Identification and Characterization of Bile Salt Hydrolase Genes from the Genome of *Lactobacillus fermentum* MTCC 8711

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Abstract *Lactobacillus fermentum* is a lactic acid bacterium of probiotic importance, which is found ubiquitously in fermented milk products. Bile salt hydrolase (BSH) has a significant role in affording probiotic properties to lactobacilli. In the present study, two *bsh* genes encoding BSH1 and BSH2 were identified from the draft genome sequence of *L. fermentum* MTCC 8711. Nucleotide comparison revealed no significant similarity between *bsh1* and *bsh2* genes, whereas the deduced amino acid sequences showed 26 % sequence similarity between both BSH1 and BSH2. Pfam analysis revealed the presence of cys-2 active site residues in the catalytic pocket of both BSH1 and BSH2 highly essential for catalysis. Phylogentic analysis of BSH1 and BSH2 revealed the possible independent origin of these proteins in *Lactobacillus*. We cloned these genes in pSLp111.3, a *Lactobacillus* expression vector with signal peptide A (*slp*A) and expressed in the native *L. fermentum* strain for overexpression and extracellular secretion. The *bsh*1 gene failed to express and to produce promising BSH activity. However, *bsh2* gene was overexpressed and the recombinant strain showed improved BSH activity. Induction of the recombinant strain with an optimal 2 % xylose concentration secreted 0.5 U/ ml of the BSH into extracellular medium. Furthermore, the recombinant strain was able to completely assimilate the 100-µg/ml cholesterol within 24 h, whereas the native strain took 72 h for the complete assimilation of cholesterol.

Keywords *Lactobacillus fermentum* · Bile salt hydrolase · Overexpression · Extracellular · Conjugated bile salts · Probiotic · Cholesterol

Introduction

The use of probiotics, in particular lactic acid bacteria (LAB), has been gaining much interest in the recent years. Gastrointestinal tract contains several LAB, which could be physiologically

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important for maintaining gut health. The LAB is widely used in the production of fermented foods, including dairy products such as yogurt, cheese, butter, buttermilk, kefir, and koumiss. Moreover, owing to its numerous and potential health-promoting benefits, there is a considerable interest in the use of probiotics as biotherapeutic agents [1]. Bile tolerance is one of the desirable criteria for probiotic lactobacilli that can survive in the intestinal environment. The cholesterol-lowering effect of probiotic lactobacilli is attributed to the presence of bile salt hydrolase (BSH) activity. The BSH belong to the choloylglycine hydrolase family of enzymes, classified as N-terminal nucleophilic (Ntn) hydrolases with an N-terminal cysteine residue, which is abundant in wide variety of gastrointestinal bacteria such as *Lactobacillus* [2], *Bifidobacterium* [3], *Bacteriodes* [4], *Clostridium* [5], and *Enterococcus* [6] species. Bile acids are conjugates of *N*-acyl amidates with either glycine or taurine, synthesized from cholesterol in the liver and secreted into the small intestine. Bile salts have the significant role in lipid metabolism, which emulsify and solubilize dietary fat and fat-soluble vitamins through its detergent activity. Bile salts are deconjugated by BSH, and the deconjugated forms are very less soluble and not absorbed by the intestinal cells. Thus, unconjugated bile salts are excreted in the feces. This homeostatic response results in the higher utilization of cholesterol for de novo synthesis of bile acids thereby lowering the serum cholesterol level [1]. Several mechanisms have been postulated for the removal of cholesterol by the probiotic bacteria, such as assimilation of cholesterol by growing cells, incorporation of cholesterol into the cellular membrane, and coprecipitation of cholesterol with deconjugated bile salts [7]. Though the cholesterol-lowering mechanism of probiotics has not yet been fully understood, the cholesterol and bile salt metabolism remain closely related to each other thus creating insights into the BSH hypothesis.

Lactobacillus fermentum is a potential probiotic organism with desirable properties such as non-pathogenic to human, increased resistance to the environment prevailing in intestine and beneficial effects to the human immune system [8]. *L. fermentum* also naturally inhabits human gastrointestinal tract and affords resistant to bile toxicity by producing BSH, which is involved in the removal of conjugated amino acid moiety of bile acids [9]. Earlier, we have reported the potential probiotic properties of *L. fermentum* MTCC 8711 [10]. Recently, we have reported the whole genome sequence of *L. fermentum* MTCC 8711 [11]. Genome sequence analysis revealed the presence of two *bsh* genes in *L. fermentum*. In this study, we report the overexpression of one of the *bsh* genes in *L. fermentum* and the improved cholesterol-lowering property of the recombinant organism.

Materials and Methods

Chemicals and Reagents

Antibiotics, sodium salts of glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA) were obtained from Sigma-Aldrich (Bangalore, India). Other laboratory-grade chemicals were purchased from Himedia Laboratories (Mumbai, India). PCR master mix, T4 DNA ligase, restriction endonucleases, DNA, and protein molecular weight markers were obtained from Fermentas (Thermo Scientific, CA, USA).

Bacterial Strains and Plasmids

L. fermentum MTCC 8711 was propagated in deMan–Rogosa–Sharpe (MRS) broth (Himedia, Mumbai, India) incubated at 37 °C without shaking for 18–22 h. *Escherichia coli* strains were

propagated in Luria–Bertani (LB) broth at 37 °C overnight with vigorous shaking. The PCR cloning vector pTZ57R/T (Thermo Scientific) was used to clone *bsh* gene. The *Lactobacillus* expression vector containing signal sequence *slp*A (pSLp111.3) was used to overexpress *bsh* genes in *L. fermentum*. The expression vector was a kind gift from Prof. Jos Seegers (Falco Biotech, Netherlands).

Genomic and Plasmid DNA Extraction

Genomic DNA was extracted from the overnight grown cells of *L. fermentum* MTCC 8711, using a DNeasy kit (Qiagen, Hildon, Germany) according to the manufacturer's instructions. Plasmid from *E. coli* was extracted using mini prep kit (Qiagen).

Mining of *bsh* Genes from Genome of *L. fermentum* MTCC 8711

The two BSH encoding genes (*bsh1* and *bsh2*) were identified from the draft genome *L. fermentum* MTCC 8711 (AVAB00000000) by BLAST search. The respective genes sequences were extracted from the draft genome using Bioedit software.

PCR Amplification and Construction of pBSH1 and pBSH2

The *bsh1* gene was PCR amplified from the genomic DNA using Bsh1F (GCCACCAT GGTAATGTGCACGGCCGTTTCC) & Bsh1R (CGATGGATCCTTAGGGTACTTGCGAT AGG) primers with *Nco*I and *Bam*HI restriction sites, respectively. Similarly, *bsh2* gene was PCR amplified using the primers Bsh2F (ACCCATGGGTATGTGCACGAGCATCAACGT CA) and Bsh2R, respectively (AAGGATCCGTTCAATTTCACCGGCGCCCAA) with *Nco*I and *Bam*HI restriction sites, respectively. The PCR reactions were performed with the following conditions: Initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min followed by a final extension of 5 min at 72 °C. PCR products were resolved by agarose gel electrophoresis (pH 8) and gel purified using QIA Quick gel extraction kit (Qiagen). The gel-purified PCR product was cloned into pTZ57R/T vector and transformed into *E. coil* DH10B cells by electroporation [12]. The transformed cells were plated on LB plates with ampicillin (100 μg/ml), X-gal (40 μg/ml), and IPTG (20 μg/ml) for blue white selection. The recombinant *E. coli* clones with pBSH1 and pBSH2 were selected and used for subsequent experiments.

Cloning of *bsh1* and *bsh2* Genes in pSLP111.3 Vector

The pTZ57R/T plasmid-harboring *bsh*1 and *bsh*2 genes (pBSH) were isolated individually from *E. coli* DH10B clones. The pBSH1 and pBSH2 was restricted with *Nco*I and *Bam*HI and ligated into the expression vector, pSlp111.3 cleaved with the same restriction enzymes. The resulting recombinant plasmids, pSLPBSH1 and pSLPBSH2 were transformed individually into *L. fermentum* MTCC 8711 by electro transformation [13]. The transformants were scored on MRS agar plates containing chloramphenicol (10 μg/ml).

Qualitative and Quantitative Determination of BSH Activity

Qualitative BSH activity of the recombinant *L. fermentum* MTCC 8711 cells was determined on MRS plate containing bile salts [14]. For plate assay, cells were grown

on MRS agar plates supplemented with 0.2 % (w/v) of bile salts (taurodeoxycholate (TDC) and glycodeoxycholate (GDC)) along with 2 % xylose and 0.37 g/l of $CaCl_2$. The plates were incubated at 37 °C for 72 h. The bile acid precipitates around the colonies (opaque halo) or the formation of opaque granular white colonies with silvery shine was considered as BSH activity. For the well assay, wells were prepared using a gel puncture on MRS agar plates with bile salts and xylose. In each well, 0.1 ml of extracellular/intracellular fractions were added and incubated at 37 °C for 72 h. BSH activity was quantified by ninhydrin assay as described by Liong and Shah [15]. The extracellular and intracellular BSH activities were measured using GDC and TDC as substrates.

Overexpression of bsh1 and bsh2 Genes in L. fermentum MTCC 8711

The L. fermentum harboring pSLPBSH1 and pSLPBSH2 plasmids was grown individually in MRS broth supplemented with chloramphenicol (10 μg ml^{-1}) and incubated at 37 °C under static condition. When the OD (600 nm) reached 0.6, the culture was induced with 0, 0.5, 1, 2, and 3 percentages of xylose, respectively and incubated for 4 h at 30 °C under static condition. After induction, the cells were harvested by centrifugation at 12,000 rpm for 5 min. The cell pellet was resuspended in lysis buffer (50 mM sodium phosphate and 300 mM NaCl, pH 7.5) and sonicated for 3 min (10 s bursts, 21 % amplitude). The cell lysate was centrifuged at 12,000 rpm for 30 min at 4 °C. The expression of recombinant BSH1 and BSH2 in both intracellular and extracellular fractions were analyzed by both qualitative and quantitative assays. The fractions were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining (R250).

Cholesterol Assimilation Assay

The assimilation level of cholesterol by L. fermentum was determined by the method described previously [10]. L. fermentum MTCC 8711 was grown in 100 ml of MRS broth supplemented with 0.3 % bile salt mixture and 10 mg of cholesterol. After incubation at 37 °C for various time intervals (24, 48, 72, and 96 h), cells were removed by centrifugation at 8,000 rpm for 10 min at 4 °C. The spent broth was collected and the cholesterol level was estimated. The uninoculated broth was considered as control. To the 1 ml of spent broth, 3 ml of 95 % ethanol followed by 2 ml of 50 % potassium hydroxide were added. The contents were mixed well after the addition of each component. The tubes were heated for 10 min at 60 °C in a water bath. After cooling, 5 ml of hexane was dispensed and vortexed for 5 min at 20-s interval. Then, 3 ml of water was added and mixed thoroughly. Tubes were allowed to stand for 15 min at 30 °C. After phase separation, 2.5 ml of hexane layer was transferred to a fresh tube and allowed to dry completely. To this, 1.5 ml of ferric chloride reagent was added and allowed to stand for 10 min. Subsequently, 1 ml of concentrated sulfuric acid was added along the sides of the tube and mixed well and allowed to stand for 45 min at 30 °C. The absorbance was measured at 540 nm in spectrophotometer (Model UV-1700, Shimadzu, Tokyo, Japan). The concentration of cholesterol was determined using cholesterol standard graph. The percentage assimilation was calculated using the following formula:

$$\% \text{ assimilation } = \frac{\text{Conc. of cholesterol in control} - \text{Conc. of cholesterol in sample}}{\text{Conc. of cholesterol in control}}$$

Computational Tools

The domain analysis of BSH1 and BSH2 were performed using BLASTp and NCBI conserved domain search tool. The in silico model structure of two BSH enzymes were predicted using SWISS MODEL server. The predicted structure was validated by protein structure validation software suite (PSVS) tool (http://psvs-1_5-dev.nesg.org/). The catalytic pocket and active site residues of BSH1 and BSH2 were predicted using CASTp server and pfam analysis. The promoter and signal sequences were predicted using BPROM and Signal P 4.1 server, respectively. The protein sequences showing hit with BSH1 and BSH2 query sequences on BLAST search were retrieved from NCBI and aligned using clustalW program. The aligned sequences were used for phylogenetic tree construction by UPMGA algorithm with boot strap replication of 1,000 using MEGA 5.1 beta 1 software.

Results

Bile Salt Hydrolase Genes in *L. fermentum*

Mining the draft genome sequence of *L. fermentum* MTCC 8711 (GenBank accession no. AP008937) showed the presence of two *bsh* genes designated as *bsh1* and *bsh2*, respectively. The 927 nucleotide long *bsh1* gene exhibited 99 % similarity with *L. fermentum* F1 BSH gene. The *Bsh2* was 978 bp long and showed 99 % homology with the *bsh* gene of *L. fermentum* NCDO394. The deduced amino acid sequences of BSH1 and BSH2 were compared using BLASTp. The BSH1 showed only 26 % similarity to BSH2 amino acid sequence with query coverage of 97 %. It is interesting to note that the two genes *bsh1* and *bsh2* showed no similarity at nucleotide level.

Phylogeny of BSH1 and BSH2

BLAST search of BSH1 and BSH2 resulted in identification of 44 different BSH sequences from NCBI database. BSH1 amino acid sequence were found to be widely distributed among organism belonging to species *Lactobacillus*, *Listeria*, *Enterococcus*, *Kurthia*, *Carnobacterium*, *Bacillus*, *Erysipelothrix*, *Fermicutes*, *Clostridium*, *Coprobacillus*, and *Anaerostipes*, whereas, BSH2 amino acid sequence were distributed among species *Lactobacillus*, *Weissella*, *Oenococcus*, *Lactococcus*, *Enterococcus*, *Bacillus*, *Clostridium*, *Carnobacterium*, *Kurthia*, and *Listeria* (Figs. 1 and 2). It was found that BSH1 showed 100 % similarity with BSH of another *L. fermentum* strain F1 and <45 % similarity with other representative organisms, whereas, BSH2 amino acid sequence showed 99 % similarity to BSH sequence of *L. fermentum* and <59 % similarity with other representative organisms in the phylogenetic tree.

In Silico Model Structure, Catalytic Site and Active Site Residues Prediction for BSH Enzymes

The in silico model of BSH1 and BSH2 were predicted using SWISS MODEL server. BSH1 structure was predicted to have 33.44 % of α-helix, 52.06 % of random coil, and 13.96 % of extended strand, whereas, BSH2 structure was predicted to contain 28.25 % of α-helix, 51.25 % of random coil, and 20.50 % of extended strand (Fig. 3a). Domain analysis of BSH enzymes revealed that BSH1 has an incomplete domain of N-terminal nucleophile (Ntn)_hydrolase superfamily (accession no. cl00467), whereas BSH2 possess the complete domain
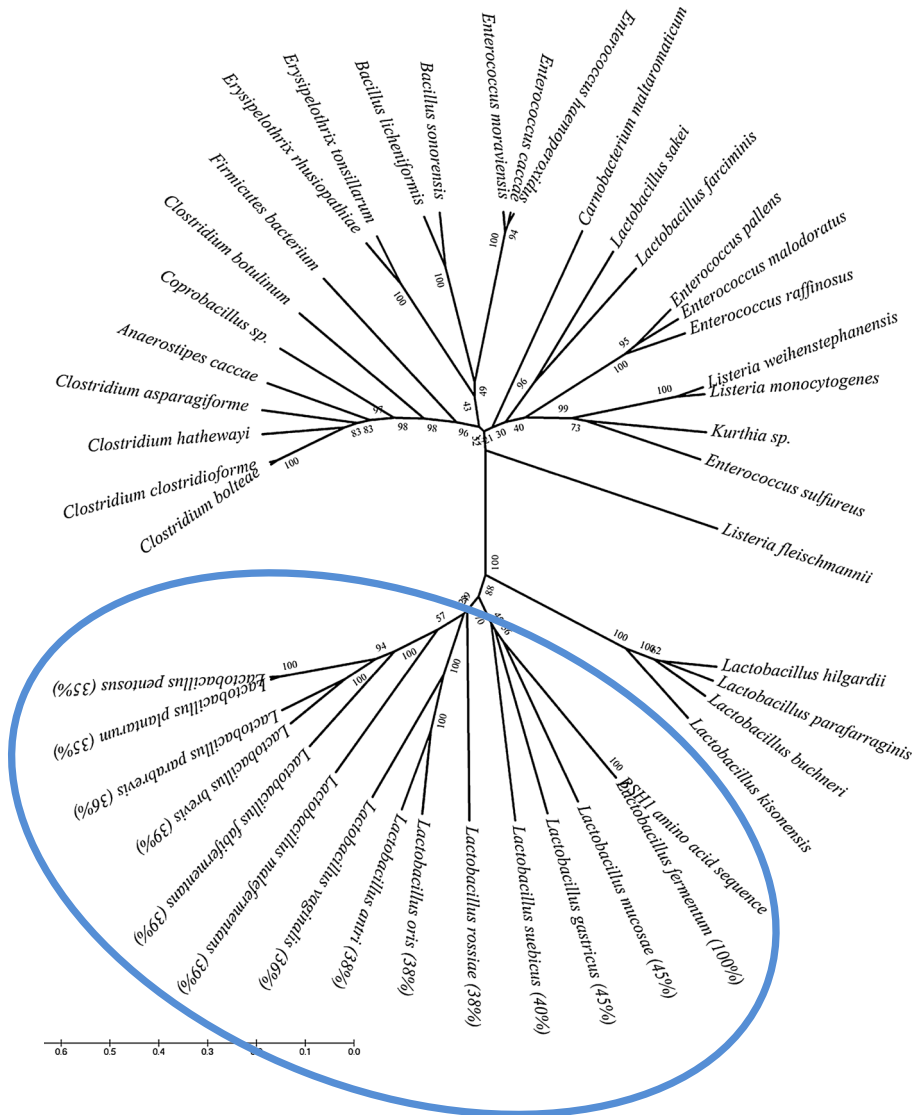
Fig. 1 Phylogeny of BSH1. The BSH1 of *L. fermentum* MTCC 8711 falls into a group of closely related BSH enzymes (*circled*). *Numbers in brackets* indicate amino acid sequence identity with BSH1. Sequences identified by BLAST searches with BSH1 as the query were aligned with MEGA 5.1 and represented in a tree structure

of Ntn_hydrolase superfamily (accession no. cl00467) and an incomplete domain specific for Ntn_penicillin V acylase (accession no. cd00542) (Fig. 3b). The catalytic site residues of both BSH enzymes were predicted using CASTp server. The results revealed that the residues forming the catalytic sites of the both the enzymes are different. However, the active site residue (Cys-2) identified by pfam analysis was conserved in both enzymes (Fig. 4). Thus, both BSH1 and BSH2 belong to N-terminal hydrolase superfamily (Ntn), members which have N-terminal
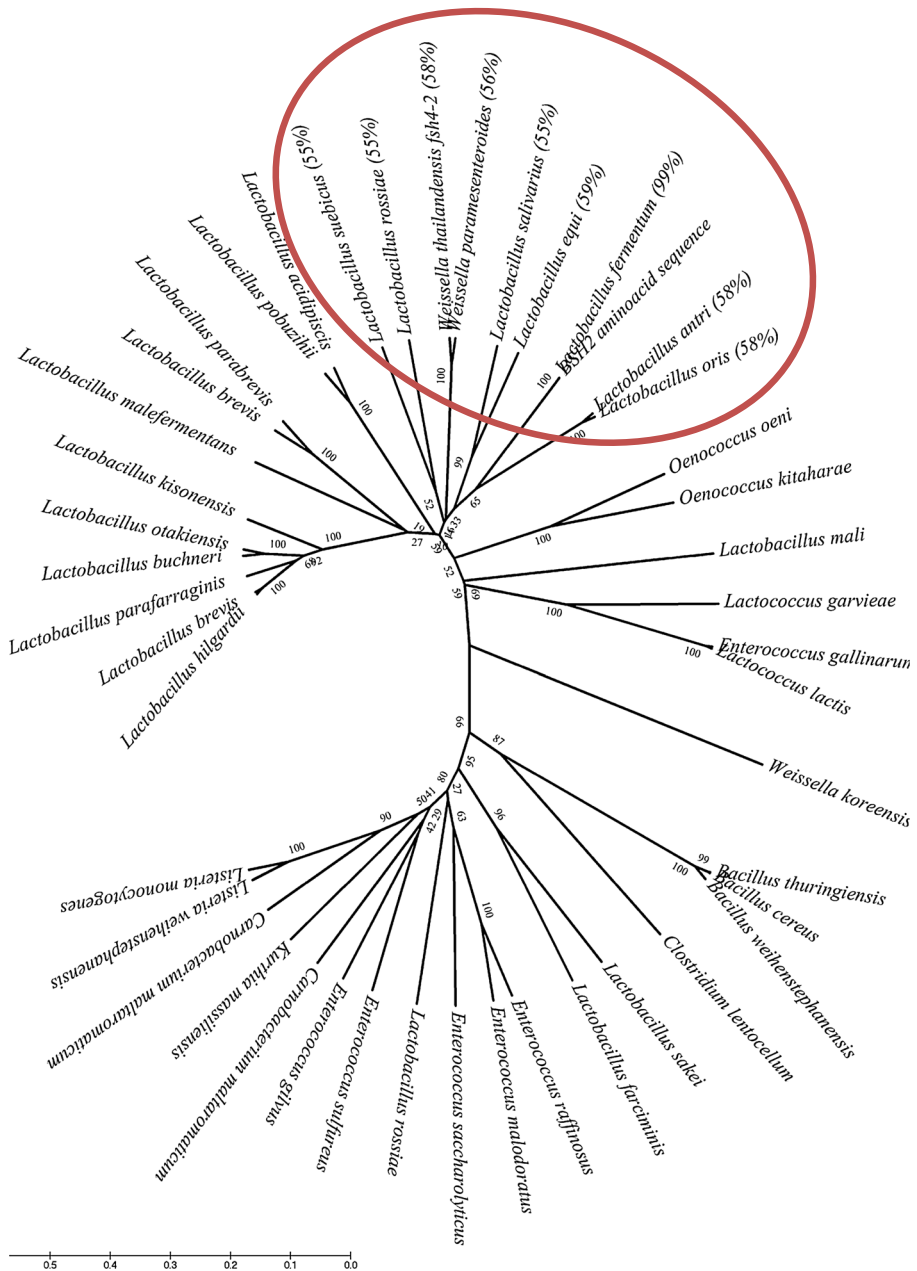
Fig. 2 Phylogeny of BSH2. The BSH2 of *L. fermentum* MTCC 8711 falls into a group of closely related BSH enzymes (*circled*). *Numbers in brackets* indicate amino acid sequence identity with BSH2. Sequences identified by BLAST searches with BSH2 as the query were aligned using MEGA 5.1 and represented in a tree structure

catalytic nucleophile that cleaves an amide bond. Both enzymes possess Cys-2 as the active site residue, which serve as the nucleophile and proton donor and is important for catalysis.
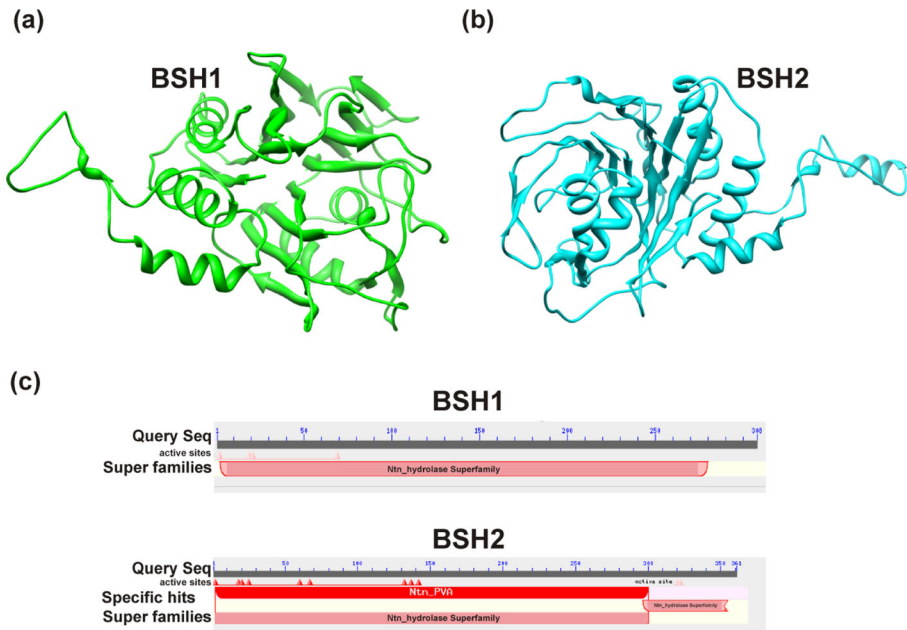
Fig. 3 In silico structure prediction and domain analysis of BSH enzymes. In silico model of BSH1 (a) and BSH2 (b) predicted using SWISS MODEL server. c BLAST searches reveal the presence of domain for Ntn_hydrolase superfamilies in both BSH1 and BSH2. The BSH1 has incomplete domain for Ntn hydrolase superfamily. BSH2 have complete domain for specific Ntn_penicillin V acylase and complete domain for Ntn hydrolase superfamily

Overexpression of BSH2 in Native *L. fermentum* MTCC 8711

Primers were designed to amplify *bsh*1 and *bsh*2 genes from the genome of *L. fermentum* MTCC 8711 with the *Nco*I and *Bam*HI restriction sites. The amplified genes were cloned individually in frame in the expression vector, pSlp111.3 and the resulting recombinant plasmids were transferred into *L. fermentum* MTCC 8711. The cloned *bsh*1 gene failed to produce BSH activity in both extracellular and intracellular fraction. The expression of the *bsh*2 gene in the recombinant *L. fermentum* strain was analyzed by the induction with different concentrations of xylose. Extracellular and intracellular fractions were collected and the BSH activity was quantified using ninhydrin assay and the proteins were analyzed by SDS-PAGE. For extracellular expression of BSH, 2 % of xylose was found to be optimal concentration, resulting with 0.5 enzyme activity (U/ml) as shown in Fig. 5. In the case of intracellular expression, 0.23 U/ml of maximal enzyme activity was obtained with 1 % xylose concentration. Extracellular BSH enzyme activity was found to be predominant over the intracellular expression. Extracellular fractions with different concentrations of xylose induction were resolved on SDS-PAGE. The Coomassie stained gel showed visible bands of approximately 36.5 kDa with increasing intensity directly proportional to the increased xylose concentration (Fig. 6). The expression of the *bsh*2 gene was also confirmed by qualitative plate assay and well assay. The freshly grown recombinant *L. fermentum* MTCC 8711 cells marked on the MRS plate with 0.2 % (*w/v*) of GDC showed a visible halo surrounding the cells after incubation, while the native cells did not (Fig. 7). For the well assay, the soluble extracellular fractions added in the wells of MRS-GDC plates showed good zone of clearance concordant to their xylose concentrations. Larger zone was visualized in the well with 2 % xylose induced fraction (Fig. 8).
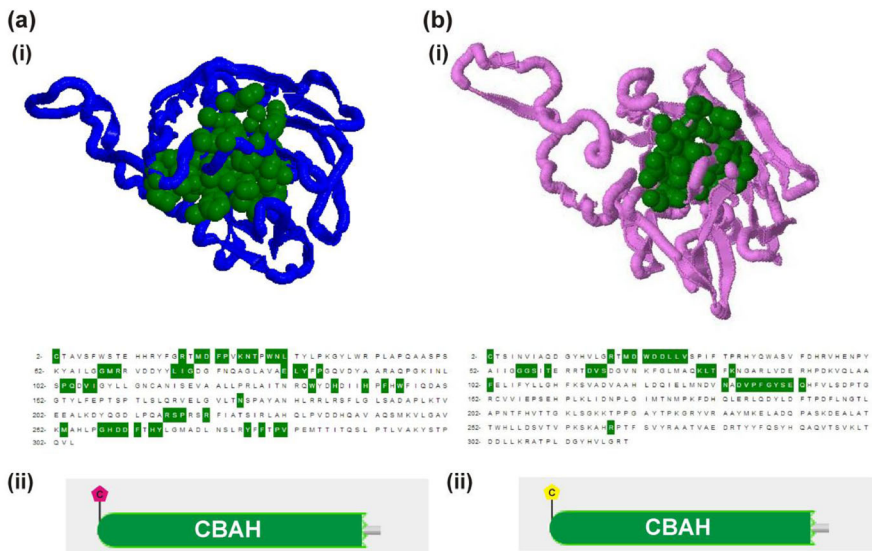
Fig. 4 Catalytic site and active site prediction for BSH enzymes. The catalytic site of BSH enzymes were predicted using CASTp server. The active site residues of both BSH enzymes were identified by pfam analysis. **a** Catalytic site and active site residue prediction of BSH1. **b** Catalytic site and active site residue prediction of BSH2. The catalytic acid residues were marked as *green color*. The residues forming catalytic site of BSH1 and BSH2 enzymes varies, but the cysteine at position 2 of BSH1 and BSH2 serves as active site residues for both the enzymes

Cholesterol Assimilation by Recombinant *L. fermentum* MTCC 8711

The cholesterol-removing ability of probiotic *L. fermentum* with or without harboring pSLPBSH2 plasmid was assessed; tested strains had the ability to remove cholesterol from laboratory media during growth. Wild-type *L. fermentum* MTCC 8711 cells grown in MRS broth supplemented with cholesterol (10 mg/100 ml) and 0.3 % GDC showed up to 60 % cholesterol assimilation after 24 h of incubation, followed by 94 % at 48 h and then reached 100 % at 72 h and maintained 100 % till 96 h of incubation. In comparison to wild type, recombinant *L. fermentum* MTCC 8711 showed approximately 100 % of cholesterol assimilation at the first 24 h of incubation after which the level was maintained the same up to 96 h of incubation.

Discussion

L. fermentum is a lactic acid bacterial strain with multifaceted beneficiary properties. *L. fermentum* has heterofermentative metabolism, possesing antagonistic ability and stabilizes the normal gut microflora to maintain the gastrointestinal health of mammals [16, 17]. Transformants showed good BSH activity and cholesterol assimilation activity. The native *L. fermentum* MTCC 8711 was not able to show the enzymatic activity on the plates as well as the quantitative assay though it possesses the *bsh* genes in its genome. This could elucidate that the amount of enzyme produced by the native strain might not be sufficient for exhibiting the activity. Hence, the recombinant strain was constructed in the native host using pSLPBSH2 to validate the BSH activity of *L. fermentum* MTCC 8711. Extracellular BSH activity was reported
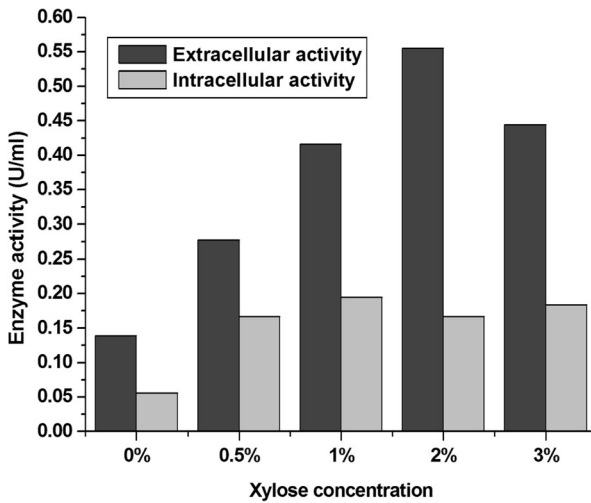
Fig. 5 Comparison of enzyme activity of both intracellular and extracellular fraction

earlier in Clostridium *perfringens* isolated from the chicken intestines [18]. Several reports are available on the cloning and characterization of *bsh* gene. Reports are also available on molecular cloning, characterization and heterologous expression of similar gene from *L. fermentum* NCDO394 in *E. coli* cells [7]. Qualitative BSH activity using plate and well assay with the recombinant *L. fermentum* MTCC 8711 harboring pSLPBSH2 showed good amount of enzymatic activity on the plates with bile substrate (GDCA). The zones obtained in the well assay were directly proportional with the increasing amounts of xylose concentrations with 2 % being the optimal concentration. Further increase in the xylose concentration did not increase the enzyme activity. The recombinant strain showed more specificity towards the GDCA upon TDCA. Similar results for substrate specificity were obtained by Kumar et al. [7] and Liong and Shah [15] where in the BSH gene was specific towards the glyco-conjugated salts than the tauro ones. The cells were able to reach 100 % cholesterol assimilation in about 24 h when compared with the native strain that took approximately 72 h to reach that point. Cholesterol assimilation is an important aspect of possessing the BSH enzyme, where in the conjugated bile salts in the presence of cholesterol show reduction in the cholesterol levels by acting upon the bile salts. The possible mechanism of the cholesterol has not yet fully understood while the proposed mechanism of cholesterol assimilation has been associated with BSH activity [19]. The *bsh1* gene

Fig. 6 SDS-PAGE Analysis of *bsh2* gene overexpression from *L. fermentum*. **a** *Lane M*, Protein marker (3.5 to 43.5 kDa); *lane 1*, no xylose induction; *lane 2*, 0.5 % xylose induction; *lane 3*, 1 % xylose induction; *lane 4*, 2 % xylose induction; and *lane 5*, 3 % xylose induction
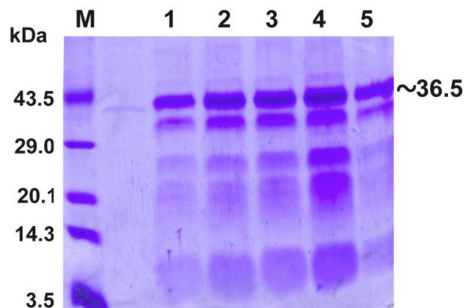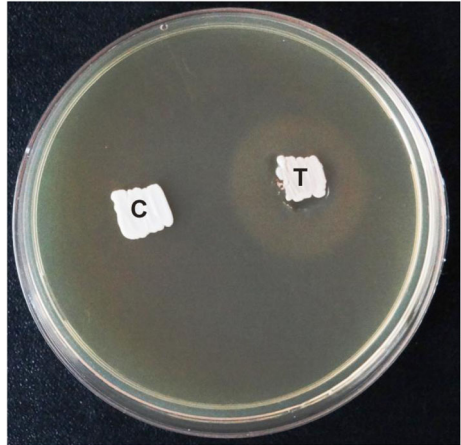
Fig. 7 Qualitative plate assay of enzyme activity of *bsh*2 gene from *L. fermentum*. *C* Control native strain, *T* test recombinant strain


retrieved from genome of *L. fermentum* MTCC 8711 failed to produce BSH activity. Sequence analysis revealed that BSH1 has only a part of for N-terminal nucleophile (Ntn)_hydrolase superfamily domain (accession no. cl00467). Both BSH1 and BSH2 possessed a Cys-2 active site residue, which serve as nucleophile and proton donor highly for catalysis. This result is consistent with the previous report on BSH enzyme from *Lactobacillus johnsonii*, which has Cys-1 active site for catalysis [20].

Earlier, the functionality of the *bsh* genes of *Lactobacillus plantarum* WCFS1 was explored using *bsh*-deficient host *Lactococcus lactis* NZ9000 by use of the NICE system [21]. Several *bsh* genes have been cloned and characterized from *Lactobacillu*s strains [21–23]. Few reports are also available on the heterologous expression of the *bsh* genes from lactobacilli in heterologous hosts such as *E. coli* [21, 7]. However, no reports are available on the overexpression of the *bsh* gene in the native lactobacilli. Preparation of fermented foods such as yogurt using lactobacilli with higher BSH activity would serve as a functional biotherapeutic agent.

In conclusion, a BSH gene from *L. fermentum* MTCC 8711 was cloned and overexpressed in the native host system. The recombinant strain showed promising BSH activity along with the

Fig. 8 Qualitative well assay of *enzyme activity of bsh2 gene from L. fermentum*. **a** *Well A*, No xylose induction; *well B*, 0.5 % xylose induction; *well C*, 1 % xylose induction, *well D*, 2 % xylose induction; *well E*, 3 % xylose induction; *well F*, negative control (transformed empty pSLP111.3)
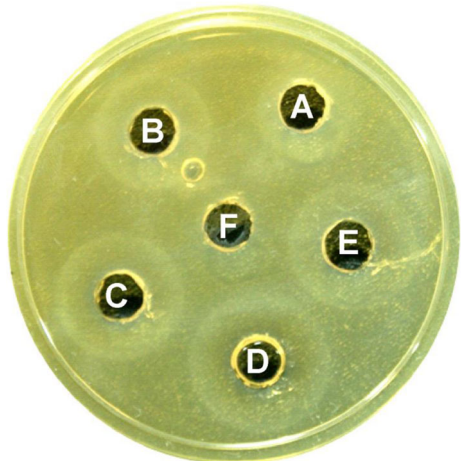
cholesterol assimilation property. Although the native strain possessed two *bsh* genes in its genome, it did not show significant activity in vitro. The recombinant strain harboring pSLPBSH2 showed promising BSH activity. The extracellular enzyme activity was higher than the intracellular fraction. The extracellular enzyme was purified and it showed higher specificity towards the glyco-conjugated bile salts. The recombinant strain was able to achieve 100 % cholesterol assimilation within 24 h under in vitro conditions. Therefore, the *bsh* overproducing *L. fermentum* MTCC 8711 could be used to develop a potential biotherapeutic agent to overcome hypercholesteremia.

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