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NEW RECOMBINATION OF *LACTOBACILLUS ACIDOPHILUS* BILE SALT HYDROLASE GENE A (BSHA) AND TRANSFORMATION IN *ESCHERICHIA COLI*

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ABSTRACT

The bile salt hydrolase gene (*bshA)*, encoding bile salt hydrolase enzyme (EC 3.5.1.24) from *Lactobacillus acidophilus Ar* probiotic isolate, which is responsible for assimilation cholesterol were studied in the present work. This gene designed contain two restriction sites (*PstI/SacI*) were added to each end of fragment and amplified by PCR techniques use specific primer to genetic analysis sequencing for gene nucleotides which were 801 bp. Gene manipulation during cloning inserted into pMGe36 vector respectively. pMGe36 vector is overexpression plasmid for *E. coli*. The resulted constructs were named as pMG36e / *bshA*. The recombinant was transferred to *E. coli* MC1022 by chemical transformation. Obtained recombinants analyzed for expression and sequences. The results were confirmed that production of bile salt hydrolase from recombinant *E.coli* MC1022 pMGe36/*bshA* found to be higher expression while compared with *E.coli* MC1022 wild type strain from 100 to 184.78 (U\mg). The recombinant plasmid also found to be stable in host organism after a few generations.

KEYWORDS: Probiotic strain *Lactobacillus acidophilus Ar, E. coli* MC1022, Bile salt hydrolase, *bshA* genes.

INTRODUCTION

In humans and other mammals, primary bile salts are produced de novo in the liver from cholesterol.[1] Following manufacture, conjugated bile salts are stored in the gall bladder and secreted via the bile duct into the small intestine.

Here, these conjugates form spontaneous micelles that trap dietary cholesterol and fats, thus facilitating their absorption by the intestinal epithelium into the blood stream.[2] While more than 95% of bile salts enter the enter hepatic circulation in humans, up to 650 mg of bile salts per day elude absorption through the intestinal epithelium.

Thus, high concentrations of these conjugates are present in the gastrointestinal tract. Certain species of the indigenous microflora, including a number of lactobacilli and bifidobacteria, have evolved the ability to deconjugate bile salts. This action is dependent on the presence of an enzyme known as bile salt hydrolase catalyzes the hydrolysis of glycine- and/or taurineconjugated bile salts into the amino acid residue and the bile acid.[3]

However, studies on the impact of BSH producing organisms in the colonized host have produced much conflicting evidence. Observations that a reduction in the levels of serum cholesterol is associated with the presence of BSH-producing organisms has led to increased interest in the possibility of their use in hypercholesterolemia individuals or to prevent elevated cholesterol levels in individuals with normal cholesterol status.[4]

Conversely, negative effects have also been reported including cases of contaminated small bowel syndrome, impaired lipid absorption, gallstone formation and increased risk of colon cancer.[5]

Lactobacillus acidophilus is a human isolate used commercially for over 25 years as a probiotic. The organism has the ability to survive in the gastrointestinal tract, adhere to human epithelial cells in vitro, utilize fructooligosaccharides, modulate the host immune response, and prevent microbial gastroenteritis.[6] Analysis of the genome sequence revealed the presence of two putative bile salt hydrolase genes. The bile-hydrolyzing capability associated with Lb. acidophilus had been previously identified by phenotypic screen. Due to the implications of the presence of bile salt hydrolase in several probiotic strains, this study was designed to further characterize this activity in through targeted gene inactivation.[7]

Probiotic *Lb. acidophilus* have bile salt hydrolysis gene responsible on the bile salt pool might be regarded as a ‘biological’ alternative to common medical or surgical interventions to treat hypercholesterolemia.[8] Thus, this study aims to: Characterization to *Lb. acidophillus Ar* probiotic strain bile salt hydrolase *bshA* gene which is capable of the hydrolysis of some glycin- glycoconjugated bile salts, due to bile salt tolerance and treat hypercholesterolemia

and bile salt hydrolase gene was sequencing analysis to identification. And cloning *Lb. acidophilus Ar* probiotic strain bile salt hydrolase gene *bshA* and overexpression of this gene in *E. coli* MC1022.

MATERIAL AND METHODS

Bacterial characterizations

1. Bacterialstrains, plasmids and growth conditions

Lactobacillus acidophilus Ar bacteria was growing in MRS broth medium at 37ºC for 42h, activated bacteria was streaked on MRS agar to isolated single colony in same condition to prepare PCR single colony. *E. coli* MC1022 cells were propagated aerobically Luria Bertani (LB) media contain 0.1 mg/ ml con. of ampicillin or Erthromycin antibiotic for plasmid detection and for bacterial growth in broth medium, all incubation carried out at 37Cº for 18 hr. by shaking at 250 rpm.[9] The bacterial strain and plasmids used in this work (table 1 and table 2).

Table 1. Bacterial strains were using.

Bacterial strains	Properties	Reference
Lb. acidophilus Ar	Probiotic strain	Commercial
E.coli MC1022	Plasmid free strain used for transformation	KSU university
E.coli	Strain for carry pMG36e plasmid	KSU university

2. Cholesterol tolerance

Lactobacillus acidophilus Ar growthing in MRS media containing cholesterol (4 mg/ml) at 37Cº for 42h.[10]

3. Bile salt tolerance

The *Lb. acidophillus Ar* strain was treated with using 0.5-0.2% (wt/vol) concentration from sodium salt of urodeoxycholic acid (SCA) and incubated at 37ºC aerobically for 42h. To test bacterial growth tolerance, *E. coli* MC1022 cells were incubated with bile salt at 37ºC for 18hr. Than bacterial growth measured spectrometrically at 600 nm for 2 h intervals during the 8 h incubation period.[11] For control MRS and LB media with / without bile salt and bacterial culture used as reference.[12]

4. Polymerase chain Reaction PCR

1. Colony PCR

Single colony selected from growing plate of *Lb. acidophilus Ar* to identified strain and prepare for gene encoding bshA into 10µl of sterile Milli Q water.[14]

A microliter of this suspension was added to PCR reaction mixture containing 32 µl of sterile Milli Q water and 7.5 µl of PCR mix contain primer for *Lb. acidophilus Ar* 16S DNA all in a 0.5 ml eppendrof tube. The PCR mix was composed of 4 µl PCR buffer which are; 1 µl of each of forward and reveres primers, 0.5 µl of Ampli taq. (4 µl buffer, 0.5 µl taq DNA polymerase, 1 µl dNTP). The amplification was performed on a DNA thermal cycler using following program; initial denaturation 3min at 94^0C, 1min denaturation at 94^0C, Annealing 30 sec at 55^0C, 30 sec at 75^0C. Final extension was at 72^0C for 1 min 4 for 35 cycles.

2. Electrophoresis of DNA Molecules and Cloning Plasmids

For electrophoresis of DNA molecule, 1% (w/v) agarose gel and for cloning fragments electrophoresis which digested with restriction enzymes, 0.7% (w/v) agarose gel in 1× TBE electrophoresis buffer (0.1 mM Tris/HCl, 0.1 mM boric acid, 0.002 mM EDTA, pH 8.3) (A=60,V=70-120) were used[15] The gel was stained in 0.5 µg ethidium bromide ml−1and after distaining the gel was photographed.[16]

3. Molecular cloning Techniques, reagents and Enzymes

Throughout this work standard molecular cloning techniques were used.[17] All restriction endonuclaces, T4 DNA ligase and Taq polymerase supplied from sigma.

4. Primers

Primers were designed by using a clone manager (demo 9.2 designer). These primers were stored at -20ºC. Primers utilized in (table 2).

Table 2. Primers used in Research.

Primers	Primer sequence 5´ to 3´
Lb. acidophilus. 16SF	5´ACTACCAGGGTATAATCC3´
Lb. acidophilus. 16SR	5´AGCTGAACCAACAGATTCAC3´
bshA F	*PstI* 5´AAGAGCTCATGTGACATCAATTATATT3´ *SacI*
bshA R	5´TTCTGCAGTTAGTTTTGATGGTTAAATTTAG3´ *PstI*

4. DNA sequencing of *bshA* gene

Sequencing of the gene was performed according to manufacturer protocol. A sequencing reaction was set up as follows; for purification 7µl *bshA* gene PCR product were used in 3 µl exo sap solution. Thermocycle were run at 37ºC for 30 min then at 80ºC for 15 min. 2 µl of sterile Milli Q water and 2 µl of *bshA* gene primers (table 2.3) with 6 µl of ABI PRISM Big Dye TM terminator Cycle ready reaction were prepared (supplied by Applied Biosystems). The sequencing reaction was than performed at 96ºC for 2 min, than 10sec at 96ºC, Annealing (5 sec at 50ºC), (4min at 60^0C) for 25 cycles in a Hybrid Omni gene Thermal Reactor. Once the sequencing reaction was completed the product was purified according to specifications provided with the ABI PRISM Big Dye ^{TM} Kit.

5. Conformation and Cloning *bshA* gene

a. Restriction enzymes

For digestion of (5 µl) p MG36e vector (fig. 1) and *bshA* gene PCR product, 9 µl sterile Milli Q water, used 2 µl *SacI* and *PstI* restriction enzyme and 4 µl enzymes buffer were mixed gently than incubated at 37ºC in water bath for 2 hr.[18] pMG36e vector were also digested with same way to use Thermo scientific clone JET PCR cloning Kit (Table 3).

b. Ligation plasmids with *bshA* gene

Ligation of (2 µl) digested plasmids with (3 µl) digested *bshA* gene were performed by using (1 µl) T4 DNA ligase, (2 µl) ligase buffer, (2 µl) sterile Milli Q water. Thermo cycle was used for incubation at 22ºC for 20 min (table 3).[19]

Table 3. Plasmids and constructs were using

Plasmids	Properties	Reference
pMG36e vector	Cloning and expression plasmid, Replicon (rep), T7promoter, Ery^R	Commercial
pMG36e/*bshA*	pMG36e vector carry ≅ 1kb fragment of *Lb. acidophillus Ar bshA* gene	

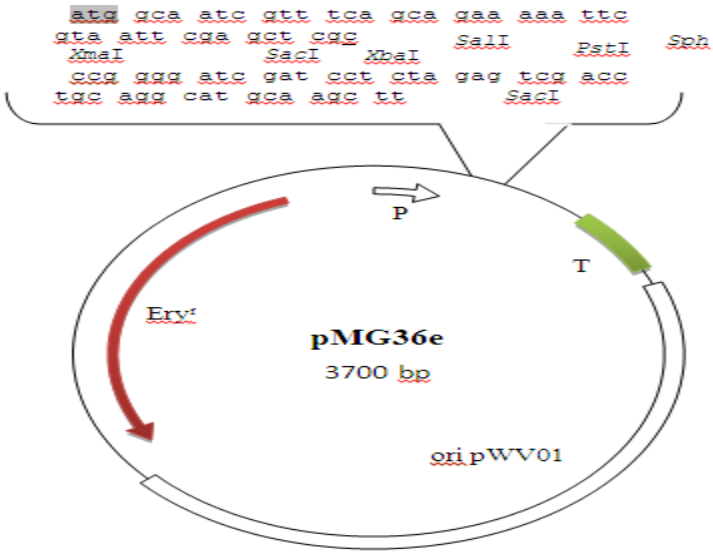

Figure 1. Physical map of pMG36e vector.

c. $CaCl_2$ transformation

1. Calcium Chloride competent cell protocol for pMG36e/*bshA* vector transformed in *E. coli* MC 2210

LB broth was inoculated with overnight culture of *E. coli* MC 1022 into 100mL falcon tube and incubated at 37°C to reach appropriate cell density (O.D. 0.350-0.600) or $1*10^{-6}$ cell/ml. cells were colded on ice for 10 mins. cells were collected by centrifugation at 3500 rpm for 10 min. Then supernatant was removed and pellet gently resuspended with 10 mL cold 0.1M $CaCl_2$. Leaved on ice for 5 mins, centrifuged at 3500 rpm for 10 min. Again supernatant discarded and pellets resuspended on 1mL cold 0.1M $CaCl_2$. Then about ~200µL of competent cells, 5µL of circular plasmid or all of a ligation reaction of plasmid DNA added into a microtube (pMG36e /*bshA* vector) was used for ligation.[20]

left on ice for 30 mins. The mixture was heat shocked at 42C˚ for 30 sec, replaced on ice for 10 min and than 1ml LB broth added, incubated (water bath) shaken at 37C˚ for 1-2 hours. Then centrifuged at 5000 rpm for 5 min, then 500 µL LB broth added to competed cell pellets, vortexes, Transformed cells 100 µl were plated out in LB agar plate containing appropriate antibiotic (Amp 5 µg/ml or LB Emr 100µg/ml).[21]

5. Cloning strategies

Cloning *E.coli* MC 1022 with pMG36e /*bshA* vector was carried bshA gene cultured in serial growth to plasmids extraction for high cloning efficiency and constructional measurement of plasmid.[22]

6. *BshA* enzyme assay

1- The best wild and cloning *E. coli* MC1022 isolate was grown in the nutrient broth medium for bile salt hydrolase production which contained 0.5% Na-urohydroxycholic acid bile salt (substrate), 2.5% glucose (pH 5.8). To examine the effect of carbon sources on the enzyme production. A 250-ml flask containing 50 ml of the medium was inoculated with shaker (120 rpm) for 18 hr at 37Cº. Control bacteria was prepared from wild type of *E. coli* MC1022 without bile salt.[23]

2- cells culture were concentrated from 50 ml liquid medium by centrifugation at 8,000 x g for 20 min. The cell pellets were resuspended in 1 ml of ice-cold condition containing 0.01M Na-Phosphate buffer pH 6,7. Cells were washed twice and resuspended with 0.01M Na-Phosphate buffer containing 0.1M cysteine and 1M EDTA to take $O.D_{600}$ for cells in (0.30-0.40)nm.

The cell lysis process used sonication with a Soniprep 150 (USA) in ice-cold condition. The sonicator was set to 16 micron amplitude for 5 minutes (1\2 min turn on and 1\2 min turn off). Cells suspension was centrifuged at 8,000 x g for 30 min, supernatant took for enzymetic activity proteins quantity measurement to calculate enzymtic specific activity in ninhydrin method (fig.2).[28] and proteins concentration measurement in Bradford method (fig 3).[24]

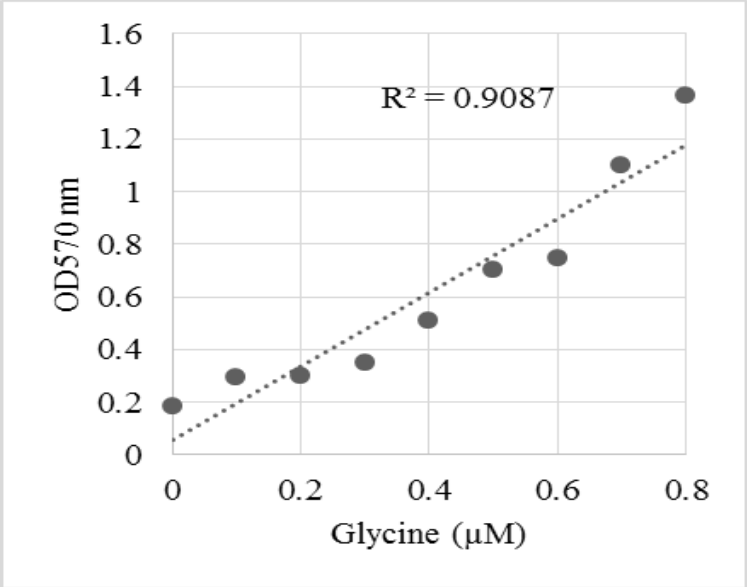

Figure 2. Glycine standard curve (ninhydrin method)

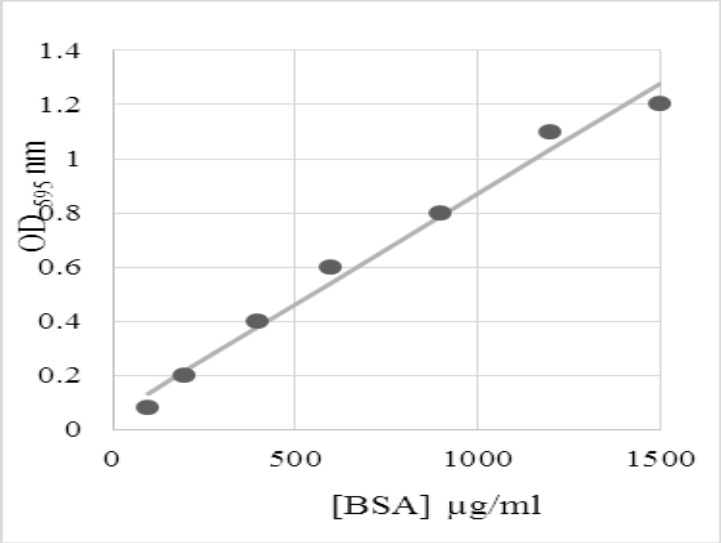

Figure 3. Bovine Serum Albumin standard curve. (ninhydrin method)

RESULTS

1. Bacterial strain characterization

Lb. acidophilus Ar probiotic strain was isolated from capsule treatment for woman candida diseases which was identified morphologically as LAB, its gram positive bacteria and has ability to remove cholesterol. Its tolerance to cholesterol was observed when growth bacteria in MRS media containing 4mg/ml cholesterol at 37ºC for 42 hr incubation (fig.4).

Figure 4. Single and group cells of *Lactobacillus acidophilus Ar* strain.

2. Bile salt tolerance

Tolerance to bile allows lactic acid bacteria to survive in the small intestine. At the beginning of the performed experiments *L. acidophilus Ar* culture O.D $_{600}$ different when the substrates with the addition of 0.5% bile salt was the increase in the number of live cells from (O.D $_{600}$= 1.25 to 1.85) cfu\ml.

3. Molecular characterization of *Lb. acidophilus Ar* probiotic strain

The probiotic strain *Lb. acidophilus Ar* was amplified in PCR reaction using the specific primers of partial DNA of 16S rRNA gene that results of strain from MRS media were detected as *Lb. acidophilus Ar* (Fig. 5 a).

4. Screening *BshA* Gene *Lb. Acidophilus Ar* Probiotic Strain And Sequencing Analysis

The *bshA* gene of *Lb. acidophilus Ar* was amplified using primers that mentioned, derived from the nucleotide sequencing of *Lb. acidophillus Ar bshA* gene (Fig 5). The length fragment was 801 bp used Master cycle PCR, big dye Terminator Cycle Sequencing standard Kit (Fig. 6).

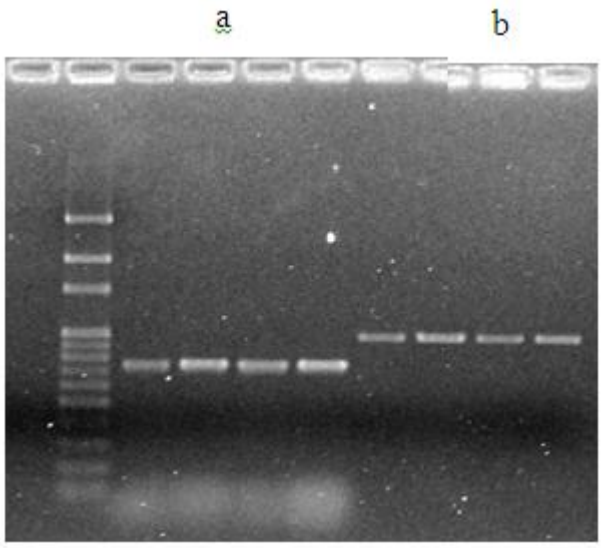

Figure 5. a) Lanes of PCR products of amplified 16S rRNA gene to identified *Lb. acidophilus Ar* strain using specific primers. b) Lanes PCR products of amplified *bshA* gene of *Lb. acidophilus Ar* strain using specific primers lanes: 100 bp DNA marker.

P*st*I revers primer

CYKATTKRTWRGGARWYCTTCTAGAAGATCTGCAGTTAGTTTTGATGGTTA

AATTTAGTTTTATCAAGCATATTATAAGWGATCAAATTGCTGCTATCTAGAT

CTTCTTTATTCATATCAACAACGTTAATTTGTTTGTTTGAATAAGTGGTGTAG
TAGAAAATACCTTTGTCTAAGTTAGTTCCATCAGAATAAATTGTATATTCAAA
TGAGTTTGGACCAACTTCATCCAGTCCCTTTTGTTGTTCAACCGAATGTAAA
ATGTGGAAGTAAGTATCAATATTTTCTTCTTCGGTTTCAGCAATTGGAGCAT
TAAATTTATTGAAAGCTACTCTGACAAAACGTGATTCAGAATCCATTCCACC
TGGTAAGTTGTGAGACCCTAATCCACGGCTGTAGCCAGCCATATTTACTTTA
TCTGAGAAGTTATTTTTAGGCATTTTTGGAGATACGTCAGCATAGTTATTTA
AATTGAATAATTGCTTTGGAAATTGTGGATTATTAGTTAAGCAGCCAACTGG
ATTATCATAAATATGCATTCCATCTTTGTCTGTTTCAACAACTAATGATGTAC
CTGTTTTATCTGCAATAAGCCAGTGAAGAGAGGAGGTTTGCATTTTTTCGCT
GAAATTTAAATCGGCGATGTTGATTCTGCTAAGTAAATCCTTTACTTCGCTA
ATAGTGCTACACTGTCCTAAAATCCAAGGGATGAATTCAAAGGAAGCAATAT
TATCTTTATTTTCTTTTTCTTCGTAATATGTAGCATTTCCTGGATAGTTGAGT
CCGGCCATACCTAAACCT.

SacI

Figure 6. *bshA* gene sequence data is 801 bp only revers strand

5. Analysis of Sequence Data

Sequencing analysis of the clone obtained from PCR reaction showed high similarity (44%) from *L. acidophilus Ar* probiotic strain (fig.6) When sequencing results were obtained in a Genetic analyzer system and they were analyzed using BLAST programmer in NCBI nucleotide database confirmed that the strain belong to *Lb. acidophillus* species.

6. The primers

Sequencing analysis results for the primers *bshA* F, *bshA* R (table 2), that *bshA* R can homology matching with revers strand of *L. acidophilus Ar bshA* gene, but *bshA* F do not contain homology matching only 5 bp from GC can homology matching occurred with forward strand of *bshA* gene appear in clone managing designer because of *L. acidophilus Ar* bshA gene was different from *L. acidophilus* strain which obtained for primer designing in BLAST program (demo 9.2) (Fig 6).

***7. E.coli* MC1022 charecterazion**

E. coli MC1022 is a Gram-negative artificial, plasmid free bacteria and can be able to express foreign gene. This bacteria can grow in LB-broth at 37ºC and sensitive to erythromycin and ampicillin antibiotics.

8. Plasmid conformation and gene fusion analysis

1. Constriction of *bshA* gene reporter gene fusion

To clone bshA gene from chromosomal *DNA of Lb. acidophilus*, primers *bshA* F and *bshA* R (table 2) were designed, the primers were engineered to contain a *PstI\SacI* restriction sites for both *bshA* F and *bshA* R respectively. After PCR amplification, resulted fragment was inserted into pMG36e vector and this new recombinants named as pMG36e */bshA* respectively. Obtained recombinants then transformed into *E.coli* MC1022 by chmical method. After transformantion 4-5 cell/100µl single colonies obtained.[20] and each plate containig transforment colonies were screened by PCR amplification (Fig.7) using *bshA* F and *bshA* R primers and restriction digest analysis was performed to ensure that the *bshA* fregment present. Purified gene fragment was then sequenced to check no erroreous base pair changes occurred during the PCR amplification process.

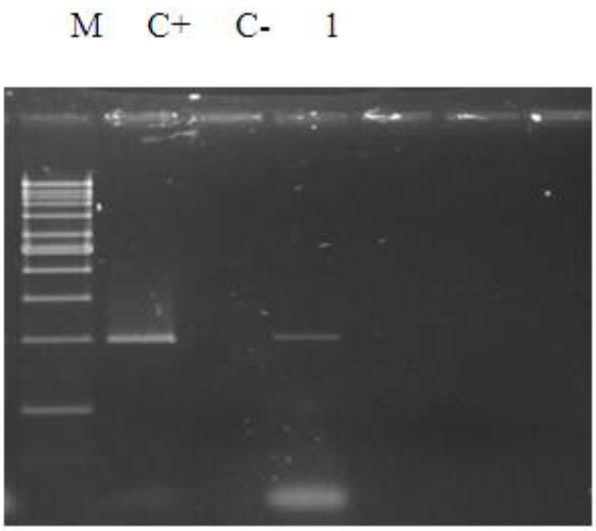

Figure 7 b- C+) lane *bshA* gene control, C-) *E.coli* colony non *bshA* gene transformation, lane (1) for cloning single colony carry *bshA* gene after $CaCl_2$ transformation of pMG36e/*bshA*, used M) 1000bp ladder.

2. Conformation of cloning plasmids with *bshA* gene

Recombinant *E.coli* MC1022 which carriy recombinant pMG36e /*bshA* vector was growth in the appropriate conditions, after that recombinant plasmid were isolated and pMG36e /*bshA* was digesed with *PstI\SacI* to to confirme presence of bshA gene by gel electrophoresis separation (Fig.8). It has been confirmed that after gel electrophoresis separation two plasmid fragments and bshA gene with control appeared.[17]

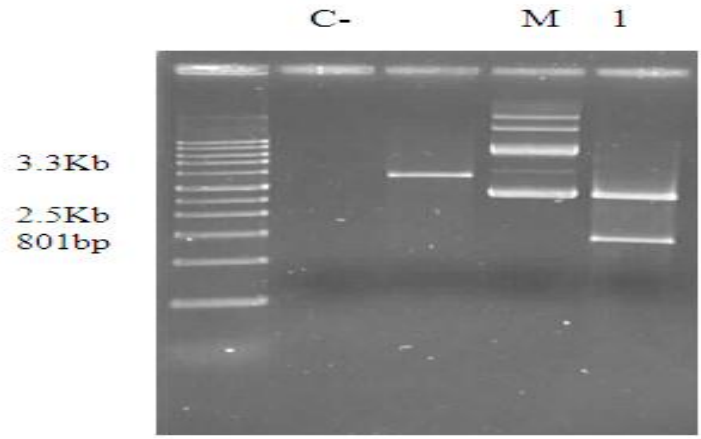

Figure 8. M) lane2, Recombinant pMG36e/*bshA* 3.3 Kb., lane 1, pMG36e vector 2.5 Kb (control). 1) Recombinant pMG36e/*bshA* digested with *PstI* *SacI* restriction enzyme to get tow fragment (2.5 Kb+ *bshA* gene 801bp).

9. Analysis of pMG36e /*bshA* vector stability in *E. coli* MC1022

A bacterial cloning system for mapping and analysis of complex genomes has been developed. It is capable of maintaining bacterial *bshA* fragment of greater than 300 kilobase pairs. Individual clones of this gene appear to be maintained with a high degree of structural stability in the host, even after many generations of serial growth arrived (30 – 40) times of plasmids extractional concentration. Because of high cloning efficiency, easy manipulation of the cloned DNA and stable maintenance of inserted DNA, pMG36e /*bshA* system may facilitate construction of DNA libraries of complex genomes with fuller representation and subsequent rapid analysis of complex genomic structure (Fig. 9).[22]

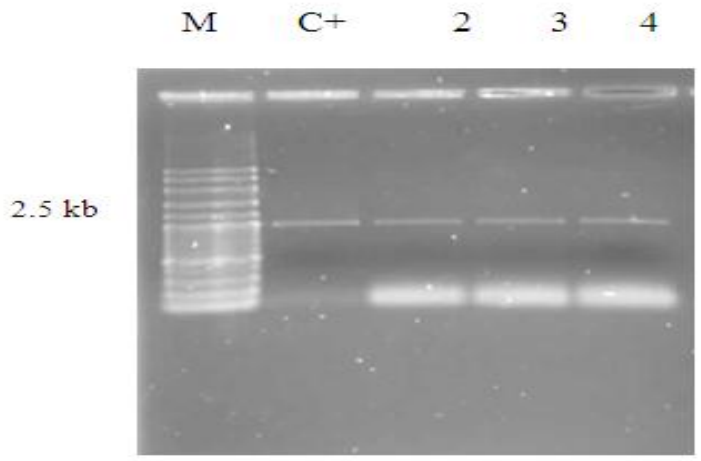

Figure 9. Lane C+ pMG36e/*bshA* vector extraction 4.5 Kb (control), Lane 1) pMG36e/*bshA* for first extraction, Lane 3,4) pMG36e/*bshA* for serial of other extraction.

10. Bile salt hydrolase enzyme assay and over expression

For assaying most bile salt hydrolase activity was measured by using ninhydrin detect with glycine curve test and proteins concentration by Bradford method (coomasia blue detect), to preserve the enzymatic activity, enzymes must retain their native form extraction capability.

The bile salt (Na-urohydroxycholic acid) concentration and fermentation substrate was used. The substrate specificity of BSH from revealed several interesting results. For instance, the former authors have demonstrated that the position of the amide bond, changes in shape and chiral nature of the amide bond and introduction of various amino acids at or around the amide bond influence the rate of hydrolysis. indicated that glycine and taurine conjugates of cholic and deoxycholic acids were hydrolyzed; however, readily as compared to glycine conjugated with ninhydrine detect and glycine standard curve (fig. 2, table 4) for bshA enzyme activity assay.

Table 4. Absorbance of enzyme extract samples at A_{595} (ninhydrin method)

Enzyme Samples	Absorbance $O.D_{570}$
E.coli MC1022 growth without bile salt	0
E.coli MC1022 growth with 0.5% bile salt	0.096
E.coli pMG36/*bshA* growth with 0.5% bile salt	0.255

These studies are in agreement with previous studies.[25] It is noteworthy that unlike previous studies where partially purified enzyme *E. coli* MC1022 pMG36e/*bshA* from culture were used as the source for BSH activity, can be used for more detailed studies on its substrate specificity using various strategies employed by.[26] Upon bile salt hydrolysis, glycine or taurine is liberated from the steroid moiety of the molecule, resulting in the formation of free (deconjugated) bile salts. Free bile salts are more easily precipitated at low pH. They are less efficiently reabsorbed than their conjugated counterparts. Since the steady state requires that the amount of bile salts extract was measured in bradford method used BSA standard curve (Fig.3, table 5) to calculate bile salt hydrolase specific activity.[27]

Table 5. Absorbance of Protein Purification Samples (ninhydrin method)

Proteins Sample	Average A_{595}
E.coli MC1022 growthing without bile salt	0.09
E.coli MC1022 growthing with 0.5% bile salt	0.32
E.coli pMG36e Ar growthing with 0.5% bile salt	0.46

The benefits s of bile salt hydrolase specific activity for comparative between recombinant *E.coli* MC1022 with wild type bile salt overexpression and bile salt effect on *bshA* gene function when bacteria was cultured with and without bile salts (table 6) (Fig.10).[25]

The result after took spectrophotometer absorbance (O.D 570,595 nm) and enforcement enzyme specific activity formula[28], these data indicate that the *bshA* gene overproducing in *E. coli* MC1022 pMG36e /*bshA* strain while same bacteria growth in broth media from non-bile salt substrate no produced enzyme for *bshA* gene function for because bile salt led to simulate gene and as enzyme substrate same time (Table 6).

Table 6. The results of overexpression data compression between cloning *E. coli* MC1022 and wild type.

Samples	Specific Activity (U\mg)
E.coli MC1022 growth without bile salt	0
E.coli MC1022 growth in 0.5% bile salt	100
E.coli pMG36e/*bshA* growth in 0.5% bile salt	184.78

These results appeared that pMG36e*bshA* vector construct active. Because its specific for *E. coli* strains and there is evidence that *E. coli* MC1022 recognizes it as original host organism. This has been shown for a *bshA* gene from *Lb. acidophilus Ar*, but may also apply to genes from other species. The expression occurs in *E. coli* ribosome binding sites are generally observed in the expected position for genes, but internal translational start points and proteolysis can complicate interpretation of cloned gene products were expressed in *E. coli* MC1022. (fig. 10) However, the importance of confirming promoter identity empirically has been demonstrated by studies in which transcriptional sites operating within *E. coli* MC1022 differed significantly from those that were active within the native bacterium.[29]

The importance of probiotic bacteria in the assimilation cholesterol processes of human fecal bacteria make them logical target for genetic manipulation to improve cholesterol balance

efficiency. Thus, introducing bile salt hydrolysis functions into non- bile salt hydrolase bacteria such as *leuccuccuse mesenteroides* or over expression occurrence for bacteria use as hypercholesterolemia treatment.[30]

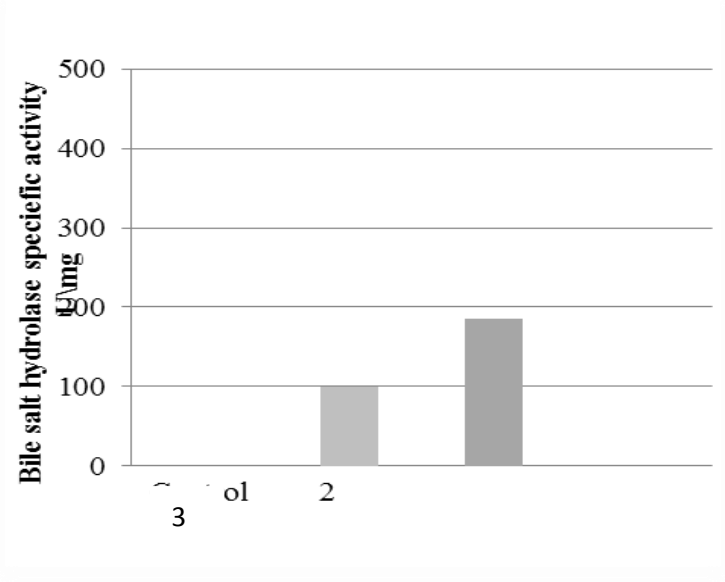

Figure 10: Bile salt hydrolase spesific activity and Overexoression values (U\mg). 1: (Control) *E. coli* MC1022 wild type growthing without 0.5% bile salt. 2: *E. coli* MC1022 wild type growthing in 0.5 bile salt. 3: Recombinant *E. coli MC1022* with pMG36e/*bshA*.

DISCUSION

1. Identification of *Lactobacillus acidophilus Ar bshA* gene and cloning in *E. coli* MC1022

The bacterial cholesterol assimilation in an important character for many organism because of its chemical and healthy benefits. The hypercholesterolemia problem is solve for human healthy and other organism energy sours by the removal cholesterol in bacterial environment by microflora, It is well Known that probiotic lactic acid bacteria like *Lb. acidophilus* is one of the major cholesterol removal organism that have (bile salt hydrolase genes) *bsh* genes responsible for this process.

The anti- candida woman disease probiotic strain *Lb. acidophilus Ar* was examined to bile salt resistance, Colonies were identified according to their morphological, cultural, physiological and biochemical characteristics, the result appeared its consist of bile salt genes resistance.

The experiments described here demonstrated that simplified in vitro mimicking of complex environmental niches can result in the identification of genes that are relevant in situ in these niches. Moreover, this approach potentially provides clues to the environmental trigger involved in the in situ regulation of specific genes to play an important role to resistance at low pH when the presence of bile salts, which should enable future unraveling of the genetic behavior of during passage through (specific parts of) the GI tract in vivo. The observed gradual decrease in the growth rate coincided with the gradually increasing severity of changes in morphology of *Lb. acidophilus Ar*.[31]

Moreover, the observed formation in vitro the increasing growth *Lb. acidophilus Ar* after addition of bile, The optical density at 600 nm change from 1.25 to 1.85 nm possibly because leakage of intracellular material from the cells and a disturbed energy balance.[32]

Identification of *Lb. acidophilus Ar* is more accurate if the whole gene is sequenced. In the early 1990s, many microbiologists have demonstrated that phylogenetic relationships among living organisms can be traced by comparing sequences of their genes and gene regions, encoding ribosomal RNAs.[33, 34] Ribosomal genes are more conservative than most of the genomic genes. The gene sequences of small (16S) rRNA subunit is particularly widely used in taxonomic studies of bacteria. The method of comparing 16S rRNA gene sequences, along with DNA typing with the use of various PCR-based techniques, is also often used for species identification in microflora of probiotic lactic acid bacteria. For instance, to identify microorganisms.

The *bshA* gene was selected from *Lb. acidophilus Ar* strain genes used PCR purification and comparing gene sequencing analysis from the corresponding sequences of strains from the NCBI database that studied it, the sequence results showed little different homology.

1. Expression of *bshA* in *E. coli MC1022*

To create the recombinant plasmids with pMG36e vector specific for *E. coli* to clone *bshA* gene, that *bshA* gene was amplified with the primers *bshA*-F and *bshA*-R (Table 2). And *SacI* site was designed in primer *bshA*-F and a *PstI* site was created in primer *bshA*-R to include the start codon sequence and the stop codon (TGA) sequence.

Cloning into the *PstI -SacI* sites of vectors resulted in the translational fusion of the *bshA* gene to the T7 promoter of pMG36e/*bshA* vector, a ribosome binding site and the start of an open reading frame are present and *E. coli* MC1022 ribosome binding site.[35]

The previously constructed *bshA* complementation library in *E. coli* MC1022, was exploited for identification of clones containing *Lactobacillus acidophilus Ar* chromosomal fragment that harbor promoter elements conditionally activated by bile salt, Which play a role in the pMG36e*bshA* vector when created in *E. coli* MC1022 and higher stability after serial sub-culturing,.[25]

2. BshA Enzyme activity

The nucleotide sequence of the *bshA* gene of *L. acidophilus Ar* was analyzed, which revealed its location and showed that it was surrounded by 801 nucleotides in a single open reading frame (ORF) and encoding a 50 amino acid in protein. BSH promoter was located upstream of the start codon. The expressed protein exhibited high homology with BSHs from other source organisms, located around the active site, were highly conserved.

The *bshA* gene was cloned in pMG36e expression vector. The produced recombinant *bshA* enzyme exhibited hydrolase activity against sodium salt of uroconjugated bile salts. The *bshA* gene, has been identified encoding bsh enzyme in the genome sequence of *L. acidophilus Ar*, where substrate specificities of enzyme was observed. Indeed, the bsh enzyme has substrate specificity, depending on the gastrointestinal ecosystem. glycine or taurine amino acid moiety.[26]

This principal due to enforce glycine standard curve and Bradford method in calculate differentiation overexpression levels for cloning *E. coli* MC1022 and wild type by know the bile salt hydrolase specific activity.

The results were confirmed that production of bile salt hydrolase from recombinant *E. coli* MC1022 pMG36e /*bshA* found to be higher compared with *E. coli* MC1022 wild type. It could be thought that pMG36e vector based constructs specific for *E. coli* strains and there is evidence that *E. coli* MC1022 recognizes promoters different from those used in the original host organism.[30]

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