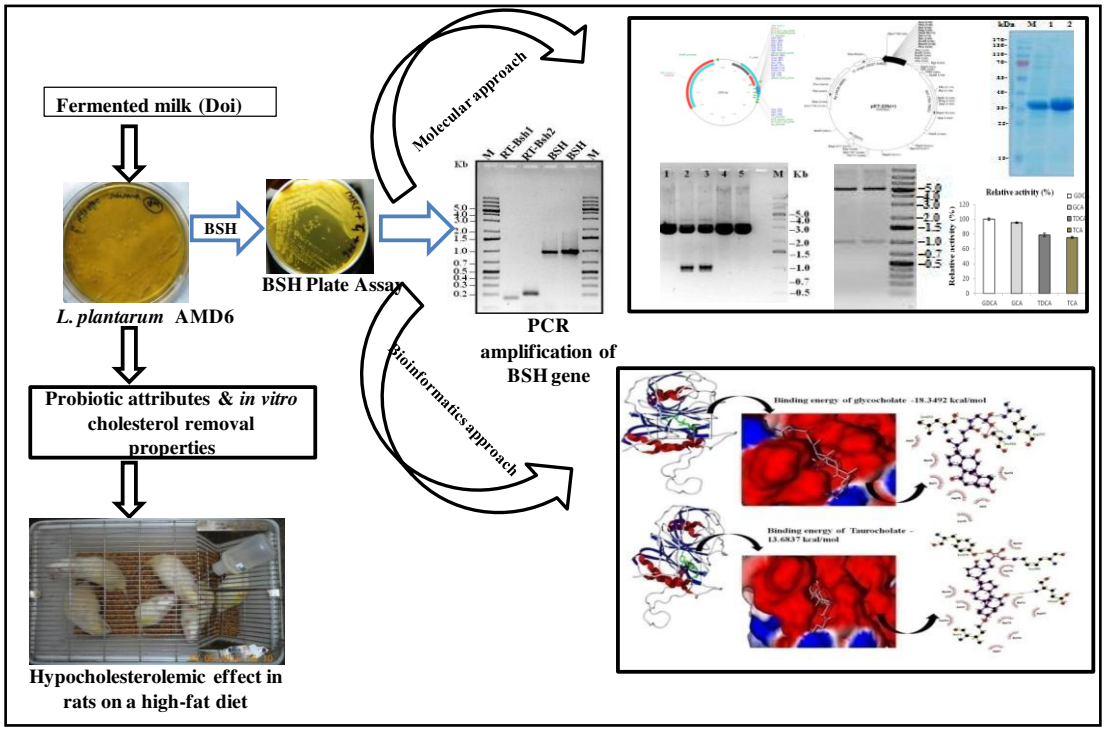
CHAPTER 4

PROBIOTIC ATTRIBUTES OF INDIGENOUS ISOLATE *LACTOBACILLUS PLANTARUM* AMD6 AND ITS CHOLESTEROL LOWERING EFFECT IN A HYPERLIPIDAEMIC RAT MODEL



Graphical Abstract

PROBIOTIC ATTRIBUTES OF INDIGENOUS ISOLATE *LACTOBACILLUS PLANTARUM* AMD6 AND ITS CHOLESTEROL LOWERING EFFECT IN A HYPERLIPIDAEMIC RAT MODEL

4.1. Abstract

Lactobacillus plantarum AMD6 isolated from traditional milk product 'Doi' displayed good tolerance to low pH and high bile salt and showed potential attributes for cholesterol assimilation. The bile salt hydrolase (BSH) gene was amplified from AMD6 and cloned. The recombinant BSH enzyme showed high preference for glycine-conjugated bile salts. The rats were divided into five groups fed on cholesterol-enriched diets for 42 days. Interestingly, the probiotic-fed -treatment group [HFD+AMD6 (10^8 CFU/ml)] exhibited a significant ($P< 0.05$) reduced body weight as compared to normal and high fat diet animals after 42 days of experiment. Similarly, the HFD+AMD6 animal showed significant reduction of serum TC, TG, and LDL-C levels to 65.45, 123.30, and 16.89 mg/dl, respectively, after 6 weeks. In conclusion, AMD6 isolate can be explored as probiotics in the management of cardiovascular diseases.

4.2. Introduction

Hyperlipidaemia is characterized by the accumulation of high level of cholesterol in the blood and it may predispose the individuals for developing cardiovascular diseases (CVDs) that is a leading cause of death in many countries. The World Health Organization has predicted that by 2030, nearly 23.3 million people will die due to CVD[1]. It has been observed that even a 1% decrease in serum cholesterol levels is estimated to reduce the risk of developing coronary artery diseases by 2-3%[2]. However, due to associated severe side effects and high cost of the currently used drugs, there is an urgent need to develop alternative strategies to reduce serum cholesterol for preventing CVDs. Several studies have shown that probiotics have broad health benefits including prevention of cardiovascular diseases and several other health problems[3, 4, 5].

The hypocholesterolemic activity of probiotics was first reported in the fermented milk of Maasai tribe of Kenya[6]. It has been previously reported that lactobacilli could reduce the serum cholesterol level through deconjugation of bile salts by enzyme bile salt hydrolase (BSH)[3]. Though many drugs are available in the

market to reduce serum cholesterol level but patients prefer non-drug treatments for hyperlipidemia for various reasons, including the undesirable effects, high prices, and personal preference for natural therapies[7]. There is a wide diversity of traditional fermented food products among the tribal people of North-East, India[8]. Curd, locally called as "Doi", is a popular fermented ethnic milk products of North-East, India. Curd is consumed by people of this region and forms an integral part of their diet[8]. A large scale isolation of microbial communities from traditional fermented milk products in our laboratory has revealed some interesting probiotic attributes of *lactobacillus*. Nevertheless, the cholesterol assimilation by probiotics reported from traditional fermented foods of North-East India has been scarcely reported. Therefore, in the present study, we have investigated the probiotic attributes of indigenous isolates, *L. plantarum* AMD6 and its cholesterol lowering effect on high fat diet induced hyperlipidaemia in rat model.

4.3. Materials and methods

4.3.1. Bacterial strains, culture media, and growth conditions

In this study *L. plantarum* AMD6 (out of 26 isolates from MRS agar) isolated from fermented milk (Doi) of Dibrugarh, Assam, India, was selected based upon promising probiotic attributes and their *in-vitro* cholesterol removal properties. The probiotic reference strain *L. plantarum* MTCC 1407 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The bacterial strain was grown in MRS agar (Himdia, India) medium under shaking conditions at 37 ºC for 24-48 h.

4.3.2. Molecular identification of isolates

16S rRNA gene sequence analysis for bacterial isolate AMD6 was performed using universal primers 27F and 1492R, whereas *Lactobacillus* genus specific 16S rRNA gene was amplified using primers (F-5'AGAAGAGGACAGTGGAAC 3' and R-5'TTACAAACTCTCATGGTGTG 3') as reported by Singh & Ramesh[9]. 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,

Switzerland). The sequence obtained was subjected to NCBI BLAST search tool in order to retrieve the homologous sequences in Genbank and the phylogenetic tree was generated using MEGA 5.05.

4.3.3. Screening of probiotic attributes

4.3.3.1. In vitro gastrointestinal stress tolerance test

The resistance of isolates to gastrointestinal stress conditions which mimic the human gastrointestinal tract (GIT) environment was checked according to the method described by Maragkoudakis et al.[10]with some modifications. Cells from an 24-48 h culture were harvested by centrifugation at 6000 x g for 5 min at 4 ºC, washed once with phosphate buffered saline (PBS) solution with pH 7.4, before being resuspended (10^8 CFU/ml) in different PBS solutions with various pH. Simulated gastrointestinal transit tolerance test was performed by resuspending cells into simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). SGF was prepared by supplementing sterilized PBS, pH 2, 3 and 4 with pepsin to a final concentration of 3 gL^{-1}. SIF was prepared by supplementing sterilized PBS, pH 6.8 and 8 with pancreatin (Sigma Aldrich, USA) to a final concentration of 1 gL^{-1}. The resistance of isolates was evaluated by counting viable colony on MRS agar plates after 0, 1, 2, 3 h for SGF and 0, 1, 2, 3, 4 h for SIF experiments, respectively.

4.3.3.2. Bile tolerance

The resistance to bile was performed according to the method described by Gilliland and Walker[11], with slight modification. Overnight bile-enriched grown culture of each isolates was inoculated in MRS broth medium supplemented with 0.1, 0.3, and 0.5% oxgall and incubated for 12, 24, and 48 h at 37 ºC. The control comprised of culture broth without oxgall. The resistance of isolates in every condition was assessed in terms of viable colony count on MRS agar plates after the treatment.

4.3.3.3. Hydrophobicity

Microbial adhesion to hydrocarbons was performed following the method reported by Rosenberg[12] with minor modification. Cells were harvested from overnight culture and washed twice with PBS, pH 7.4. Cell count was adjusted approximately to 10^9 CFU/ml by taking absorbance at OD_{600}. Then equal volume of

cell suspension was mixed with n-hexadecane and ethyl acetate separately by vortexing for 2 min. The two phases were allowed to separate for 1 h and the aqueous layer was gently pipetted out and OD_{600} was measured. 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.

4.3.3.4. Autoaggregation and Coaggregation

Autoaggregation ability of the isolate was evaluated with slight modification of Del Re et al.[13]. Briefly, 4 ml of cell suspension of cell count approximately 10^9 CFU/ml was vortexed for 10 sec at room temperature. A 100 µl aliquot was taken out from the upper surface at 2, 4 and 24 h post incubation at 37 ºC, mixed with 900 µl of PBS (pH 7.2) and OD_{600} was measured. Autoaggregation percentage was expressed as:

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

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

For Co-aggregation assay, an equal volume of the isolate (2 ml) and pathogenic indicator cell suspension (~10^9 CFU/ml) were mixed, incubated at 37 ºC and OD_{600} was recorded after 4 h. Coaggregation was calculated as described in previous chapter (Section 3.3.3.4 and 3.3.3.5).

4.3.3.5. Cell culture

The human colorectal adenocarcinoma Caco-2 cell line obtained from the National Centre for Cell Science, Pune, India was used to study the adhesion capability and the inhibition of food borne pathogenic bacterial adhesion. The cell line was routinely grown and maintained by following the procedure previously described (Section 3.3.3.6). Media and reagents were purchased from Sigma (India) and Gibco® (Life Technologies).

4.3.3.6. Adhesion and inhibition of pathogen adhesion to Caco-2 cells

The adhesion study was evaluated with slight modification by following the method described by Garcia-Cayuela et al.[14] as discussed in previous chapter (Section 3.3.3.7). *L. plantarum* MTCC 1407 was used as a reference strain to study adhesion and inhibition of pathogens to Caco-2 cells. For adhesion assay Caco-2 cell monolayers were inoculated with isolated strain and for the inhibition of pathogen adhesion, mixed inoculums containing isolate and MTCC food spoilage indicator strain (*Salmonella enterica typhimurium* MTCC 1252) was inoculated in MEM and incubated for 1 h and 2 h, respectively at 37 °C, 5 % CO_2.

4.3.4. Screening of bile salt hydrolase activity and deconjugation of conjugated bile salts

A direct plate assay method was employed for detection of bile salt hydrolase (BSH) activity. *L. plantarum* AMD6 was streaked on MRS agar supplemented separately with 0.2% glycodeoxycholic acid and 0.5% taurodeoxycholic acid, and incubated anaerobically for 48 h at 37 ºC[15]. *L. plantarum* MTCC 1407 was used as a reference strain. Sterile MRS containing L-Cysteine hydrochloride and 0.1% pancreatin supplemented with 6 mM sodium glycocholate, 6 mM sodium taurocholate, a combination of 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate was used in deconjugation assay. Deconjugation of bile salt was determined as the amount of cholic acid released using the method of Irwin, Johnson, and Kopalo[16]. The activated culture in this medium was adjusted to pH 7.0 with 1 M NaOH and centrifuged at 10,000g at 4 °C for 10 min. The supernatant was collected and adjusted to pH 1.0 with 10 M HCl. The supernatant was mixed with ethyl acetate, vortexed and further ethyl acetate layer was evaporated under nitrogen. The residue was dissolved in 1 ml glacial acetic acid, then 1% (v/v) furfuraldehyde and 8 M H_2SO_4 were added. The mixture was then vortexed and heated at 65 °C for 10 min. Glacial acetic acid was added upon cooling and the concentration of cholic acid was measured spectrophotometrically at 600 nm. The standard graph was prepared using known concentration of cholic acid (10 -100 mg/ml).

4.3.4.1. Identification of *L. plantarum* AMD6 BSH gene using colony PCR

The presence of BSH in *L. plantarum* AMD6 was confirmed by gene specific PCR using primers BSHF- 5'CGGCTGGATCCGATGTGCACTAGTCTAAC 3' and BSHR -5'ATACTCGAGATGGGCCGCTGGCAAGGTG 3' as reported by Gu et al.[17] followed by sequencing. The template DNA was obtained by lysing a single bacterial colony using 20 mM NaOH at 95˚C for 10 min followed by centrifugation at 10,000 rpm for 10 min. The PCR reaction was carried out in a total volume of 30 µl containing 2 µl of each forward and reverse primers, 15 µl of Dream Taq PCR Master Mix (2X, Thermo Scientific, #K1071), 10 µl nuclease free water (Thermo Scientific, #R0581) and 1 µl DNA template. The PCR reaction was carried out in a thermal cycler (Eppendorf Mastercycler, Germany). The reaction conditions required for the primers used are provided below in Table 4.1.

Table 4.1 PCR conditions for amplification of BSH gene

Primer	Denaturation (°C)	Final denaturation (°C)	Annealing (°C)	Extension (°C)	Final extension (°C)	Store (°C)
BSH	94	94	55	72	72	4
Time (in minute)	4	0.40	0.30	1.30	10	∞

The PCR amplified DNA fragments were resolved by 1% agarose gel electrophoresis and stained with ethidium bromide (0.5 mg/ml) followed by visualization under UV light.

4.3.5. Cloning of bile salt hydrolase (BSH) gene

The amplified BSH gene was purified using PCR KlenZol purification kit and was cloned into pBluescript KS(+) (Fig. 4.1) between *Bam*HI and *Xho*I restriction sites.

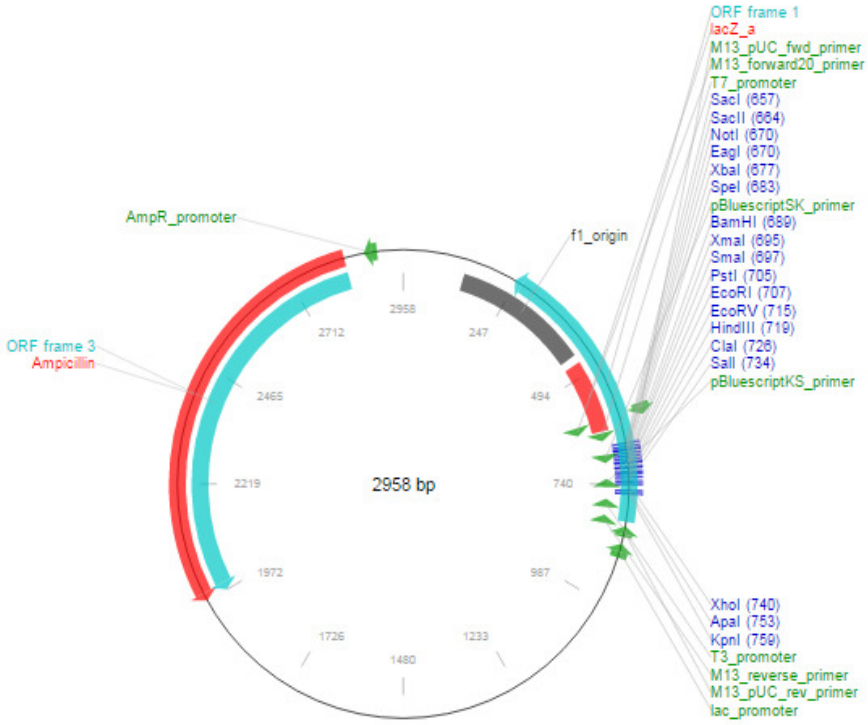


Fig.4.1 Novagen pBluescript KS (+) vector map**.**

4.3.5.1. Restriction digestion of pBluescript KS(+)

pBluescript KS(+) was double digested with *Bam*H1(Fermentas) and *Xho*1(Fermentas). The reaction conditions were set up as mentioned in Table 4.2.

Table 4.2 Double digestion of pBluescript KS(+)

Mixture	Volume (µl)
pBluescript KS(+)	3
*Bam*HI buffer	5
*Bam*HI	2
*Xho*I	2
Nuclease Free water	38
Total	**50 µl**

The mixture was incubated at 37 ˚C for 4 hours. The digested vector was run in 1% agarose gel and gel purified using QIAGEN gel extraction kit.

4.3.5.2. Restriction digestion of BSH gene

The insert (BSH) was double digested with *Bam*H1(Fermentas) and *Xho*1(Fermentas). The reaction conditions were set up as mentioned in Table 4.3

Table 4.3 Restriction digestion of BSH gene

Mixture	**Volume (µl)**
BSH gene	5
*Bam*HI buffer	5
*Bam*HI	2
*Xho*I	2
Nuclease Free water	36
Total	**50 µl**

The mixture was incubated at 37˚ C for 4 hours. The digested insert was run in 1% agarose gel and gel purified using QIAGEN gel extraction kit.

4.3.5.3. Ligation of pBluescript KS(+) and BSH gene

The double digested pBluescript KS(+) and BSH gene were ligated using T4 DNA ligase. The reaction conditions were set up as mentioned in Table 4.4.

Table 4.4 Ligation reactions

Mixture	**Volume (µl)**
pBluescript KS (+)	2.5
Insert	6
ligase buffer	2
T4 DNA ligase (5U)	0.2
Nuclease Free water	9.3
Total	**20 µl**

The ligation mixture was incubated at 16˚C for 20 hours.

4.3.5.4. Transformation

The ligated product was transformed into *E. coli* DH5α. The protocol is given below.

4.3.5.4.1. Preparation of competent cells

i. Glycerol stock of *E. coli* DH5α was revived on LBA plate.

ii. A single colony of *E. coli* DH5α was inoculated in 5ml LB broth and incubated overnight at 37 ˚C.

iii. 100 µl of overnight grown culture was inoculated into 250 ml conical flask containing 50 ml LB media and incubated at 37 ˚C till the O.D reached 0.4 at 600 nm.

iv. The culture was transferred into oakridge tubes and kept on ice for 30 minutes.

v. The cells were harvested by centrifugation at 3000 rpm for 10 minutes at 4 ˚C.

vi. The pellet was resuspended in 25 ml ice-cold $CaCl_2$ buffer (100mM $CaCl_2$ and 10mM Tris pH 7.4) and incubated on ice for 45 minutes.

vii. The cells were again harvested by centrifugation at 4000 rpm for 10 minutes at 4 ˚C.

viii. The pellet was again resuspended in 3 ml ice-cold $CaCl_2$ buffer.

ix. 200 µl of this suspension was aliquoted into pre-chilled eppendorf tubes and were used for transformation.

4.3.5.4.2. Transformation of recombinant vector into *E. coli* DH5α

i. 2 µl of the ligation mixture was added to 200 µl of the competent cells.

ii. The tubes were incubated in ice for 45 minutes and mixed after every 10 minutes.

iii. Heat shock was given at 42 ˚C for 45 seconds and the tubes were immediately transferred to the ice bucket for 2 minutes.

iv. 800 µl of LB media was added and incubated at 37 ˚C for 1 hour with shaking. It was followed by centrifugation at 10,000 rpm for 30 seconds and the supernatant was discarded till the volume of culture was approx. 100 µl.

v. The pellet was resuspended and plated on LB agar with ampicillin (100 µg/ml) and incubated at 37 ˚C for 16 h.

4.3.5.5. Screening of positive clones

Colonies of each transformant were screened by restriction digestion. The clones showing gene of interest after digestion was further confirmed by colony PCR and DNA sequencing. The plasmid was isolated from overnight grown culture of transformed colonies prior to the restriction digestion as described below.

4.3.5.5.1. Isolation of Plasmid DNA

i. A colony from the transformed plate was inoculated in 6 ml LB broth containing ampicillin (100 µg/ml) and incubated at 37 ˚C overnight.

ii. Glycerol stock of the overnight grown culture was prepared and stored in -80 ˚C.

iii. The remaining cells were harvested by centrifugation in an eppendorf at 12000 rpm for 30 seconds.

iv. The tubes were kept inverted on tissue paper to dry and then the pellet was resuspended in 200 µl TGE buffer (25 mM Tris pH 8.0, 10 mM EDTA, 0.1 mM glucose) and vortexed properly to suspend the cells.

v. This suspension was centrifuged at 12000 rpm for 30 seconds and the pellet was again resuspended in 200 µl TGE buffer and vortexed.

vi. 400 µl of lysis buffer (0.2 N NaOH, 1 % SDS) was added and mixed gently up and down for homogeneity and kept on ice for 10 minutes.

vii. 250 µl of 7.5 M ammonium acetate was added and mixed gently to observe the white precipitate.

viii. The tubes were kept on ice for 15 minutes and then spun at 12000 rpm for 10 minutes.

ix. The supernatant was transferred to a fresh tube and added 600 µl of isopropanol, mixed thoroughly and left at -20 ˚C for 60 minutes.

x. The solution was centrifuged at 12000 rpm for 10 minutes and the pellet was washed with 70% ethanol and air dried.

xi. The pellet was suspended in 100 µl TE buffer (10mM Tris pH 8, 1mM EDTA) and properly suspended the pellet with little tapping.

xii. 50 µl of ammonium acetate was added and mixed thoroughly and kept on ice for 30 minutes. It was followed by centrifugation at 12000 rpm for 10 minutes.

xiii. The supernatant was transferred to a fresh eppendorf tube and 150 µl of isopropanol was added and mixed thoroughly and kept at -20 ˚C for 60 minutes.

xiv. The mix was spun at 12000 rpm for 10 minutes and the pellet was washed with 70% ethanol and the pellet was vaccum dried.

xv. The pellet was resuspended in 100 µl TE buffer and mixed thoroughly.

xvi. 2 µl of RNase (10 µg/ml) was added, mixed thoroughly and spun down for 2 seconds and incubated at 37 ˚C for 30 minutes.

xvii. 50 µl of ammonium acetate and 150 µl of isopropanol were added and mixed properly and left the tubes at -20 ˚C for 60 minutes.

xviii. Centrifuged the tubes at 12000 rpm for 10 minutes and then washed with 70% ethanol and vaccum dried the pellet.

xix. 25 µl of TE buffer (10mM Tris pH 8, 0.1mM EDTA) was used to suspend the pellet. Plasmid was stored at -20 ˚C.

4.3.5.5.2. Restriction Digestion of recombinant pBluescript KS(+)

The recombinant pBluescript KS(+) was double digested with *Bam*H1(Fermentas) and *Xho*1(Fermentas). The reaction conditions were set up as mentioned in Table 4.5.

Table 4.5 Restriction Digestion of recombinant plasmid

Mixture	Volume (µl)
Recombinant pBluescript KS(+)	3
*Bam*HI buffer	2
*Bam*HI	0.5
*Xho*I	0.5
Nuclease Free water	14
Total	**20 µl**

The mixture was incubated at 37 ˚C for 3 hours in water bath. The digested plasmid was run in 1% agarose gel.

4.3.6. Subcloning of BSH gene in pET22b(+)

The fragment corresponding to BSH gene was released by double digestion of recombinant clones with *Bam*HI and *Xho*I and further sub-cloned into pET-22b (+) (Fig. 4.2).

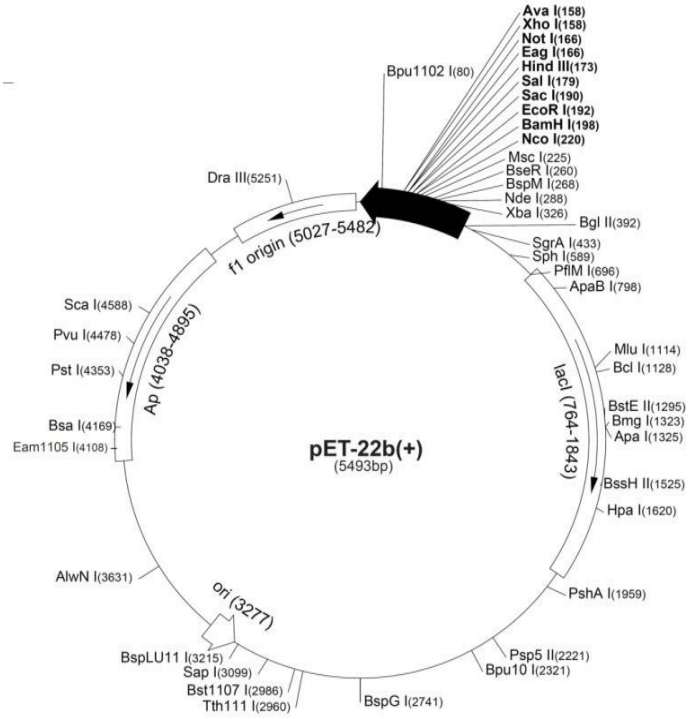


Fig. 4.2 Novagen pET-22b(+) vector map

4.3.6.1. Restriction digestion of recombinant pBluescript KS(+)

The recombinant pBluescript KS(+) was double digested with *Bam*H1(Fermentas) and *Xho*1(Fermentas). The reaction conditions were set up as mentioned in Table 4.6.

Table 4.6 Restriction digestion of recombinant pBluescript KS(+)

Mixture	Volume (µl)
Recombinant pBluescript KS(+)	3
*Bam*HI buffer	5
*Bam*HI	2
*Xho*I	2
Nuclease Free water	38
Total	**50 µl**

Mixture was incubated at 37 ˚C for 4 hours. The digested vector was run on 1% agarose gel and the BSH gene was gel purified using QIAGEN gel extraction kit.

4.3.6.2. Restriction digestion of pET-22b(+)

The pET-22b(+) was double digested with *Bam*H1(Fermentas) and *Xho*1(Fermentas). The reaction condition were set up as mentioned in Table 4.7

Table 4.7 Restriction digestion of pET 22b(+)

Mixture	Volume (µl)
pET-22b(+)	10
*Bam*HI buffer	5
*Bam*HI	2
*Xho*I	2
Nuclease Free water	31
Total	**50 µl**

Mixture was incubated at 37˚C for 4 hours. The digested vector was run on 1% agarose gel and gel purified using QIAGEN gel extraction kit.

4.3.6.3. Ligation of BSH gene into pET-22b(+)

The double digested pET-22b(+) and BSH gene were ligated using T4 DNA ligase. The reaction conditions were set up as mentioned in Table 4.8.

Table 4.8 Ligation Reaction

Mixture	**Volume** (µl)
pET-22b(+)	4
Insert	7
Ligase buffer	2
ATP (1 mM)	0.2
DTT (10 mM)	1
$MgCl_2$ (5 mM)	4
T4 DNA ligase (5U)	0.5
Nuclease Free water	1.3
Total	**20 µl**

The mixture was ligated at 16˚C and 22˚C for 24 hours. The ligase enzyme was heat inactivated at 65˚C for 10 minutes before transformation.

4.3.6.4. Transformation of recombinant vector into *E. coli* DH5α

The competent cells were prepared by $CaCl_2$ method. The recombinant pET-22b(+) vector was then transformed into *E. coli* DH5α competent cells by heat shock method.

4.3.7. Heterologous expression and enzymatic activity of recombinant BSH

The recombinant pET-22b(+) containing BSH was transformed into *E. coli* BL21 (DE3) cells and the culture at OD ~0.6 was induced with 0.1 mM IPTG for 4 h at 30 ºC. The expression of recombinant BSH protein was analyzed on 14 % SDS-PAGE followed by staining with Coomassie brilliant blue. Since the recombinant BSH protein was mainly found in inclusion bodies, the proteins were solubilized with 8 M Urea and purified by Ni^{2+} -NTA affinity chromatography (Qiagen). The recombinant BSH bound to the column was eluted with 10 mM Tris, 300 mM Nacl, 8M Urea, 250 mM Imidazole, pH 7.4. The concentration of purified protein was measured using Bradford method (Fermentas #R1271). The urea denatured BSH protein was refolded by gradient dialysis method using buffer containing different concentration of Urea (6M Urea, 4M Urea, & 2M Urea) and without Urea. The enzymatic activity of purified recombinant BSH (rBSH) was determined as described previously by Wang et al.[18] with slight modification against four different substrates, glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycocholic acid (GCA) and taurocholic acid (TCA) purchased from Sigma. 178 µl of reaction buffer (0.1 M sodium-phosphate, pH 6.0), 10 µl of purified rBSH (1 µg/µl), 10 µl of a specific conjugated bile acid (10 mM), and 2 µl of 1M dithiothreitol (DTT) were mixed gently and incubated at 37 °C for 30 min. A 50-µl aliquot of the reaction mixture was then immediately mixed with 50 µl of 15% (w/v) trichloroacetic acid to stop the reaction, followed by centrifugation for 5 min at 12,000 x g at room temperature to remove the precipitate. 50 µl of the supernatant was thoroughly mixed with 950 µl of ninhydrin reagent mix (0.25 ml of 1% [w/v] ninhydrin in 0.5 M sodium-citrate buffer (pH 5.5), 0.6 ml of glycerol, and 0.1 ml of 0.5 M sodium-citrate buffer [pH 5.5]). The reaction mix was incubated in a boiled water bath for 14 min, followed by cooling in ice water for 3 min and the absorbance was recorded at 570 nm. A control without added BSH was set up in each independent experiment. BSH

activity was expressed as µmol of amino acids released from the substrate per minute per mg of BSH.

4.3.8. Molecular docking of AMD6 bile salt hydrolase with glycocholate sodium and sodium taurocholate

The deduced amino acid sequence of the enzyme bile salt hydrolase was further considered for BLAST. Top scoring function sequences were selected for multiple sequence alignment using T-coffee and used for determining the phylogenetic tree of the closely related family of the enzyme bile salt hydrolase. The structure of bile salt hydrolase was predicted using *HHpred,* which is a HMM based homology modeling server. The obtained similar sequences were aligned using MEGA 6.0 software. The steric quality of the modelled structure was validated by using RAMPAGE, PROCHECK and VERIFY3D in terms of *QMEAN6* score, *QMEAN Z-Score* and *VERIFY3D quality score. SolvX* automated server is used to determine the solvent accessibility to protein core, which is a very important criteria to determine the biological functionality of a protein. Structure of conjugated bile acids sodium glycocholate (CID 23670522) and sodium taurocholate (CID 6675) was retrieved from PubChem. The enzyme bile salt hydrolase was docked with conjugated bile acids to evaluate the binding affinity of the enzyme towards the acids. All docking studies were performed in *Molegro Virtual Docker 5.0. LigPlot+ version 1.4.5* was used to analyse the active site residues of bile salt hydrolase binding to the conjugated bile acids and also for 2D diagrammatic representation of ligand- protein interaction. MolDock scoring function and number of hydrogen bonds for each interaction of ligand with the receptor were used to determine the best ligand interactions.

The MolDock scoring function E_{score} is defined by the following energy terms:

$E_{score} = E_{intra} + E_{inter}$, *w*here, E_{inter} is the ligand- protein interaction energy, and

$E_{intra} = \sum_{i \in ligand} \sum_{j \in ligand} E_{PLP}(r_{ij}) + \sum_{flexible\ bonds} A[1 - \cos(m.\theta - \theta_o)] + E_{clash}$

*w*here, PLP= Piecewise Linear Potential and E_{clash} is the clash potential[19].

4.3.9. Assimilation of cholesterol

The ability to assimilate cholesterol was performed by following the method of Gilliland et al.[20]. Water-soluble cholesterol (Polyoxyethanyl-cholesterylsebacate,

Sigma, United States) at the concentration of 5 mg/mL was added to MRS-Thio broth supplemented with 0·3% Ox-bile followed by inoculation and incubation anaerobically at 37 °C for 20 h. Cells were harvested from the broth by centrifugation at 12,000 rpm at 4 °C. The cell pellet was resuspended in a equal volume of distilled water to that of original broth culture. The amount of cholesterol removed from the cells and spent broth was determined by *o*- pthaladehyde method as described by Rudel and Morris[21]. Uninoculated sterile broth containing cholesterol was used as control. The attachment of cholesterol particle onto cell surface was observed using scanning electron microscopy (SEM). The cell pellet obtained after centrifugation was fixed with 2.5% glutaraldehyde for 6 h. The samples were then centrifuged and the pellet was resuspended in 1X PBS, pH 7.4 for 1 h containing 1% Osmium tetroxide (Sigma–Aldrich). Further cells were dehydrated in graded concentration of ethanol. Then specimens were platinum coated using JEOL JFC-1600 auto fine coater and observed under SEM (JEOL model JSM-6390 LV) at 20 kV.

4.3.10. Cholesterol-lowering ability of isolates in Wistar rat model

L. plantarum AMD6 and *L. plantarum* MTCC 1407 were examined for their cholesterol-lowering ability on high fat experimental diet induced rat model. The isolates were separately mixed with experimental diets to achieve the final concentration of approximately 10^8 CFU/g. A total of 42 adult Wistar rats (mean body weight 150 g) were used in this study and maintained at Defense Research Laboratory (DRL), Tezpur, India (Animal ethical approval no. 1AEC/01/2015). All animals were acclimatized by feeding on a basal diet (standard rat chow) for one week provided at DRL. The animals were divided into five experimental groups, (i) normal diet (ND), (ii) hypercholesterolaemic diet (HFD), (iii) HFD with statin (Atrovastatin Tablets IP, Macleod, India), (iv) HFD with *L. plantarum* (MTCC 1407), and (v) HFD with *L. plantarum* (AMD6). Each category had six rats in each groups as shown below (Fig. 4.3).

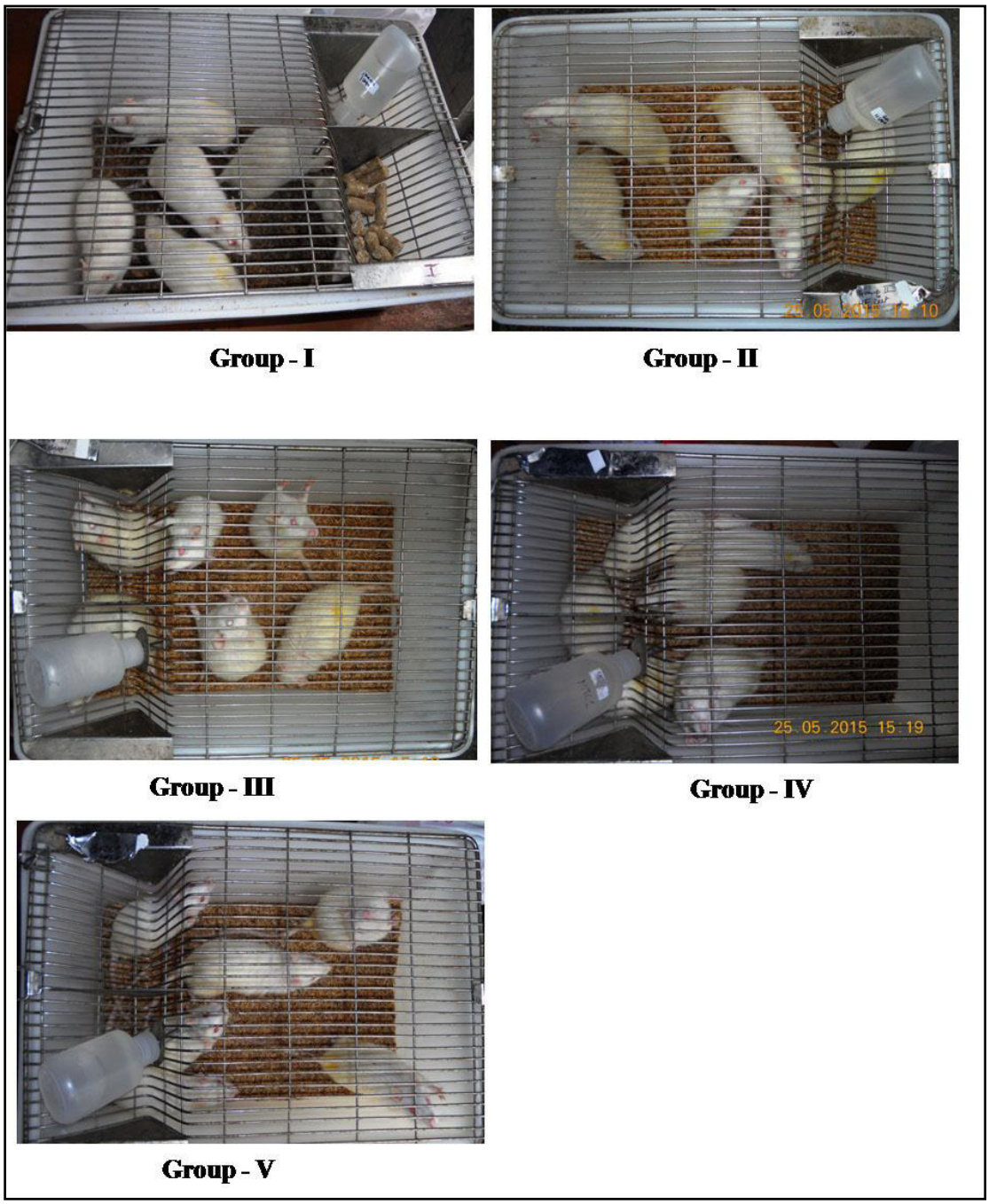


Fig. 4.3 Wistar rats used for experimental purpose

The animals were housed in individual cages and maintained under a constant 12 h light–12 h dark cycle with a controlled temperature at 25 °C and a relative humidity of 56–60%. The body weight of animals was measured weekly. The experimental high fat diet (Table 4.9) was formulated and fed to animals for 21 and 42 days with slight modification of the method reported earlier[22].

Table 4.9 Composition of experimental high-cholesterolaemic diet

Constituents	g/100g
Cholesterol	0.50
Dalda (saturated fat)	30.0
Refined soy oil	10·0
Wheat Flour	50.0
Sodium cholate	0·12
Vitamin mixture	1·01
Mineral mixture	4·00

4.3.11. Analysis of serum lipid profile

Blood samples were obtained from the retro-orbital sinus of overnight fasted animals into pre-chilled tubes at 21 and 42 days for serum lipid analysis. The collected blood samples were centrifuged at 2000 g for 15 min at 4 °C. The serum obtained were analyzed for total cholesterol (TC), triglycerides (TAG), and HDL-cholesterol using commercial enzymatic kits (Autopak, M/s Siemens Diagnostics Limited). Friedewalds equation[23] was applied to analyse the following other serum lipid fractions:

(1) LDL-cholesterol = total cholesterol - HDL-cholesterol - (TAG/5). All the concentrations are given in mg/dl.

(2) VLDL-cholesterol = The quotient (TAG/5) is used as an estimate of VLDL-cholesterol.

4.3.12. Statistics

Results were expressed as mean ± standard deviation and the data were analyzed by GraphPad 5.00, (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests was used to compare the differences among various groups. A value of $P < 0.05$ was considered to be statistically significant.

4.4. Results and Discussion

4.4.1. Preliminary Screening for probiotic attributes of isolated strains

The viability of twenty-six strains isolated from MRS agar was primarily screened for acid and bile tolerance in MRS broth adjusted to pH 2.0 with 1.0 N HCl and 0.3 % bile (Oxgall) containing MRS medium respectively (Appendix-15). The

growth was monitored on MRS agar plates using spread plate technique after 24-48h. One strain (AD6) was found to significantly resist the gastrointestinal stress conditions.

4.4.2. Molecular identification of isolates

The AD6 bacterial isolate was identified based on sequencing of 16S rDNA and the genus-specific 16S rDNA (Fig. 4.4A), and subsequently named as *L. plantarum* AMD6 (KJ867175).

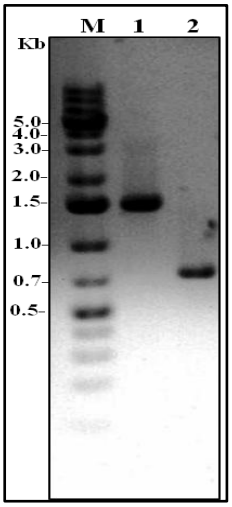


Fig. 4.4A PCR amplicon of 16S rDNA of AMD6 isolate. Lane M: 1 kb+ marker DNA, Lane 1: 16S rDNA amplicon of *L. plantarum* AMD6; Lane 2: genus specific 16S rDNA amplicon of *L. plantarum* AMD6.

The phylogenetic studies revealed that AMD6 showed maximum sequence similarity with *L. plantarum* FT731 (Fig. 4.4B).

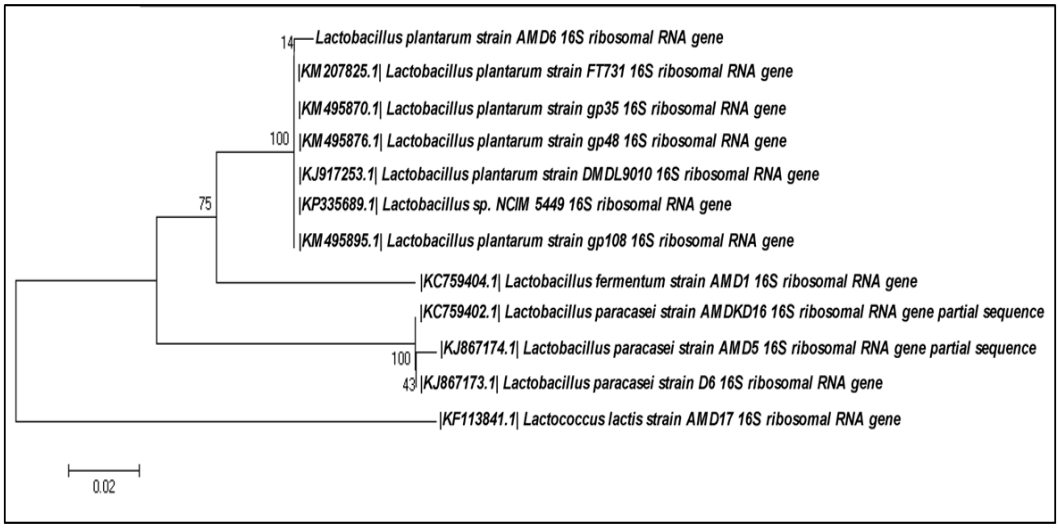


Fig. 4.4B Phylogenetic studies of AMD6 isolate with closely related species based upon 16S rDNA gene sequence. Bootstrap values (1,000 replicates) are indicated at branch nodes.

4.4.3. Probiotics attributes

4.4.3.1. Simulated gastrointestinal tract and bile tolerance

Survivability under gastrointestinal stress is a very important criterion for the selection of probiotic bacteria since the pH of the stomach varies from 2-3, and the bacterial isolate has to pass through it for a period of 2–3 h. Probiotic bacteria have to withstand this type of variation accompanied by different types of digestive enzymes such as pepsin and pancreatin. Viability under such conditions varies from species to species as per available literature[24]. When exposed to pepsin supplemented SGF of pH 2 for 3 h, the *L. plantarum* AMD6 cell count decreased to nearly 2 log units (Table 4.10A). This finding suggests that these isolates are able to propagate under laboratory stimulated gastrointestinal tract condition (Table 4.10). The viability of bacterial isolates increased considerably when exposed to pH 4 for 3 h as compared with pH 2. The reference strain MTCC 1407 showed viability loss up to 3.5 log units at pH 2 of SGF indicating that the isolate used in this study has better survivability under stress conditions.

Table 4.10A Simulated gastric fluid (SGF) tolerance test

Time (hr)	pH 2.0		pH 3.0		pH 4.0	
	AMD6	MTCC	AMD6	MTCC	AMD6	MTCC
0	7.65±0.038^{a}	8.35±0.15^{a}	7.67±0.026^{a}	7.53±0.0805^{a}	7.77±0.006^{a}	8.45±0.12^{a}
1	5.5±0.28^{b}	4.58±0.135^{b}	6.55±0.052^{b}	4.543±0.052^{b}	7.45±0.467^{b}	8.149±0.128^{ac}
2	5.27±0.178^{b}	4.63±0.036^{b}	6.45±0.045^{bc}	4.7±0.067^{b}	7.32±0.023^{b}	7.95±0.045^{bc}
3	5.17±0.082^{b}	4.56±0.578^{b}	6.25±0.058^{c}	4.73±0.197^{b}	7.32±0.060^{b}	7.94±0.022^{bc}

Values are represented as mean ± S.D. (n=3). Different letters along the same column represent significant difference (P<0.05). MTCC: *L. plantarum* MTCC 1407; AMD6: *L. plantarum* AMD6

Table 4.10B Simulated intestinal fluid (SIF) tolerance test

Time (hr)	pH 6.8		pH 8.0	
	AMD6	MTCC	AMD6	MTCC
0	7.96±0.041^{a}	8.25±0.02^{a}	7.98±0.14^{a}	8.27±0.02^{a}
1	7.97±0.008^{a}	8.19±0.35^{a}	7.84±0.15^{a}	7.95±0.06^{bc}
2	7.94±0.036^{a}	8.09±0.01^{a}	7.85±0.06^{a}	7.99±0.03^{b}
3	7.89±0.064^{a}	8.13±0.21^{a}	7.85±0.01^{a}	7.89±0.03^{c}
4	7.9±0.04^{a}	7.64±0.05^{a}	7.83±0.02^{a}	7.73±0.04^{d}

Values are represented as mean ± S.D. (n =3). Different letters along the same column represent significant difference (P<0.05). MTCC: *L. plantarum* MTCC 1407; AMD6: *L. plantarum* AMD6

Interestingly, isolates showed no significant loss of viability when exposed to SIF, which is less than 1 log CFU (Table 4.10B). The viable count of isolate was more than 70% after exposure to SGF having acidic pH and hence these isolates are considered tolerant to simulated GIT conditions. After gastric transit probiotic bacteria are exposed to the bile salts that are secreted by gall bladder to duodenum. Considering normal bile concentration (0.3%) of human GIT, we have used 0.1 %, 0.3 %, and 0.5 % oxgall to evaluate resistance towards bile. AMD6 and MTCC 1407 showed survival rates of more than 100% when incubated for 24 h at 0.5% bile (Table 4.10C). A slight reduction in viability of AMD6 isolate was observed after exposure to bile for 48 h. This finding suggests that AMD6 is a bile tolerant lactic acid bacterium, which corroborates with those reported by Oh and Jung[25]. The resistance to bile salt of the bacterial isolates might be induced by the presence of BSH and some proteins such as GshR1, GshR4, Cfa2, Bsh1, OpuA, and AtpH as reported in *L. plantarum*[26].

Table 4.10C Effect of bile salt (Oxgall) concentration on the viability of AMD6 and MTCC (Results expressed as log10 CFU/ml)

Time (h)	Control		MRS broth + 0.1 % oxgall	
	AMD6	**MTCC**	**AMD6**	**MTCC**
0	8.168±0.125^{a}	6.84±0.08^{a}	7.452±0.213^{a}	7.33±0.04^{a}
12	9.79±0.19^{b}	9.69±0.005^{c}	9.26±0.02^{c}	9.48±0.09^{c}
24	11.2±0.11^{c}	9.74±0.035^{c}	10.72±0.03^{d}	9.64±0.013^{c}
48	9.79±0.006^{b}	9.71±0.069^{c}	9.65±0.03^{e}	9.55±0.11^{c}
Time (h)	**MRS broth + 0.3 % oxgall**		**MRS broth + 0.5 % oxgall**	
	AMD6	**MTCC**	**AMD6**	**MTCC**
0	7.24±0.34^{a}	7.48±0.03^{a}	7.45±0.21^{a}	7.44±0.15^{a}
12	8.93±0.08^{b}	8.58±0.088^{b}	8.78±0.116^{b}	8.62±0.17^{bcd}
24	10.64±0.008^{c}	9.6±0.13^{c}	9.58±0.05^{c}	9.07±0.047^{d}
48	9.47±0.02^{b}	9.39±0.078^{c}	9.35±0.055^{c}	9.197±0.054^{d}

Values are represented as mean ± S.D. (n =3). Different letters along the same column represent significant difference (*P<0.05*). MTCC: *L. plantarum* MTCC 1407; AMD6: *L. plantarum* AMD6

4.4.3.2. Autoaggregation and coaggregation

The ability of probiotics to aggregate among themselves (autoaggregation) has been correlated with their adhesion capability to the intestinal mucosa and a prerequisite for persistence in the gastrointestinal tract. The aggregation capacity with genetically different species (coaggregation) is important for the probiotic strains to kill pathogens as the pathogens come into contact with the antimicrobial substances produced by the probiotics[27] . The highest autoaggregation attribute was exhibited by MTCC 1407 (39.79 ± 0.27 %) followed by AMD6 (38.32 ± 0.63 %). The co-aggregation capability with *S. enterica typhemurium* did not differ significantly

among the isolates tested in this experiment. The highest co-aggregation capacity was observed with AMD6 (42.86 ± 0.5) as depicted in Table 4.11.

Table 4.11 Autoaggregation, co-aggregation and hydrophobicity of *L. plantarum* AMD6 and *L. plantarum* MTCC 1407

Isolates	**Autoaggregation (%)**	**Co-Aggregation (%)**	**Hydrophobicity(%)**
		S. enterica typhimurium	**n-Hexadecane**
L. plantarum AMD6	38.32 ± 0.63[a]	42.86 ± 0.5[a]	42.74 ± 0.52[a]
L. plantarum MTCC 1407	39.79 ± 0.27[a]	38.00 ± 0.02[a]	36.11 ± 2.35[b]

4.4.3.3. Microbial hydrophobicity, probiotics adhesion and inhibition of *Salmonella enterica typhimurium* adhesion to Caco-2 cells

The affinity of microbial cell surface towards hydrocarbons (MATH) is considered as a key factor for adhesion and plays a crucial role in intestinal colonization and inhibition of pathogen through steric interactions[28]. ADM6 exhibited highest hydrophobicity (42.74±0.52 %) for n-hexadecane (Table 4.11). Microbial cells exhibiting higher hydrophobicity are likely to form strong contact with epithelial cells. The adhesion of isolates to Caco-2 cell line is considered as one of the selection criteria for probiotic strains. The adhesion of isolates to Caco-2 cells was examined microscopically prior to quantitative plate assay (Fig. 4.5A). In the present study, the *L. plantarum* MTCC 1407 strain and *L. plantarum* AMD6 showed 8-11% adherence to the Caco-2 cells respectively, Fig. 4.5B). Similar observation of adhesion abilities of probiotics has been documented previously[29]. In addition, AMD6 significantly ($P < 0.05$) reduced the adherence of *S. enterica typhimurium* to Caco-2 cells by 53% , whereas the reference strain *L. plantarum* MTCC 1407 displayed inhibition of *S. enterica typhimurium* adherence to Caco-2 cells by 32% (Fig. 4.5C). Therefore, our isolates can be considered as a potential probiotic to prevent pathogenic infection including the *Salmonella* associated gastroenteritis[30, 31].

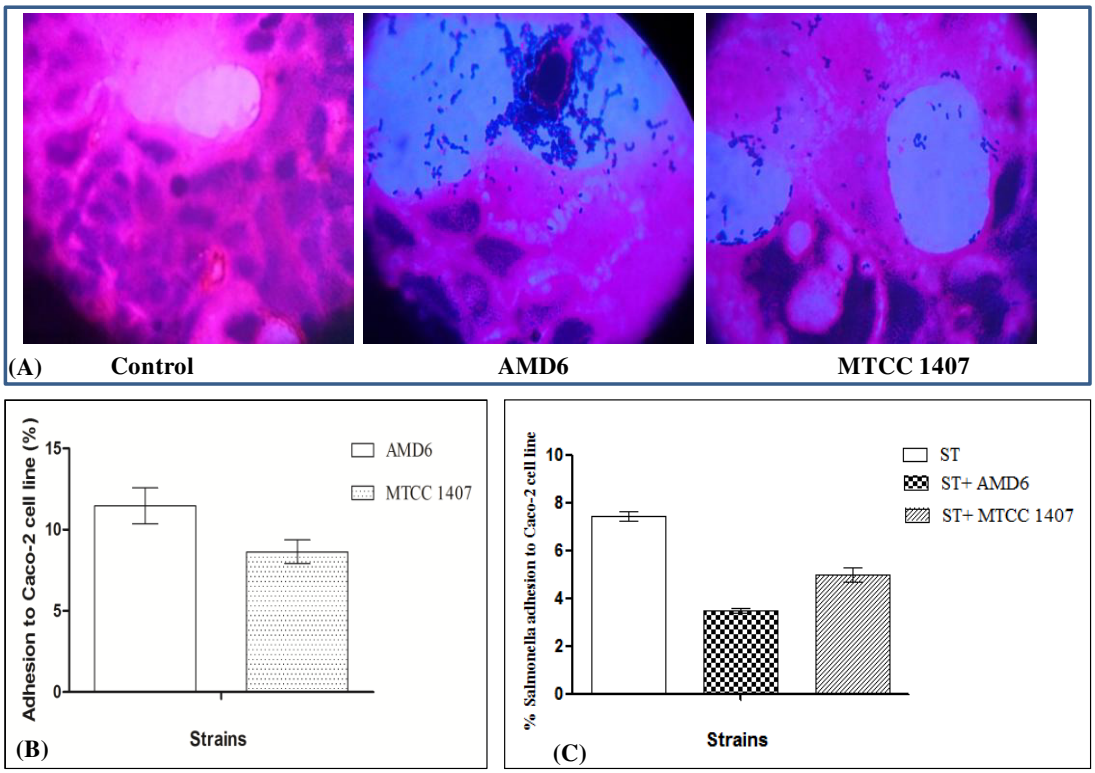


Fig. 4.5 (A) Adhesion ability of AMD6 and MTCC 1407 to Caco-2 cells as observed by Gram-staining under microscope. **(B)** Percentage adhesion of probiotic isolate to Caco-2 epithelial cells compared with the reference strains. **(C)** Percentage of *Salmonella* adhesion to Caco-2 cells in the presence and absence of probiotic isolate. ST: *Salmonella enterica typhimurium*; MTCC: *L. plantarum* MTCC 1407; AMD6: *L. plantarum* AMD6.

4.4.4. Screening of bile salt hydrolase activity, identification of BSH gene in AMD6 and deconjugation of conjugated bile salts

Bile acid is synthesized in the liver from cholesterol and secreted in the form of conjugated amino acids, with an amide bond between the bile acid carboxyl group and the amino group of either glycine or taurine. The hydrolysis of this amide linkage is catalyzed by a class of microbial enzymes referred collectively as conjugated bile acid hydrolases family[32]. *Lactobacilli* have been reported to possessed bile salts deconjugation activity as a defensive mechanism against the toxicity of conjugated bile acids[33].

AMD6 isolate showed bile salt hydrolase activity towards glycine- and taurine- conjugated bile substrates as analysed by the precipitation or the formation of opaque granular colonies with a silvery shine on MRS agar supplemented with bile salts (Fig. 4.6).

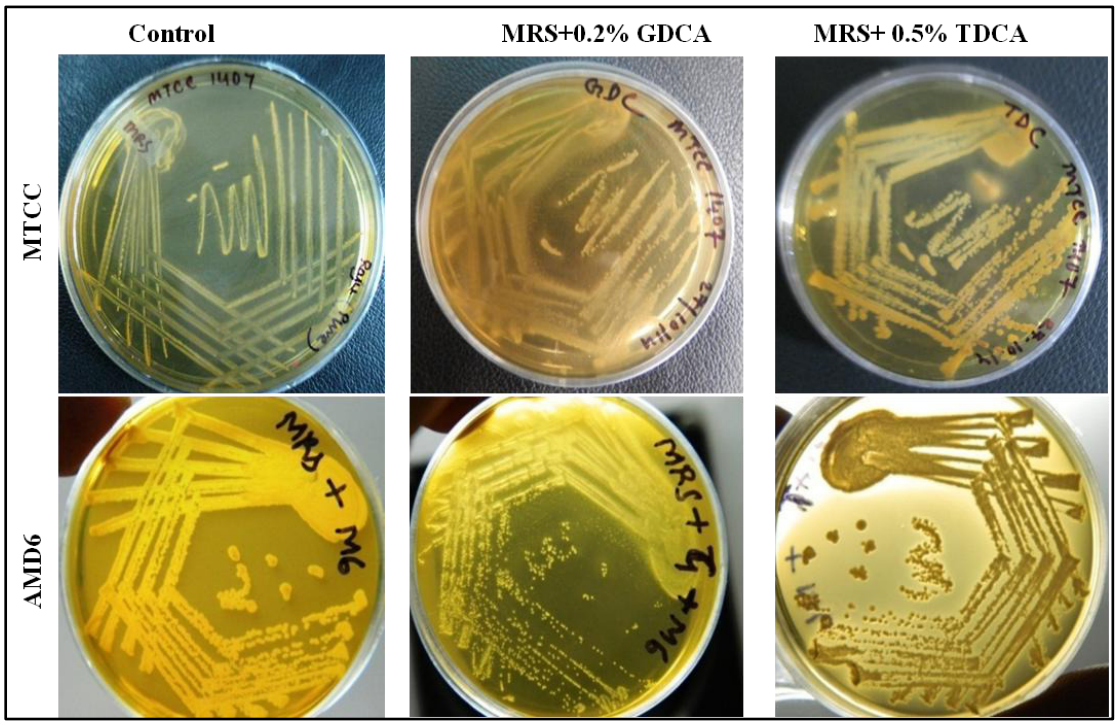


Fig. 4.6 Screening of BSH activity. Plates was incubated anaerobically for 72 hr at 37 °C. The control medium plate contains MRS agar and the assay medium plate contained 0·5% sodium salts of taurodeoxycholic acid and 0.2 % sodium glycodeoxycholate. The precipitation or the formation of opaque granular colonies with a silvery shine on the agar is indicative of bile salt hydrolase activity.

The gene encoding bile salt hydrolase enzyme was PCR amplified (~975 bp, Fig. 4.7) and the sequence has been deposited to NCBI GenBank (KP404632).

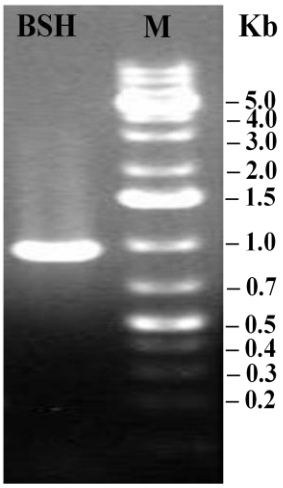


Fig. 4.7 PCR amplification of *Lactobacillus plantarum* AMD6 BSH gene. Lane 1: PCR amplification of AMD6 BSH gene; lane 2: GeneRuler^{TM} 1 Kb Plus DNA ladder (Fermentas).

The deduced 324 amino acid sequence obtained from nucleotide sequence of AMD6 BSH gene showed close relationship to *Bifidobacterium longum* bile salt hydrolase (Fig. 4.8A).

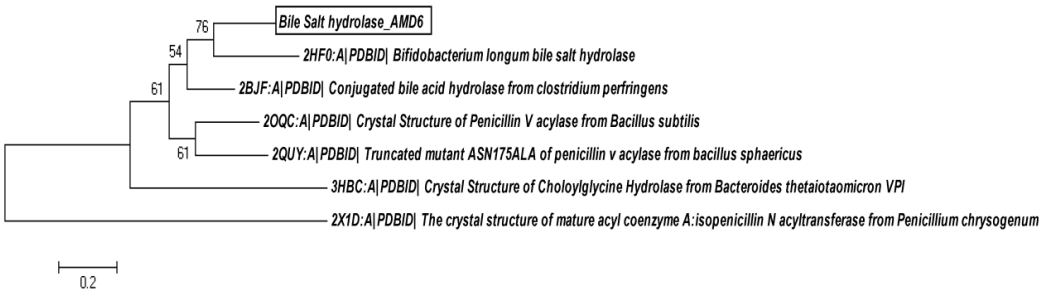


Fig. 4.8A Phylogenetic studies of AMD6 BSH gene. The evolutionary history of AMD6 BSH gene was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

A comparison of AMD6 BSH with other reported BSH protein sequences showed conservation of 20 consensus amino acid residues as indicated in the box, which is present in the conjugated bile acid hydrolases family (Fig. 4.8B).

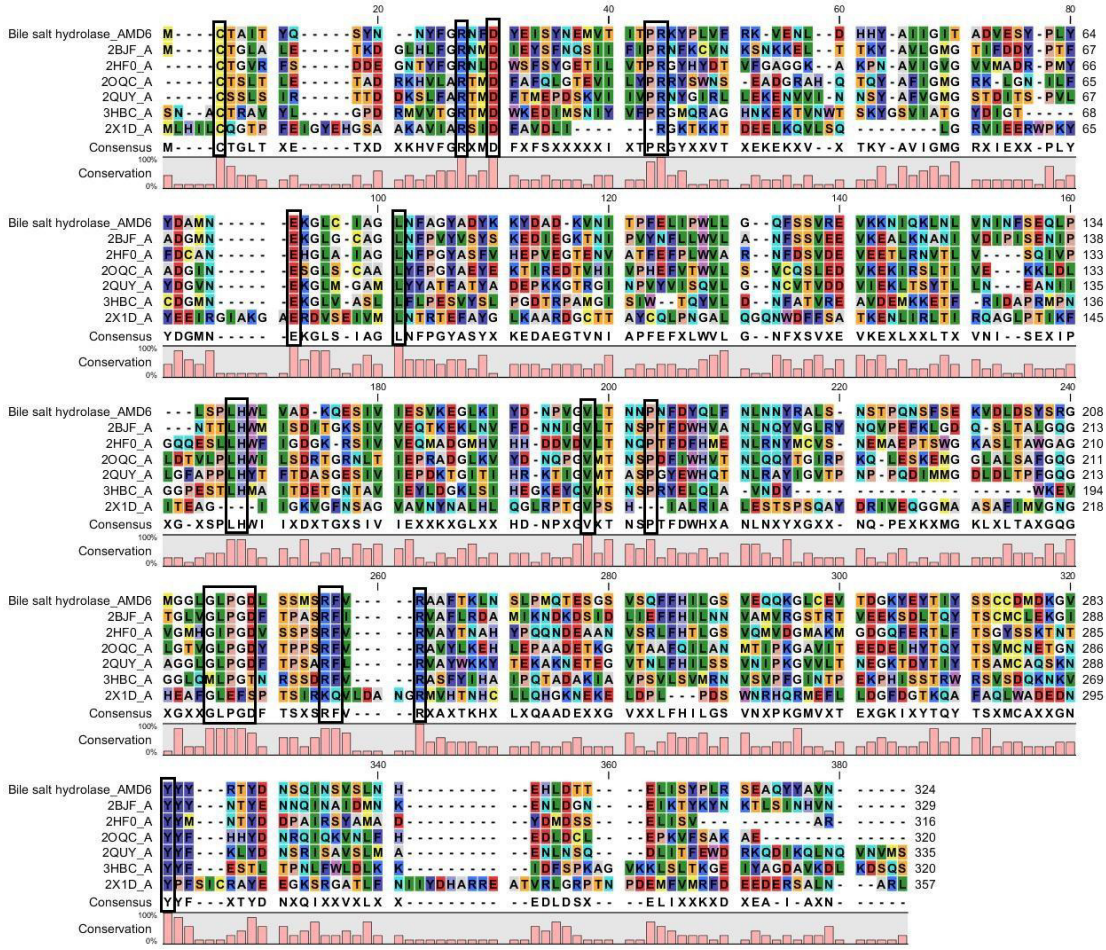


Fig. 4.8B Multiple sequence alignment of AMD6 BSH gene using MEGA 6.0. Conserved amino acids are indicated in box.

Further, the deconjugation of sodium glycocholate and sodium taurocholate by AMD6 was determined spectrophotometrically. AMD6 isolate could release cholic acid from the conjugated bile salts ranging from 0.73±0.34 to 5.87±1.38 mM, whereas the reference strain *L. plantarum* MTCC 1407 exhibited deconjugation ranging from 1.47±1.38 to 5.14±0.35 mM (Table 4.12). Moreover, it appeared that AMD6 showed higher deconjugation of sodium glycocholate compared to sodium taurocholate.

Table 4.12 Deconjugation of glycine or taurine conjugated bile based on release of cholic acid on MRS broth supplemented with 6 mM sodium glycocholate; 6 mM sodium taurocholate; 2.8 mM sodium glycocholate and 1.2 mM sodium taurocholate

Isolates	Cholic acid released (mM)		
	Sodium taurocholate	Sodium glycocholate	Sodium glycocholate + Sodium taurocholate
AMD6	0.73±0.34[a]	5.87±1.38[b]	2.69±0.35[d]
MTCC	1.47±1.38[a]	5.14±0.35[c]	2.45±0.69[d]

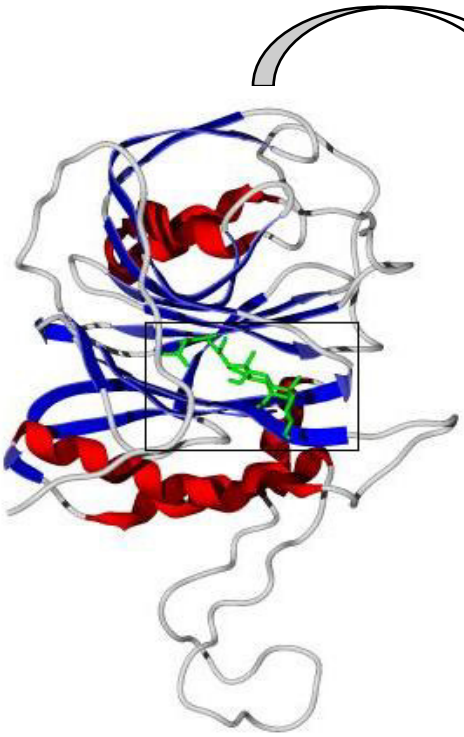
Results are expressed as means ± standard deviation of means. Means in the same column with different lowercase superscript letters are significantly different (P< 0.05).

The substrate preference was substantiated by computational docking of BSH with sodium glychocholate (-18.3492 kcal/mol, Fig. 4.9A) and sodium taurocholate (-13.6837 kcal/mol, Fig. 4.9B). However, the deconjugation ability of our isolate and the reference strain showed lesser deconjugation in a mixture of both conjugated bile salts.

4.4.5. Molecular docking of AMD6 bile salt hydrolase with sodium glycocholate and sodium taurocholate

PredictProtein server (https://www.predictprotein.org) analysis revealed that the AMD6 BSH protein comprises of nearly 20.7% helix, 24.1% β-strands and 55.2% loops. Molecular docking showed the presence of four putative residues of AMD6 BSH (Asn 25, Thr 288, Ile 272 and Asp 290) that contacts with sodium glycocholate (-18.3492 kcal/mol, Fig. 4.9A) and sodium taurocholate (-13.6837 kcal/mol) through hydrophobic interaction (Fig. 4.9B). The presence of hydrophilic interaction was also noticed in the docking studies. Similar observation was reported by Choi et al. 2014[34]

where binding energy with glycocholate and taurocholate was found to be -10.77 kcal/mol and -10.23 kcal/mol, respectively.



Binding energy of Sodium glycocholate -18.3492 kcal/mol

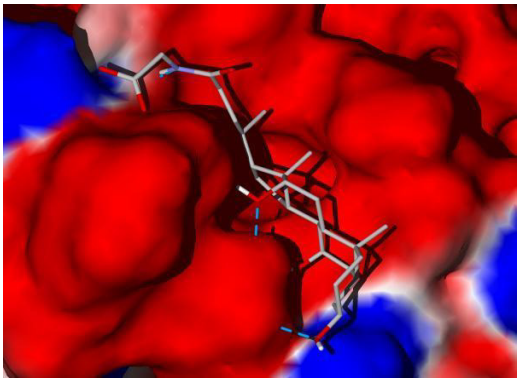


Fig. 4.9A (ii). Binding of sodium glycocholate in the catalytic site of bile salt hydrolase.

Fig. 4.9A (i) Docking of bile salt hydrolase with sodium glycocholate

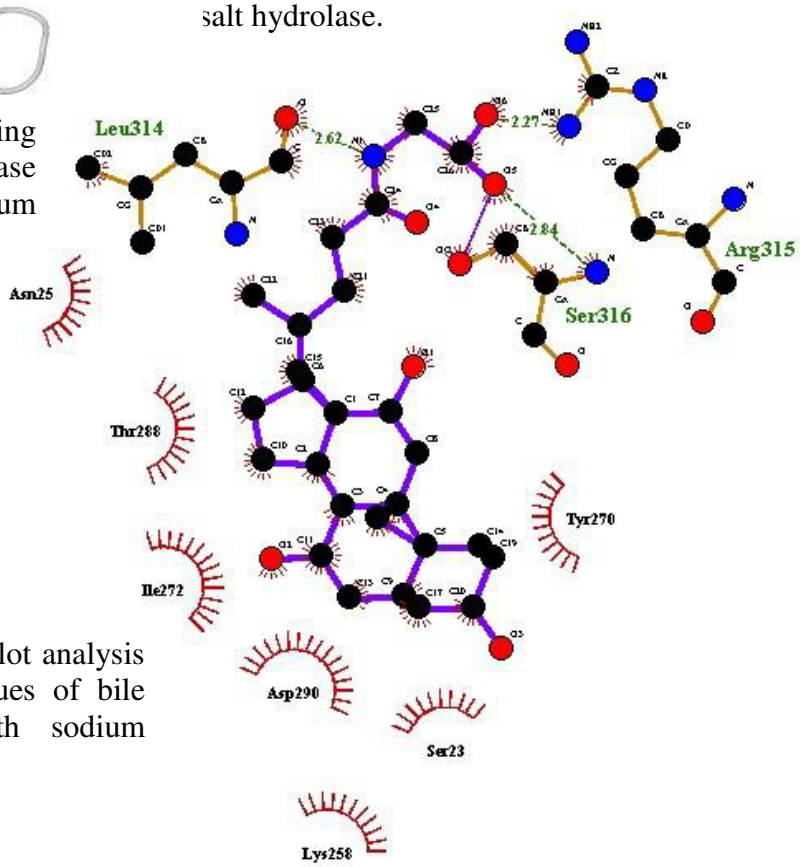


Fig. 4.9A (iii). LigPlot analysis of interacting residues of bile salt hydrolase with sodium glycocholate.

Fig. 4.9A Docking of AMD6 BSH gene with sodium glycocholate salt. Putative interacting amino acid residues are shown in relation to the bile salt substrate.

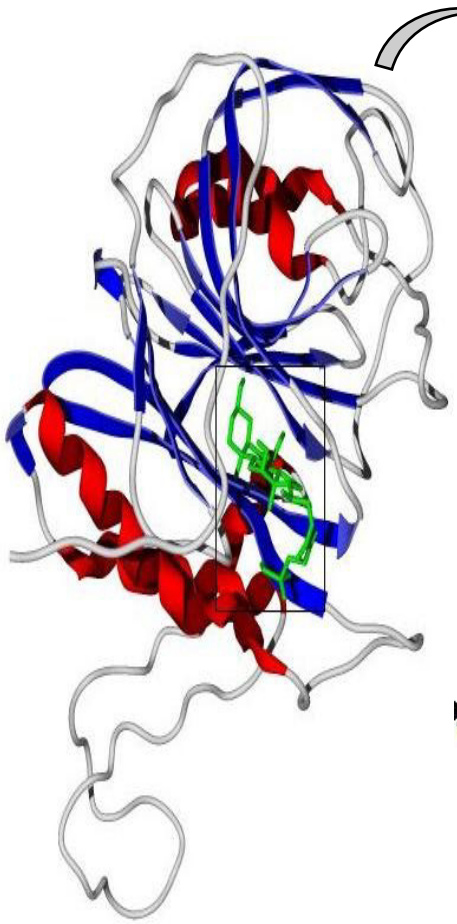


Fig. 4.9B(i). Docking of bile salt hydrolase with sodium taurocholate.

Binding energy of Sodium taurocholate - 13.6837 kcal/mol.

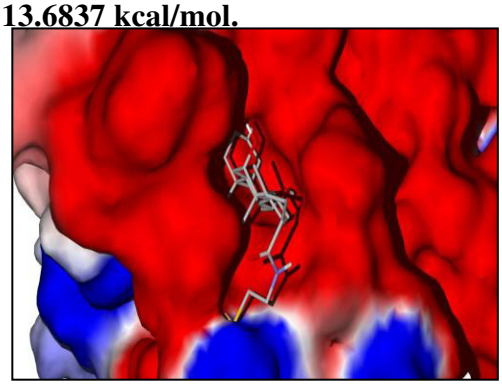


Fig. 4.9B(ii) Binding of sodium taurocholate in the catalytic site of bile salt hydrolase.

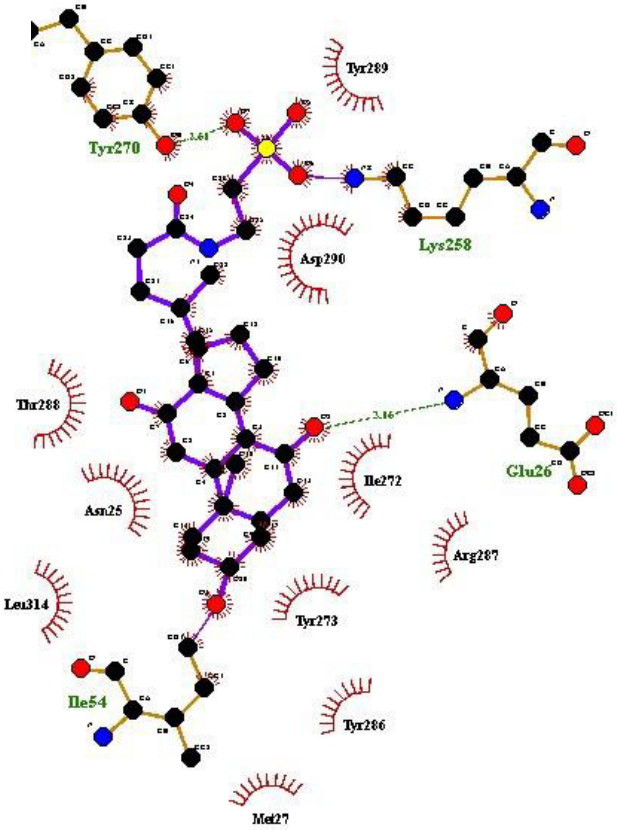
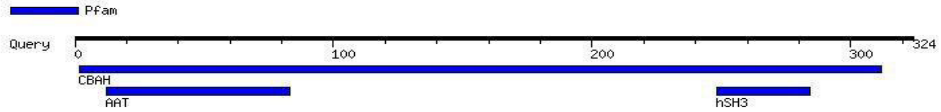


Fig. 4.9B(iii). LigPlot analysis of interacting residues of bile salt hydrolase with sodium taurocholate.

Fig. 4.9B Docking of AMD6 BSH gene with sodium taurocholate salt. Putative interacting amino acid residues are shown in relation to the bile salt substrate.

Analysis of AMD6 BSH in Motif Finder online server showed the presence of three distinct motifs viz. 6-aminopenicillinic acid acyl-transferase (amino acid position 12-83), choloylglycine hydrolase (aa position 2-312) and helically extended SH3 domain (aa position 248-284) which belongs to choloylglycine hydrolase family (Fig. 4.10).



Pfam (3 motifs)

Pfam	Position(Independent E-value)	Description
CBAH	2..312(2.9e-127)	PF02275, Linear amide C-N hydrolases, choloylglycine hydrolase family
hSH3	248..284(0.075)	PF14603, Helically-extended SH3 domain
AAT	12..83(0.13)	PF03417, Acyl-coenzyme A:6-aminopenicillanic acid acyl-transferase

Fig. 4.10 Motifs present in AMD6 BSH protein identified using Motif Finder bioinformatics tool.

4.4.6. Cloning and characterization of BSH gene

A full length BSH (~975 bp) was PCR amplified and successfully cloned into pBluescript KS (+) (Fig. 4.11A). The recombinant pBS-BSH clone was double digested with *Bam*HI and *Xho* I and the released BSH fragment was sub-cloned into expression vector, pET22 b (+) (Fig. 4.11B).

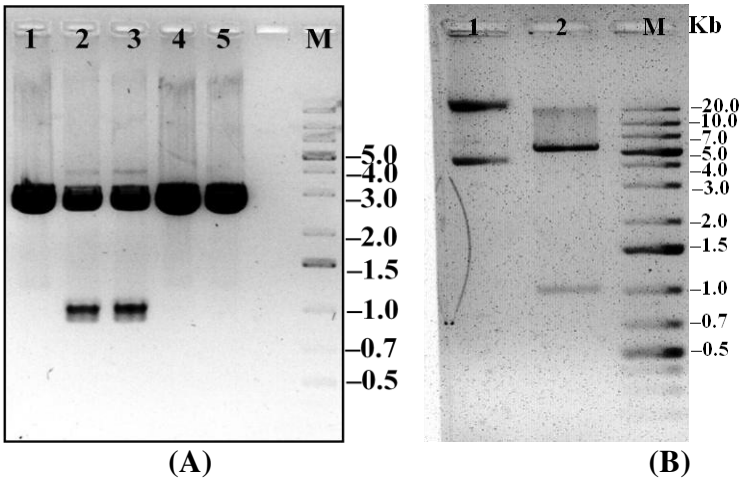


Fig. 4.11 Agarose gel electrophoresis image showing the release of DNA fragment corresponding to BSH gene (975 bp) upon double digestion of pBluescript KS(+) and pET 22b (+) with restriction endonuclease**. (A)** Lanes 2 and 3: positive clones; Lane 1, 4 and 5: negative clones. **(B)** Lane 1: undigested recombinant pET22b(+); Lane 2: recombinant pET22b(+) digested with restriction enzymes (*Bam*HI and *Xho*I); M, indicates GeneRuler™ 1 Kb Plus DNA ladder (Fermentas).

As shown in Fig. 4.12A, the recombinant BSH with C-terminal His tag (BSH-His) revealed apparent molecular weight of ~37 kDa on SDS-PAGE upon induction with IPTG which correlates with the predicted molecular mass of AMD6 BSH gene using ExPASy online tool. In this study, the majority of BSH-His was found in the inclusion bodies and SDS-PAGE analysis showed the purity of Ni-NTA affinity column eluted BSH-His under denaturing condition (Fig. 4.12B). SDS-PAGE analysis of purified BSH-His in the presence (Fig. 4.12B; Fig. 4.12C, Lane 1;) and absence of β-mercaptoethanol (Fig. 4.12C, Lane 2) in the sample loading buffer revealed barely detectable disulfide linkages which corroborates with the DISULFIND prediction tool (Fig. 4.13). Bioinformatics analysis of BSH using DISULFIND server showed the presence of four cysteine residues having low predicted disulfide bonding state[35].

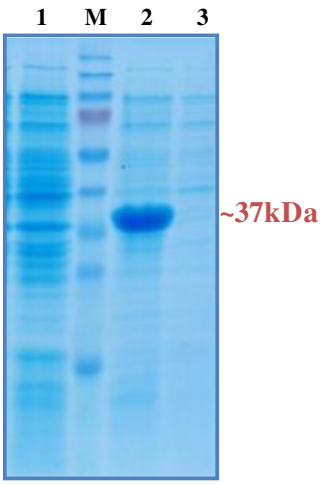


Fig. 4.12A SDS-PAGE (14%) analysis showing the over-expression of BSH in *E. coli* BL21 (DE3) upon induction with IPTG. Lanes 1: un-induced cells; Lane 2: indicates pellet fraction of BSH-His clones. Lane 3: supernatant fractions of induced BSH-His clones. Lane M indicates pre-stained protein molecular mass marker (ThermoFisher Cat. 26616). Protein bands were visualized by staining with Coomassie blue.

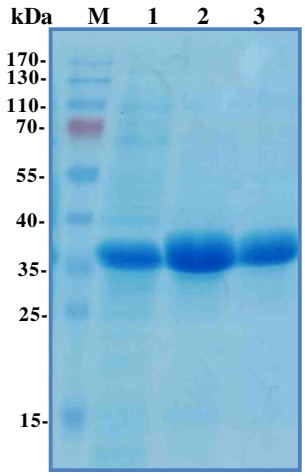


FIg. 4.12B Purification of recombinant BSH-His protein by Ni-NTA affinity chromatography. Lane 1, induced cell lysate of BSH clone; Lanes 2, & 3 indicates eluted fraction of BSH clone. Lane M represent marker as indicated earlier.

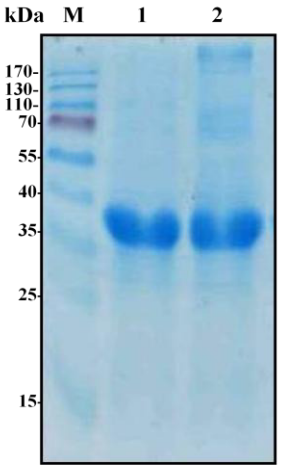


Fig. 4.12C Analysis of disulfide bond formation using SDS-PAGE. Lane 1, BSH-His in the presence (Lane 1) and absence of β-mercaptoethanol (Lane 2) in the sample loading buffer heated at 100 °C prior to gel electrophoresis. Lane M indicates pre-stained protein molecular mass marker as indicated.

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         .........10........20........30........40........50........60........70........
AA       MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGITADVESYPLYYDAMNEKGLCIAGLN
DB_state 0                                                                           0
DB_conf  7                                                                           9

         80........90........100.......110.......120.......130.......140.......150......
AA       FAGYADYKKYDADKVNITPFELIPWLLGQFSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGL
DB_state
DB_conf

         .160.......170.......180.......190.......200.......210.......220.......230.....
AA       KIYDNPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPGDLSSMSRFVRAAFTKLNSLPM
DB_state
DB_conf

         ..240.......250.......260.......270.......280.......290.......300.......310....
AA       QTESGSVSQFFHILGSVEQQKGLCEVTDGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVSLNHEHLDTTELISYPLRS
DB_state                         0               00
DB_conf                          9               88

         ...320..
AA       EAQYYAVN
DB_state
DB_conf
```

Fig. 4.13 Cysteines disulfide bonding state and connectivity predictor using DISULFIND prediction tool. AA indicates amino acid sequence, DB_state predicted disulfide bonding state (1=disulfide bonded, 0=not disulfide bonded); DB_conf: confidence of disulfide bonding state prediction (0=low to 9=high).

The enzymatic activity of refolded BSH-His was determined using conjugated salts (GDCA, GCA, TDCA and TCA). The purified recombinant BSH-His (4 µg) showed broad substrate specificity (Fig. 4.13) with a high preference for glycine-conjugated bile acids (GCA, 8.38±0.95 µM/min/mg; GDCA, 8.80 ± 1.63 µM/min/mg; Table 4.13) was observed as compared to tauro-conjugated bile salts as reported in the case of BSH of *Bifidobacterium longum* and *L. plantarum* CK 102[36, 37]. The presence of equal BSH-His protein loading in an enzymatic assay is indicated by the Commassie brilliant blue staining of the SDS-PAGE (Fig. 4.14, inset).

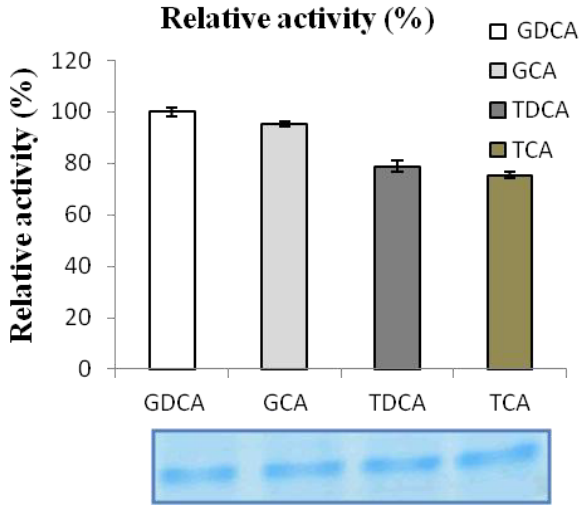

Fig. 4.14 The enzymatic activity of BSH-His using different bile substrates, glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycocholic acid (GCA) and taurocholic acid (TCA). The relative activity was calculated using GDCA as a standard (Percentage). Reaction was initiated by the addition of 4 µg of the purified BSH-His at 37 ºC for 30 min. Coomassie blue stained SDS-PAGE serve as equal BSH protein loading control (See inset).

Table 4.13 The specific activity of BSH-His using different bile substrates, glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycocholic acid (GCA) and taurocholic acid (TCA)

Substrate	**Specific activity (µM/min/mg)**
GDCA	8.80 ± 1.64
GCA	8.38 ± 0.96
TDCA	6.94 ± 2.16
TCA	6.64 ± 1.22

The broad-substrate activity of recombinant BSH-His is consistent with the activity as observed in a qualitative plate assay.

4.4.7. In vitro assimilation of cholesterol and scanning electron microscopy studies of cholesterol binding to cellular surface

The isolates were further investigated for their ability to assimilate cholesterol under simulated intestinal conditions. The cholesterol assimilation efficiency of spent broth and cells are provided in Table 4.14. A significant assimilation of cholesterol ($P < 0.05$) was exhibited by the resuspended AMD6 bacterial cells that ranged from 49.26 ± 8.96 µg/ml to 53.75 ± 5.89 µg/ml at 12 h and 48 h, respectively, under conditions that mimic the human gastrointestinal tract.

Table 4.14 *In vitro* cholesterol assimilation by AMD6 and MTCC 1407 with 0.3% bile salts (oxgall) after 48 h

Time (hr)	Strains	Cholesterol (µg/ml)		
		Control broth	Spent broth	Resuspended cells
12	AMD6	59.36±4.8[a]	9.56±3.96[a]	49.26±8.96[a]
	MTCC	59.36±4.8[a]	10.96±3.11[a]	47.39±7.97[a]
24	AMD6	59.36±4.8[a]	5.96±4.52[a]	53.10±9.65[a]
	MTCC	59.36±4.8[a]	4.16±2.54[a]	54.89±2.09[a]
48	AMD6	59.36±4.8[a]	5.36±1.41[a]	53.75±5.89[a]
	MTCC	59.36±4.8[a]	3.96±1.69[a]	55.31±2.99[a]

Assimilation of cholesterol by bacterial isolates (MTCC and AMD6) in de Man, Rogosa and Sharpe-medium (MRS) containing cholesterol and 0.3 % oxgall. Values are represented as mean ± S.D. (n =3). Different letters along the same column represent significant difference (P <0.05). ND : not determined.

As reported by Miremadi et al.[38], the cholesterol assimilation appears to be growth dependent as evident from this study. Scanning micrographs showed that cholesterol was adhered to the cellular surface of the isolates (Fig. 4.15). The attachment of water soluble cholesterol particles on the cell surfaces resulted into the appearance of roughness of cell morphology[39].

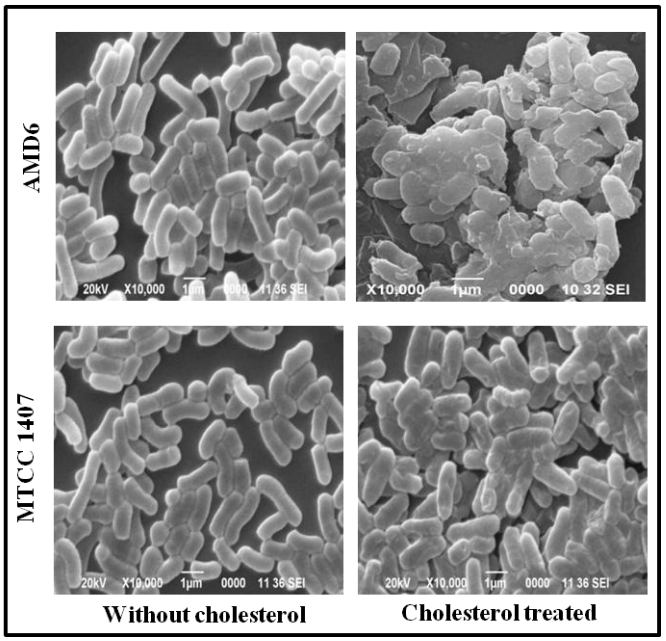

Fig. 4.15 SEM micrograph of AMD6 and *L. plantarum* MTCC 1407 grown in the absence or presence of water soluble cholesterol at 37 °C.

4.4.8. Cholesterol-lowering ability of probiotic isolates in Wistar rat model

L. plantarum AMD6 and *L. plantarum* MTCC 1407 were evaluated for their cholesterol-lowering ability using the Wistar rats as the animal model system. All the

experimental rats used in the study remained healthy, and their body weight gain after each week were calculated and recorded as indicated in Table 4.15.

Table 4.15 Faecal cholic acid concentration and body weight gain of rats fed with cholesterol-enriched experimental diets or supplemented with isolates AMD6 and reference probiotic strains

Groups/Week	Body weight (g)							Cholic acid released (mM)
	0 day	1 week	2 week	3 week	4 week	5 week	6 Week	42 d
ND	206.66±8.16^{a}	215±8.94^{a}	227.5±10.83^{ab}	234.16±7.36^{ab}	247.50 ± 5.24^{ab}	265.00 ±7.74^{a}	268.33±5.16^{a}	134.95±7.896^{a}
HFD	201.66±9.30^{a}	217.5±6.89^{a}	233.33±4.08^{a}	245±8.94^{b}	260.00 ± 9.49^{b}	298.33 ±2.58^{b}	314.16±5.84^{be}	112.93±5.939^{b}
HFD + STATIN	199.16±6.64^{a}	214.16±9.70^{a}	224.16± 9.70^{ab}	229.16±8.01^{a}	250.83 ± 6.65^{a}	280.83 ±5.85^{c}	295.83±9.70^{cd}	207.34±3.874^{c}
HFD + MTCC	205±7.74^{a}	216.66±10.32^{a}	223.33±6.83^{ab}	231.66±7.53^{ab}	252.50 ±9.35^{ab}	285.83 ±4.92^{c}	300.83±5.84^{bd}	133.64±8.18^{a}
HFD + AMD6	200±10.95^{a}	207.5±5.24^{a}	214.16±3.76^{b}	230.00±8.37^{ac}	251.66 ± 9.31^{ac}	282.50 ±4.18^{c}	296.66±9.83^{cd}	129.19±6.6^{ab}

Values are represented as mean ± SD (n=6). Means with different letters in the same row are significantly different (P<0.05) checked by Tukey's multiple comparison test, GraphPad prism, ver. 5.0. ND: Normal Diet; HFD: High fat diet; MTCC: *L. plantarum* MTCC 1407; Statin: Atrovastatin (Macleod); AMD6: *L. plantarum* AMD6.

Statistical analysis revealed that body weight gain did not differ among the groups significantly (P>0·05) after 1 week of feeding on cholesterol-enriched diet. The hypercholesterolaemic diet control animals (HFD), which were fed on high cholesterol diet, showed a relatively higher body weight (245 ± 8.94 g) after 3 weeks as compared to other experimental groups. Interestingly, the probiotic fed treatment groups, HFD+AMD6 exhibited a significant (P < 0.05) decrease of body weight to 296.66 g, when compared with high cholesterol fed group (314.16 g) after 42 d of experiment (Table 4.16). As shown in Table 4.16, faecal cholic acid excretion levels differed significantly among the HFD group and HFD+AMD6 treatment group. On supplementation of HFD with BSH containing AMD6, a substantial increase in the excretion of cholic acid (129.19 mM) in the faeces was observed after 42 d of feeding which is comparable to faecal cholic acid excretion (207.34 mM) observed in standard positive control group (HFD+Statin). The faecal cholic acid content of HFD

animal group was found to be 112.93 mM, which was lower than that of AMD6 dietary treatment. This attribute could be ascribed to deconjugation abilities of bile salts by AMD6 as observed in the present study.

Table 4.16 Total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels in the serum of rats fed a high-cholesterol diet alone (HFD) or supplemented with HFD+AMD6 and HFD+MTCC probiotic isolates for 6 weeks.

Lipid profile (mg/dL)	HDL-C		LDL-C		VLDL-C		TC		TG	
Group/Days	21	42	21	42	21	42	21	42	21	42
ND	12.0±2.19^{ab}	11.67±2.08^{ac}	31.25±6.003^{a}	29.06±2.32^{a}	16.18±3.19^{a}	19.07±1.97^{a}	59.43±5.47^{a}	59.8±2.52^{a}	80.9±7.07^{a}	95.37±9.83^{a}
HFD	14.6±3.20^{a}	7.00±2.82^{ac}	43.49±13.63^{ac}	41.81±10.43^{b}	20.38±11.636^{a}	51.37±13.45^{b}	78.48±3.40^{a}	98.0±4.72^{b}	101.92±5.799^{a}	256.87±8.91^{b}
HFD and Statin	12.83±2.44^{ab}	13.33±4.16^{ac}	43.33±6.24^{ac}	27.95±2.01^{a}	18.9±5.163^{a}	24.55±3.43^{a}	75.06±7.72^{a}	65.83±1.06^{ad}	94.53±8.48^{a}	112.90±9.05^{a}
HFD and MTCC	13.5±4.13^{a}	13.0±1.41^{ac}	39.15±2.87^{ad}	28.74±11.45^{a}	15.68±5.802^{a}	23.98±4.81^{a}	68.33±5.99^{a}	65.72±7.97^{ad}	78.4±10.07^{a}	108.53±9.41^{a}
HFD and AMD6	10.6±2.19^{ab}	20.75±2.63^{b}	40.62±7.44^{ac}	16.89±6.37^{a}	17.976±5.47^{a}	27.81±13.08^{ac}	69.2±3.8^{a}	65.45±2.095^{ad}	89.88±10.57^{a}	123.30±3.90^{a}

Values are represented as mean ± S.D. (n=6). Means with different letters in the same column are significantly different (P<0.05) checked by Tukey's multiple comparison test, GraphPad prism, ver. 5.0. HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; TC: Total cholesterol; TG: Triglycerides; ND: Normal diet; HFD: High fat diet; MTCC: *L. plantarum* MTCC 1407; Statin: Atrovastatin (Macleod); AMD6: *L. plantarum* AMD6.

The feeding of high-cholesterol diet to Wistar rats for 42 days significantly increased the levels of serum total cholesterol, triglycerides, low-density lipoprotein-cholesterols and very low density lipoprotein-cholesterols (Table 4.16).

In comparison to the control group (HFD), the dietary treatment rats (HFD+AMD6) showed significant reduction of the serum TC, TG, and LDL-C levels to 16.89, 123.30, and 27.81 mg/dl, respectively, after 42 d of probiotic treatment in the diets (Table 4.16). In this study, statin, a cholesterol lowering drug has also been included in the dietary preparation which served as a positive control for the experiments. As expected, HFD+Statin treatment significantly lowered serum TC, TG, and LDL-C levels to 65.83, 112.90, and 27.95 mg/dl, respectively, after 42 d of experimentation. Similarly, the reference probiotics formulated diet group

(HFD+MTCC) exhibited reduction in serum levels of TC, TAG, LDL-cholesterol levels after 42 d of experiments. Our data showed that the probiotic-containing diet treatment (HFD+AMD6) could lower the serum cholesterol with efficiency comparable to that of standard control treatment (HFD+Statin). At 21 d of the experimental period, there was no significant difference in the levels of HDL-C among all the groups. After 42 d of dietary treatment, VLDL-C levels in the HFD+AMD6 and HFD+Statin groups were reduced (P<0.05) to 27.81 and 24.55 mg/dl, respectively, as compared to control HFD rats (51.37 mg/dl). AMD6 isolate exhibited bile salt hydrolase activity and showed higher substrate specificity towards glycine-conjugated bile than taurine-conjugated bile. Brashears et al.[40] reported that sodium glycocholate preferred BSH-active strain may have far more prospects to decrease serum cholesterol levels if *in vivo* bile salt hydrolysis contributes significantly. Bile salts are biological surfactants that could lower the surface and interfacial tension, leading to increased cellular attachment of cholesterol[41]. Previous studies have reported that probiotic *Lactobacillus* strains have hypocholesterolaemic effect in rats and mice after administration of high cholesterol diet[7, 42]. The existing mechanism of cholesterol removal by probiotics include assimilation of cholesterol by growing cells, binding of cholesterol to surface, incorporation of cholesterol into the cellular membrane, deconjugation of bile via bile salt hydrolase and co-precipitation of cholesterol with deconjugated bile[3].

4.5. Conclusion

In conclusion, the findings of the present study indicated that *L. plantarum* AMD6 have the potential abilities to regulate lipid metabolism (TC, TG, LDL-C, and HDL-C). AMD6 also exhibited very good tolerance to low pH, high bile salt, and showed cholesterol assimilation under experimental conditions that mimic the intestinal tract. Hence, this indigenous isolates *L. plantarum* AMD6 has the potential to be explored for the preparation of functional foods and management of cardiovascular diseases.

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