^{a}
	L. rhamnosus MTCC 1408	75.47 ± 1.94^{a}	78.83 ± 2.39^{cegh}	91.02 ± 2.62^{a}

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.

Table 5.1B Percentage viability of *B.amyloliquefaciens* AMS1 and *L. rhamnosus* under simulated Intestinal fluid (SIF) transit

	Strains	pH 6.8 (SIF)	pH 8.0 (SIF)
1 h	*B. amyloliquefaciens* AMS1	98.27±2.19^{a}	92.66±1.00^{a}
	L. rhamnosus MTCC 1408	90.1±2.12^{bd}	80.33±5.59^{bg}
2 h	*B. amyloliquefaciens* AMS1	98.88±0.44^{a}	89.75±0.41^{ah}
	L. rhamnosus MTCC 1408	89.81±2.58^{cd}	70.42±2.87^{ci}
3 h	*B. amyloliquefaciens* AMS1	95.1±1.08^{a}	82.01±3.86^{dgh}
	L. rhamnosus MTCC 1408	91.69±2.54^{ad}	70.62±1.78^{ei}
4 h	*B. amyloliquefaciens* AMS1	94.69±3.02^{ad}	84.75±2.84^{ag}
	L. rhamnosus MTCC 1408	92.77±2.66^{ad}	66.32±2.02^{fi}

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.

5.4.3.2. Antibacterial activity

The isolate showed antibacterial activity against two indicator strains tested (Table 5.2). The highest zone of inhibition (11 mm) was formed against *Listeria monocytogenes* (MTCC 839) followed by 10 mm zone against *K. pneumonia* (MTCC 618).

Table 5.2 Antimicrobial activity

Indicator strain	Zone of inhibition
Listeria monocytogenes (KF894986)	11 mm
K. pneumoniae (MTCC 618)	10 mm
Yersinia enterocolitica (MTCC 859)	–
Salmonella enterica typhimurium (MTCC 1252)	–
Bacillus cereus (MTCC 430)	–

Zone of inhibition showed are mean of three replicates, – indicates no zone of inhibition.

5.4.3.3. Cell surface hydrophobicity

Hydrophobicity of a bacterial strain measures the capacity to adhere to hydrocarbons, and holds a strong relationship with ability to adhere to the epithelium

of digestive tract[34]. The percent cell surface hydrophobicity of *B. amyloliquefaciens* AMS1 was compared with *L. rhamnosus* and it was found that *B. amyloliquefaciens* AMS1 showed maximum hydrophobicity with n-hexadecane (68.5-77.9%) followed by chloroform (63.5-74.3%). These values are comparable to those of *L. rhamnosus* MTCC 1408 (Fig. 5.3). Islam et al.[35] also reported ≥50 % cell surface hydrophobicity of *B. amyloliquefaciens* using xylene.

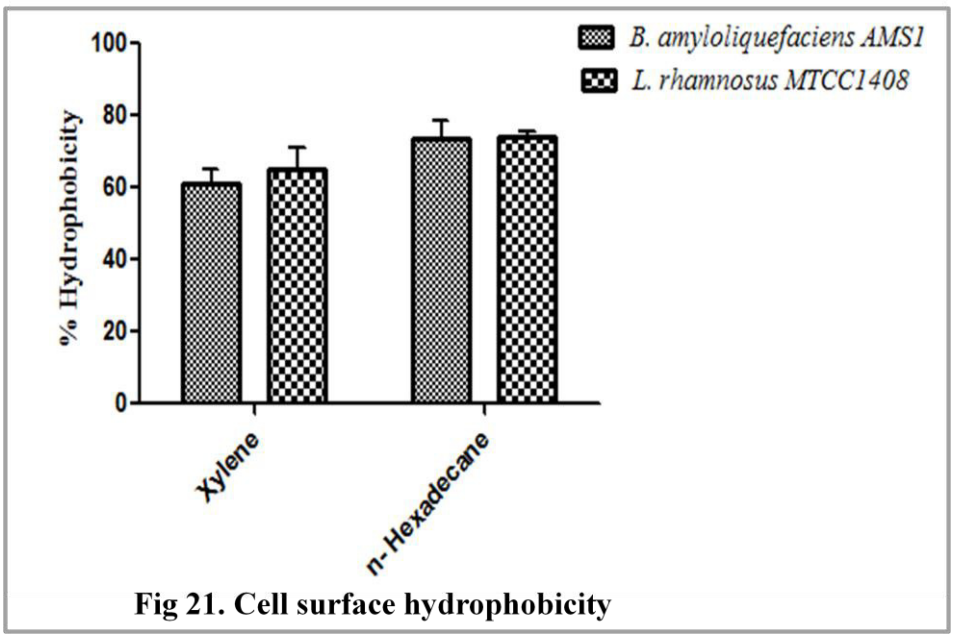


Fig. 5.3 Cell surface hydrophobicity of *B. amyloliquefaciens* AMS1.

5.4.3.4. Autoaggregation and Co-aggregation

It has been previously reported in case of *L. rhamnosus*[36] that with an increase in incubation time the auto aggregation increases rapidly. In the present study, *B. amyloliquefaciens* AMS1 showed a significant auto aggregation pattern with highest degree of auto aggregation after 24 h incubation (65.5–75.5%). These values were comparable with those of the reference strain *L. rhamnosus* MTCC 1408 (Fig. 5.4).

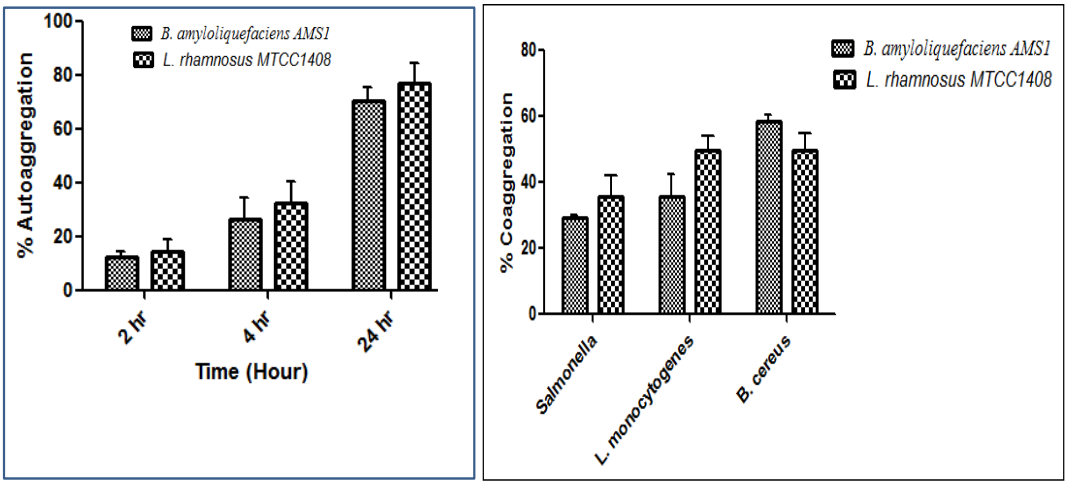


Fig. 5.4 Auto aggregation and co-aggregation of *B. amyloliquefaciens* AMS1.

The isolate showed co-aggregation with all the tested pathogens and the highest was observed against *B. cereus* MTCC 430 (56.6-60.3%). The reference strain showed comparable co-aggregation abilities except against *L. monocytogenes* MTCC 839 which differed significantly at $p< 0.05$ (Fig. 5.4).

5.4.3.5. Adhesion and inhibition of pathogen adhesion to Caco-2 cells by *B. amyloliquefaciens* AMS1

The adhesion of probiotic to epithelial cells such as Caco-2 cell line is regarded as one of the most important attribute. *B. amyloliquefaciens* AMS1 was evaluated for its capability to adhere to Caco-2 cells using *L. rhamnosus* MTCC1408 as a reference strain. Fig.5.5A showed microscopic images of adhesion between Caco-2 cells and probiotic *B. amyloliquefaciens* strain after gram staining. The adhesion results showed that *B. amyloliquefaciens* AMS1 (7.99 ± 0.36%) was able to adhere to Caco-2 cells with no considerable difference in comparison with the reference strain (10.13 ± 0.54 %) (Fig. 5.5B). Our study suggested that *B. amyloliquefaciens* AMS1 reduced the adherence of *S. enterica typhimurium* (MTCC 1252) to Caco-2 cells by 8.95 ± 28.14 % whereas *L. monocytogenes* AMDK2 (KF894986, local isolate) adherence was reduced by 30.06 ± 0.23 %. *L. rhamnosus* MTCC 1408 also showed inhibition of *L. monocytogenes* AMDK2 and *S. enterica typhimurium* (MTCC 1252) adherence by 24.26 ± 0.43% and 13.72 ± 0.82%, respectively (Fig. 5.5C). Anti-adherent evaluationof novel probiotics is of significance for the inhibition of pathogen infections.

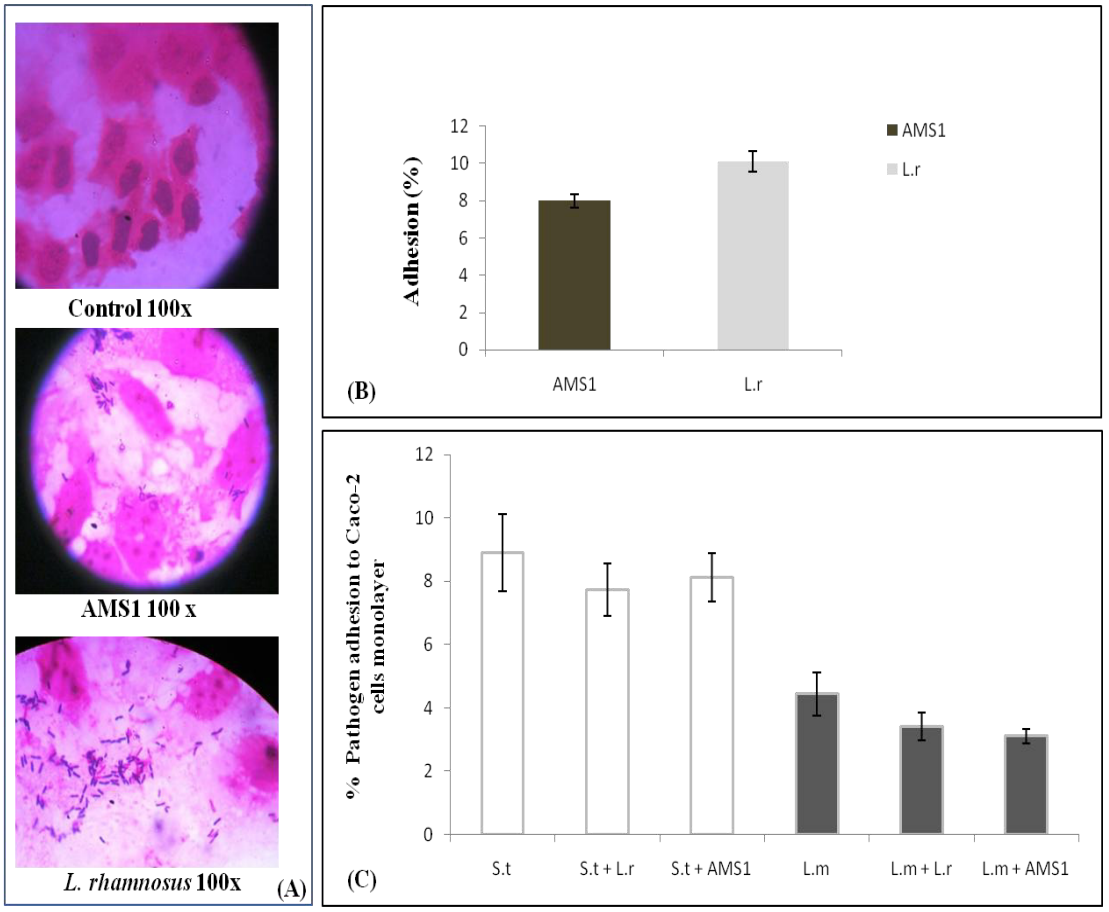


Fig. 5.5 (**A**) Adhesion of *B. amyloliquefaciens (*AMS1) and *L. rhamnosus* MTCC 1408 (L.r) to Caco-2 cell line observed by Gram staining under bright field microscope at 100 x. Control indicates only gram stained Caco-2 cells. (**B**) Adherence (%) of *B. amyloliquefaciens (*AMS1) and *L. rhamnosus* MTCC 1408 (L.r) to Caco-2 monolayer. (**C**) Percentage of *Listeria monocytogenes* AMDK2 (L.m) and *Salmonella enterica typhimurium* (S.t) adhesion to Caco-2 monolayer in the presence and absence of probiotic.

5.4.4. Antibiotic susceptibility test

Antibiotic susceptibility test indicated that *B. amyloliquefaciens* AMS1 is sensitive to all antibiotics included in the Table 5.3 except penicillin G (1 unit) and ampicillin (10 mcg). Resistance to a given antibiotic can be inherent to a bacterial species or genus. Therefore, resistance to penicillin G and ampicillin may be an intrinsic characteristic of *B. amyloliquefaciens* and might be useful for the *Bacillus* taxonomy. Hoa et al.[37] also reported the resistant nature of probiotic *Bacillus* against penicillin and ampicillin.

Table 5.3 Antibiotic susceptibility test

Antibiotics	**Zone of inhibition (mm)**
Ampicillin (10 mcg)	–
Chloramphenicol (25 mcg)	+++
Penicillin G (1 unit)	–
Streptomycin (10 mcg)	++
Sulphatriad (300 mcg)	+
Tetracyclin (25 mcg)	+++

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.

5.4.5. Cellulolysis

The isolate showed a significant cellulolytic activity on CMC agar plate.

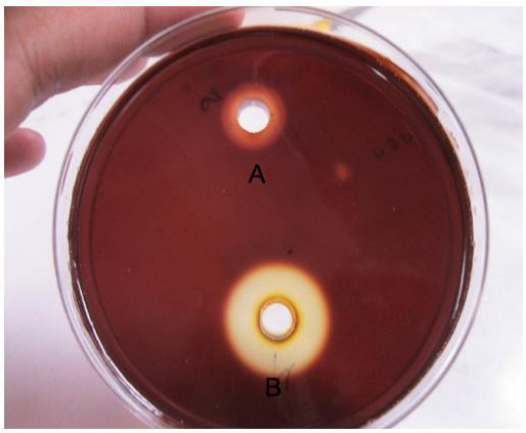


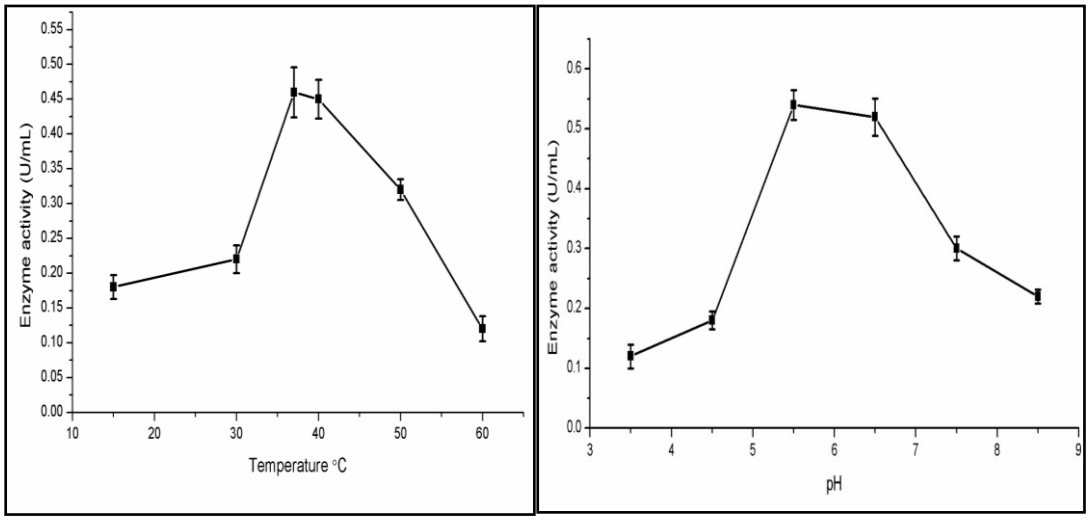
Fig. 5.6 Screening of cellulase production at 37 ˚C by plate assay. 'A' and 'B' represent enzyme activity before dialysis and after dialysis respectively.

The crude protein fraction after dialysis displayed a substantial increase in its cellulolytic activity (Fig. 5.6).

5.4.6. Optimization of cellulase production at different temperatures and pH

Enzyme activity recorded at different temperatures revealed that *B. amyloliquefaciens* AMS1 yielded maximum cellulase production at 37 ˚C (Fig. 5.7A). Negative effects were observed on the production by increase or decrease in the temperature.

B. amyloliquefaciens AMS1 was allowed to grow in media of different pH ranging from 3.5 to 8.5. Maximum enzyme activity was observed in medium of pH 5.5–6.5 (Fig. 5.7B).



(A) **(B)**

Fig. 5.7. Optimization of cellulase production at different (A) temperatures and (B) pH.

5.4.7. SEM analysis of the degradation of maize straw

The morphological changes of the maize straw powder after treatment with dialyzed supernatant containing cellulase were recorded by SEM. As shown in Fig. 5.8, structure of the guard and subsidiary cells in corn leaves is shown in the control figure whereas treated sample was more disordered and the peeling off of the linters was clearly observed. The solid structure of cellulose was swollen and had become loose by imbibition of dialyzed supernatant liquid of probiotic culture showing cellulase activity at different temperatures (37 °C and 60 °C). The total cellulolytic activity was found to be 0.43 ± 0.006 U/ml and 0.18 ± 0.003 U/ml at 37 and 60 °C respectively.

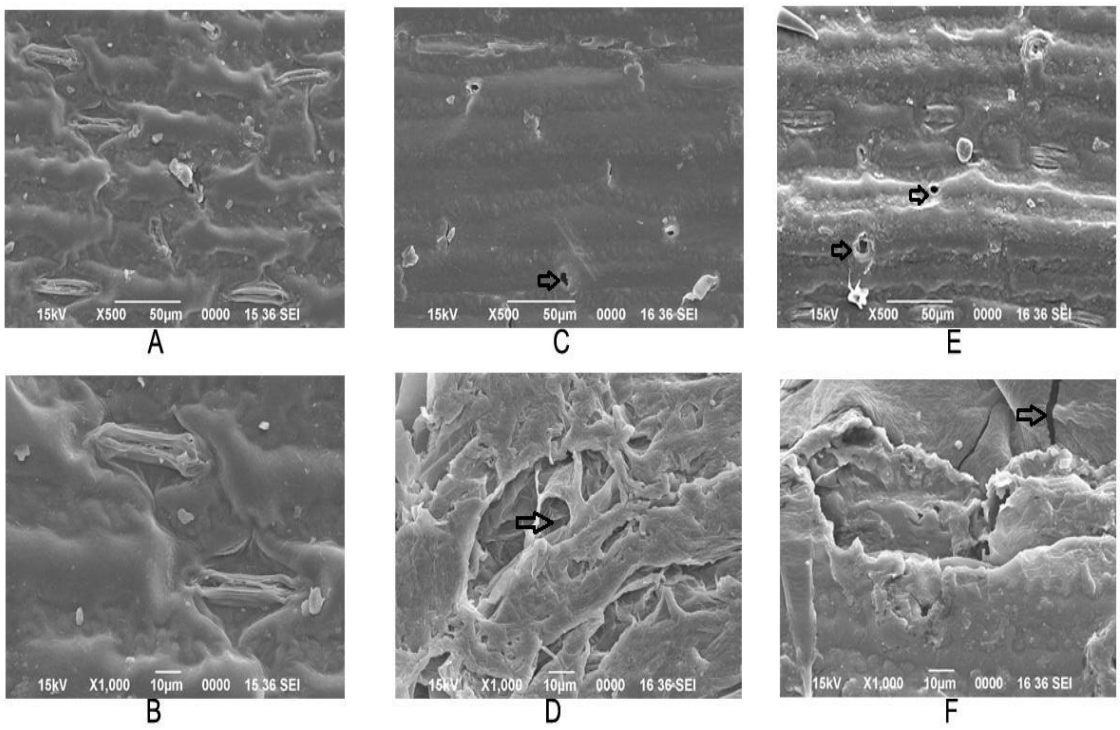


Fig. 5.8 Scanning electron micrograph showing cellulose degradation of maize straw at different temperatures. A) Control contains only maize straw at 500x, B) Control contains only maize straw at 1000x, C) Maize straw incubated for 37 °C with dialyzed culture supernatant at 500x, D) Maize straw incubated for 37 °C with dialyzed culture supernatant at 1000x, E) Maize straw incubated for 60 °C with dialyzed culture supernatant at 500x, F) Maize straw incubated for 60 °C with dialyzed culture supernatant at 1000x.

The isolate showed a remarkable cellulolytic activity as SEM revealed different levels of degradation; colonized cellulose substrates showed cavity formation and erosion of cell wall as reported by Hamed[38] and hollow degradation patterns, similar to that observed with *Micromonospora* pure cultures[39] and in the form of pits in the fiber surface, which appeared to be due to breakdown of complex polysaccharide with the enzymes produced by *B. amyloliquefaciens* AMS1.

5.4.8. Filter paper degradation

Filter paper degradation results showed that Whatman no.1 filter paper (1 x 6 cm) was completely disintegrated by *B. amyloliquefaciens* AMS1 within 96 h of incubation (Fig. 5.9). This shows the tremendous cellulolytic potential of the strain under investigation.

Fig. 5.9 Filter paper degradation by *B. amyloliquefaciens* AMS1 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.

5.5. Conclusions

In the present work, *B. amyloliquefaciens* AMS1 showed potential probiotic characteristics as well as a significant cellulolytic activity *in vitro*. It proved to be sufficiently robust to survive the harsh physico-chemical conditions present in the gastrointestinal tract. The ability to degrade CMC, maize straw and filter paper conferred cellulolytic potential on the bacterium isolated from a fermented food, Soybean. Generally the animal feeds of plant origin have higher cellulose contents which could be hydrolyzed by cellulolytic probiotics in conjunction with the rumen microbes, forming a source of energy for the animal rather than it being passed in the undigested feces.

The demand for enhanced productivity of animals and digestibility of animal feed by means of different functional foods is increasing. Such demands can be fulfilled by feeding animals with diets that contain nutritionally rich, beneficial-for-health, and lignocellulose-degrading elements. Further, the stability of gut microbiota is essential to intestinal health. Probiotics as modulators of the gut flora enhance the well-being of the animal. If these probiotics interact with the host by producing lactic acid or enzymes that might be of importance to the animal such as cellulose degradation, then the animal not only will have a better intestinal health, it can also result in better digestion of the feed, thus improving the feed conversion rate.

5.6. References

1. FAO. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. In: The joint FAO/WHO expert consultation report on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, October 1-4. Cordoba, Argentina, 2001. (http:// www.fao.org/es/ESN/probiot/probio.htm).
2. Verschuere, L., et al. Probiotic bacteria as biological control agents in aquaculture, Microbiol Mol Biol Rev **64**, 655--671, 2000.
3. Balcazar, J.L., et al. The role of probiotics in aquaculture, *Vet. Microbiol.* **114**, 173--186, 2006.
4. 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.
5. 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.
6. 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.
7. 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.
8. Maragkoudakis, P.A., et al. Lactic acid bacteria efficiently protect human and animal intestinal epithelial and immune cells from enteric virus infection, *Int. J. Food Microbiol.* **141**, S91--S97, 2010.
9. Ji, J., et al. Probiotic *Bacillus amyloliquefaciens* mediate M1 macrophage polarization in mouse bone marrow-derived macrophages, *Arch. Microbiol.* **195**, 349--356, 2013.

10. Das, A., et al. Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*), *Fish Shellfish Immunol.* **35**, 1547--1553, 2013.
11. Lee, Y.J., et al. Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull, *Bioresour. Technol.* **99**, 378--386, 2008.
12. Wizna., et al. Improving the quality of Tapioca by-products (Onggok) as poultry feed through fermentation by *Bacillus amyloliquefaciens*, *Pakistan Journal of Nutrition* **8**, 1636--1640, 2009.
13. Morris, E.J., & van Gylswyk, N.O. Comparison of the action of rumen bacteria on cell walls from Eragrostistef, *J. Agric. Sci. (Camb.)* **95**, 313--323, 1980.
14. Varga, G.A., & Kolver, E.S. Microbial and animal limitations to fiber digestion and utilization, *J. Nutr.* **127**, 819S--823S, 1997.
15. Iyer, B.K., Singhal, R.S., & Ananthanarayan, L. Characterization and in vitro probiotic evaluation of lactic acid bacteria isolated from idli batter, *J. Food. Sci. Technol.* **50**, 1114--1121, 2013.
16. Opazo, R., et al. Reduction of soybean meal non-starch polysaccharides and alpha-galactosides by solid-state fermentation using cellulolytic bacteria obtained from different environments, *PLoS One* **7**, e44783, 2012.
17. Guo, C.F., et al. Screening of bile salt hydrolase-active lactic acid bacteria for potential cholesterol-lowering probiotic use, *Adv. Mater. Res.* **345**, 139--146, 2012.
18. 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.
19. Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783--791, 1985.

20. 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.
21. Charteris, W.P., et al. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract, *J. Appl. Microbiol.* **84**, 759--768, 1998.
22. Maragkoudakis, P.A., et al. Probiotic potential of *Lactobacillus* strains isolated from dairy products, *Int. Dairy J.* **16**, 189--199, 2006.
23. 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.
24. Rosenberg, M. Microbial adhesion to hydrocarbons: twenty-five years of doing MATH, *FEMS Microbiol. Lett.* **262**, 129--134, 2006.
25. Del Re, B., et al. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*, *Lett. Appl. Microbiol.* **31**, 438--442, 2000.
26. Handley, P.S., et al. A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate strains of *Streptococcus salivarius*, *J Gen Microbiol* **133**, 3207--3217, 1987.
27. García-Cayuela, T., et al. Adhesion abilities of dairy Lactobacillus plantarum strains showing an aggregation phenotype, Food Res. Int. 57, 44--50. 2014.
28. Bauer, A.W., et al. Antibiotic susceptibility testing by a standardized single disk method, Am. J. Clin. Pathol. **45**, 493--496, 1966.
29. 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.
30. Ghose, T.K. Measurement of cellulase activity, *Pure Appl. Chem.* **59**, 257–268, 1987.
31. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.* **31**, 426--428, 1959.

32. Saitou, N., & Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol. Biol. Evol.* **4**, 406--425, 1987.
33. 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.
34. Kos, B., et al. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92, *J Appl. Microbiol.* **94**, 981--987, 2003.
35. Islam, H.V., et al. Isolation and characterization of putative probiotic bacterial strain, *Bacillus amyloliquefaciens*, from North East Himalayan soil based on *In Vitro* and *In Vivo* functional properties, *Probiotics. Antimicrob. Proteins.* **3**, 175--185, 2011.
36. Pithva, S., et al. Probiotic attributes of autochthonous *Lactobacillus rhamnosus* strains of human Origin, *Appl. Biochem. Biotechnol.* **173**, 259--277, 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. Hamed, S.A.M. In-vitro studies on wood degradation in soil by soft-rot fungi: *Aspergillus niger* and *Penicillium chrysogenum*, *Int. Biodeter. Biodegr.* **78**, 98--102, 2013.
39. 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.