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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

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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 h of incubation. A full length cellulase gene of AMS6 was amplified using degenerate 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 *B. subtilis* AMS6 as a promising candidate envisaging its application as an animal feed additive for enhanced fiber digestion and gut health of animal.

1. Introduction

Probiotics are live microbes, which when administered in adequate amounts confer a health benefit to the host (Araya et al., 2002). 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 (Guo et al., 2015). 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 (Kaushik et al., 2009). In addition, they should possess good surface hydrophobicity and aggregation properties for colonization in gut (Del Re et al., 2000). Lactic acid bacteria have been extensively studied as a potential probiotic for ruminants as compared with *Bacillus* probiotics (Brashears et al., 2003; Maragkoudakis et al., 2010; Peterson et al., 2007; Tabe et al., 2008; Younts-Dahl et al., 2004). However, *Bacillus* sp. also has potential probiotic as well as other attributes (Ji et al., 2013). 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 (Cutting, 2011) 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 wean-

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ing; at the beginning of the lactation period; and after a dietary shift from high forage to high readily fermentable carbohydrates (Chaucheyras-Durant and Durant, 2010). 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.

2. Materials and methods

2.1. Sampling and isolation of bacteria

Churpi, an indigenous fermented soybean product, was collected from Bomdila, Arunachal Pradesh, India, located at Latitude 27.25 N and Longitude 92.4 E, and stored at 4 °C until processed. The bacterial strain used in the study was isolated from Churpi and it was tested for its probiotic attributes and cellulose degrading capability.

2.2. Identification of the isolate

The morphological and biochemical characterization of the isolate was performed according to the standard procedure of Bergey's Manual of Systematic Bacteriology (Boone et al., 2001).

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 (Guo et al., 2010). The PCR amplification was performed in Eppendorf thermocycler in a total volume of 25 μL reaction mixture according to Manhar et al. (2015). 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 (Kimura, 1980; Felsenstein, 1985; Tamura et al., 2011).

2.3. Screening of probiotic properties

2.3.1. In vitro *gastrointestinal stress tolerance test*

Overnight culture of *B. subtilis* AMS6 and probiotic reference strain *Lactobacillus 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) in different PBS solutions with various pH. Resistance to simulated gastric fluid (SGF) was tested as reported earlier (Charteris et al., 1998). SGF was prepared by suspending pepsin (0.3 mg/mL; HiMedia) and NaCl (0.5%, w/v) in sterilized PBS adjusted to pH 2–4. Tolerance to small intestinal fluid (SIF) condition was tested in PBS solution adjusted to pH 6.8 and 8.0, containing pancreatin (0.1 mg/mL, Sigma) and 0.3% (w/v) Oxgall (HiMedia) (Maragkoudakis et al., 2006). Cells in SGF were incubated at 37 °C for 0, 1, 2, and 3 h, and those in bile solution and SIF were incubated at 37 °C for 0–4 h. The resistance of isolates in every condition was assessed in terms of viability on respective agar plates expressed in percentage after the treatment.

$$\text{Viability}\,(\%) = \left(\frac{N_t}{N_0}\right) \times 100 \quad (1)$$

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

2.3.2. 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 7185), *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 (Aslim et al., 2005).

2.3.3. Autoaggregation and Co-aggregation

Autoaggregation assay was performed according to Del Re et al. (2000) with slight modifications. Briefly, bacterial cell suspension (2 mL) was vortexed for 10 s and incubated at 37 °C. An aliquot of 0.1 mL collected from the upper surface at regular time interval was mixed with 0.9 mL PBS and its A_{600} was measured. Autoaggregation percentage was expressed as:

$$\text{Autoaggregation}\,(\%) = 1 - \frac{A_t}{A_0} \times 100 \quad (2)$$

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

The bacterial cell suspensions preparation protocol for co-aggregation assay was the same as that for autoaggregation assay. Equal volumes (2 mL) of each cell suspension (isolate and pathogen cell suspension) were mixed together in pairs by vortexing for 10 s. Control tubes were set up at the same time, containing 4 mL of each bacterial suspension. The absorbance (A_{600}) of the suspensions was measured followed by mixing and 5 h of incubation at room temperature. Samples were taken in the same way as in the autoaggregation assay. The percentage of co-aggregation was calculated using the equation of Handley et al. (1987):

$$\text{Coaggregation}\,(\%) = \frac{\left[(Ax + Ay)/2\right] - A(x+y)}{Ax + Ay/2} \times 100 \quad (3)$$

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

2.3.4. Cell surface hydrophobicity

The cell surface hydrophobicity of the selected bacterial isolate was measured by the method described by Rosenberg (2006) with slight modifications. Briefly, overnight grown culture in Luria Bertani broth at 37 °C was pelleted (6000*g*, 5 min) and then washed twice and re-suspended in phosphate buffer. The absorbance of the suspended pellet 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. *L. rhamnosus* MTCC 1408 grown in MRS broth was used as probiotic reference strain. 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:

$$\text{Hydrophobicity}\,(\%) = 100 \times \frac{Abs_{initial} - Abs_{final}}{Abs_{initial}} \quad (4)$$