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KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE

CLONING and OVEREXPRESSION of *Lactobacillus acidophilus* BILE SALT HYDROLASE A GENE (*bshA*) in *Escherichia coli*

ATHEER AHMED MAJEED

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ATHEER AHMED MAJEED

Thesis submitted in candidature for
the degree of Master in
Department of Bioengineering and Sciences

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Cloning and overexpression of *Lactobacillus acidophilus* bile salt hydrolase A gene (*bshA*) in *Escherichia coli*

(M.Sc. THESIS)

Atheer Ahmed Majeed

ABSTRACT

The bile salt hydrolase gene (*bshA*), encoding bile salt hydrolase enzyme (EC 3.5.1.24) from probiotic isolate *Lactobacillus acidophilus Ar* strain which is responsible for assimilation cholesterol were studied in the present work. About 801 bp in length DNA fragment of *Lb. acidophilus Ar* strain was amplified by PCR techniques. Two restriction sites (*PstI/SacI*) were added to each end of that fragment for manipulation of DNA during cloning. Amplified fragment inserted into *pJET*1.2\blunt end vector and *pMG36e* vector respectively. *pJET*1.2\blunt end vector is overexpression plasmid for *E. coli MC1022*, and *pMG36e* vector is a shuttle vector which is able to replicate in both *E. coli* and lactic acid bacteria. The resulted constructs were named as *pJET*/*bshA* and *pMG36e*/*bshA* respectively. Both recombinants were 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 pJET*/*bshA* found to be higher while compared with *E. coli MC1022* wild type and recombinant *E. coli MC1022 pMG36e/bshA* strain. However production of bile salt hydrolase from recombinant *E. coli MC1022 pMG36e*/*bshA* found to be higher while compared with *E. coli MC1022* wild type. The recombinant plasmid also found to be stable in host organism after a few generations.

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

KahramanmaraşSütçü İmam University

Graduate School of Natural and Applied Sciences

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***Lactobacillus acidophilus'* un safra tuzu hidrolaz geninin (*bshA*) *Escherichia coli* ye aktarılması ve aşırı üretimi**

(MASTIR TEZİ)

Atheer Ahmed Majeed

ÖZET

Safra tuzu hidrolaz enzimini (EC 3.5.1.24) kodlayan Safra tuzu hidrolaz geni (*bshA*), kolesterol düşürücü etkisi olan *Lactobacillus acidophilus Ar* suşu bu çalışmada kullanılmıştır. Yaklaşık olarak 800 bç. uzunluğundaki DNA parçası *Lb. acidophilus Ar* suşundan PZR tekniği ile çıkarılmıştır. DNA'nın işlenmesi ve klonlanması için her iki uca kesme enzimi (*PstI/SacI*) kodu eklenmiştir. Elde edilen DNA parçası *E. coli* MC1022 ekspresyon vektörü pJET1.2\blunt end ve Mekik vektör pMG36e ye yerleştirilmiştir. Elde edilen yeni recombinant plasmitlere sırasıyla pJET/*bsh*a ve pMG36e/ *bshA* adı verilmiştir. Her iki recombinant molekül daha sonara kimyasal metotla *E. coli* MC1022 ye aktarılmıştır. Elde edilen rekombinantlar daha sonar enzim üretimi ve gen dizi analizleri için kullanılmıştır. Elde edilen sonuçlara gore gen transferi yapılan, pJET/*bsh*a içeren *E. coli* MC102, bakterilerinin, pMG36e/ *bshA* içeren *E. coli* MC102, bakterilerinden ve bu geni taşımayan *E. coli* MC102, bakterilerinden daha fazla Safra tuzu hidrolaz enzimi ürettikleri gözlemlenmiştir. Ayrıca pMG36e/*bshA* içeren *E. coli* MC102, bakterilerinin bu geni taşımayan *E. coli* MC102, bakterilerinden daha fazla Safra tuzu hidrolaz enzimi ürettikleri gözlemlenmiştir. Rekombinant plasmitlerin taşıyıcı bakterilerde bir kaç generasyon kaldıkları da gözlemlenmiştir.

Key words: Probiyotik, *Lactobacillus acidophilus Ar, E. coli,* Safra tuzu hidrolaz enzimi, *bshA* geni.

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LIST OF SYMBOLS AND ABBRIVIATIONS

LAB **:** Lactic acid bacteria.

***BshA* gene** **:** bile salt hydrolyase gene

pMG36e/*bsh*a : pMG36e vector carry 0.801kb fragment of *Lb. acidophillus Ar bshA* gene

pJET/*bsh*a : pJET vector carry 0.801kb fragment of *Lb. acidophillus Ar bshA* gene .

Inc **:** Incompatability.

Ori **:** Origin of replication.

Amp^r **:** Ampicillin resistance

Ery^r **:** Erythromycin resistance

O.D **:** Optical density

RNA **:** Ribonucleic acid.

ATP **:** Adenosine triphosphate.

GTP **:** Guanosine triphosphate.

G+C **:** guanine + cytosine

Bp **:** Base pair.

FAO **:** Food and Agriculture Organization.

WHO **;** World Health Organization.

GIT **:** Gastro intestinal tract.

LDL **:** Low density Lipopolysaccharide.

UV **:** Ultra violet.

Gly **:** glycine

BSA **:** Bovine Serum Albumin

Δ Y : Y-axil for absorbance measurement (O.D)

Δ X : X-axil for time measurement (min)

1. INTRODUCTION

In humans and other mammals, primary bile salts are produced *de novo* in the liver from cholesterol (Ahn, *et al* 2003). 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 bloodstream (Buck,*et al* 1994). 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 taurine-conjugated bile salts into the amino acid residue and the bile acid (Ziarno, *et al* 2007). 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 (Begley,*etal* 2006). 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 (Gotteland,2006).

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 (*Savage, et al 1996*). 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 (Olivia, *et al* 2005).

It is well known that the presence of lactobacilli is important for the maintenance of the intestinal microbial ecosystem. They have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp., and others (Jason, *et al* 2006).

This inhibition could be due to the production of inhibitory compounds such as organic acids, hydrogen peroxide, bacteriocins or reuterin or to competitive adhesion to the epithelium. In order to survive in and colonize the gastrointestinal tract, probiotic bacteria should express high tolerance to acid and bile and have the ability to adhere to intestinal surfaces (*Savage, et al 1996*). Survival in and temporary colonization of the human gastrointestinal tract have been demonstrated for some lactic acid bacteria.

2. LITERATURE REVIEW

2.1. Probiotic definition and History

Probiotics microorganisms are usually refer to bacteria found in the human and other mammalian gut, which are beneficial to health. They're also called "friendly" or "good" bacteria. They are ubiquitous and symbiotic throughout the gastrointestinal system, where they have an important and protective role. Probiotics are not just normal and helpful, but essential to prevent illness and improve function. Lactic acid bacteria and *bifidobacteria* constitute the major types of colon flora. *Lactobacillus acidophilus* has a long history of safe use in the dairy industry and exists naturally in the human gastrointestinal tract infections and inflammatory bowel disease, antagonizing pathogens and conferring antiallergenic properties on the immune system (Hummel AS, 2007).

Lactic acid bacteria conferred a type of protection from, or reversal of, the intestinal auto-intoxication. Mitchnikoff had observed that rural dwellers in Bulgaria lived to very old ages, despite extreme poverty and harsh climate. They had an average lifespan much greater than wealthier Europeans, and he noted that they drank fermented milk products. Metchnikoff surmised that the lactic acid bacteria associated with fermented milk products had anti-aging health benefits. He named the organism "*Lactobacillus bulgarius*". Metchnikoff went on to publish "The Prolongation of Life: Optimistic Studies", stating that "Ingesting microorganisms could have substantial health benefits in humans (Gill**,** 2004).

During an outbreak of shigellosis (shigella is a bacteria that causes severe diarrhea), due to Nissle isolated a new strain of *Escherichia coli* from the feces of a World War I soldier who was afflicted with shigella but did not develop the diarrheal illness. The new bacterial strain was named "*Escherichia coli* Nissle 1917." Nissle used the strain to treat intestinal diseases, like shigella and salmonella, with great success as prebiotic. Nissle's namesake probiotic actively interacts with the body's immune system, and is still in use today (Reid, 2008).

However, Rettger also demonstrated that other bacteria which naturally exist in the gut could be effective as probiotics, helping to restore normal bacterial colonization when introduced into the human digestive tract. One of these bacteria, *Lb. acidophilus*, has been shown to be an effective treatment for constipation.

The word probiotics comes from the Latin pro ("for") and the Greek bios ("life"). Metchnikoff is generally considered to be the "father of probiotics," but there are numerous other scientists who have individually been credited with coining the word itself – for example Kollath (1953), Organization and the Food and Agriculture Organization of the United Nations developed in 2001 a widely used definition that "Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host" seems to be the most accurate. The probiotic bacteria selected should have the following main characteristics: (Holzapfel *et al*., 2001).

• They should have "non-pathogenic' activity

• They should be "resistant to bile salts" and gastric acids

• They should have the "desired technological and organoleptic properties"

• They should have "biological efficiency" on humans, including 'adhesion to epithelial cells' in the intestine.

• They should interact with "enteropathogenic" bacteria

• They should be able to "colonize" in the gut

• They should be able to "stimulate the immune system".

2.2. Lactic acid bacteria as probiotic

Lactic Acid Bacteria, or Lactobacillus, is the most common type of bacteria found in probiotics. Used for nearly 5000 years to culture and ferment food, lactic acid bacteria, is most commonly found in yogurt, kefir, cheeses and other dairy products. These bacteria, usually found in decomposing plants and lactic products, produce lactic acid as the major metabolic end-product of carbohydrate fermentation **(**Die ,1918).

This trait has, throughout history, linked LAB with food fermentations, as acidification inhibits the growth of spoilage agents. *Proteinaceous bacteriocins* are produced by several LAB strains and provide an additional hurdle for spoilage and pathogenic microorganisms. Furthermore, lactic acid and other metabolic products contribute to the organoleptic and textural profile of a food item. The industrial importance of the LAB is further evinced by their generally

recognized as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces (Rattanacha, 2010).

The genera that comprise the LAB are at its core *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus* as well as the moreperipheral *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*; these belong to the order Lactobacillus (Marco, *et al* 2006). Potentially beneficial, bacterial cells to the gut ecosystem of humans and other animals, whereas prebiotics are indigestible carbohydrates delivered in food to the large bowel to provide fermentable substrates for selected bacteria. Strains of LAB are the most common microbes employed as probiotics. Two principal kinds of probiotic bacteria, members of the genera *Lactobacillus* and *Bifidobacterium*, have been studied in detail.(Servin AL 2004)

Most probiotic strains belong to the genus *Lactobacillus*. Probiotics have been evaluated in research studies in animals and humans with respect to antibiotic-associated diarrhea, travellers' diarrhea, pediatric diarrhea, inflammatory bowel disease, and irritable bowel syndrome. In the future, probiotics possibly will be used for different gastrointestinal diseases, vaginosis, or as delivery systems for vaccines, immunoglobulin, and other therapies (Nase, *et al* 2001).

To understand the distribution and range of bile salt hydrolase activity in lactic acid bacteria. Probiotic lactic acid bacteria which have ability to bile tolerance and resistance to acids were screened. More than 300 strains of the genera *Bifidobacterium* and *Lactobacillus* and the species *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Streptococcus thermophilus* were screened for that aim. Results obtained for 273 strains showed that bile salt hydrolase activity is common in *Bifidobacterium* and *Lactobacillus* but absent in *L. lactis*, *Leu. mesenteroides*, and *S. thermophilus*. Nearly all *bifidobacteria* species and strains have bile salt hydrolase activity, whereas this activity can only be found in selected species of lactobacilli. (Bravo,*et al* 2011) , the truth observed between the habitat of a genus or species and the presence of bile salt hydrolase activity. Most often bile salt hydrolase activity is found in strains that have been isolated from the intestines or from feces from mammals--an environment rich in conjugated and unconjugated bile acids (Sonomoto, 2011). Strains and species from other habitats like milk or vegetables--environments from which bile salts are absent-do normally not

have bile salt hydrolase activity. In two independent assays, we established that bile salt hydrolase activity in *Lb .acidophillus* is, in general, much higher than in other *lactobacilli* spp **(**Millette,*et al* 2007).

2.3. *Lactobacillus acidophilus*

For centuries lactic acid bacteria have been used in the preservation of food for human consumption. In 1906, Nobel piece winner Dr. Illya Metchnikoff attributed the long life span of the Balkan people to the ingestion of large quantities of fermented foods—foods rich in lactobacilli and other lactic acid producing organisms which inhibit pathogens and detoxify one's system. In the late 1950s, Dr. Khem Shahani began studying *L. acidophilus, B. bifidum,* and other lactic acid producing cultures to determine their dietary effect on humans (Sui**,** 2002) Lactic acid bacteria (LAB) such as *Lactobacillus acidophilus* are important microorganisms in the intestines of healthy humans , in LAB are considered to beneficial microorganisms, and have been associated with several probiotic effects in both humans and animals ,(Buck L.M., 1994).", Today, hypercholesterolemia is a major challenge for human health worldwide and a central cause of coronary heart diseases. It is treated with pharmacological drugs, which are very expensive and cause a number of redundant side effects. Probiotic-based oral therapy has proved remarkable efficacy to reduce the blood cholesterol level up to 33% ,where BSH activity is significantly liable for the benefit. Human feces isolate *L. plantarum*CK 102 has been found to reduce the blood cholesterol level, triglyceride, LDL-cholesterol, and free-cholesterol in rats . Cholesterol lowering activity of BSH by probiotic organisms has been widely discussed (Sridevi, N.,2009).

Cell free supernatant from *L. acidophilus* ATCC 43121 exhibited cholesterol removing activity, which was different from earlier reports, suggesting this mechanism from live cells. The molecular mass of a protein with cholesterol-removing activity was estimated at 12 kDa . Infant feces isolate *L. plantarum* PH04 was able to reduce the serum cholesterol and triglycerides to levels of 7%and 10%, respectively, when it was fed to hyper-cholesterolemic mice at numbers of 107CFU per mouse per day for 14 days (Nguyen, *et al* 2007).

L. acidophilus was used in the form of acidophilus milk to treat constipation and diarrhea in the 1920s and 1930sin the USA. It was widely administered, often with positive

results. So commercial acidophilus tablets were marketed, but they were decried by serious researchers for their lack of any viable *L. acidophilus* bacteria (Vouloumanou *et al*. 2009).

When the public derived no benefits from the inert "acidophilus tablets", interest waned had:

1-Resistant to stomach acid bile salts.

2-Higher cholesterol.

3- Friendly bacteria to support the natural environment of the gut.

4-Great support for when the body is under pressure.

5-Contains the most prolific resident strains.

6-Vaginal yeast infections.

7-Sensitivity to milk (lactose-intolerance). (Gauri D., *et al* 2013)

2.4 .Bile salt effective on *Lactobacillus acidophilus* genome

Lactobacillus acidophilus bile tolerance is crucial for this strain's ability to survive and persist in this environment. A functional genomics approach was used to identify and characterize mechanisms of bile tolerance in *L. acidophilus*. The effects of bile on gene transcription of strains. The studies showed that after bile exposure, genes involved in carbohydrate metabolism were generally induced, while genes involved in other aspects of cellular growth were mostly repressed. Genes found in this operon were shown to contribute to both bile tolerance and bile sensitivity, as well as mediating tolerance to other inhibitory compounds. The results demonstrate a regulatory network operating between carbohydrate metabolism and bile exposure, and describe the effects of bile on gene transcription and adherence phenotypes of *L. acidophilus* (Ann E.P.,2009).

2.5. Genetics function for *Lb. acidophillus* bile salt tolerance

A wealth of information exists on the molecular mechanisms employed by bacteria to sense and resist physiologically relevant stresses, for example the low pH stress of the stomach or the elevated osmolality of the gastrointestinal tract. In contrast, the genetics of bile resistance

is poorly understood, particularly in Gram positive organisms. The modes of action of bile on bacterial cells Given the complicated nature of this stress, the ability of an organism to tolerate bile will presumably require a wide array of proteins, including many which govern cell envelope architecture or maintenance of intracellular homeostasis (Duary, et *al* 2011).

Proteins that take up or extrude bile, or enzymes that modify and transform bile salts are also likely to play important roles in bile resistance. This study will include the information available on the effects of bile on bacterial protein expression and the genes that have been shown to contribute to bile stress survival. (Begley**,.** 2005). Bacterial bile salt deconjugation was result of numerous studies of this type are during passage through the caecum and colon, conjugated bile acids can be transformed by the indigenous bacterial flora (Kim, 2008).

Some of the enzymes involved in bile salt modifications (Azcarate-Peril**,** 2006). Therefore, there are two mechanisms and anatomical sites of bile acid biosynthesis. The first is de novo synthesis of so-called primary bile acids from cholesterol in the liver. The second is the production of secondary bile acids due to modification of primary bile acids by bacterial enzymes in the intestine (Liong, 2005).

2.6. *Lb. acidophilus* Bile salt hydrolases enzyme

The enzymes are (EC 3.5.1.24) that catalyze and hydrolyse bile acids and that these enzymes and BSHs share substrates (Lundeen, 1992). but this has not yet been investigated. BSHs are generally intracellular enzymes have a slightly acidic optimal pH usually between pH 5 and 6), the BSH of *L. acidophilus* which requires low oxidation–reduction potential , This increase is due to induction of BSH by an uncharacterized extracellular factor (Corzo, 1999).

1- description of the enzyme in a pathogenic species that is not considered a member of the normal enteric flora. Interestingly, the authors note that the G + C content of the *bsh* gene is lower than neighboring genes and suggest that it may have been acquired from low G + C content bacteria such as lactobacilli (Dussurget *et al*2002).

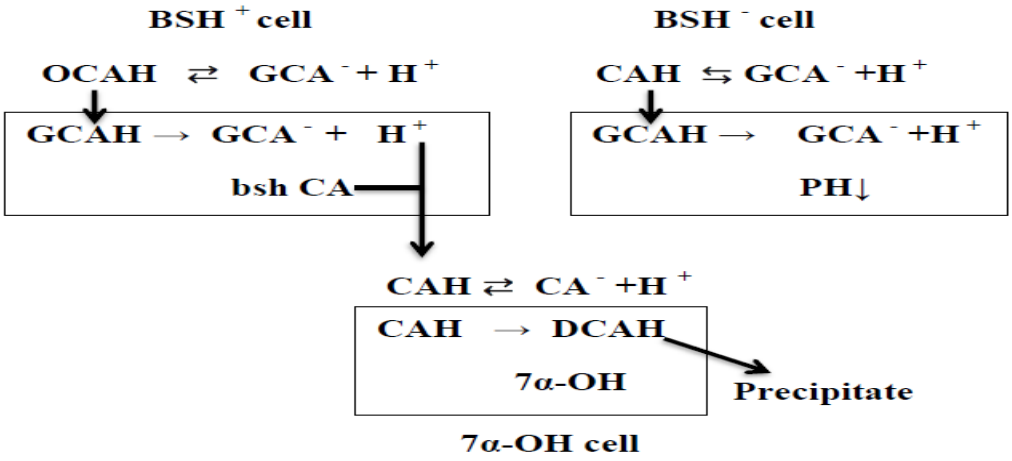
2- Function(s) of BSH. The ecological significance of microbial BSH activity is in three major hypotheses have been advanced.

Firstly, it has been proposed that deconjugation may confer a nutritional advantage on hydrolytic strains as liberated amino acids could potentially be used as carbon, nitrogen and energy sources.

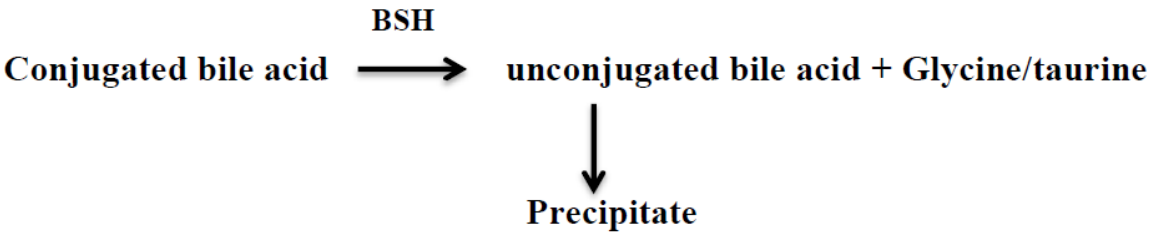
That lactobacilli do not utilize the steroid moiety of bile salts as cellular precursors suggesting that this is not a universal function of BSHs (Ahn, 2003).

-Secondly, it has been proposed that BSHs facilitate incorporation of cholesterol or bile into bacterial membranes. This incorporation may increase the tensile strength of the membranes or may change their fluidity or charge in a way that could affect sensitivity to a-defenses and other host defense molecules (fig. 2.2) (Liong MT, 2005).

-Finally, it is possible that de-conjugation of bile salts may be a detoxification mechanism and BSH enzymes may play a role in bile tolerance and consequently in survival in the gastrointestinal tract. Numerous investigators have refuted this hypothesis.



A



B

Figure 2.1. A,B chemical interaction to convert bile salt as substrate by bile salt hydrolase enzyme

2.7. Genetic Analysis of Two Bile Salt Hydrolase Activities in *Lactobacillus acidophilus*

Two genes, *bshA* and *bshB*, encoding bile salt hydrolase enzymes (EC 3.5.1.24) were identified in the genome sequence of *Lactobacillus acidophilus* . Targeted inactivation of these genes via chromosomal insertion of an integration vector demonstrated different substrate specificities for these two enzymes (Varcoe, 2003).

Analysis of *Lb. acidophilus* genome sequence revealed the presence of two putative bile salt hydrolase genes (Altermann**,** *et al*2005.). The bile-hydrolyzing capability associated with *L. acidophilu*s had been previously identified by molecular screen in electrophoresis migration. 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. To identify the molecular foundations of the relationship between probiotic organisms and their hosts, genetic characterization of strains is essential (McAuliffe**,** 2002.)

Thus, *L. acidophilus* was chosen for whole-genome sequencing. The 2.0-Mb genome sequence of *L. acidophilus* has recently been elucidated. A gene was designated as *bshB*. Analysis of the genomic organization of the region surrounding *bshA* (Sui, 2002.). However, the G-C contents of both genes are similar to those of other genes in the *L. acidophilus* genome. Therefore, it is possible that *bshA* and *bshB* may have originated from different sources through horizontal gene transfer from a closely related genome, but we have no tangible evidence for this at the present time. Another possibility is that these genes may have arisen from a duplication event, following which the genes have subsequently diverged to encode enzymes with different substrate specificities (Barrangou, 2003).

So that the results suggest that:

(A) *BshA* activity is dictated by the steroid nucleus of the conjugated bile salt

(B)While the specificity of *bshB* is determined by the presence of taurine in the bile salt structure. The study take *_bshA* gene was capable of the hydrolysis of some tauro- and glycoconjugated bile salts (Walker, 1993).

2.8. Genetic function of Bile Salt Hydrolase gene (*bshA*) in *Lactobacillus acidophilus*

Lactobacillus acidophilus bshA gene responsible about activity for hydrolysis and acidification that mean is capable coding to Bile Salt Hydrolase enzyme to hydrolysis of some tauro- and glycoconjugated bile salts, to hydrolyze the glycine-conjugated compounds such as glycodeoxycholic acid and glycocholic acid as the steroid moiety , to conversely steroid nucleus of the conjugated bile salt (Fig. 2.2) (Olivia, *et al* 2005).

2.9. Aims of the study

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 (De Smet,*et al* 1998).Thus , this study aims to:

1. Characterization to *Lb. acidophillus* probiotic strain bile salt hydrolase *bshA* gene which is capable of the hydrolysis of some tauro- and glycoconjugated bile salts, due to bile salt tolerance and treat hypercholesterolemia.
2. Cooperation *Lb. acidophilus Ar* probiotic strain bile salt hydrolase gene sequence with other *Lb. acidophilus* probiotic strain gene sequences.
3. Cloning *Lb. acidophilus Ar* probiotic strain bile salt hydrolase gene *bshA* and transformation and overexpression of this gene in *E.coli* MC1022.

3. MATERIAL AND METHODS

The gene *bshA* , encoding bile salt hydrolase enzyme (EC 3.5.1.24) responsible for cholesterol removal was identified in the genome sequence of *Lactobacillus acidophilus Ar* strain which isolated from probiotic pure strain capsules consist of 100 billion CFU use anti candida of vigenal woman diseases . This strain was treated by different kinds of antibiotics to resistance test and treated by different kinds of bile salt concentrations to tolerance test (Erika A. ,2009), addition make co factors to inducer bile salt hydrolase gene to cholesterol assimilation.

3.1. Chemical materials

All chemicals were obtained from Sigma (Germany); Merck (England) and Favorgen(Taiwan) unless otherwise stated.

3.2. Media

3.2.1. MRS medium

MRS broth was used for the enrichment, cultivation and isolation of all species of *Lactobacillus* from all types of material according to DeMan, Rogosa and Sharpe (MRS) at 30ºC and the pH most range as pH 5.7 +/- 0.2 .

Table 3.1.Typical composition of MRS for 1 liter of medium. (De Man, J.D.; 1960)

Ingredients	**g/l**
Peptone from casein	10 g
Meat extract	8 g
Yeast extract	4 g
D(+) Glucose	20 g
di-potassium hydrogen phosphate	2 g
Tween* 80	1 g
di-Ammonium hydrogenocitrate	2 g
Sodium acetate	5 g
Magnesium sulfate	0.2 g
Manganese sulfate	0.04 g

3.2.2. Luria-Bertani (LB) medium

LB media formulations have been an industry standard for the cultivation of *Escherichia coli* as far back as the 1950s.These media have been widely used in molecular microbiology applications for the preparation of plasmid DNA and recombinant proteins. It continues to be one of the most common media used for maintaining and cultivating laboratory recombinant strains of *Escherichia coli*. For physiological studies however, the use of LB medium is to be discouraged .Measure out the following:

- 10 g tryptone

- 5 g yeast extract
- 10 g NaCl
- 15g agar if need

to make a total of 1 liter PH = 7.0. Autoclave at 121 °C for 20 mins.(Nikaido, H. 2009).

3.2.3. Nutrient Broth

Nutrient Broth is used for the general cultivation of less fastidious microorganisms, can be enriched with blood or other biological fluids.

Table 3.2.Typical composition of Nutrient Broth for 1 liter of medium

Ingredients	**g/l**
Peptic digest of animal tissue	5 g
Sodium chloride	5 g
Yeast extract	1.5 g
Beef extract	1.5 g
Final pH (at 25°C)	7.4±0.2

Directions

Suspend 13 grams in 1000 ml distilled water. Heat, if necessary, to dissolve the medium completely. Dispense as desired andsterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Downes F. P. and Ito K., (Ed.), 2001) .

3.3. Equipment

Microscope slides (Sailing Boat); Microscope (Olympus);Analytical balance (Vibra);Heating magnetic stirrer (Velp); Beaker (Iso lab); Ph meter (JP Selecta); bunsen burner;PCR (eppendorf) Gel electrophoresis apparatus (Cole-parmer); Microwave oven

(Vestal);Water bath (JP Selecta); Fume hood (Nüve); Vortex (Velp); Pipette (Biohit); incubator(Nüve); Microtiter plate; Digital Camera (Canon); Termobloque TMR (Bunsen); autoclave(Nüve); Transilluminator (UVP); Thermal cycle (Favorgen); Graduated Cylinder (Kartell);Genetic analyzer (applied Biosystem/Hitachi); mastercycle PCR (AppliedBiosystem/Eppendolf).spctrophotometer (scientific) ,Nanodrop (2000 thermo) gel showing system (Transilluminator UV) ,centerfuge (sigma), water bath (memert) , balance of weight (bioneer), microfuge (sigma),Ultrasonicater (sinoperp 150).

3.3. The solutions

3.3.1. Dibasicsodium Phosphate buffer

Preparation of (0.1 M,0.01 M) and pH 6.5 from $Na_2HPO_4.H_2O$ was solved in 1L Distilled water(Atlas ,*etal* .1995).

3.3.2 **Exo SAP solution**

Exonuclease plus Shrimp Alcaline Phosphatase (ExoSAP) clean up, as any other PCR purification method aims to remove PCR reagents that unincorporated primers and dNTPs from the PCR reaction so that these do not interfere in a downstream cycle sequencing reaction. Exonuclease will degrade unincorporated primers, while Shrimp Alcaline Phosphatase will dephosphorylate unincorporated dNTPs. PCR products can be purified by adding Exo Sap working solution to a PCR product in a ration of 4:10.

3.3.2. Sodium citrate buffer

Preparation of buffer (0.5 M) and pH 5.6from :-

a- citric acid 9.6 g(0.5 M) was solved in 100mlDistilled water.

b- Sodium citrate $C_6H_5O_7Na_3.2H_2O$14.7 g (0.5 M) was solved in 100 ml Distilled water mixed 13.7 ml from (a) solution and 36.3 ml from (b) solution for complete to 100 ml distal water (Atlas ,*etal* .1995).

3.3.3. Cysteine solution

Prepared 0.1 M from 1.21 g cysteine in 100 ml Distilledwater.

3.3.4. Tricholateaceticacid(TCA) 15%

Solved 2.5 g from TCA in 100 ml distal water and saved in dark container.

3.3.5. Ethylen diamine tetra acetic acid (EDTA)

Solved 27.2 g from EDTA in 1000 ml Distilled water and saved in 4C^{o}.

3.3.6. Sodiumurodeoxycholic acid solution (substrate)

Preparation 0.04 M from solved 0.36 g sodium urodeoxycholic acid salt (SCA) in 10 ml from 0.1 M phosphate buffer and pH 6.5 .

3.3.7. Ninhydrin solution 1%

Solved 1.78 g from Ninhydrin in 100 ml sodium citrate 0.1 M pH 5.6, saved in dark container**.**(lee and takahashi, 1966)

3.3.8. Ninhydrin reagent

Preparation 0.5 ml ninhydrine solution 1% with 1.2 ml glycerol and 0.2 ml sodium citrate 0.5 M pH 5.6 (Robyt, J.F., 1990.).

3.3.9. Bovine serum albumin stock solution (BSA)

BSA was made purchased in crystalline form 0.1 g of BSA was dissolved in 10 ml of distal water at room temperature.

3.3.10.10% phosphoric acid solution

10ml phosphoric acid prepared in 100 Distilled water.

3.3.11.40% methanol solution

40 l methanol prepared in 100 Distilled water.

3.3.12. Coomassie blue reagent (Bradford reagent)

Coomassie (Bradford) Protein Assay Reagent, 950mL, containing coomassie G-250 dye, 40% methanol, 10% phosphoric acid and solubilizing agents in 50% distal water. Store at 4°C. Caution (Sorensen, K. 1994).

3.3.13.Tris-HCl buffer

Prepared 0.1 M ,pH8.0 solution from 122 g Trizma Base 1000 ml Distilled water .

3.4. Bacterial characterizations

3.4.1. Bacterialstrains , plasmids , and growth conditions:

Lactobacillus acidophillus 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 (Sarah J., 2010). The bacterial strain and plasmids used in this work (table 3.3 and table3.4).

Table 3.3. Bacterial strains used in thesis.

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

Table 3.4. Plasmids and constructs used in thesis

Plasmids	**Properties**	**Reference**
pJET 1.2 \ blunt vector	Cloning and expression plasmid ,Replicon (rep),T7promoter,Amp^R	Commercial
pMG36e vector of *E.coli*	Cloning and expression plasmid ,Replicon (rep), T7promoter,Emr^R	Commercial
pMG36e/*bshA*	pMG36e vector carry ≅ 1kb fragment of *Lb. acidophillus Ar bshA* gene	
pJET/*bshA*	pJET vector carry ≅ 1kb fragment of *Lb. acidophillus Ar bshA* gene	

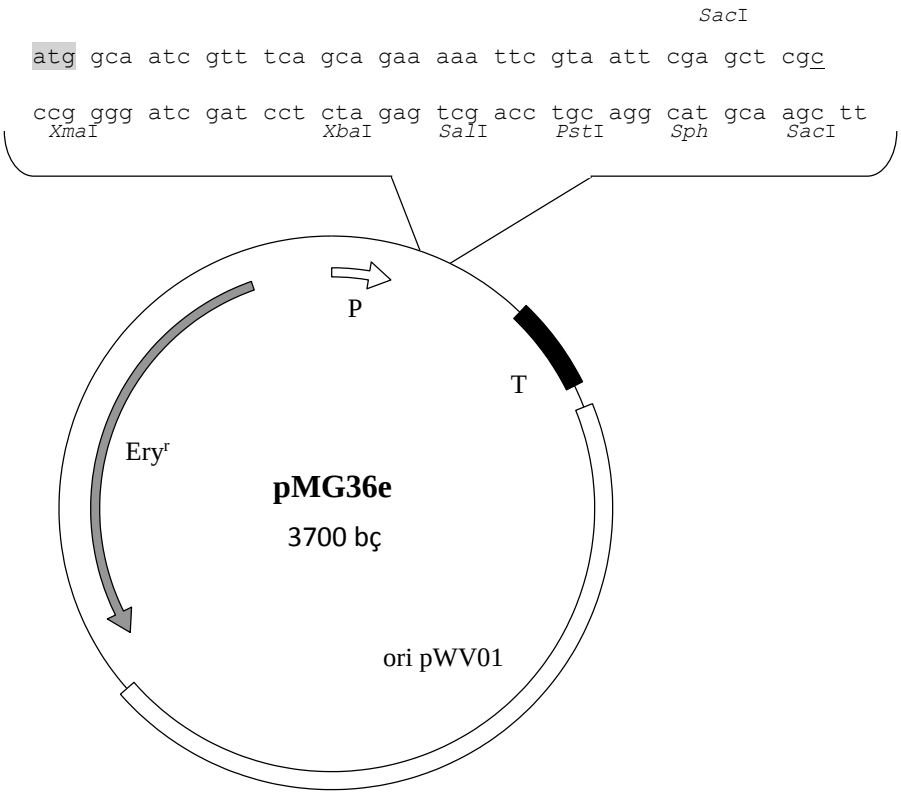


Figure 3.1 Physical map of pMG36e vector

3.4.2. Cholesterol tolerance

Lactobacillus acidophillus Ar were isolated from MRS media growing bacteria which containing cholesterol (4 mg/ml) at 37Cº for 42h.(Małgorzata., 2007)

3.4.3. Bile salt tolerance

The *Lb. acidophillus Ar* strain was treated with (sodium salt of urodeoxycholic acid) (SCA) using 0.5-0.2% (wt/vol) concentration and incubated . at 37ºC aerobically for 42h. To test bacterial growth tolerance, *E.coli* MC1022 cells were incubated with bile salt at 37 ºC for 18hr. Than bacterial growth measured spectrometrically at 600 nm for 2 h intervals during the 8 h incubation period (Gauri D.,2013). For control MRS and LB media with / without bile salt and bacterial culture used as reference. (Kamila G.,2007).

3.4.4. Antibiotics

Lactobacillus acidophillus Ar bacteria was treated with 125mg/ml final concentration of different antibiotics (such as; gentamycin , streptomycin, erythromycin, tetracycline , vancomycin , rifampicin) by simply adding to 5 ml MRS broth (Danielsen, M., 2003).

3.5. Polymerase chain Reaction PCR

3.5.1. 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 .(Grunenwald, H. 2001)

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 Amplitaq. (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 ^{0}C) , 1min denaturation at 94 ^{0}C, Annealing (30 sec at 55 ^{0}C), (30 sec at 75 ^{0}C). Final extension was at 72 ^{0}C for 1 min 4 for 35 cycles .

3.5.2. 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 (Syrovy, L., 1991). The gel was stained in 0.5 µg ethidium bromide ml^{-1} and after distaining the gel was photographed (Grunenwald, H. 2000).

3.6. Molecular cloning Techniques, reagents and Enzymes

Throughout this work standard molecular cloning techniques were used (Noreen E.,*et al* 1979). All restriction endonuclaces,T4 DNA ligase and Taq polymerase supplied from sigma .

3.7. Primers

Primers were designed by using a clone manager (cm dome 9.2 designer). These primers were stored at -20ºC. Primers utilized in this chapter can be found in table 3.5.

Table 3.5. Primers used in thesis.

Primers	**Primer sequence 5´ to 3´**
Lb. acidophilus. 16SF	5´ACTACCAGGGTATAATCC3´
Lb. acidophilus. 16SR	5´AGCTGAACCAACAGATTCAC3´
bshA F	5´AAGAGCTCATGTGACATCAATTATATT3´ *SacI*
bshA R	5´TTCTGCAGTTAGTTTTGATGGTTAAATTTAG3´ *PstI*
pJET 1.2 vector F	5´CGACTCACTATAGGGAGAGCGGC 3´
pJET 1.2 vector R	5´AAGAACATCATTTTCCATGGCAG 3´

3.8. DNA sequencing of *bshA* gene

Sequence 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 30min then at 80 ºC 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 BigDye ™ terminator Cycle ready reaction were prepared (supplied by Applied Biosystems). The sequencing reaction was than performed at 96 ºC for 2 min, than 10sec at 96 ºC, Annealing (5 sec at 50ºC), (4min at 60 ^{0}C) 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 BigDye ™ Kit.

3.9. Conformation and Cloning *bshA* gene

3.9.1. Plasmid DNA preparation

The *E.coli* pMG36e strain from overnight culture was harvested by centrifugation at 5000 rpm for 10 min . Isolation of plasmid was performed according to the protocol provided with the GeneJET Plasmid Miniprep Kit, and d Thermo scientific clone JET PCR cloning Kit for pJET 1.2 \ blunt vector was used.

3.9.2. Restriction enzymes

For digestion of (5 µl) plasmids or *bshA* gene PCR product, 9 µl sterile Milli Q water, 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. (Sambrook, J., 1989). pJET 1.2/blunt vector and pMG36e plasmids were also digested with same way to use Thermo scientific clone JET PCR cloning Kit .

3.9.3. 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 . (Aitken A., 2012).

3.9.4. $CaCl_2$ transformation

3.9.4.1. Calcium Chloride competent cell protocol for pJET/*bshA* and pMG36e/*bshA* vectors 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 (pJET /*bshA* and pMG36/*bshA* vectors) were used for ligation). (Doulas H., 1991) left on ice for 30 mins. The mixture was heat shocked at 42 ^{0}C for 30 sec, replaced on ice for 10 min and than 1ml LB broth added, incubated (water bath) shaken at 37 ^{0}C for 1-2 hours.

Then centrifuged at 5000 rpm for 5 min, 500 µL LB broth plates added to competed cells, vortexed, Transformed cells 100 µl were plated out in LB agar plate containing appropriate antibiotic (Amp 5 µg/ml or LB Emr 100µg/ml). (Sriram P., 2010)

3.10.Cloning strategies

Cloning *E.coli* MC 1022 with pJET/*bshA* and pMG36e/*bshA* vectors were carried *bshA* gene cultured in serial growth to plasmids extraction for high cloning efficiency and constructional measurement of plasmid. (Shizuya H, et al.1992)

3.11 Cloning and overexpression of *bshA* gene in *E. coli* MC1022 cells

3.11.1 Measurement of cloning *E. coli* MC1022 growth curve

The bile salt (0.5% (w/v) of sodium salt of urodeoxycholic acid (SCA) were used in the growing medium of recombinant *E .coli* MC1022 with pJET*/bshA* and pMG36e*/bshA* and non-recombinant *E .coli* MC1022 for *bshA* expression. About 107 cfu/ml culture inoculated into culture medium and incubated aerobically at a 37ºC for 18 h. Bacterial growth was measured by spectrophotometer at 600 nm at 2hr intervals, during the 8 h incubation period. Appropriate controls (LB broth in the bile salt and Medium without culture and bile salt) were used as a reference blank. Overexpression measurement of cultures were calculated, according to the formula:

$$A_{600}\text{ nm control} = (A_{600}\text{ nm control} - A_{600}\text{ nm bile salt}):$$

Then :

A600 nm control - optical density of the culture broth without bile salt.

A600 nm bile salt - optical density of the broth containing bile salt, measured at the same time Simultaneously, the number of live bacteria (cfu/ml) was determined. The test was duplicated for overexpression (Kamila G.,2007).

3.11 .2 *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 37 Cº. Control bacteria was prepared from wild type *E. coli* MC1022 without bile salt. (Robyt, J.F., 1990)

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 10mM 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 enzyme specific activity in ninhydrin method and proteins concentration measurement in Bradford method.(Venisse, J.-S., 2001)

3.11.2.1 Glycine curve

1- Preparation of (glycine) standards from the stock (gly) solution was diluted to span the 0-8 µM range used Na- phosphate buffer 0.1 M, in the (table 3.6).

2- 1.9 ml of ninhydrin reagent added to 100µl above glycine dilutions and this repeated twice for each concentration allowing two measurements to be made for each concentration of standard. Each sample was allowed to incubate at 100 ^{0}C water bath for 3 min , let to be cold before being measured (Rahman, 2003).

3-The absorbance of each standard was measured at 570 nm against a blank that was composed of 1ml of Na- phosphate buffer and 1.9ml µL of ninhydrin reagent (table 3.6). Absorbance was plotted against (gly) and an equation for the line was generated (fig.3.2) .

Table 3.6.Standard curve of glycine stock solutions preparation and Absorbance Measurements

Sample Number	gly Stock mg/ml	Na-phosphate buffer	Average A_{570}
1	0	1	0.182
2	0.1	0.9	0.295
3	0.2	0.8	0.300
4	0.3	0.7	0.350
5	0.4	0.6	0.510
6	0.5	0.5	0.702
7	0.6	0.4	0.750
8	0.7	0.3	1.100
9	0.8	0.2	1.365

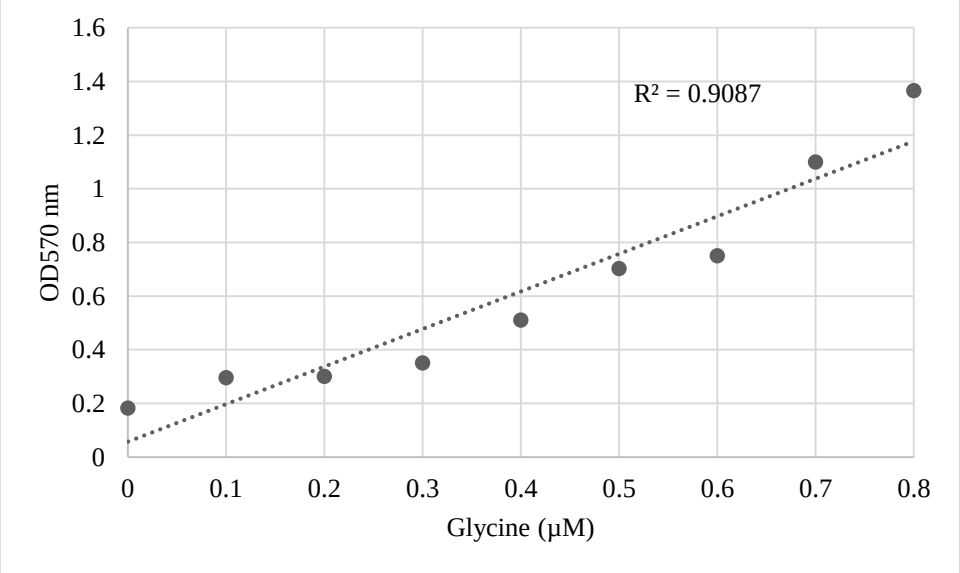


Figure 3.2. Glycine standard curve

3.11.2.2 Detectin of *BshA* enzyme activity by Ninhydrin method assay

Intracellular bile salt hydrolase were detected in ninhydrin method which has two stepes:

The first step: 200 µl solution (0.06 M of Na-urohydroxycholic acid salt) substrate incubated in 37 ^{o}C water bath for 5 min. It added to 800 µl 0.01M Na-Phosphate buffer pH6.5 with 200µl cells extracts and the mixture was then incubated at 37C^{o} for 30 min. The enzyme reaction was stopped by using 500 µl 15% TCA solution. Then microcenterfugated for 15 min and supernatant taken for enzyme assay. The blank was prepared by same way as mentioned above without addition of enzyme (Al-wendawi ,2008).

The second step: 200µl last step supernatant added to 800µl distilled water for each samples enzyme with blank for equal zero in spectrophotometer and added 1.9 ml ninhydrin reagent, left at 100 ^{o}C in water bath for 15 min. The absorbance took at O.D 750 nm .(Al-wendawi, 2008). Calculation of enzyme activity in comparative O.D 750 nm values of samples enzyme (table 3.7) with glycine curve and followed formula:

$$\text{Slope} = \Delta Y / \Delta X$$

Enzyme activity = OD_{570} sample value / Enzyme velum * slope value * enzyme reaction time

Enzyme Specific Activity = Enzyme activity U/ml from ninhydrin method / Proteins con. Mg/ ml from bradford method.

Table 3.7. Absorbance of enzyme extract samples at A_{595}

Enzyme Samples	**Absorbance**
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
E.coli pJET/*bshA* growth with 0.5% bile salt	0.345

3.11.2.3 Bradford method for *BshA* enzyme concentration

- Preparation of (BSA) standards from the stock BSA solution was diluted to span the 100-800µg/ml range in the (table 3.8).
- 60 µL of each standard was mixed with 940 µL of Bradford reagent in (3.5.12). This was repeated twice for each concentration allowing three measurements to be made for each concentration of standard. Each sample was allowed to incubate at room temperature for 10 minutes and no longer than ½ hour before being measured.
- The absorbance of each standard was measured at 595 nm against a blank that was composed of 60 µL of water and 940 µL of Bradford reagent.
- Absorbance was plotted against BSA and an equation for the line was generated.(fig.3.3)
- A sample from 400 µL the cell extract and the first stage of a current add preparation of 500 µL Tris- Hcl buffer 2.5 ml Bradford reagent and was prepared and measured in the same manner as the standards. Twice identical measurements were made for each samples.(table 3.9) (Ninfa, A.J & Ballou, D.P. 1998).

Table 3.8. Standard BSA solution preparation and absorbance measurements at A_{595}

BSA µg/ml	**Volume (µL) of 10 mg/ml BSA Stock**	**Volume (µL) of distal water**	**Absorbance**
100	5	495	0.20
200	10	490	0.4
400	20	480	0.6
600	30	470	0.8
800	45	455	1.1

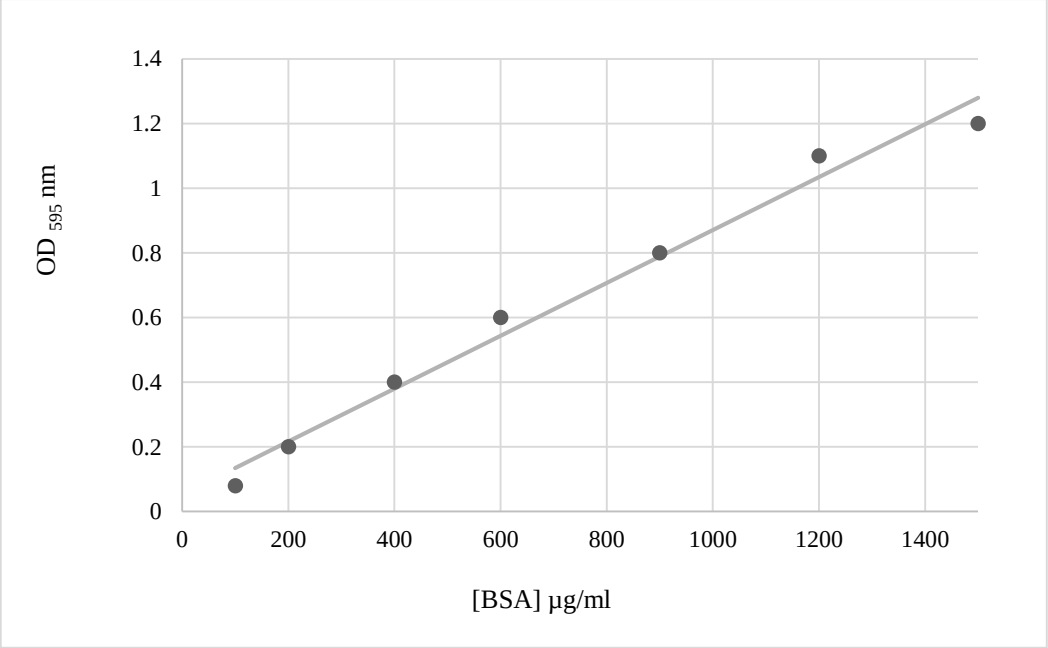


Figure 3.3. Bovine Serum Albumin standard curve.

Table 3.9. Absorbance of Protein Purification Samples

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 pMG36Ar growthing with 0.5% bile salt	0.46
E.coli pJETAr growthing with 0.5% bile salt	0.30

3.12. Bacterial Storage

All *Lb. lactobacillus Ar* strain and recombinant *E.coli* MC1022 with pJET/*bshA* and pMG36e/bshA vectors stoke made from overnight growth cultures and stocked in 30% glycerol containing medium to freez at – 20 ºC. (Fonsece, 2006).

4. RESULTS

4.1.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 37 ºC for 42 hr incubation (fig.4.1). This strain has ability to resistance different antibiotics as well as the showing distinct biochemical characteristics (Table 4.1).

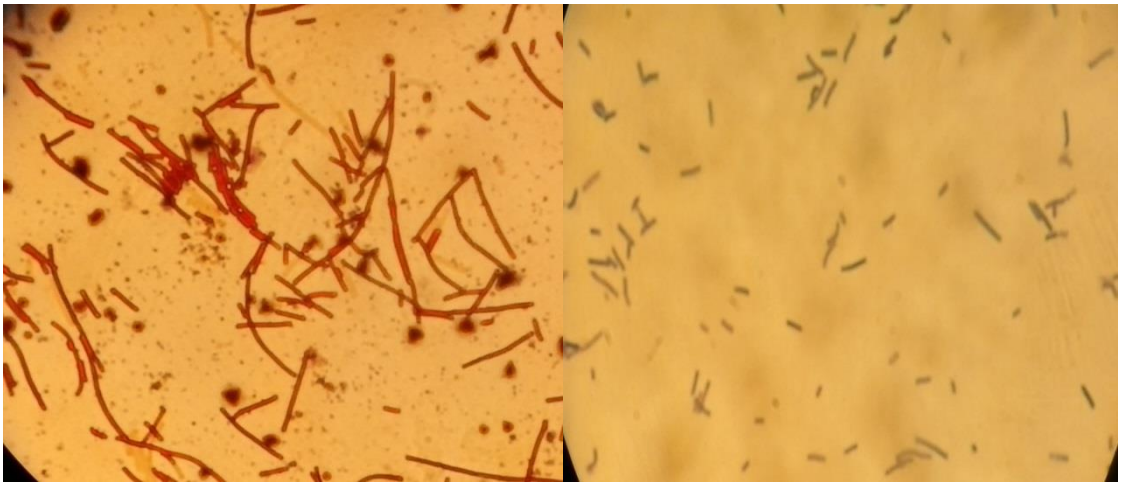


Figure 4.1. Single and group cells of *Lactobacillus acidophilus* Ar strain.

4.1.1. 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.

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

The probiotic strain *Lb. acidophilus Ar* was identified to use PCR reaction using the specific primers of partial DNA of 16S rRNA gene that mentioned (Chapter 2) Our results indicate that strain from MRS media were detected as *Lb. acidophilus Ar* (Fig. 4.2) .

Table 4.1. Show antibiotics sensitively of *Lb. acidophilus Ar* strain

Antibiotic types	**Antibiotics con.**	**Resistance test**
Refambicine	125 mg\ml	R
Erthromycin	125 mg\ml	R
Tetracyclin	125 mg\ml	R
Vancomycin	125 mg\ml	S
Gentamycin	125 mg\ml	S

Setrptomycin	125 mg\ml	S

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

The *bshA* gene of *Lb. acidophilus Ar* was amplified using primers that mentioned (Chapter 3) (Fig 3.2 b), derived from the nucleotide sequencing of *Lb. acidophillus Ar bshA* gene. Its was successfully amplified as 801 bp fragment used Master cycle PCR, big dye Terminator Cycle Sequencing standard Kit (Fig. 4.3).

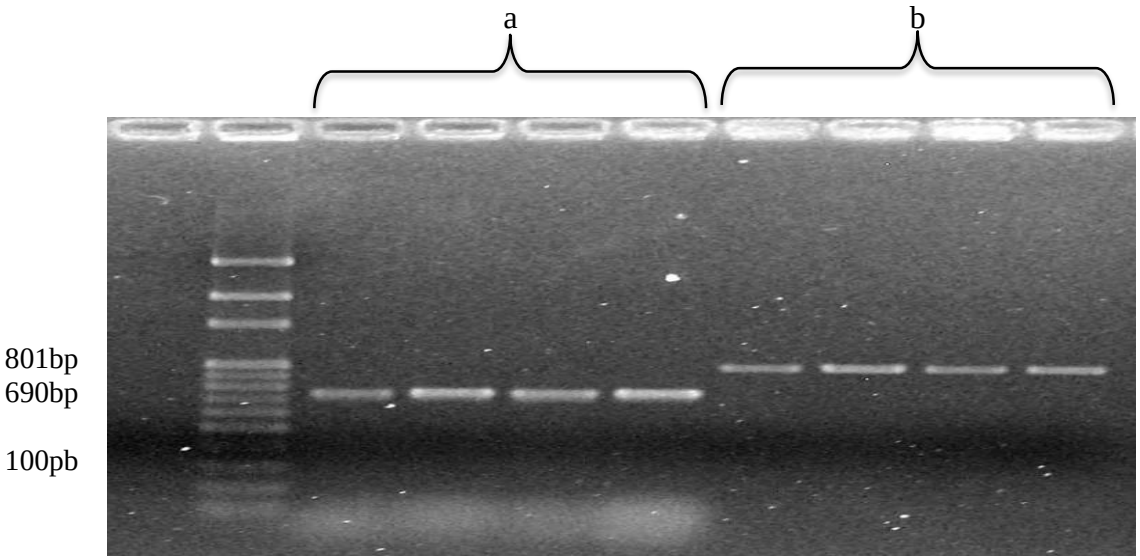


Figure 4.2. a) Lanes of PCR products of amplified 16S rRNA gene to identified *Lb. acidophilus Ar* strain using specific primers. b) Lanes PCR products of amplified *bsha* gene of *Lb. acidophilus* Ar strain using specific primers lanes: 100 bp DNA marker.

PstI revers primer

CYKATTKRTWRGGARWYCTTCTAGAAGATCTGCAGTTAGTTTTGATGGTTAAATTTAGTTTTATCAAGCATATTA

TAAGWGATCAAATTGCTGCTATCTAGATCTTCTTTATTCATATCAACAACGTTAATTTGTTTGTTTGAATAAGTG

GTGTAGTAGAAAATACCTTTGTCTAAGTTAGTTCCATCAGAATAAATTGTATATTCAAATGAGTTTGGACCAACT

TCATCCAGTCCCTTTTGTTGTTCAACCGAATGTAAAATGTGGAAGTAAGTATCAATATTTTCTTCTTCGGTTTCA

GCAATTGGAGCATTAAATTTATTGAAAGCTACTCTGACAAAACGTGATTCAGAATCCATTCCACCTGGTAAGTTG

TGAGACCCTAATCCACGGCTGTAGCCAGCCATATTTACTTTATCTGAGAAGTTATTTTTAGGCATTTTTGGAGAT

ACGTCAGCATAGTTATTTAAATTGAATAATTGCTTTGGAAATTGTGGATTATTAGTTAAGCAGCCAACTGGATTA

TCATAAATATGCATTCCATCTTTGTCTGTTTCAACAACTAATGATGTACCTGTTTTATCTGCAATAAGCCAGTGA

AGAGAGGAGGTTTGCATTTTTTCGCTGAAATTTAAATCGGCGATGTTGATTCTGCTAAGTAAATCCTTTACTTCG

CTAATAGTGCTACACTGTCCTAAAATCCAAGGGATGAATTCAAAGGAAGCAATATTATCTTTATTTTCTTTTTCT
TCGTAATATGTAGCATTTCCTGGATAGTTGAGTCCGGCCATACCTAAACCT

SacI

Figure 4.3.*bshA*gene sequence data is 801 bp only revers strand

4.3. Analysis of Sequence Data

Sequencing analysis of the clone obtained from PCR reaction showed high similarity (44%) from *L. acidophilus Ar* probiotic strain (fig.4.4 a) . Sequencing results were obtained in a Genetic analyzer system and they were analyzed using BLAST programmer in NCBI nucleotide database. Each of the sequence results showed little non- homology with the *bshA* gene sequences of *Lb. acidophilus Ar* these results confirmed that the strains belong to *Lb. acidophillus* species (fig.4.4 b).

4.4. The primers

Sequencing analysis results for the primers *bshA* F ,*bshA* R which minted in chapter 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 (dome 9.2)(Fig 4.3).

```
Bsha Ar          36 ttagttttgatggttaaatttagttttatcaagcatattataagwgatca
LA 11978          978 ..................................c.....t.....

Bsha Ar          86 aattgctgctatctagatcttctttattcatatcaacaacgttaatttgt
LA 11978         928 .................................................

Bsha Ar          136 ttgtttgaataagtggtgtagtagaaaataccttgtctaagttagttcc
LA 11978         878 .................................................

Bsha Ar          186 atcagaataaattgtatattcaaatgagtttggaccaacttcatccagtc
LA 11978         828 .................................................

Bsha Ar          236 ccttttgttgttcaaccgaatgtaaaatgtggaagtaagtatcaatattt
LA 11978         778 .................................................
```

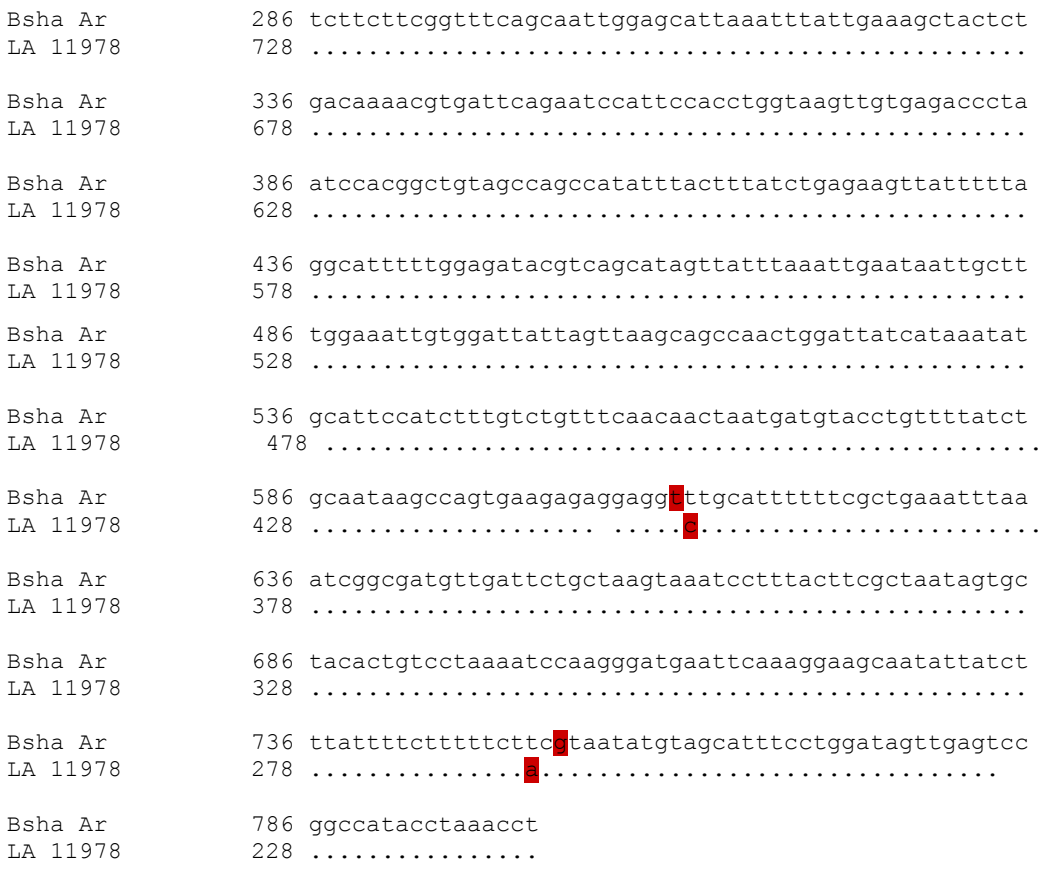


Figure 4.4. Show non-homology sites (red color) between *bshA* gene sequence when insert data to nucleotide blast *bshA* gene of *Lb acidophilus* LA11 strain to compared with Gene bank data base

4.5. *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 37 ºC and sensitive to erythromycin and ampicillin antibiotics.

4.6. Plasmid conformation and gene fusion analysis

4.6.1. Constriction of *bshA* gene reporter gene fusion

To clone *bshA* gene from chromosomal *DNA of Lb. acidophilus*, primers *bshA* F and *bshA* R (see Matherial and methods) 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 both pJET1.2\blunt and pMG36e Easy vectors and this new recombinants named as pJET/*bshA* and pMG36e/*bshA* respectively. Obtained recombinants then transformed into *E.coli* MC1022 by chmical method. After transformantion 4-5 cell/100µl single colonies obtained (Douglas H, 1991), and each plate containig transforment colonies were screened by PCR amplification (Fig.4.5 a,b) using *bshA* F and *bshA* R primers and restriction digest analysis was performed to ensure that the *bshA* fregment present. Purified gen fragment was then sequenced to check no erroneous base pair changes occurred during the PCR amplification process.

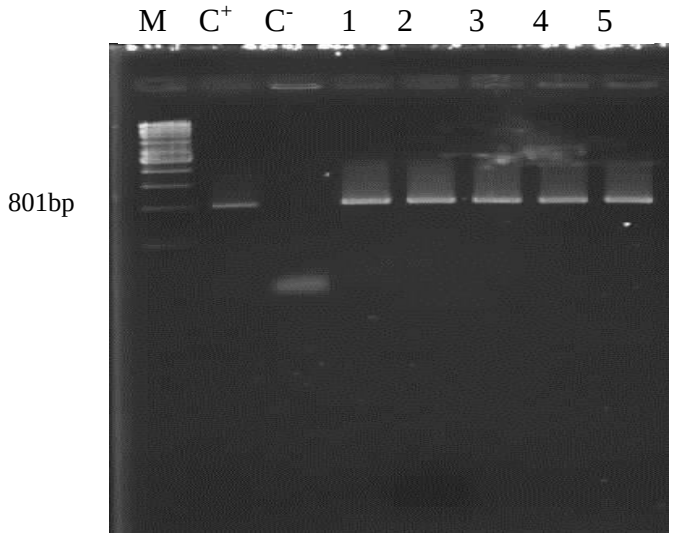


Figure 4.5. C+ *bshA* gene control all lane (1-5) for cloning single colony carry *bshA* gene after CaCl transformation of pJET/*bshA* M) used 1000pb ladder.

M C+ C- 1

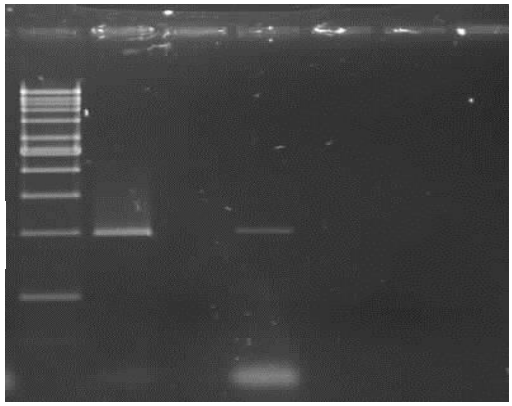


Figure 4.5. C+) lane *bshA* gene control ,C-) *E.coli* colony non bshA gene transformation , lane (1) for cloning single colony carry *bshA* gene after $CaCl_2$ transformation of pMG36e/*bshA*, used M)1000pb ladder

4.6.2. Conformation of cloning plasmids with *bshA* gene

Recombinant *E.coli* MC1022 which carriy recombinant plasmids pJET/*bshA* or pMG36e/*bshA* were growt in the appropriate conditions, after that recombinant plasmids were isolated. Isolated recombinant plasmids pJET/*bshA* or pMG36e/*bshA* were digesed with *PstI\SacI* to to confirme presence of *bsha* gene by gel electrophoresis separation (Fig.4.6 a,b) (Fig.4.7). It has been confirmed that after gel electrophoresis separation two plasmid fragments and *bsha* gene with controls appeared.（ Noreen E., *et al* 1979)

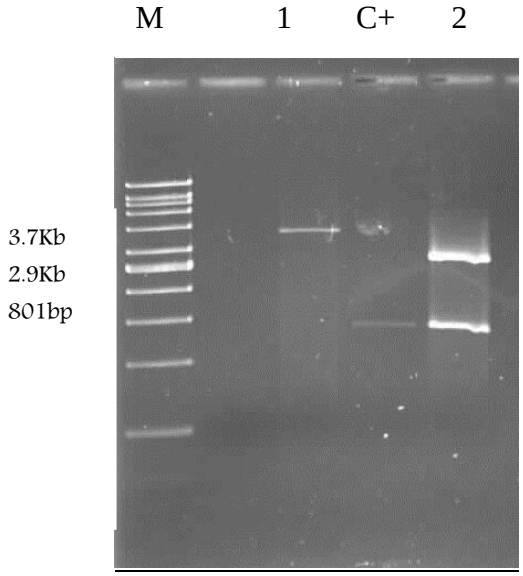


Figure 4.6. a) Lane 1, pJET/*bshA* vector 3.7Kb, Lane C+, PCR reaction of *bshA* gene 801bp (control) Lane 2) pJET/*bshA* vector digested with *PstI/SacI* restriction enzyme to get tow fragments (2.9 Kb+ 801bp),M) 1000bp ladder.

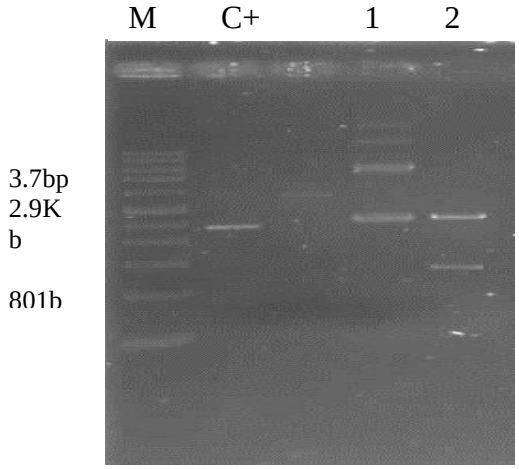


Figure 4. 6. M) 1000 bp ladder lane C+) pJET1.2 vector 2.9Kb (control) , lane 1) pJET/*bshA* vector 3.7 bp and pJET1.2 vector 2.9Kb (control) lane 2, pJET/*bshA* vector digestived use *PstI* *SacI* restriction enzymes to get tow fragments (2.9 Kb+ *bshA* gene 801bp).

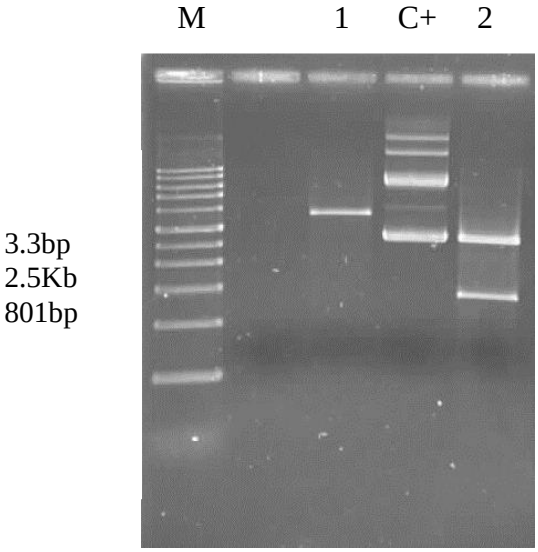


Figure 4.7. Lane 1) Recombinant pMG36e/*bshA* 3.3 bp., lane C+: pMG36e vector 2.5 Kb (control) Lane2) Recombinant pMG36e/*bshA* digested with *PstI* *SacI* restriction enzyme to get tow fragment (2.5 Kb+ *bshA* gene 801bp).

4.7. Analysis of pJET/*bshA* and pMG36e/*bshA* 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, pJET/*bshA* or pMG36e/*bshA* system may facilitate construction of DNA libraries of complex genomes with fuller representation and subsequent rapid analysis of complex genomic structure (Fig. 4.8) . (Shizuya H, et al 1992)

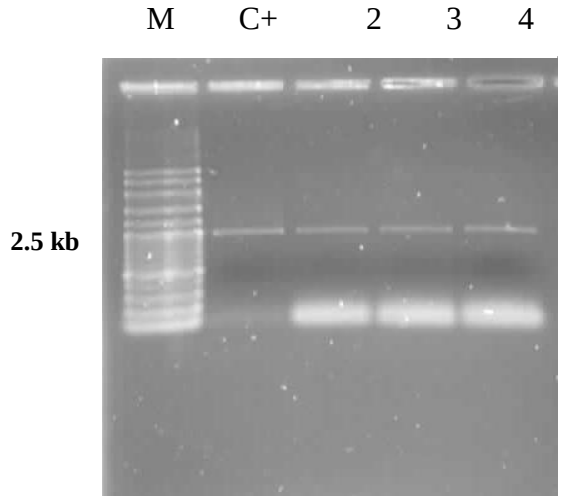


Figure 4.8. Lane C+ pMG36e/*bshA* vector extraction 4.5 Kb (control), Lane 1) pMG36e/*bshA* for first extraction, Lane 3,4) pMG36e/*bshA* for serial of other extraction

4.8. Bile salts effects on cloning and wild type *E. coli* MC1022 growth

For present experiment growth of recombinant and control *E. coli MC1022* were measured at 600nm. Although addition of 0.5% bile salt in the growing medium slightly increase

in cell number can be observed while these increase in cell number more clear and higher in plasmids pJET/*bshA* or pMG36e/*bshA* carried *bsha* gene of *E. coli MC1022* (Fig4.9).

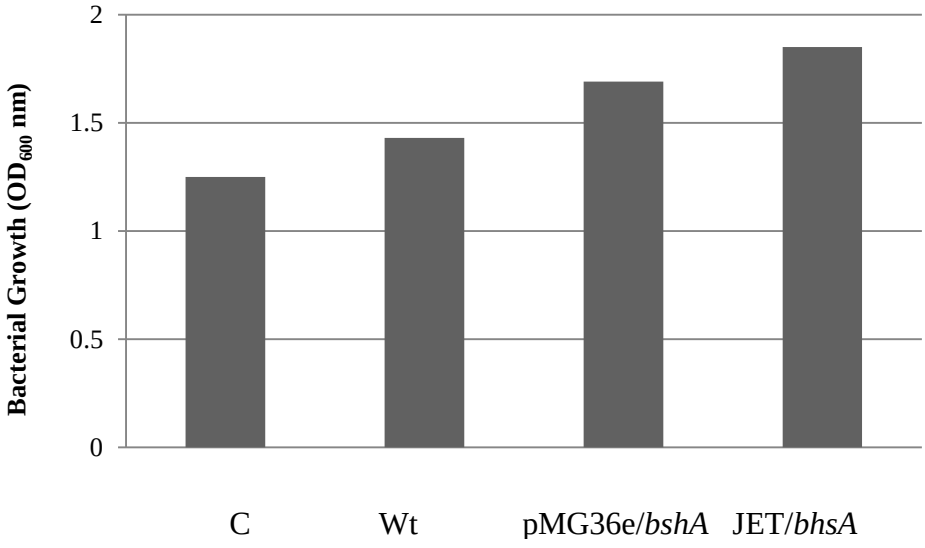


Figure 4.9. Growth rate (O.D) of *E. coli MC1022* without and with recombinant vectors in the presence of 0.5% bile salt. Lane C) Control of *E. coli* MC1022 culture without bile salt, Lane Wt) of *E. coli* MC1022 in 0.5% bile salt, Lane of *E. coli* MC1022 with pMG36e/*bshA* vector, Lane of *E. coli* MC1022 with pJET/*bshA* vector.

4.8.1. Bile salt hydrolase enzyme assay

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. 3.1, table 3.4) for *bshA* enzyme activity assay.

These studies are in agreement with previous studies **(**Michael P.,1988**).** It is noteworthy that unlike previous studies where partially purified enzyme *E. coli* MC1022 pJET/*bshA* or pMG36e/*bshA* cultures were used as the source for BSH activity, can be used for more detailed studies on its substrate specificity using various strategies employed by (Batta et al. 1984**) .**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.3.2, table 3.6) to calculate bile salt hydrolase specific activity.(Huijghebaert S.,1989)

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.(Michael P.,1988)

After took spectrophotometer absorbance (O.D $_{570,595\ nm}$**)** and enforcement enzyme specific activity formula (3.12.2.3 material and method) ,these data indicate that the *bshA* overproducing in *E. coli* MC1022 pJET/*bshA* strain comparison with *E. coli* MC1022 wild type then *E. coli* MC1022 pMG36e/*bshA* strain and no enzyme for *bshA* gene function for bacteria growth in broth media from non-bile salt substrate (Table 4.1).

Table 4.2. The results of overexpression data compression between cloning *E. coli* MC1022 and wild type, read at $A_{595\ nm}$

Samples	**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
E.coli pJET/*bshA* growth in 0.5% bile salt	383.3

These results appeared that pJET/*bshA* construct more active than pMG36e/*bshA* construct. Because its specific for *E. coli* strains and there is evidence that *E. coli* MC1022 recognizes promoters different from those used in the 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* MC1022 in the absence of any obvious ribosome binding site, and ribosome binding may be dependent on secondary structure. 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. 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 (Özköse *et al.* 2004).

Proven promoter sequences have shown similarity to the consensus sequences of *E. coli*MC1022, and other little consensus sequences that function in *E. coli* MC1022 pMG36e/*bshA* (Fig. 4.10) but proven promoter sequences have shown similarity to the consensus sequences in other lactic acid bacteria completely.(Ekinci, 1999)

The importance of lactic acid 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 sale hydrolysis functions into non- bile salt hydrolase bacteria such as *leuccuccuse mesenteroides* or over expression occurrence for bacteria use as hypercholesterolemia treatment (Tanaka *et. al,* 1999).

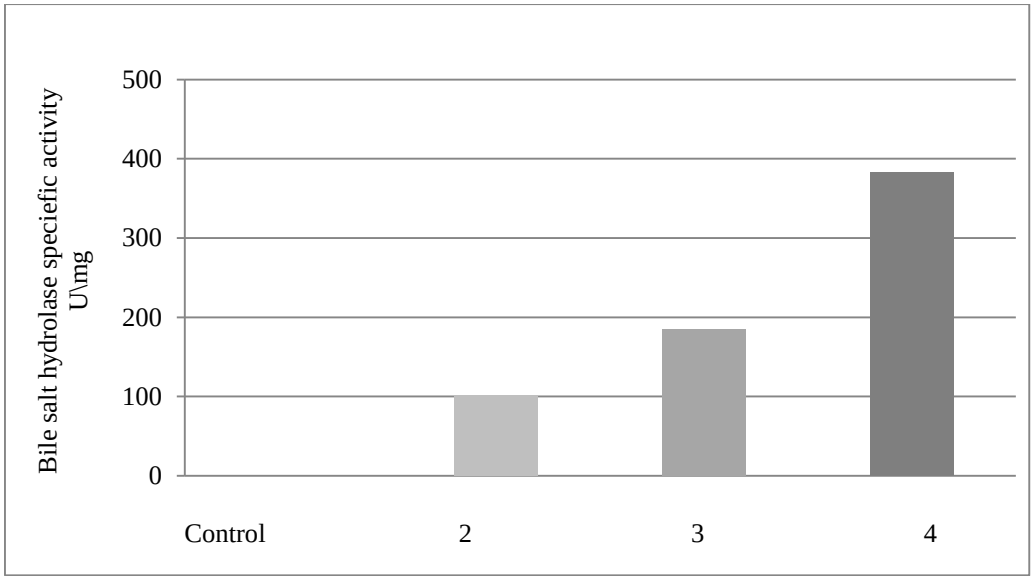


Figure 4.8. Bile salt hydrolase spesific activity and Overexoression values (U\mg). 1: (Control) *E. coli* MC1022 wild type growthing without 0.5% bile salt. 2: *E. coli* MC1022 wild type growthing in 0.5 bile salt. 2: Recombinant *E. coli MC1022* with pMG36e/*bshA* 4: Recombinant *E. coli MC1022* with pJET/*bshA* .

4.9. Analysis of bile salt enzyme amino acid Sequence Data

Amino acids Sequence analysis of *bshA* enzyme encoding gene from *L. acidophilus Ar* probiotic strain was done. Sequencing results were obtained by using BLAST programmer in NCBI nucleotide database. Each of the sequence results showed after at least 50 amino acids found stop codon. The amino acids sequence results of gene encoding *bshA* enzyme of *Lb. acidophilusAr*, confirmed that the strains belong to *Lb. acidophilus* species (fig.4.11).

```
1
61  XIXXEXF*KI   CS*F*WLNLV   LSSIL*XIKL   LLSRSSLFIS   TTLICLFE*V   V**KIPLSKL
121 VPSE*IVYSN EFGPTSSSPF CCSTECKMWK *VSIFSSSVS AIGALNLLKA TLTKRDSESIPPGKL*DPNP
181 RL*PAIFTLS EKLFLGIFGD TSA*LFKLNN CFGNCGLLVK QPTGLS*ICIPSLSVSTTND VPVLSAISQ*
241 REEVCIFSLK FKSAMLILLS KSFTSLIVLH CPKIQGMNSKEAILSLFSFS S*YVAFPG*L SPAIPKP
```

Figure 4.119. Amino acids sequencing Bile salt hydrolase isolated in the present work.

5. DISCUSION

5.1. 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 *Lactobacillus acidophilus* is one of the major cholesterol removal organism that have (bile salt hydrolase genes) *bsh* genes responsible for this process .

The anticandida woman disease probiotic *Lactobacillus acidophilus Ar* strain 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* .(Peter . *et al* 2004).

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 cfu /ml possibly because leakage of intracellular material from the cells and a disturbed energy balance.(Breton *et al* 2002).

Identification of *Lb. acidophilus Ar* strain 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 (Gutell *et al.,* 1994, Olsen *et al.,* 1991). 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.

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

To create the recombinant plasmids with pJET1.2\ blunt end and pMG36e vectors specific for *E. coli* to clone *bshA* gene, the *bshA* gene was amplified with the primers *bshA*-F and *bshA*-R (Table 2). And S*acI* site was designed in primer *bshA*-F and a P*stI* site was created in primer *bshA*-R to include the start codon sequence and the stop codon (TGA) sequence.

Cloning into the P*stI* -S*acI* sites of vectors resulted in the translational fusion of the *bshA* gene to the T7 promoter of pJETAr1.2 and promoter (P) of lambda phage in pMG36e, a ribosome binding site and the start of an open reading frame are present and *E. coli* MC1022 ribosome binding site.(Ekinci,1999)

The previously constructed *bhsA* complementation library, 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 plasmid pJET/*bshA* more than pMG36e/*bshA* were created in *E. coli* MC1022 and higher stability after serial sub-culturing, and prepared for cloning to other lactic acid bacteria (LAB).(Michael P. , *et al* 1988)." Transformed into *E. coli* MC1022 to perform the overexpression study in vitro for the overexpression of *E. coli* MC1022 cells after *bshA* transferring , the optical density at 600 nm, increase in *E. coli* MC1022 wild type from 0.199 cfu/ml to 0.381 cfu\ ml when recombinant g *E. coli* MC1022 with pMG36e/*bshA* and became 0.6145 for recombinant *E. coli* MC1022 with pJET/*bshA*.

5.3. 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 into the both pJET1.2\ blunt expression vector and pGM36e vector. The produced recombinant *bshA* enzyme exhibited hydrolase activity against sodium salt of uroconjugated bile salts .The gene, *bshA* 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 (Batta, A. K.,1984).

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 pJET/*bshA* found to be higher while compared with *E. coli* MC1022 wild type and recombinant *E. coli* MC1022 pMG36e/*bshA* strain. However production of bile salt hydrolase from recombinant *E. coli* MC1022 pMG36e/*bshA* found to be higher while compared with *E. coli* MC1022 wild type. It could be thought that pJET 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.(Tanaka, H., K., 1999)

6. CONCLUSION

1. Lactic acid bacteria are live microorganisms present in food and dietary supplements that beneficially affect the individual by improving the intestinal microbial balance properties. Their market value and biological potential is enormous because of their health-promoting properties. Therefore, comparative studies on probiotic potential of one selected strain of *Lactobacillus acidophilus Ar* were carried out during this work.(Hatakka, K.; *et al* 2001).

2. The bacteria interfere with the recycling of bile salt (a metabolic product of cholesterol) and facilitate its elimination, which raises the demand for bile salt made from cholesterol and thus results in body cholesterol consumption.

3. Fermentation products of lactic acid bacteria inhibit cholesterol synthesis enzymes as bile salt hydrolase (*bshA* ,*bshB*) and thus reduce cholesterol production ,This in turn led to the cholesterol, HDL, and LDL levels in serum were decreased in the probiotic group , which favorable LDL to HDL .

4. In this study bile salt hydrolase gene (*bshA* gene) of probiotic lactic acid bacteria *Lb. acidophilus Ar* strain which used to treatment for anti-candida women diseases was investigated. Biochemical characters was studied for bacteria special bile salt toxicity tolerance and effects which refers to *bshA* gene present in *Lb. acidophilus Ar* strain. PCR technique used to amplify *bshA* gene by using *bshA* primers. According to sequences analysis showed that *bshA* gene consist of 801 bp length.

5. To identify the gene(s) encoding *bshA* activity in *Lb. acidophilus Ar*, an P*stI* \ S*acI* digested genomic library was created in pJET1.2 and screened in *E. coli* MC1022 cells. the colonies on the selective medium (LB) supplemented with 0.3µl of ampicillin/ ml and insert to constructed With pMG36e specific prepare transfer to other lactic acid bacteria, used in (LB)media 0.3 µl of erythromycin/ml .the result appeared high stability for cloning plasmids occurred in *E. coli* MC1022 after many serial sub culture especial with pJET/*bshA* vector.

6. Expression of *Lb. acidophilus* Ar *bshA* gene occurs in *E. coli* MC1022 in the absence of any obvious ribosome binding site, and ribosome binding may be dependent on

secondary structure. Ribosome binding sites are generally observed in the expected position for gene isolated *Lb. acidophilus Ar* , but internal translational start points and proteolysis can complicate interpretation of cloned gene products expressed in *E. coli* MC1022 . 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 promoters and vectors have been developed to allow quantitative modulation of gene expression over a range of levels but were overexpression within pJET/*bshA* vector more than pMG36e/*bshA* vector .(Tanaka, H., K. ,1999)

7. RECOMMENDATIONS

According to results of this study, the following recommendations can be made:-

1. Transformation and expression of bile salt hydrolase gene to other lactic acid bacteria (LAB) which have not this gene, *as L. acid lactis* and *Leuconostoc mesenteroides* .
2. More analysis of genomic sequence to bile salts function and effects on *Lb. acidophilus* probiotic genes and other lactic acid probiotic genes which important in achieving bile tolerance in this species and same the major facilitator super family genes (MFS).
3. Study bile salts effect on genes responsible about gradually severity of changes in lactic acid probiotic bacteria morphology cells including bulky structures on the cell surface.
4. Bile salt gene overexpression in animal tissue for safety food engineering products.
5. Bile salt gene overexpression in probiotic bacteria for gallbladder stones treatment and fragmentation replace laser and other surgery.

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