BOLU ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

CLONING OF BILE SALT HYDROLASE GENE (*BSH*) FROM HUMAN ORIGINATED *LACTOBACILLUS PLANTARUM* AND CHARACTERIZATION OF BSH ENZYME BY SITE DIRECTED MUTAGENESIS

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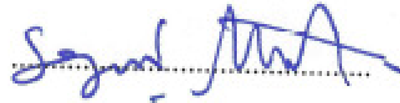
Member Prof. Dr. Sezai TÜRKEL Bursa Uludağ University	
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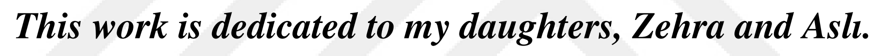

This work is dedicated to my daughters, Zehra and Aslı.

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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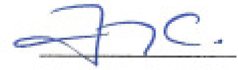

ABSTRACT

CLONING OF BILE SALT HYDROLASE GENE (*BSH*) FROM HUMAN ORIGINATED *LACTOBACILLUS PLANTARUM* AND CHARACTERIZATION OF BSH ENZYME BY SITE DIRECTED MUTAGENESIS
PHD THESIS
CANSU ÖNAL
BOLU ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
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(SUPERVISOR: ASSOC. PROF. DR. MEHMET ÖZTÜRK)

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The gastrointestinal microbiota plays an important role in human physiology and is liable for nutritional, physiological and immunological functions of the host. Microbial colonization of gastrointestinal system significiantly affect the genetic factors of colorectal cancer etiology. The microbial products of the toxic secondary bile acids such as lithocholic acid and deoxycholic acid caused colon cancer by influencing inflammation, DNA damage and apoptosis induction. The hydrolysis of glycine or taurine-conjugated bile acids is the gateway reaction for the occurrence of secondary bile acids. BSH enzymes (EC 3.5.1.24) from various sources rather differ in characteristics, substrate preference and specificity. To show relationship between BSH enzymes and toxic secondary metabolites, structure of the BSH must be better understood, but the structure and reaction mechanism of such an important BSH enzyme are not known very well. Site-directed mutagenesis is used to understand the structure and function of the enzyme due to the importance of the substitution of the codons coding amino acids supposed to be responsible for substrate specificity. In this study, *Lactobacillus plantarum* B14 BSH gene encoding 324-amino acids was cloned and the codons of the partially conserved amino acids, Phe-18, Tyr-24, Asn-79, Leu-138 and Asn-180 supposed to be responsible for substrate specificity, substituted by site directed mutagenesis. All mutant BSHs were purified using an *E. coli* BLDRE3-pET22b expression system. The stability of the mutant enzymes and molecular weight 37 kDa were confirmed by SDS–PAGE. The high BSH activity of *Lb. plantarum* B14 was determined qualitatively by Direct Plate Assay and quantitatively by Ninhydrin assay. The effects of the mutations on substrate specificity and catalytic activities of BSH were investigated by partially purified of mutant BSH enzymes with six different bile salts. The study showed that *Lb. plantarum* B14-BSH exhibited greater hydrolysis toward glyco-conjugated bile salts compared to tauro-conjugated bile salts and the BSH activities of mutant enzymes showed signifciantly decreasing alternations towards different bile salts.

KEYWORDS: *Lactobacillus plantarum*, Bile Salt Hydrolase (BSH), Site Directed Mutagenesis, Substrate specificity.

ÖZET

İNSAN KAYNAKLI *LACTOBACILLUS PLANTARUM* KÖKENLİ SAFRA TUZU HİDROLAZ (*STH*) GENİNİN KLONLANMASI VE STH ENZİMİNİN YÖNLENDİRİLMİŞ MUTAGENEZ İLE KARAKTERİZASYONU
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Gastrointestinal mikrobiyota insan fizyolojisinde önemli bir rol oynar ve konağın beslenme, fizyolojik ve immünolojik fonksiyonlarından sorumludur. Gastrointestinal sistemin mikrobiyal kolonizasyonu, kolorektal kanser etiyolojisinin genetik faktörlerini önemli ölçüde etkiler. Litokolik asit ve deoksikolik asit gibi toksik sekonder safra asitleri, inflamasyon, DNA hasarı ve apoptoz indüksiyonunu etkileyerek kolon kanserine neden olmaktadır. Glisin ya da taurin ile konjuge safra asitlerinin hidrolizi, sekonder safra asitlerinin ortaya çıkması için bir geçiş reaksiyonudur. Çeşitli kaynaklardan elde edilen STH enzimlerinin (EC 3.5.1.24) karakterizasyonu, substrat tercihi ve spesifisiteleri farklılık göstermektedir. STH enzimi ve toksik sekonder metabolitler arasındaki ilişkiyi anlamak için, STH'nin yapısı daha iyi anlaşılmalıdır. Ancak STH enziminin yapısı ve çalışma mekanizması çok iyi bilinmemektedir. Enzimin yapısını ve işlevini anlamak için substrat özgüllüğünden sorumlu olduğu düşünülen amino asitleri kodlayan bölgeye yönelik yönlendirilmiş mutagenez kullanılır. Bu çalışmada, 324-amino asitten oluşan *Lactobacillus plantarum* B14 STH geni klonlanmış ve kısmen korunmuş ve substrat özgüllüğünden sorumlu olduğu düşünülen Phe-18, Tyr-24, Asn-79, Leu-138 ve Asn-180 amino asitleri, yönlendirilmiş mutagenez ile değiştirilerek, enzim aktivitelerine bakılmıştır. Tüm STH 'ler *E. coli* BLDRE3-pET22b ekspresyon sistemi kullanılarak saflaştırılmıştır. Mutant enzimlerin ve her biri aynı molekül ağırlığı (37 kDa) ve stabilitesi SDS-PAGE ile doğrulanmıştır. *Lb. plantarum* B14'ün, STH aktivitesi, nicel olarak Direkt Plate Testi ve nitel olarak Ninhidrin Testi ile belirlenmiştir. Mutasyonların substrat özgüllüğü üzerindeki etkileri, altı farklı safra tuzları ile mutant STH enzimlerinin kısmen saflaştırılmasıyla araştırılmıştır. Araştırmada, *Lb. plantarum* B14 STH'ın tauro-konjuge safra tuzlarına kıyasla glisin ile konjuge safra tuzlarına daha fazla hidroliz sergilediği ve mutant enzimlerin STH aktivitelerinin, farklı safra tuzlarına farklı ölçüde azalan değişimler gösterdiği tespit edilmiştir.
ANAHTAR KELİMELER: *Lactobacillus plantarum*, Safra tuzu hidrolaz (STH), Yönlendirilmiş mutagenez, Substrat özgüllüğü.

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LIST OF ABBREVIATIONS AND SYMBOLS

A : Adenine
Ala : Alaline
Amp^R : Ampiciline resistance
BA7 : Bile acid 7a/b-dehydroxylation
bp : Base pair
BLAST : Basic Local Alingment Search Tool
BSA : Bovine Serum Albumin
BSH : Bile Salt Hydrolase
CA : Cholic Acid
CBAH : Conjugated Bile Acid Hydrolase
CDCA : Chenodeoxycholic Acid
CDI : Clostridium Difficile Infection
CHD : Coronary Heart Disease
DCA : Deoxycholic Acid
DTT : Dithiothreitol
EDTA : Ethylenediaminetetraacetic acid
FAO : Food and Agriculture Organization
FDA : Food and Drug Administration
GCA : Glycocholic Acid
GCDCA : Glycochenodeoxycholic Acid
GDCA : Glycodeoxycholic Acid
GIT : Gastrointestinal Tract
GPBAR1 : G-protein-coupled Bile Acid Receptor 1 (TGR5)
GRAS : Generally Recognized As Safe
HDL : High-Density Lipoproteins
IgA : Immunoglobulin A
IBD : Inflammatory Bowel Disease
IBS : Irritable Bowel Syndrome
IBS : Irritable Bowel Syndrome
IMAC : Immobilized metal ion affinity chromatography

IPTG : Isopropyl β-D-1-thiogalactopyranoside
Kbp : Kilo base pair
kDa : Kilo Dalton
L : Leucine
L : Liter
Leu : Leucine
LAB : Lactic Acid Bacteria
LB : Luria-Bertani Broth
LCA : Lithocholic Acid
LDL : Low-Density Lipoproteins
M : Molar
mM : Millimolar
mg : Milligram
ml : Milliliter
MRS : de Man, Rogosa and Sharpe
Ni^{+2} : Nickel (II)
Ntn : N-terminal nucleophilic
OD : Optic Density
PAGE : Polyacrylamide Gel Electrophoresis
PEG : Polyethylene Glycol
PCR : Polymerase Chain Reaction
rpm : Rotation per minute
SDS-PAGE : Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIB : Swiss Institute of Bioinformatics
TCA : Taurocholic Acid
TCA : Trichloroacetic Acid
TCDCA : Taurochenodeoxycholic Acid
TDCA : Taurodeoxycholic Acid
TGR5 : G-protein-coupled Bile Acid Receptor 1 (GPBAR1)
Tween20 : Polyethylene Glycol Sorbitan Monolaurate
U : Enzyme unit
µg : Microgram
UTIs : Urinary Tract Infections
µl : Mikroliter

µM **:** Micromolar

WHO **:** World Health Organization

X-gal **:** 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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1. INTRODUCTION

Colorectal cancer (CRC) is located in the top ranked of cancer deaths worldwide. It is now well known that it is highly associated diets with higher animal protein and saturated fat. Saturated fats increase intestinal bile secretion, which then changes the bile acid pool to form a population of intestinal microorganisms. Intestinal microbiota influences host physiology and growth performance through food digestion and modulation of host immunity (Xu et al., 2016) by increasing the tumor-promoting some secondary bile acids; lithocholic acid (LCA) and deoxycholic acid (DCA). Mechanisms of secretory bile acids promoted by CRC have been investigated and studies showed that CRC is related with high DCA levels in feces, bile and serum (Ridlon et al., 2016b). In addition to this, the intestinal Bile salt hydrolase (BSH), found commonly in intestinal species of Lactobacillus and Bifidobacterium, catalyzes the hydrolysis of glycine or taurine conjugated bile acids into the amino acid residues and the secondary bile acids. BSH catalyzes the fundamental gateway reaction for the alteration of bile acids in the intestine and plays a significant role in host metabolism and energy harvesting (Begley et al., 2006; Jones et al., 2008; Joyce et al., 2014; Martoni et al., 2015). BSH enzymes from various sources of ecological diversity vary in characteristics, substrates specificity and different catalytic activity. To show the relationship between BSH enzyme and toxic secondary metabolites, structure of the BSH must be better understood, but the structure and reaction mechanism of such an important BSH enzyme was not known very well. In addition to the three dimensional structure of BSH enzyme, site-directed mutagenesis is used to understand the structure and function of the enzyme. It is obvious, the importance of the substitution of the amino acids supposed to be responsible from substrate specificity (Begley et al., 2006; Chand et al., 2018).

1.1 Microbiome

Microbiome is composed of microbiota, the symbiotic microorganisms that colonize human body, mainly bacteria, also archaea, fungi, protozoa, viruses and their complete genetic elements (genomes) and environmental impact to the host. A defined environment of microbiome can be the gastro intestinal tract (GIT) of a human or only a soil sample. In recent years, many researchers paid attention to the bacteria in the intestinal environment and focused on the gastrointestinal pathogens and the way they cause the disease. So, there is a considerable increase in the studies of the commensal microorganism implications on the mammalian gut (Sekirov et al., 2010; Marizzoni et al., 2017).

Intestinal microbiota plays a very important role for human physiology with a wide variety of microorganisms. The human column is an ecosystem have various living bacteria in approximately 10^{12} cells/g contents and belonging to more than 1,000 different species. In healthy adults, fecal microbiota identified into the three dominant phyla that Firmicutes, Bacteroidetes and Actinobacteria. These contents can be changed with diet and there is a large scale variation in the species composition between individuals.

Combined genomes (microbiome) products cover a wide range of metabolic functions and activities critical to several aspects of human health, such as providing colonization resistance toward potential pathogens, and stimulating the immune function of the host, by regulation of the host epigenetic machinery, nutrient and drug metabolism (Hamer et al., 2011, Louis et al., 2014). On the basis of all these capacities, the microbiota can be considered as an additional organ (Marizzoni et al., 2017).

1.2 Gastrointestinal Microbiota

The Gastrointestinal Microbiota (GI) is an extremely complex ecosystem, containing more than 3 million genes and involved in the production of enzymes generating metabolites that can induce wellness as well as disease.

The rapid increase in chronic inflammatory and metabolic diseases suggests that the hypothesis that the major contraction of bacterial diversity has a role in the recent evolutionary process of large-scale urban life. Antibiotic uses, diet changes and infectious diseases cause changes in the microbiota of the host. As a result, the contraction of organism diversity can also trigger possible functional deficits, leading to a loss of metagenomic components (Hand et al., 2016). The type and amount of metabolites derived from colonic microbial fermentation depends largely on the microbial composition, transit time and substrates variation. Some of these fermentation products have been shown to be protective of the colon epithelium, but some metabolites have been shown to have proinflammatory or prokarsinogenic effects (Hamer et al., 2011). ... Metabolites produced by microbiota can be helpful to health or pathogenic. Microbiota affects the immune processes throughout the body, including the central nervous system. Microbiota can cause neurodegeneration and inflammation through oxidative toxicity (free radicals). It is known that the intestinal brain axis is bi-directional and influenced by the genetic structure of the host.

Consistently increasing literature shown that there are relationship between disease and intestinal microbiota resulting in host intestinal immune imbalance, such as Irritable Bowel Disease (IBD) which consists of two major forms: Crohn's Disease (CD) and ulcerative colitis (Rapozo et al., 2017; Basson et al.,2016); atopic diseases such as atopic dermatitis, allergic rhinitis and asthma (Park et al., 2018); neurodegenerative diseases such as Parkinson's Disease (PD), Alzheimer's Disease (AD), multiple sclerosis and amyotrophic lateral sclerosis (Quigley, 2017; Westfall et al., 2017); cancer (Kelly et al., 2015; Abreu and Peek JR, 2014); diabetes and obesity (Million et al., 2012; Li et al., 2013; Han and Lin, 2014). For example, a reduction in the abundance and diversity of Firmicutes is frequently associated with IBD and Irritable Bowel Syndrome (IBS) (Basson et al., 2016). These related studies have mainspring related that alterations with the intestinal microbiota and some important disease (Hamer et al., 2011).

Factors that determine intestinal microbiology include host genetics, dietary supplementation, secondary metabolites, fatty acid composition and

amount in dietary, toxins and chemicals that can be taken with food (Yokota et al., 2012). Bile tolerance is one of the main selective factors affecting the commensal microbicide composition, which has numerous advantages in Gastro Intestinal Tract (GIT) and is determined by host bile secretion (Horackova et al., 2017).

Occupancy of the GIT by microbes generates cancer risk by inducing oxidative and nitrosative DNA damage which in return cause chronic inflammation, increased cell proliferation, change to stem cell differentiation, and production of procarcinogenic metabolites. Deterioration in the composition, distribution, or metabolism of the colon microbiota might change the homeostatic environment of the colon against inflammation, dysplasia, and cancer. Recent studies have identified microbial and environmental factors, such as nutrition and way of life (western diet), that can promote colon cancer (Abreu and Peek, 2014). There have been many studies that compare the composition to determine whether changes in the gut microbiota of the microbial community in patients with colorectal cancer (CRC) (Biagi et al., 2010; Candela et al., 2013).

Metabolomic studies provide new insight into the response of microbial metabolite profiling and controlled diet manipulation in patients with different CRC risks. For this reason, we need more knowledge to better understand and anticipate the effects of dietary individuals on the microbial metabolism. Analysis of isolate cultures and metagenomic data helps identify which bacteria are responsible for different metabolic activities, but interactions between metabolites and multiple organisms are often not investigated. In addition, such studies may explain the effects of dietary variation linked to the microbiota composition and metabolite profiles. However, the quantitative contribution of different metabolites is important not only to take into account the metabolite fluxes, but also to factor in the intestinal transit, absorption and tissue distribution in the body at the same time. For this reason, theoretical modeling with microbial ecology and physiology studies in the gut will be studied that may contribute to the understanding of complex interactions within the microbial community as well as between microbiota and host (Lois et al., 2014).

1.3 The Ecology of the Gastrointestinal Tract

Hippocrates said that in 400 B.C. “Death sits in the bowels” and now, the ecology of the GIT is currently hot research topic. The interrelation of GI and microorganisms has been such an interesting and important topic for health researchers and is theorized to play a key role in good wellness (Kelly et al., 2015). There is a highly variable, complex interaction between microorganisms and intestinal cells. A pathogenic effect can occur as well as competitive or symbiotic interactions. This interaction has a vital role in the physiological and immunological functions of the host in normal nutrition. Therefore, many probiotic and prebiotic products have been developed to maintain the GIT health stable (Tannock et al., 2005). GIT Microbiota plays many important roles in human physiology. Firstly, it is responsible for metabolism and energy harvesting from undigested foods and plays a role in the synthesis of vitamins such as B and K and in the metabolism of polyphenols. Than, it provides colonization by resistance against to potential pathogens, also plays a role in bile metabolism and stimulates the immune function of the acids of the host (Chand et al., 2017).

Bile tolerance ability is an important predictive factor for colonization in the human GIT. It is widely known that strains isolated from GIT are more resistant to the effects of bile salts than those obtained from other sources. In addition to this, to find new probiotic strains in recent years have increased the efforts to test bile tolerance in strains obtained from various fermented dairy products or non-dairy fermented products and also from different types of animal GIT derived bacteria, mainly lactic acid bacteria (Horackova et al., 2017).

1.4 Lactic Acid Bacteria (LAB)

LABs are found in almost all of human cavities, mouth, stomach and other part of the GIT and the skin. The genera of LABs are include Lactobacilli, Lactococci, Streptococci, Enterococci, Oenococci, Pediococcus, Leuconostoc, and in addition to Carnobacterium, Tetragenococcus, Vagococcus, and Weisella genera (Douillard and Voss, 2014). *Lactobacillus* the most abundant and known

genus of LAB family also called Döderlein's found in the GIT belong to genera Bacillus. *Lactobacillus* is a genus of Gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria. Lactobacillia are major part of the lactic acid bacteria group that they named Lactic acid bacteria because most of its members convert lactose and other sugars to lactic acid. In humans, they are present in the vagina and the GIT. *Lb. plantarum*, *Lb. johnsonii*, *Lb. gasseri*, *Lb. rhamnosus*, *Lb. casei*, *Lb. paracasei* ssp. *paracasei*, *Lb. crispatus*, *Streptococcus salivarius*, *Enterococcus faecalis*, and *Lb. iners* were isolated from both rectal and oral mucosa of individuals (Ahrne et al., 1998; Douillard and Voss, 2014).

LABs are generally recognized as safe food-grade microorganisms. They are classified as generally recognized as safe (GRAS) microorganisms and are technologically suitable for industrial processes, so they are used as starter cultures for many foods naturally. The most of LAB used as starter cultures in dairy fermentations are *Lactococcus lactis*, *Streptococcus thermophilus*, *Lb. delbruekii* subsp. *bulgaricus*, and also some *Leuconostoc* or other *Lactobacillus* spp. (Douillard and Voss, 2014). LAB strains are also used in the food industry for formation of flour, preservation and the production of fermented products (Bi et. al., 2016; Xu et. al., 2016).

At the present time, many probiotic bacteria both in the food (raw milk, cheese, yoghurt and butter) and pharmaceutical market belong to the genera *Lactobacillus* and *Bifidobacterium* (Öner et al., 2014). *Lactobacillus* occupy an important place different from other lactic acid bacteria because of several scientifically established and/or clinically proofs from healthy diet for humans and animals; some *Lactobacillus* species are commonly used reduction and prevention of diarrhea or constipation (De Vrese et al., 2001), healing of the equilibrium status of the microbiota in the intestine using antimicrobial, cholesterol reduction (Shehata et al., 2016), reduction of the symptoms of the lactose intolerance, hindrance of food allergy, support to the immune system, and antitumourigenic capacities with acid and bile tolerance (Klerebezem et. al., 2010; Liong et al., 2005).

1.4.1 The Genus of *Lactobacillus*

Lactobacillus, are a group of rod-shaped, Gram-positive, non-spore-forming, generally nonmotile, facultative anaerobic or microaerophilic bacteria of the Lactobacillaceae family. Lactobacilli are characterized by their ability to produce lactic acid from lactose and other monosaccharides to other genera in the family. *Lactobacillus* can survive in both aerobic and anaerobic environments. Since *Lactobacillus* are symbiotic population of animal and human gut flora, they can be found from mouth to vagina (Figure 1.1). A wide range of species of *Lactobacillus* are used commercially during the production of sour milks, cheeses, and yogurt. Moreover, and they have an important role in the manufacture of fermented vegetables (pickles and sauerkraut), drinks (wine and juices), sourdough breads, and some sausages. *Lactobacillus* is also used in commercial probiotic preparations, especially to prevent imbalance of microbiota caused by antibiotic treatment. The genus *Lactobacillus* encompasses a considerable number of different species that display a relatively large degree of diversity that are widely used in food product fermentation. Some of the well characterized *Lactobacillus* strains are; *Lb. acidophilus, Lb. brevis, Lb. casei, Lb. johnsonii, Lb. paracasei, Lb. plantarum* and *Lb. rhamnosus*. They have substantiated health-beneficial effects.

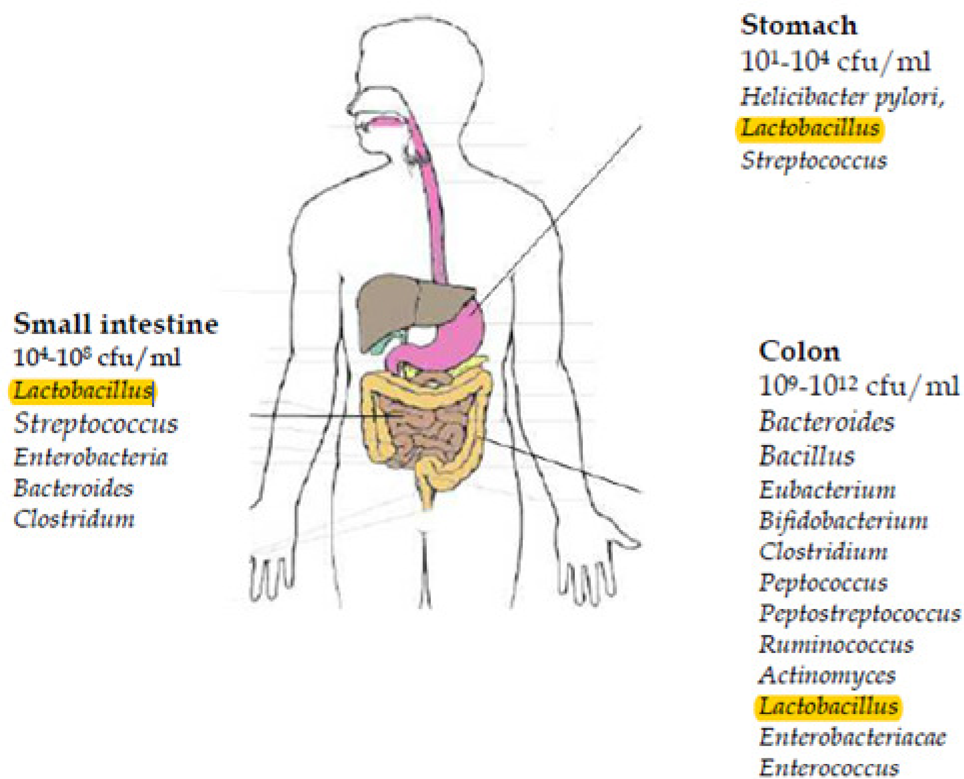

Figure 1.1. *Lactobacillus* and other species distribution in the human GIT (Radulovic et al., 2012).

Many experiments have been carried out in vitro or *in vivo* to investigate the cholesterol-lowering effect of lactic acid bacteria. Especially *Lactobacillus* strains have been reported to have a hypocholesterolemic effect in humans and animals (Shehata et al., 2016; Tsai et al., 2014). Recently, several strains of *Lb. salivarius*, a highly preferred probiotic strain, exhibited good probiotic properties such as antimicrobial activity, modulation of inflammation and even reduction of neoplastic lesions (Lv et al., 2016). In high fat diets of rats fed, antioxidant inhibition and hepatosity were determined by flow cytometric analysis of Nrf2 expression and translocation, which was markedly promoted in those treated with *Lb. plantarum FC225* (Gao et al., 2013). Walia and friends showed that the study of *Lb. plantarum* and *Lb. rhamnosus GG* supplemented daily to 1,2-dimethylhydrazine (DMH) induced colon carcinogenesis rats, these bacterias can provide protection against oxidative stress and apoptotic-related protein dysregulation during experimentally induced colon carcinogenesis (Walia et al., 2018). The genomes of *Lb. plantarum* WCFS1, *Lb. johnsonii* NCC 533, and *Lb. acidophilus* NCFM have all been completely sequenced. *Lb. plantarum* WCFS1

genome include 3.3 kbp long and 3.052 open-reading frames, and a 44.5% G+C content. *Lb. plantarum* has ecologically many different niches in the environment with this huge genome.

1.4.2 *Lactobacillus plantarum*

Lactobacillus plantarum (Lb. plantarum) is a sophisticated species can be found in a very different environment including some dairy producs as kefir, yoghurt or many vegetable or other plant fermentations (De Vries et. al., 2006). *Lb. plantarum* can ferment many different carbohydrates. Moreover, *Lb. plantarum* is frequently encountered as a natural inhabitant of the human GIT. It has a high tolerance to low pH and it is also known to having beneficial effects for health, there are some commercial forms as probiotics (Chou and Weimer, 1999). Among the lactic acid bacteria, *Lb. plantarum* can take place in a very large ecosystem with one of the largest known genomes (Kleerebezem et al., 2003). The whole genome sequence has already been completed and is now available as public domain in the NCBI database (Kleerebezem et al., 2003).

Lb. plantarum is a Gram-positive aero-tolerant bacterium that can grow at 15°C to 45°C and specificly produces lactic acid, the end product of oxygen-free glycolysis. Various strains of *Lb. plantarum* have been genetically defined. Thus, genetic tools have been developed for such species, including vectors that can be used for gene expression and gene degradation or deletion variants (Kleerebezem et al., 2003).

Lb. plantarum can produce antimicrobial agents that have significant effects on both Gram-positive and Gram-negative bacteria and thus survive in the human GIT (Moser and Savage, 2001). *Lb. plantarum* GD2 strain have higher cholesterol removing activity and bile resistance which was isolated from breast-fed human infant feces and identified by API 50CHL kit and 16S rRNA gene sequence analysis method (Yıldız et al., 2011).

1.4.3 *Lb. plantarum* B14

Previous studies showed that *Lb. plantarum* had four *bsh* genes, *bsh*1-4 and biodiversity analysis suggests that BSH1 is generally responsible for BSH activity. The conserved appearance of the *bsh*2, *bsh*3 and *bsh*4 genes among *Lb. plantarum* strains reveals an important role for these genes in the physiology and lifestyle of *Lb. plantarum* species (Gu et al., 2014; Öztürk et al., 2018). Analysis of these other *bsh* related genes in *Lb. plantarum* WCFS1 indicated that they can code extensively penicillin acylases and they will have the effects of converting other substrates than the bile acids in the natural environment (Lambert et al., 2008a). *Lb. plantarum* B14 have been chosen on the basis of its high Bile Salt Hydrolase (BSH) activity due to previous studies.

1.5 Bile

Bile is a digestive juice produced by hepatocytes and poured into the duodenum by means of bile duct. About 97% of the bile fluid is water. The rest is directly bilirubin, biliverdine, bile salts, bile acids, lipids (phospholipids, colostral, and triglycerides), electrolytes and some enzymes (Begley et al., 2006). The oils taken with food are small fat droplets when they come to the small intestine. The lipase enzyme that plays a role in fat digestion cannot affect the oils in this case. Bile salts in bile fluid adhere to oil droplets, bringing them into fine particles (emulsions), thereby ensuring that the oils are digested and absorbed. During this absorption, oil, D, E and K vitamins are also absorbed. Bile salts also show antimicrobial activity against a multiplicity of foodborne microbes (Boyer, 2013).

1.6 Bile Acids/Bile Salts

Bile acids are formed by the oxidation of cholesterol by two different enzymatic reactions, alternative or classical pathway. Specifically, it contains 24 carbons with two or three hydroxyl groups and a side chain terminating in a carboxyl group. Bile acids are synthesized in a multistage, multi-organelle way in the liver. The most common bile acids are colic acid and chenodeoxycholic acid.

They are called "primary bile acids" regardless of whether they are conjugated or nonconjugated forms (Figure 1.2).

N-acyl amidination of bile acids is catalyzed by two consecutive enzymatic steps in the liver. Firstly, a cholyl-CoA synthetase, resulting in an acyl-CoA thioester followed by N-acyl transferase (BAT), leads to N-acyl bile acid conjugation as a result of the transfer of either the glycine or taurine from the CoA-thioester to the bile acid group. BAT shows high substrate specificity for both glycine (Km 5 mM) and taurine (Km 1 mM). Therefore, the rate of bile acid taurine: glycine conjugation is diet dependent (Ridlon et al., 2016-2). The proportion of glycine in the bile to taurine forms can vary in animals and humans, but in humans is approximately 3: 1. The conjugation of bile acids with glycine or taurine results in the presence of a carboxyl group (a glycine) or a sulfonate group (taurine) which is wholly ionized at physiological pH; Thus, conjugated forms are called bile salts (Ferrier, 2013).

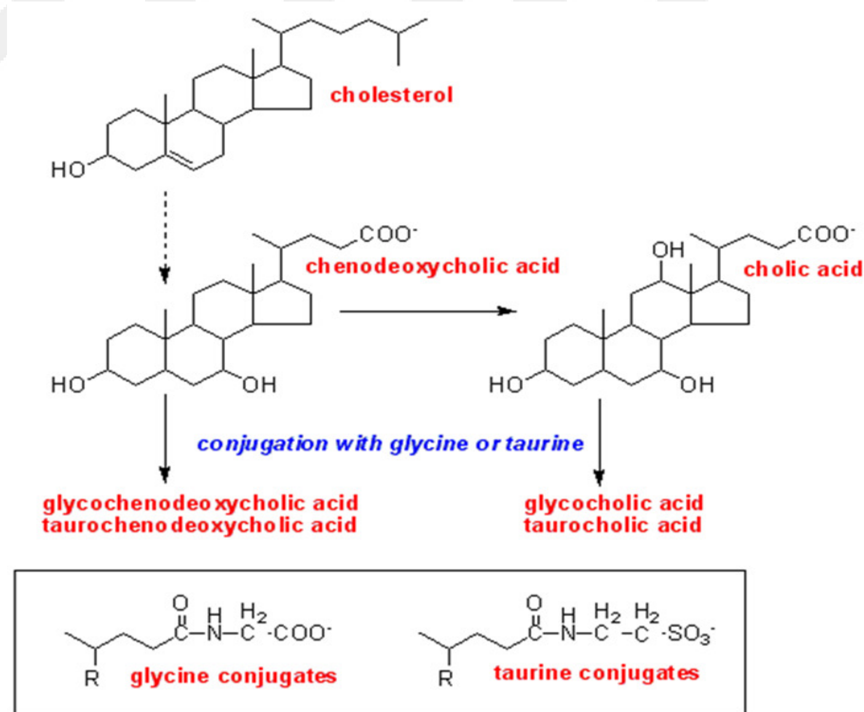

Figure 1.2. Bile acids
(http://david-bender.co.uk/metonline/lipids/bile/bile12.htm)

In humans, the salts of taurocholic acid and glycocholic acid (derivatives of cholic acid) represent approximately eighty percent of all bile salts. Bile acids are amphipathic due to the hydrophobic methyl groups and hydrophilic hydroxyl groups in the steroid ring of they contain (Figure 1.3). The main function of bile acid is to facilitate the formation of micelles, which promotes the processing of dietary fat (De Smet et al., 1994). Bile acids are influential detergents due to their improved amphipathic character.

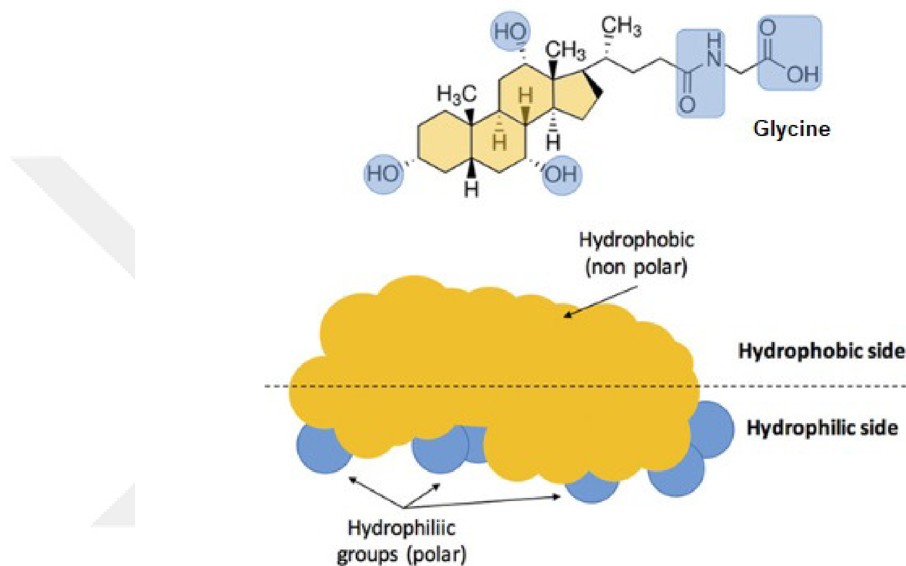

Figure 1.3. Structure of bile acid (modified by Urdaneta and Casadeus, 2017)

1.6.1 Secondary Bile Acids

The bacterial enzyme 7-α dehydroxylase in the intestine can convert primary bile acids into "secondary" bile acids by removing a hydroxyl group, producing deoxycholic acid from cholic acid and lithocholic acid from chenodeoxycholic acid (Table 1.1.) (Ferrier, 2013). It can be said that bile acid deconjugation is one of the most important transformation reactions in intestinal bacteria.

The specific enzymes that catalyze the hydrolysis of glycine- and/or taurine-conjugated bile salts into the amino acid residue and the bile acid by gut microorganism (Liong and Shah, 2005). Lactobacilli and bifidobacteria are particular species of the autochthonous gut microorganisms, they had improved the talent to hydrolysis of conjugate bile salts This catalytic activity is linked in having of an enzyme known as bile salt hydrolase (BSH; cholylglycine hydrolase).

Table 1.1. Alterations of the Bile Salts

Bile Salt Conjugates	→	Bile Acids	→	Secondary Bile Acids
Glycocholic acid	BSH	Cholic acid	7α Dehydroxylase	Deoxycholic acid
Glycodeoxycholic acid	BSH	Deoxycholic acid		
Glycochenodeoxycholic acid	BSH	Chenodeoxycholic acid	7α Dehydroxylase	Lithocholic acid
Taurocholic acid	BSH	Cholic acid	7α Dehydroxylase	Deoxycholic acid
Taurodeoxycholic acid	BSH	Deoxycholic acid		
Taurochenodeoxycholic acid	BSH	Chenodeoxycholic acid	7α Dehydroxylase	Lithocholic acid

1.6.2 Enterohepatic Circulation of Bile Acids

Enterohepatic circulation (EHC) refers to the fact that most of the bile salts secreted into the intestine are reabsorbed (aproximatelly 95%) together with the nutrients at the end of the digestion, delivered to the liver and re-released therefrom. Conjugated bile acids are poorly absorbed by passive diffusion in the small and large intestines and mainly absorbed at the terminal ileum by the active transport mechanisms which is called ileum bile acid transporter (IBAT) (Alrefai and Gill, 2007). Almost 600-800 ml of bile is produced every day. The bile is re-secreted 6 to 15 times a day and a total of 0.2 to 0.5 g of bile acid is lost with feces. This amount is equalized by the synthesis of new bile acids in the liver in each secretion. (Kim et al., 2004, 2005; Baptissart et al., 2012).

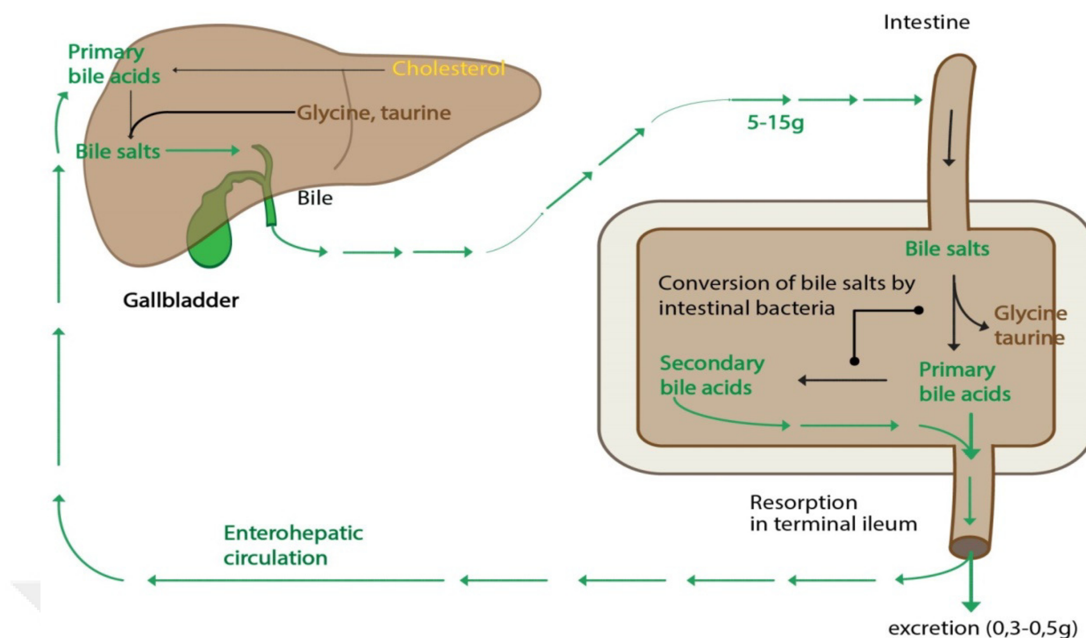

Figure 1.4. Enterohepatic circulation of bile salts (http://fblt.lf3.cuni.cz/fblt.cz/en/skripta/ix-travici-soustava/5-jatra-a-biotransformace-xenobiotik/index.html)

1.7 Bile Salt Hydrolase (BSH)

BSHs, called cholylglycine hydrolases (BSH; E.C.3.5.1.24) and are included in the classes of microbial enzymes, catalyze the hydrolysis of conjugated bile salts with they share the name conjugated bile salt hydrolases (Batta et al., 1984). Bile salt deconjugation by hydrolyzing the amide bond to produce free amino acids and unconjugated bile acids; is a significant gateway reaction to further bacterial alternations. Bile salts, such as oxidation or dehydration of bacteria in the intestine, may result in an undesirable amount of accumulation or secondary toxic metabolite formation (Sridevi et al., 2009; Begley et al., 2006; Ridlon et al., 2006). A wide array of variants of BSH enzymes have been purified from both gastrointestinal and different environment's microorganisms. In the microorganism studies performed in germ free mice, lactobacilli were responsible for the majority of bile salt hydrolase activity (Tannock et al., 1989). As a result of the evolutionary analysis of BSH gene sequences, BSH has been classified into five groups, BSHA, BSHB, BSHC,

BSHD and E, with BSHA and BSHB subtypes having high hydrolysis activity (Liang et al., 2018).

1.8 Bile Salt Hydrolase Active Genus

Most microorganisms are usually intolerant to the acidic conditions in the stomach, and later to the luminal media of bile and pancreatic secretions. These antibacterial properties authenticated higher sensitivity to Gram-positive due to Gram-negative bacteria (Hofmann and Eckmann, 2006). BSH enzymes have been identified in various bacterial species and strains from different sources. It can be mentioned that there is a relationship between the presence of BSH activity and the environment in which a genus or species lives. BSH-producing strains are commonly found in Gram-positive bacteria as primarily Firmicutes, followed by Bacterioidetes and Actinobacteria (Stellwag and Hylemon, 1976; Jones et al., 2008; Lambert et al., 2008). Functional and comparative metagenomic analysis showed that a high level of BSH active strains identificated that isolated from the intestines or feces samples from the mammalians. The flora in which these strains are found is rich in both conjugated and unconjugated bile acids. BSH active strains also isolated from bile free habitats as dairy products and some vegetables (Kim et. al., 2005; Tanaka et. al., 1999; Begley et. al., 2006).

Briefly, not all Lactobacilli strains have BSH activity, but BSH activity is more frequently found in isolates from mammalian intestines or feces, especially where bile salts are abundant (Jones et al. 2008; Tanaka et al. 1999). The fact that BSH activity differs significantly from intestinal bacteria also strongly suggests that it is a host-based selection. BSH alleles detected in the human microbiome during the studies are significantly different from those isolated from other media such as murine gut. This difference is determined by species-specific differences in host bile acid pools (Jones et al., 2008). Although the *bsh* gene sequence analysis of some of the commonly used probiotics is reported, the interactions between *bsh* gene distribution, evolutionary association and BA hydrolysis activity in Lactobacillaceae are still unclear (Liang et al., 2018).

1.9 Dispersion of BSH Enzymes in Microorganisms

BSH enzymes are found in a diffuse large range of microbial species across most phyla, previously they have also been reported to be found in commensal intestinal, gram positive bacteria such as in *Lactobacillus* (Corzo and Gilliland, 1999; Elkins et al., 2001; McAuliffe et al., 2005; Lambert et al., 2008; Ren et al., 2011; Wang et al., 2012; Chae et al., 2012; Gu et al., 2014; Jayashree et al., 2014;), *Bifidobacterium* (Grill et al., 1995, 1995b; Tanaka et al., 2000; Kim et al., 2004, 2004b, 2005), *Enterococcus* (Wijaya et al., 2004), and *Clostridium* spp (Gopal-Srivastava and Hylemon, 1988; Coleman and Hudson, 1995; Rossocha et al., 2005). BSH was identificated in small number of gram negative bacteria like *Bacteroides* spp (Stellwag and Hylemon, 1976; Kawamoto et al., 1989). Because of the specific activity of BSH, studies are more common in gastrointestinal microorganisms. For example, the pathogen microorganisms of *Listeria monocytogenes* is BSH active, with the adequacy to withstand more easily in the intestine during infection (Dussurget et al., 2002; Begley et al., 2005) quite differently, *Xanthomonas maltophilia* was found in soil (Dean et al., 2002; Pedrini et al., 2006) and thermophilic *Brevibacillus* sp isolated from hot water source (Sridevi et al., 2009). This demonstrates that the function may be a common feature in bacteria that are actually adapted to different habitats. Interestingly several strains may contain more than one BSH allele, even up to 4 different alleles were found in certain isolates (Lambert et al., 2008a). Although there is no activity of all detected BSHs, there are strongly evolutional demonstrations, especially in the horizontal transition between intestinal bacteria (Jones et al., 2008a). More than one different BSH has been reported in some bacterial strains. Four BSH were isolated in *Lb. plantarum* WCFS1 (Lambert et al., 2008a), *Lb. plantarum* ST-III (Ren et al., 2011) and *Lb. plantarum* GD2 (Öztürk et al., 2018). *Lb. johnsonii* PF01 contains three BSH's (Chae et al., 2012). Two BSH with different substrate specificity were isolated from the *Lb. acidophilius* NCFM (McAuliffe et al., 2005). Additionally, Two BSH (BSH1 and BSH2) were found in the *Lb. salivarius* UCC118 and analysis showed that BSH2 was related to penicillin V acylase (PVA) enzymes family (Fang et al., 2009).

Table 1.2. List of the BSH enzymes identified in bacteria from various sources (BSH specifity refers maximum BSH activity that identified from referance litherature) (CBAH: Conjugated Bile Acid Hydrolase, H: Hydrolase)

Source of strain	BSH enzyme	BSH specifity	Referance
Bacteroides fragilis ATCC 25285	BSH	GCA	Stellwag and Hylemon, 1976
Clostridium sordellii 4709	BSH	GCDCA	Masuda, 1981
Bacteroides vulgatus VI 31	Chenodeoxycholyltaurine H	TCDCA	Kawamoto et al., 1989
Lb. plantarum 80	CBAH	GCA	Christianes et al., 1992
Lb. acidophilus L1	BSH	GCA	Corzo and Gilliland, 1999a,b
Bifidobacterium longum SBT2928	BSH	GCDCA	Tanaka et al., 2000
Lb. buchneri JCM1069	BSH	TDCA	Mosser and Savage, 2001
Listeria monocytogenes	BSH	ND	Dussurget et al., 2002
Xanthomonas maltophilia CBS 827.97	Cholylglycine H	ND	Dean et al., 2002
Bifidobacterium bifidum ATCC 11863	BSH	GCA	Kim et al., 2004a,b
Enterococcus faecium FAIR-E 345	BSH	TDCA	Wijaya et al., 2004
Bif. adolescentis ATCC 15705	BSH	ND	Kim et al., 2005
Clostridium perfringens 13	CBAH	TCA	Rossocha et al., 2005
Lb. acidophilus NCFM	BSH	TDCA	McAuliffle et al., 2005
Lb. buchneri ATCC 4005	BSH	ND	Sridevi et al., 2009a
Brevibacillus sp.	BSH	GDCA	Sridevi et al., 2009b
Lb. acidophilus LA11	BSH-A/ BSH-B	GCA	Jiang et al., 2010
Lb. salivarius NRRL B-30514	BSH	GCDCA	Wang et al., 2012
Lb. jonsonii PF01	BSH-A/ BSH-B	TCA /GDCA	Chae et al., 2012
Lb. fermantum NCDO394	BSH	GCA	Kumar et al., 2012
Bif. animalis DN-173 010	BSH	GCDCA	Lepercq et al.,2014
Staphylococcus epidermidis RM1	BSH	GDCA	Mukherji and Prabhune, 2015
Lb. reuteri NCIMB 30242	BSH	ND	Martoni et al., 2015
Lb. casei J57	BSH	TCA	Gonzalez and Vazquez, 2015
Pediococcus pentosaceus KID7	BSH	ND	Damodharan et al., 2015
Lb. rhamnosus E9	BSH	GCA	Kaya et al., 2017
Lb. gasseri ATCC 33323	BSH	GDCA	Ba and Ozturk, 2018
Enterococcus faecalis	BSH	GCA	Chand et al., 2018
Lb. plantarum WCSF1	BSH1	GCA	Lambert et al., 2018
Lb. Plantarum GD2	BSH1	GCA	Öztürk et al., 2018
Lb. Plantarum B14	BSH1	GCDA	In this study

1.10 Functional Advantages of BSH to the Bacteria

It is clear that, for normal biological activity of the intestine it is highly necessary for conjugated bile salts (CBAs) to emulsify and dissolve fat. In addition to this, CBAs also display antimicrobial activity due to acidic feature and they can breakdown of bacterial membranes by changing cellular homeostasis (Begley et al. 2005; Jones et al., 2008; Yang et al., 2017). In line with this, it has been suggested that deconjugation of bile salts may be a possible detoxification mechanism and increase bile tolerance in the gastrointestinal tract and thus increase the viability of microorganisms in the intestine (Dussurget et al., 2002). On the other hand, the real effects of BSH activity to microorganisms and bile salt tolerance are not clear. For this reason, the investigation and comparison of biochemical and physiological character of varied BSHs is important to reveal their functions. BSHs catalyze the transition reaction to more extensive steps of bile acid modification by intestinal microbiota.

1.10.1 Bile Detoxification or Bile Tolerance

Although the physiological effect of BSH is uncertain, studies have shown that LABs containing BSH are more resistant to conjugated bile salts. (Bi et al., 2016). Biofilm formation is a significiant sign of bile resistance mechanism indicated that in many studies, including LABs (Ambalam et al., 2012), *Bacteroides fragilis* (Pumbwe et al., 2007) and *Vibrio cholera* (Hung et al., 2006). Knowing that BSH triggers the formation of a bile acid biofilm on the surface of *Lb. lactis*, which in turn results in increased bile resistance (Bi et al., 2016). *Leuconostoc citreum* is a common lactic acid bacterium in several fermented foods, but it is not accepted a probiotic because of a lack of bile salt resistance. Constructing of a bile salt resistant *Leuconostoc citreum* by cloning a *bsh* gene from *Lb. plantarum* indicated that the percentage of survival of the transformant were increased from 0.8% to 67.6% in 10 mM bile salt (Cho et al., 2015).

1.10.2 Nutritional

Whereby the cholate part is not further metabolised, it is thought that microrganisms do not use steroids. In addition, the physiology of glycine or taurine has not been found clearly.On the other hand, some studies showed that lactobacilli do not exploit the steroid moiety of bile salts so this reaction is not in force for BSHs (Tannock et al., 1989). BSH is estimated to occur in relation to an enzyme pathway that has another role in bacteria.

The deconjugation is a hydrolytic reaction that provides nutrition; carbon nitrogen and energy source to microorganisms. According to studies by Huijghebaert et al. (1982) and Van Eldere et al. (1988) the reactions of products includes, glycine and taurine that can be metabolized to ammonia and carbon dioxide; and taurine also metabolized to sulphate. In addition to this, given the growth rates of *Clostridium* colonies isolated from rat, taurine does not induce growth of sulfur compounds and sulfhydryl-containing amino acids after removal of bile salts containing sulfur (Huijghebaert et al., 1982). Clostridia used liberation taurine as an electron acceptor.

The other known advantage of bile salt hydrolase is the feeding of bacteria and the preservation of membrane integrity (Begley et al., 2006). Entirety of membrane can be supported as low pH and this cause coprecipitation of cholesterol with deconjugated bile salts that can trigger host's cholesterol to penetrate into the bacteria membrane and cholesterol reduces the immunological acceptance of the host's bacterial cell (Taranto et al., 2003). Today, it is clear that BSH plays a major role in cholesterol catabolism in the transport, digestion and absorption of nutrients, fats and vitamins in mammals (Martinot et al., 2017).

1.11 Probiotic Preference of BSH Positive Strains

BSHs catalyze the "gateway" reaction in a further pathway of bile acid alternation by the gut microbiota. Microorganisms have to resist not only bile salts but also other antimicrobial substances in the bile to survive and colonize the

gastrointestinal tract. Therefore, biliary tolerance is one of the most important features and selection criteria for probiotic bacteria because it determines their ability to survive and grow in the small intestine and ultimately to function as probiotics (Ruiz et al. 2013).

Co-aggregation ability studies with *E. coli* are also an important selection criterion for probiotics. The catalytic reaction of BSH with Taurocholic acid or Glycocholic acid substantiate further that it can act as an excellent source for multiple probiotic curations (Yadav et al., 2017). In vitro studies with *Lb. plantarum* p98lb1 strain showed that exhibiting the more probiotic features compared to the other strains with remarkable tolerance to bile and cholesterol reduction due to BSH activity (Seddik et al., 2017). Lundeen and Savage (1992) suggested that BA deconjugation might generate a detoxification influence in *Lactobacillus* strains by dissociating conjugated BA that could enter the cell by passive diffusion or by active transport and they may be converted to their weaker deconjugated counterparts (pKa 5). These latter compounds can then recapture the cotransporting proton so that excessive ATP is avoided and the pH is preserved. The internal pH of *Lactobacillus* strains were quantified using the fluorescent probe according to Kurdi et al. (2006). On its way out, it has been suggested that the relationship between BSH activity and LAB tolerance is partially related to intracellular pH variations (Bustos et al., 2012).

It has been shown that bile is damaging to bacterial membrane integrity and BSH reduces toxicity to bacteria by converting conjugated bile acids to deconjugate form. Compared to the conjugate counterparts, the de-conjugated bile acids, produced as a result of BSH activity, are reduced in solubility and detergent activity and thus less toxic to intestinal bacteria. In addition, the deconjugated bile acids may indirectly cause changes in the membrane, such as an increase in unsaturated fatty acids, to increase their flexibility and strength, depending on the change in membrane fluidity. It has been suggested that the participation of cholesterol in bacterial membranes is through the formation of a binding network between bile salts and membrane fatty acids and molecules. It is known that intestinal epithelium secretes mucin as a defense mechanism and to facilitate nutrition. Klinkspoor et al. (1999) in their studies on adenocarcinoma cells, they

found that unconjugated (hydrophobic), bile salts increased the release of mucin compared to those conjugated (hydrophilic) with taurine via by proteinkinase C mechanism. For this reason, it is thought that the bacterial BSH activity may have been gained for self-defense and colonization in the gastrointestinal system during the evolutionary process (Chand et al., 2017).

1.12 Constructive Effects of BSH in the Intestine

Bile acids may have both positive and negative direct effects on intestinal bacteria (Begley et al. 2005). BSH activity has the potential to greatly influence host physiology with regulate energy expenditure, glucose and lipid metabolism, thyroid hormone signaling, and cellular immunity. Modification of primary bile acids to secondary bile acids is associated with increased hydrophobicity and binding to membrane lipids (Ridlon et al., 2016).

1.12.1 Antiparasitic or Antimicrobial Effects

The detergent characterization of bile acids, concentration and bile acid hydrophobicity play an important role in antimicrobial effects (Ridlon et al., 2016). Additionally, it has been shown that even very low concentrations of bile acids can alter the lipid composition of the membrane, dissolve the membranes and cause degradation of membrane proteins resulting in leakage of the cell membrane content (Taranto et al., 2006).

Some specific *Lactobacillus* species synthesize antimicrobial compounds related to the bacteriocin classes which include strength of mucosal barrier, competition for adhesion, and immunomodulatory mechanisms. According to a recent study by Boyanova et al., (2017) the bacteriocin-like inhibitory substances with a strong anti-*Helicobacter pylori* activity of seven *Lactobacillus delbrueckii* subsp. *bulgaricus* strains, can be use in the control of *H. pylori* infection which resistance to antibiotics as valuable probiotics. A healthy microbiota metabolizing bile acids may also have protective effects against the development of *C. difficile*

infection. In healthy intestine, secondary bile acids be dominate in feces, they can repress the sprouting of *C. difficile* spores. The hypothesis that antibiotics reduce the metabolic capacity of microbes by decreasing a subset of bacteria with the enzymatic character of producing secondary bile acids has been put forward. A reduction in bile salt metabolizing enzymes due to an increase in primary bile salts that allow germination of *C. difficile* spores resulting in Clostridium Difficile Infection (CDI) (Allegrettii et al., 2016).

BSH activities of some probiotic strains like *Lactobacillus* species have shown anti-giardial effect both in invitro and invivo studies (Travers et al., 2016; Allain et al., 2018). The mechanisms are not exactly known but they may include the host immunomodulation to extracellular compounds released by the bacteria (Perez and Briz, 2009).

1.12.2 Cholesterol Lowering Effect of BSH

Coronary artery disease is among the first causes of death as the most important reason for the increase in cholesterol in the blood. Cholesterol-lowering drugs are expensive and side effect as a result of using BSH active probiotic has become the focus of increasing attention (Al-Saleh et al., 2006; Kumar et al., 2010).

The deconjugated form of bile salts is more hydrophobic than the conjugate ones, so they are less absorbed than the intestines. As a result, bile salts taken up by feces are recovered by de nova synthesizing from cholesterol. This leads to a decrease in serum cholesterol levels (Choi et al., 2015). The other mechanism is that de-conjugated bile acids have further binding affinity to the farsenoid X receptor (FXR), to be a suppressed transcription of the enzyme cholesterol 7-alpha hydroxylase (7AH), that is responsible in bile acid synthesis from cholesterol (Choi et al., 2015).

The importance of BSH for probiotic microorganisms is that it provides an advantage for reducing serum cholesterol and preventing hypercholesterolemia

(Jones et al., 2013). The cholesterol lowering efficacy of a microencapsulated bile salt hydrolase-active *Lb. reuteri* NCIMB 30242 in yoghurt formulation was reported in hypercholesterolemic adults (Jones et al., 2012).

The recent studies indicate that manipulation of culture conditions resulted in 2.9-fold increase in the production of BSH from *L. buchneri* and the isolated enzyme has the potential to decrease serum cholesterol amount and could relieve the risk for coronary heart disease. In addition, there is a need for further study to better understand the mechanism of action and the greater effect of bile salt hydrolase intake (Sridevi et al., 2009). In vitro studies have shown that *Lb. plantarum* ECGC has high BSH activity, ability to lower cholesterol, high resistance to stomach, pancreatic and bile acids, and high resilience in freeze drying. Systematic approaches such as probiotic selection of BSH active microbial strains for treatment with biomarker diseases known to be mechanisms such as cholesterol are becoming increasingly common (Costabile et al., 2017).

1.13 Potential Side Effects due to High-Level BSH Activity in the Intestine

Bile acids are signal molecules in addition to their physiological roles in helping the digestion of fats. Bile acids have well-defined molecular properties, such as specific signaling pathways and activation of receptors, which play a role in the regulation of many biological functions such as lipid, glucose and energy metabolism. (Martinot et al., 2017). Many studies have showed that various pathological diseases caused by BA such as cholestasis, bile duct obstruction, liver fibrosis and cirrhosis, liver and colon cancer, and irritable bowel syndrome due to changes in bile acid profile and accumulation of toxic bile acids. (Hofmann, 1999). However, it is known that bile acids generally have a negative effect on cell integrity, possibly leading to cell death due to an increase in membrane permeability (De Boever et al., 2000). In the formation of cell damage, the hydrophobicity and structure of bile acids is also important. Hydrophobic bile acids and those with more than three hydroxy groups have been shown to be more detrimental to membrane integrity than other bile acids (Hofmann, 2001).

1.13.1 Secondary Bile acids and BSH

The intestinal bacteria can perform numerous biotransformation of bile salts during enterohepatic circulation. The important biotransformations include: hydrolysis of conjugated bile acids to free bile acids and glycine or taurine by BSH; 7α-dehydroxylation of CA and CDCA yielding deoxycholic acid (DCA) and lithocholic acid (LCA) respectively and bile acid 7β-dehydroxylation of Ursodeoxycholic (UDCA) to LCA. The bile acid 7α/7β-dehydroxylations performed by anaerobic gut bacteria include a multi biosynthesis steps. In addition, the intestinal bacteria are both able to the oxidation and epimerization of hydroxy groups at the C3, C7 and C12 position of bile acids to isobile (β-hydroxy) acids (Ridlon et al., 2010; 2016).

Although probiotics containing BSH are known to have beneficial effects, the carcinogenic effects of secondary metabolites have also been shown (Kim et al., 2005). Increased bile salt hydrolase activity leads to the formation of toxic or mutagenic secondary bile salts by increasing the next step 7α-dehydroxylase activity (Marteau et al., 1995).

The bacterial enzyme 7α-dehydroxylase in the intestine can convert “primary bile acids” into “secondary bile acids” by hydroxylation, deoxycholic and lithocholic acid are formed from colic acid and chenodeoxycholic acid, respectively (Ferrier et al., 2013). Bile salt deconjugation is one of the most important biotransformation reactions of intestinal microorganisms (Liong and Shah 2005). Some specific gut bacteria, such as lactobacilli and bi-dobacteria, have the ability to deconjugate bile salts. This activity depends on the existence of an Bile Salt Hydrolase (BSH) enzyme activity. In addition to this, bile acids, glycine or taurine conjugated bile salts, 7-α-dehydroxylated derivatives of bile acids as deoxycholic acids and lithocholic acids can be found togetger in human intestinal bile (Gilliand et al., 1977).

1.13.2 Cholestasis

Bacterial transmission may incite lithogenesis to inducing cholestasis. The contamination of intestinal microorganisms in lithogenic bile possibly was shown of elevated in gut permeability when biliary ducs obstruction occur and also, stimulate to increased inflammatory defense reaction and gall stone genesis (Ziar et al., 2013; Choi et al., 2015; Lion et al., 2015).

1.13.3 Gallstone Formation

In patients with cholesterol gallstones (GS), excessive deoxycholic acid (DCA) is common in bile acid pools with bile cholesterol supersaturation. The increased formation of CA appears to significantly increase the amount of bile-secreted cholesterol with a corresponding increase in cholesterol saturation index (Berr et al., 1996).

1.13.4 Irritable Bowel Syndrome/ Disease (IBS/IBD)

IBD is categorized as Crohn's disease (CD) and ulcerative colitis (UC) based on pathophysiological characteristics. While UC is an inflammatory disease limited to the colonic mucosa, CD has a potential for development throughout the whole gastrointestinal tract with a higher formation in the small and large intestines (Yamamato and Matsumoto, 2016).

Research on the relationship between bile acids and IBD has focused mostly on how the disease affects both intestinal and serum bile acids. Terminal ileum resection is a common complication of IBD. In the GI tract, terminal ileum is responsible for the reabsorption of bile acids, for this reason, the removal of this site of the small intestine can lead to bile acid malabsorption which typically characterized by bile acid-mediated diarrhea or, in large resections of the terminal ileum, a lack of fat digestion (Cima and Pemberton 2001; Gothe et al. 2014). In some studies, changes in the composition of bile acids have been noted in the bile,

and in feces of patients, there has been shown an increase in conjugated bile acids and a decrease in secondary bile acids (Duboc et al., 2013; Staley et al., 2017).

1.13.5 Liver Diseases

Primary sclerosing cholangitis (PSC) occur with or without IBD and is a cholestatic disease causing the bile canal devastation cause to liver failure as cirrhosis (Hirschfield et al., 2013). Inflammation and obstruction of biliary ducts result in alteration of bile flow and a decrease in resistance to bile acid toxicity (Hohenester et al., 2012; Hirschfield et al., 2013).

Changes in the bile acid assortment or some of the specific bile acids may give as biomarkers related with the potential pathogenesis of liver diseases (Yang et al., 2017). Bile acids directly damaged the liver by detergent cytolytic effects, since very low levels of toxic bile acids killed hepatocytes directly when added to these cells in vitro. In addition, serum and tissue levels of toxic bile acids uncommonly reach these submillimolar levels in pathophysiological conditions, suggesting that their cytolytic properties may not be responsible for liver cell death. Subsequently, it was proposed that bile acids induced apoptosis in hepatocytes (Li et al., 2017).

1.13.6 Weight Gain (obesity) & Lipid Metabolism

BSH activity effect the structure of both gastrointestinal mucosa and hepatic functions. Study with germ free and conventionally-raised animal models it showed that BSH activity in intestine results in corollary retrofit in lipid and cholesterol metabolism and have an effect on signaling functions and weight gain (Joyce et al., 2014). Another study indicated that a single cloned BSH in considerably reduced weight gain in animal models. Given the potential role of microbial BSH activity in weight gain, it shows that BSH could be used as an adjunct to treatment of obesity and metabolic diseases (Joyce et al., 2014). In addition to this, recent studies have demonstrated that high level BSH activity

including microorganism can blunt the FXR response in mice (Degirolamo et al., 2014; Kim et al., 2016). On the other hand, the association of TGR5, another bile acid receptor, can reduce body weight and show a fine interaction between FXR and TGR5, which deserves a greater work (Watanabe et al., 2006).

1.13.7 Diabetes

Type 2 diabetes is a risk factor for the formation of gall bladder disorders and is often associated with hypertriglyceridemia (De Santis et al., 1997; Ruhl and Everhart, 2000). Studies indicated that there is connection between hypertriglyceridemia and the formation of vesicular stones (Boland et al., 2002). All the same, the composition of the bile acid pool was differentiated in some Type 2 Diabetes Mellitus patients (Nguyen and Bouscarel, 2008). FXRa is known to control homeostasis of bile acids and triglycerides therefore studies have been assume to determine the presence of a potential link between Farnesoid-X-Receptor alpha (FXRa) and carbohydrate metabolism. A study with a diabetic rat model, show that the expression of FXRa is modulated by the glycemic variations and liver expression is indeed decreased (Duran-Sandoval et al., 2004; Nguyen and Bouscarel, 2008).

1.13.8 Interrelation of FXR and TGR5

Bile salts are also the natural ligands such as the farnesoid-X nuclear receptor (FXR). Therefore, they are considered important regulators of gene expression in the liver and intestines (Hylemon et al., 2009; Torchia et al., 2001). In addition, activation of bile acid synthase is regulated by the negative feedback control mechanism by the FXR nuclear receptor located in the ileum and liver (Sayın et al., 2013).

The activation of signaling functions and mucosal defenses to Bile salts are mediated primarily through two receptors. Activation of FXRa or NR1H4 plays an important action in mucosal defense mechanism to against bacterial

invasion in the small intestine and also affect on cholesterol and triglyceride homeostasis. On the other hand, G-Protein-Coupled Bile Acid receptor-1 (GPBAR-1) or TGR5 activation is responsible for energy and possibly glucose homeostasis (Jones et al., 2008). FXR, which may be stimulated by unconjugated bile acids, not only regulates lipogenesis and triglyceride synthesis, but also regulates glucose homeostasis by increasing glycogen synthesis or decreasing glycolysis (Geng and Lin, 2017). There is a relationship between T-beta-MCA (FXR inhibitors) and CDCA and the secondary bile acids DCA and LCA (FXR promoters). Studies have shown that intestinal microbiota not only regulates secondary bile, they also inhibit bile acid synthesis in the liver by reducing FXR inhibition in the ileum (Sayın et al., 2013). Furthermore, the FXR found in the liver regulates the expression of the cholesterol enzyme 7 α-hydroxylase (CYP7A1), a limiting factor in bile acid synthesis from cholesterol (Chiang, 2009).

1.13.9 Colon Cancer and BSH

Colorectal Cancer (CRC) is the third most common malignant tumor worldwide and in our country, with over 1.2 million new cases of CRC and 609.000 estimated deaths in 2008, as reported by the International Agency for Research on Cancer and the World Health Organization (WHO). On average, a million people get colon cancer every year, and unfortunately five hundred thousand of them die. In other respects, only about 15% of CRC incidence can be explained by heredity alone (Ridlon et al., 2016) and diet plays a significant role the risk of CRC. Almost, up to 75% of cases being thought to be associated with diet. An important consideration is focused on nutritional supplements that may affect intestinal microflora as a strategy for the prevention of CRC (Rafter et al., 2003). A study by Duan et al. (2014) showed that, serum bile acid levels of patients with colon cancer were determined and compared with healthy volunteers, the free bile acids were increase, and the conjugated bile acids were decrease in patients with colon cancers. These data provide a new approach to the potential biomarkers for colon cancers (Duan et al., 2014).

The researches has detected that CRC progression was influenced not only by the existance of specific pathogens but also affected by the gut microbiota diversity and that the microbiota also has a role in the etiology of many types of cancer by induce inflammation, DNA damage and apoptosis (Lois et al., 2014). Nutritions and seconder metabolites plays an initiation or progression role of colorectal cancer.

Accumulation at high levels of secondary bile acids (BAs) as deoxycholic acid (DCA) and lithocholic acid (LCA) in the bile acid complex, have long been known from pointed patients diagnosed with colonic carcinomas on elevated levels of fat because of Western diets (Degirolamo et al., 2011; Ridlon et al., 2014). It has been known that these bile acids are responsible for carcinogenic or cocarcinogenic compounds produces by microorganisms (Hill et al., 1975; Nagengast et al., 1995; Bernstein et al., 2005). Although the mechanism of carcinogenicity was not be clearly clarified, there were evidences of DNA damage and apoptosis induction (Bernstein et al., 2005). In the vast majority of studies, investigating the role of bile acids in colon cancer and it has been found that cancer patients have much more fecal bile acids than healthy controls or other diseases (Hill et al., 1975; Nagengast et al., 1995). In addition, there was a significant increase in concentrations of 7α-dehydroxylase from Clostridia strain in the feces of patients with colon cancer. These results suggest that there is a link between these bacteria and disease status (Hill et al., 1975).

Bile acids are a group of acidic steroid molecules that have their own physical-chemical and biological properties. At high concentrations, they become toxic to mammalian cells and their presence is associated with various liver diseases and colon cancer pathogenesis. Bile acids cytoxic affect to membrane damage, but also, can cause oxidative stress and apoptosis with have nondetergent effects (Amaral et al., 2009). Population-based studies indicated that individuals who consume high-fat and high-bovine energy in patients with colonic carcinoma had at the same time high levels of fecal secondary BA, mostly DOC and LCA (Ajouz et al., 2014). LCA facilitates the development of cancer by practically eliminating the ability of the colonies to pass apoptosis (Korzoni et al., 2000). Secondary bile acids have also been suggested as potential causative agents of

cancers in different body parts (Bernstein et al., 2005). Increased secondary BA concentrations have deleterious effects on DNA as oxidative damage, on the colonic epithelial structure. They act through multiple mechanisms including inflammation, nuclear factor-kappa B (NFKB) activation and increased cell proliferation (Degirolamo et al., 2011).

Ajouz et al. (2014) described briefly high bile acid exposure can cause cancer by;

- Production of reactive oxygen species (ROS)
- Production of reactive nitrogen species (RNS)
- Distribution of the cell membrane and mitochondria
- DNA damage induction
- Apoptosis and mutation
- Apoptosis decreasing
- Chronic exposure ability

1.14 Structure and Biochemical Characteristics of BSH

BSHs commonly found to be located intracellularly and oxygen sensitive or insensitive, identified as a homotetrameric feature and that tetramer assembly is essential for the activity of the enzyme. Online software ExPASy (http: //web.180expasy.org/compute_pi/) analysis indicated that the deduced protein of *Lb. plantarum* B14 BSH, enzymes had theoretical molecular weight of 37.077 kDa and pI values of 5.21 (Öztürk et al., 2018). Each similar monomers of ~316–328 amino acids and the optimal pH for BSH activity is relatively acidic, ranging from 3.5 to 6 (Öztürk et al., 2018). The BSH activity was maintained at up to 60°C. SDS-PAGE analysis of putative BSH and gel filtration revealed that the analyzed protein is probably a tetramer formed of four monomers native molecular weight of this enzyme is about range from 28 to 50 kDa (~37 kDa) (Lundeen and Savage, 1992; Kumar et al., 2004).

BSH recognize different bile salts in the steroid parts and also in amino groups. BSHs mostly identified as having narrow substrate spectrum and exhibit

higher hydrolyzing activity to glycine-conjugated bile salts than taurine-conjugated bile salts (Coleman and Hudson, 1995; Smet et al., 1995; Tanaka et al., 2000; Kim et al., 2004; Liong and Shah, 2005). On the other hand, some of BSH enzymes exhibit a previously taurine-conjugated preference bile salts, such as both of BSH enzymes in *Lb. jonsonii* PF01 (Chae et al., 2013) and the BSH enzymes from *Lactobacilli* strains (Jiang et al., 2010). In a study by Moser and Savage, showed that *Lb. buchneri* JCM1069 strain have much more BSH activity to taurodeoxycholic acid than taurocholic acid (Moser et al., 2001). Taurodeoxycholic acid and taurocholic acid both contain taurine as a conjugated part acid but differ in steroids backbone. In addition, the inactivation of the *bsh*A of *Lb. acidophilus* NCFM strain reduced to the hydrolyze capacity both taurine and glycine conjugated bile salts containing chenodeoxycholic as steroid moiety. However, most of the studies available in the literature have been found to be characterized by more amino acid moieties than substrate preference, and most BSH show more activity in glucoconjugate bile salts than tauroconjugated bile salts.

Lactobacilli contain more than one BSH gene locus or BSH homolog, which results in the presence of different BSH activities (Begley et al., 2006). Sequence analysis shows that the BSHs are mostly protected from the amino acids in the active site, whereas the majority of the amino acids in the other region, which are considered to be responsible for substrate recognition, are not particularly protected. Leu142 (L138 in *Lb. plantarum* B14) in the substrate recognition region is fully conserved, but its importance is not clear. Further studies and structural analyzes with BSHs from various species will undoubtedly discover the essential amino acid compositions of the active site and substrate binding pocket and also provide clearer information about the substrate preference of BSHs enzymes (Begley et al., 2006).

BSHs (EC 3.5.1.24) are included in the Cholyglycine hydrolase (CGH) enzyme family, similar to penicillin V acylases (PVA) (EC 3.5.1.11), which convert 6-aminopenicillin acid (6-APA) with penicillin hydrolase activity. Accordingly, PVA commonly used in the semisynthetic antibiotics industry. Both BSHs and PVAs have been defined as N-terminal nucleophilic (Ntn) hydrolases

with an N-terminal cysteine aminoacids. One of the common characteristics of the Ntn-hydrolase family is that in the auto proteolytic process, Cys-1 becomes a center of catalytic activity after removal of the initial methionine amino acid (Kumar et al., 2006). This classification was confirmed by Cys1Ala exchange by site-directed mutagenesis, resulted in an inactive protein in *Bifidobacterium bifidum* (Kim et al., 2004) and *Bifidobacterium longum* (Tanaka et al., 2000; Kumar et al., 2006) species (Figure 1.5). BSH Cys-1 with the nucleophilic amino acids serine or threonine which has a hydroxyl group instead of a thiol group abolishes BSH activity and exchange of Cys-1 with alanine in the *B. longum* BSH results in an inactive protein.

However, in a study with *L. lactis*, having BSH expression increased strain bile salt tolerance, but excessive increase in bile acid biosynthesis in the cytoplasm inhibited growth. Therefore, when BSH is used as a potential food-grade component for bile salt tolerance of probiotic bacterial strains, the efficiency-related activity of bile acid should be considered (Bi et al., 2016).

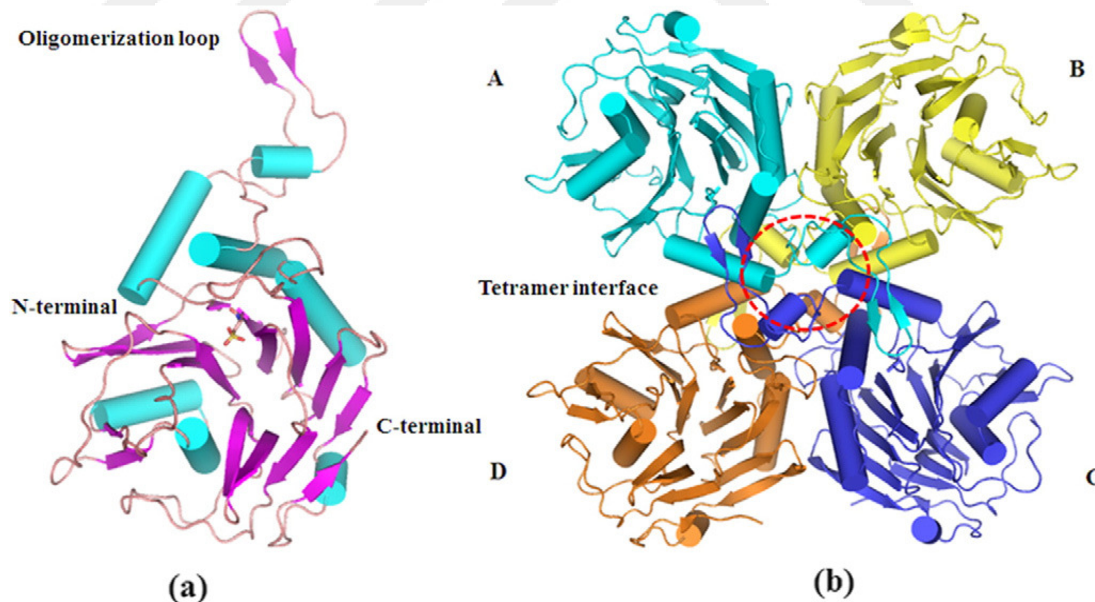

Figure 1.5. (a) Structure of the monomer subunit of the *Bifidobacterium longum* BSH, (b) Tetramer assembly of BSH enzyme from bacteria (Chand et al., 2017).

Bile salt hydrolase is a constitutive, usually intracellular, enzyme that catalyzes the hydrolysis of amide bonds (glycine or taurine) to liberate it from the

bile acid steroid nucleus. The cysteine residue which has been shown to be essential for BSH as a catalytically active site at the N-terminus, the thiol (SH) group (Jones et al., 2008; Begley et al., 2006; Rossocha et al., 2005). Penicillin V amidase isolated from *Bacillus sphaericus* shows a high similarity to the amino acid sequence of the BSH enzyme (Ridlon et al., 2006). Optimum conditions for this enzyme may show some differences in relation to the source as growing temperature, 37°C to 45°C in lactobacilli (Sridevi et al., 2009a) or 50°C in *Enterococcus faecalis* (Chand et al., 2018). Some food additives used in industrially produced products, e.g. riboflavin and salts (KIO3, NaIO3, CuSO4, CuCl2) or certain antibiotics, which has been identified as significantly (up to 100%) inhibitors of BSH activity in *Lb. acidophilus* PF01 (Lin et al., 2014).

1.15 BSH Activity

1.15.1 Catalytic Activity

Evolutionary phylogenetic analyzes show that species with the N-terminal nucleophile (Ntn)-CGH like protein family in the intestines develop BSH activity against conjugated bile acids (Jones et al., 2008). In the catalytic activity of the Ntn-hydrolases, a nucleophilic attack occurs on the amide bond with the N-terminal catalytic domain, followed by a tetrahedral intermediate that is stabilized with an oxyanion hole. In addition to this, comparative genomics and structural studies have shown that some special residues be responsible for the catalytic activity which are completely conserved throughout Ntn-hydrolases. The crystal structures of *Clostrodium perfrigens* BSH (Rossocha et al., 2005), *Bifidobacterium longum* BSH (Kumar et al., 2006), *Lb. salivarius* BSHA and BSHB (Xu et al., 2016), *Enterococcus faecalis* BSH (Chand et al., 2018) and penicillin V acylase of *Lysinibacillus sphaericus* (bsPVA) (Suresh et al., 1999) found out a relation structural and catalytic similarity. Ntn-hydrolase like folding among Ntn-hydrolases with compared to active site structure. Cysteine nucleophile (Cys2) is conserved in catalytic active center containing and its orderly circumjacent amino acids site residues such as Arg16, Asp18, Asn79,

Asn170 and Arg223 (amino acid numbering given according to *Lb. plantarum* BSH) are all strickly conserved in most of BSHs. It has been suggested that the amino acid region corresponding to Gln82 or Asn82 as a result of sequence analysis is the critical region that is responsible for the enzyme activity and BSH enzymes with this amino acid region are more advantageous than other BSH enzymes with low BA hydrolysis activity (Liang et al., 2018). Cys2 at the N-terminal forms catalytic Ntn-diad together with Asp21 (Homology to Asp18 of *Lb. plantarum* B14). The alpha-amino group of Cys2 is incorporated into the nucleophilic cysteinyl sulfur bridge by hydrogen bonding with the water molecule. The sulfhydryl group that stabilized with Arg18 make a nucleophilic attack on the amide bond of the specific conjugated bile salts (Chand et al., 2018). The differences at the amino acid present in the loops produce altered binding pocket conformation, allowing the variation of enzyme-substrate relationships (Xu et al., 2016).

1.15.2 Substrate Specifitiy

BSH can catalyze glycine or taurine conjugated bile acids. There are so many studies focused on substrate specificity which related to amino acids moieties (Tanaka et al., 2000; Kim et al., 2004). There are eight conjugated bile acids; Glycocholic acid (GCA), taurocholic acid (TCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycolithocholic acid (GCLA), taurolithochoic acid (TLCA), glycochenodeoxycholic acid (GCDA), and the taurochenodeoxycholic acid (TCDA), commonly acting the main emulsification function in the human small intestine. The percentage of bile content varies widely among species, although this difference is not precisely known, but varies with diet. In addition to this, BSH enzymes from diverse sources have differences in substrate specificity (Table 1.2), optimal study temperature and pH for enzymatic activity (Begley et al., 2006).

1.15.2.1 Taurine/ Glycine Affinitiy

Many studies indicated that BSH have further substrate specificity for glyco-conjugated bile acids than tauro-conjugated bile acids (Colleman and Hudson, 1995; Tanaka et al., 2000; Nguyen et al., 2007; Ramasamay et al., 2010). In addition to this there are differences according to the cholate property as Taurodeoxycholic acid activity but not taurocholic acid hydrolase activitiy. Moser and Savage (2001) found out that *Lb. buchneri* JCM1069 exhibit activity to taurodeoxycholic acid and did not exhibit activity to taurocholic acid. These two acids have same amino acid but their steroid moieties have difference at the 7α position (Moser and Savage, 2001). BSH-A inactivation of *Lb. acidophilus* NCFM decrease the strain's ability to hydrolyze bile salts including chenodeoxycholic as the steroid moiety, e.g., TCDCA and GCDCA (Mc Auliffe et al., 2005). *Lb. plantarum* GK81, shown that resistant to bile as well as gastric acid and exhibited high BSH activity to both sodium glycocholate and sodium taurocholate (Sung-Me M and Ahn DH, 2012). BSH preferred taurine-conjugated salts than gluco-conjugated bile salts as substrates can be explained in two ways. Firstly, the steric difficulty occurred by sulfur structure in the taurine. Secondly, it is possible to synthesize the glycine amino acid naturally and the glyco-conjugated bile salts are more common in bile complex. The specificity of the enzyme activity is not dependent on the cholyl structure (Chae et al., 2012). In order to understand the various substrate preferences and functions of the BSH enzymes, it is important to fully explain the structural changes that occur during substrate binding (Chand et al., 2017).

Differences were observed in the docking results of BSH obtained from *Lb. plantarum* with both taurocholic acid and glycol-cholic acid. However, the least binding energy involved in the interaction with Lys88 and glyco-cholic acid. This means that Lys plays an important role in the active site of BSH. Further, Asn12, Ile8 and Leu6 residues were found to play a role in the BSH-substrate relationship when appropriate protein ligand stabilization model analysis was performed by molecular dynamic simulations. However, there was a relation with Lys88 amino acid to tauro-cholic acid and Asp126 amino acid to glyco-cholic acid had been found. These amino acid residues is thought to be have a special

role in the binding of the ligands with the enzyme because of the docking was carried out with stabilized BSH in protein structure (Yadav et al., 2017).

Studies showed that most of the *Lactobacillus* strains, for instance *Lb. casei* BF1, deconjugated GCA more than TCA and several other researchers had reported similar results (Corzo and Gilliland 1999; Liong and Shah 2005). Another theory indicated that GCA is more toxic than TCA so the deconjugation of GCA important and would provide better survivability of the *Lactobacillus* strains in the intestine. In addition to this, after deconjugation, taurine can be metabolized from other bacteria that lead to highly toxic production, hydrogen sulfide (Ridlon et al., 2006). These findings can explain that strains have a strong ability to deconjugate glycine conjugates, but little or no taurine conjugates to be good probiotics candidates (Ramasamy et al., 2010).

1.15.2.2 Amino Acids Moieties

It is known that, CGHs (Callan–Giddings–Harvey–Strominger model) shows that both BSH and PVA includes conserved amino acid sequences that in the active center and diverge amide bonds however, they are display as a significantly different substrate preference (Jones et al., 2008; Kumar et al., 2006). BSH and the PVA have 29% sequence similarities exhibited, there are quite a few differency in the aminoacid residues and structural units including in binding of substrate. The amino acids inside of the binding pocket are unspecific (Xu et al., 2016). Previous studied of structural and functional studies and comparative genomics have identified many conserved, amino acid residues participating in the four substrate binding loops contiguous in the active site (Chand et al., 2017; Kumar et al., 2006). Being establish the larger volume steroid core in the active part, the loops are commonly shorter in BSHs. First of all, they described inside of the substrate binding pockets; loop I contained 8 amino acids from 20 to 27 (LEISFGEH) and 15 aminoacids for loop II, 124-138 (LVDINFSKKLQLSPL) (Rani et al., 2017). Loop I come into being 20–27 amino acids and Tyr24 in *Lb. salivarus* BSH (equaled to Phe26 in *Clostridium perfringens* BSH), along with Phe65 (equaled to Ala68 in *Clostridium perfringens* BSH), located into the

binding complex. These informations in *Clostridium perfringens* BSH show that these amino acids can be forced to bind a different orientation by turning the substrate 90° and display strength it sit in the binding pocket which leads to different enzyme and substrate interference and is clearly different (Rossocha et al., 2005). In loop II, the hydrophobic Leu134 aminoacid in the *Lb. salivarus* BSH enters the pocket and intensifies the substrate binding pocket entry. Phe130 can also support to this restricted spatial configuration (Xu et al., 2016). It is known that loop III that 129 to150 residues is more dynamic and contains more hydrophilic amino acids; these residues to be a good match for the hydroxyl group of bile salts to the suitable catalytic domain (Lambert et al., 2008b).

In the binding complex, deoxycholate is enfolded in Phe61 and Ile137 on the part of the deoxycholyl loop I and by Met20, Phe26 and Ala68, on the part of the isovaleric acid side chain of deoxycholate. In addition, hydrophobic interactions are done by the Ile133 and deoxycholyl loop II and Leu142 and loop IV (Rossacha et al., 2005).

For instance, Tyr24 is submit in loop I as observed in *Lb. salivarus* BSH; the larger hydrophobic amino acid, Trp21 (Leu20 in *Lb. salivarus* BSH and Ile22 in *Clostridium perfringens* BSH) appears to make this tyrosine to sit outside of the binding pocket. When compared to *Lb. salivarus* BSH with *Clostridium perfringens* BSH and *Bifidobacterium longum* BSH also defined differences in other encompassing amino acids in *Lactobacillus salivarus* BSH, including Leu63 (substituted for Thr66 in *Clostridium perfringens* BSH and Met65 in *Bifidobacterium longum* BSH) and Ile56 (substituted for Thr59 in *Clostridium perfringens* BSH and Val58 in *Bifidobacterium longum* BSH) located at the base of the binding pocket, and Phe22 (substituted for Tyr24 in *Clostridium perfringens* BSH and Phe23 in *Bifidobacterium longum* BSH) and Leu18 (substituted for Met20 in *Clostridium perfringens* BSH and Leu19 in *Bifidobacterium longum* BSH) located in loop I. These differences may lead to different enzyme and substrate interactions and determine the nature of the substrate preference. In addition, unlike the binding pocket in *Clostridium perfringens* BSH that exhibits an open entrance with a shallow bottom. A number of amino acid residues in *Lb. salivarus* BSH make the enzyme to exhibit a narrow

inlet to the coupling pocket and an increased internal capacity of the coupling pocket (Xu et al., 2016).

Polar complementarity of conjugated bile acids has suggested an important aspect of substrate specificity (Batta et al., 1984). *Bifidobacterium longum* BSH showed good hydrogen bonding interaction and polar complementarity for GCA substrates of all three hydroxyl groups. However, the polar complementarity of *Clostridium perfringens* BSH and BSH activity were found to be lower than that of *Bifidobacterium longum* BSH (Kumar et al., 2006; Chand et al., 2017).

1.16 Methods of the Measurement for BSH Activity

The BSH activity is found in specific bacteria, easily detected by several methods (Sedlackova et al., 2015). There are various in vitro tests for the selection and study of functional properties of a probiotic strains. BSH activity is mostly determined by detecting the free amino acids; taurine or glycine from enzyme activity or detected precipitation of bile salts.

1.16.1 Qualitative Analysis

1.16.1.1 Direct Plate Assay

As a screening method of the deconjugation within the strains, a differential medium supplemented with tauro- or glyco- conjugated bile acids is used for detecting BSH-active colonies, based on the Dashkevicz and Feighner's method (1989) the accumulation of free salts as halos around colonies or granular, opaque and white colonies were formed (Christiaens et al., 1992; Fang et al., 2009; Moser and Savage, 2001).

1.16.1.2 TLC (Thin Layer Chromatograpy)

Guo and collegues (2011) performed an experiment that indicated when phosphomolybdic acid was used as a visualization reagent to TLC assay discriminating a higher sensitivity for screening of BSH activity. TLC assay does not require that strains grow in the existence of bile salts, and, thus, has a wider application range than the plate assay (Guo et al., 2011).

1.16.1.3 Native PAGE

Native PAGE and SDS-PAGE different in that using a nondenaturating acrylamide gel with Laemmli buffer system except SDS (Laemmli, 1970). After electrophoretic separation of protein sample, the gel was washed then incubated with sodium salt of glycodeoxycholic acid or taurodeoxycholic acid. The BSH activity detected in the gel that form a white opaque band, precipitate of deoxycholic acid (Jarocki et al., 2014).

1.16.1.4 TEM (Transmission electron microscopy)

Vesicle-like components become in the cytoplasm of cells expressing BSHs, and also formed on the membrane peripheral can be detected by TEM (Bi et al., 2016).

1.16.2 Quantitative Analysis

1.16.2.1 Liquid Chromatography Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry-Ion Trap-Time of Flight (LC–MS-IT-TOF), done atmospheric pressure ionization together with IT and TOF technologies and delivers high mass accuracy and resolution free from the MS

mode can be detect bile acid component, inside of the bacterial cells as according to its mass and retention time (Joyce et al., 2014; Bi et al.,2016).

1.16.2.2 High Performance Liquid Chromatography (HPLC)

BSH activity can be measured by degradation of sodium glycocholate and sodium taurocholate from the reaction mixture using HPLC. The amount of sodium glycocholate and sodium taurocholate decreases as comparatively as the amount of sodium cholate that resultant in the mixture can be detected by HPLC (Corzo and Gilliland, 1999).

1.16.2.3 Ninhydrin Assay

The Ninhydrin assay is based on the spectrophotometric determination of the liberated amino acids resulting from the BSH activity. It consists of a series of reaction chains. The main steps can be summarized as; incubation of the conjugated bile salts with in cell-free extracts followed by the coloring of the free amino acids that reacts with Ninhydrin reagent (Christiaens et al., 1992). One unit of BSH activity was defined as the amount of enzyme required to catalyze the release of 1 mol taurine/glycine from the substrate per min (Tanaka et al., 1999).

2. AIM AND SCOPE OF THE STUDY

Bile salts are amphipathic molecules mainly derived from the cholesterol, conjugated with glycine or taurine amino acids after the synthesis in the liver, lay in the gall bladder than released into the duodenum of small intestine. They are responsible for the lipid digestion in gastrointestinal tract of human and other vertebrates. In the intestine, bile salts action as a detergent, promote solubilization, digestion and absorption of dietary lipids and lipid-soluble vitamins. Bile salts were absorbed via active transport by the distal ileum cells at the end of the small intestine and then reach the liver by the blood stream and secreted again in new bile complex. This process is known as the entrohepatic circulation (EHC). Approximately 5% of bile salts escapes from the small intestine than reaches into the large bowel, and conjugated bile salts are hydrolysed by members of endogenous microbiota that they include Bile Salt Hydrolase (BSH) (Batta et al., 1990). BSH was found and isolated from different bacterial species such as; *Lactobacillus* spp. (Gilliand and Speck, 1977), *Bifidobacterium* spp. (Grill et al., 1995) (Kim et al., 2004a), *Clostridium* spp. (Srivastava et al., 1988) and *Bacteroides* spp. (Stelwag et al., 1976).

It is clear that the BSH enzyme plays an important role in the host bowel physiology, colonization of microorganisms at the gastointestinal tract. The physiological advantages of BSHs are not exactly understood and they wide variety between bacterial genera of GIT. In vivo studies showed that cholesterol level was reduced between %23 and %33 by the oral application of *Lactobacillus* spp. in human (Nguyen et al., 2007), mouse, rat and pigs (Tannock et al., 1989; Liong et al., 2006; Tok and Aslım, 2007); These findings are supported by invitro studies of Yıldız et al., (2010). On the other hand, high degree of BSH activity causes dehydroxylation of primary bile salts and composes secondary bile salts, especially deoxycholic and lithocholic acids. The secondary bile acids produced by the gut bacteria can have a toxic and mutagenic effect by reaching high levels in the enterohepatic circulation and in the intestine (Marteau et al., 1995).

Accumulation of toxic secondary bile acids can cause oxidative damage, mitochondrial dysfunction and cancer genesis (Degirolamo et al., 2011). An elevated level of unconjugated bile salts results with malabsorption of lipids and causes steatorrhea and gallstones (Berr et al., 1996). Furthermore, secondary bile acids contribute to the pathogenesis of colon cancer and other gastrointestinal (GI) diseases (Debruyneet al., 2001; Kurdi et al., 2003; Baptissart et al., 2012 and Ou et al., 2012).

The emphasis of the site, ion charge, body and chirality of different of tauro-and glyco- conjugates on the incidence of catalysis by BSHs has also been researched (Huijghebaert and Hofmann, 1986). BSHs may be different in size and amino acid compound. The activity of enzyme had different in kinetics, substrate preference and gene regulation (Kumar et al., 2006). X-ray crystallography and analogy studies of enzyme structure have provided comprehension into the mechanisms of bile salt transformation reactions. In addition to three dimensional structure of BSH enzyme, site-directed mutagenesis is one of the methods to be used to understand structure and function of the enzymes. Cysteine (Cys) is strictly conserved in all members of Ntn-hydrolase superfamily enzymes. Site directed mutagenesis of Cys2 showed that the significance of the SH- group in the N-terminal cysteine was confirmed by changing Cys amino acid with other potential nucleophilic residues, Ser or Thr, results in an inactive enzyme (Kim et al., 2005). Other strictly concerved amino acids, Arg16, Tyr19, Arg170 and Arg223 are thought to play role in catalytic site of the BSH enzyme (Rossacha et al., 2005).

The characteristics of BSH enzymes are widely different among genus or species and interestingly, even in the strains *Lb. plantarum,* frequently encountered in the human intestine. BSH enzymes from different *Lb. plantarum* strains can deconjugate bile salts in diverse ratios or some of them have no activity against some of bile salts.

To show the relationship between BSH enzymes and their substrate specifities, structure of the BSH must be known better, but the structure and working mechanism of such an important enzyme is not known very well. There

are two possible hypotheses that explain how BSHs recognize bile acids. According to first hypothesis, substrates are recognized by the amino acid moiety and BSHs prefer glucoconjugated bile salts more than tauroconjugated bile salts (Kim et al., 2004; Tanaka et al., 2000). It was also proposed that BSH recognize bile acids on both the cholate steroid center and the conjugated amino acid part (Begley et al., 2006). Second hypothesis argued that some key amino acid residues in the substrate binding complex are responsible for the substrate specificity of BSH enzyme (Kumar et al., 2004; Rossacha et al., 2005).

Is BSH active probiotics, advantageous or deleterious to the host? Today we do not know the answer of this question. There are many research about serum cholesterol reducing effect of BSH enzyme activity. On the other hand, many recent researches indicate that there is strong relationship between colon cancer and some secondary bile salts induced by BSH to hydrolysis of bile salts. BSH enzymes have a different substrate affinity and as a result they produce different secondary metabolites some of which are toxic some of which are not. In this situation, determination of substrate specificity of the BSH enzymes is important event. Therefore, this study was concentrate on substrate specificity of the BSH enzyme. Here we mentioned that five amino acids are responsible for substrate specificity and probiotic strains developed in accordance with the information obtained from the BSH study can help to improve bowel function and health. BSH activity already used as a selection criteria for the some probiotics by the feature of reduction serum cholesterol level. On the other hand, we should learn about which type of BSH enzyme produce which type of secondary metabolites, toxic or non-toxic, and which type of BSH result in colon cancer.

The aim of this study was to investigate the human source of *Lb. plantarum* B14 strains with high BSH activity were used to better understand the substrate specification of the BSH enzyme. For this purpose, 975 bp *bsh* gene was amplified from *Lb. plantarum* B14 strain by using species specific forward and reverse primers. Then amplified *bsh* genes were cloned into pBluescript vector. The *bsh* gene nucleotide residues in the clones having BSH activity were determined by sequencing and nucleotide residues were compared with the sequence that of Gene Data Bank. The partially conserved amino acids, Phe-18,

Tyr-24, Asn-79, Leu-138 and Asn-180 amino acids that are supposed to have role in substrate specificity, were substituted for Leu-18, Phe-24, Val-79, Glu-138 and His-180 amino acids respectively by site directed mutagenesis. Mutant enzymes were analyzed by Direct Plate Assay and then BSH enzyme were purified by protein purification kit and gel filtration techniques. Finally, to better understand substrate specify of mutant BSH enzymes were incubated with six different bile salts; glycocholic acid, glycodeoxycholic acid, glycochenedeoxycholic acid, taurocholicacid, taurodeoxycholic acid and taurochenedeoxycholic acid.

3. MATERIALS AND METHODS

3.1 Bacterial Strains, Plasmids and Culture Conditions

The bacterial strains and plasmids used in this study were listed in Table 3.1. *Lactobacillus plantarum* B14 propagated aerobically at 37°C in MRS (Man-Ragosa-Sharpe) medium (Merck) was with 0.05% (wt/vol) cysteine HCl, for overnight (16 hours). *Escherichia coli* cells were inoculated at 37°C for 16-18 hours in Luria-Bertani (LB) broth with vigorous shaking or on LB medium solidified with 1.5% agar and supplemented appropriate ampicillin (100 µg/ml) as needed. The plasmid pBluescript (Stratagene, USA) and pET22b (Novagen, USA) were used as the vectors for subcloning and over expression of his-tagged proteins for purification, respectively.

Table 3.1. Bacterial strains and plasmids

Strain	Genotype – Origin	Phenotype	Reference
Lb. plantarum B14	Human origin (feces)		This work
Escherichia coli	**Genotype – Origin**	**Phenotype**	**Reference**
XL1-Blue	rec A end A 1 gyr A986 thi-1hsdr17supE44 rel A1 lac		Stratagene
BLR (DE3)	F-ompT gal dcm lon hsdSB (rB - mB-) λ(DE3)		Novagen
Plasmid	**Genotype**	**Phenotype**	**Reference**
pBluescript	SKII+	Amp^r	Fermentas
pCON1	pBluescript SKII+ with 1.0 kb *bsh* gene insert	Amp^r	This work
pET22b(+)	pelB, T7 lac, ApR, pBR322 ori (C-terminal His-tagged protein)	Amp^r	Novagen
pCON2	with 1.0 kb *bsh1* gene insert and fragment from MCS site of pET22b(+)	Amp^r	This work
pCON1F18L	Substitution of phenylelanine for leucine in pCON1	Amp^r	This work
pCON1Y24F	Substitution of tyrosine for phenylelanine in pCON1	Amp^r	This work
pCON1Y24L	Substitution of tyrosine for leucine in pCON1	Amp^r	This work
pCON1N79V	Substitution of asparagine for valine in pCON1	Amp^r	This work
pCON1L138E	Substitution of leucine for glutamic in pCON1	Amp^r	This work
pCON1N180H	Substitution of asparagine for histide in pCON1	Amp^r	This work
pCON2F18L	Substitution of phenylelanine for leucine in pET22b(+)	Amp^r	This work
pCON2Y24F	Substitution of tyrosine for phenylalanine in pET22b(+)	Amp^r	This work
pCON2Y24L	Substitution of tyrosine for leucine in pET22b(+)	Amp^r	This work
pCON2N79V	Substitution of asparagine for valine in pET22b(+)	Amp^r	This work
pCON2L138E	Substitution of leucine for glutamic in pET22b(+)	Amp^r	This work
pCON2N180H	Substitution of asparagine for histide in pET22b(+)	Amp^r	This work

3.2 Construction of pCON1 in *E. coli* XL-1 Blue Cells

3.2.1 Genomic DNA Isolation

A freshly grown single colony (1.5–2 mm diameter) from *L. plantarum* B14 strain was used to inoculate 5 ml culture of MRS medium supplemented with 0.05% (wt/vol) cysteine HCl and grown at 37°C for 16-18 hours. One and a half milliliters of MRS inoculated with fresh culture of strain (30 µl, 2% of MRS medium) and incubated for 16 h at 37°C. One and a half milliliters of each 16 h

stationary-phase culture collected by centrifugation at 13.000 *g* and 25°C for 2 minutes (Micro120, Hettich, Germany). Genomic DNA was isolated by Genomic DNA Isolation Kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions.

3.2.2 Amplification of *bsh* Gene

Approximately 1.0 kb (975 bp) *bsh* gene was amplified by PCR using species specific primers; BSH1-plntF, 5'-ATGTGTACTGCCATAAC-3' (17 bp) and BSH1-plntR, 5'-GCTTCTGATCGTAATG-3' (16 bp). The species specific forward and reverse primers were designed on the basis of the alignment of the all published complete available genome sequence of *Lb. plantarum* in NCBI Gene Data Bank for amplification of the *bsh* gene. Optimized PCR reactants and condition are summarized in Table 3.2 and Table 3.3, respectively.

The resulting amplicons were visualized and separated by 1% (w/v) agarose gel electrophoresis at 70 V for 1 h using Thermo EC 250-90 minicell primo electrophoretic Gel System and then followed by Ethidium bromide staining (0.5 µg/ml). After visualization, products were purified from agarose gel to remove unincorporated primers, nonspecific products and unwanted chemicals. 1.0 kb PCR products (*bsh* gene) was purified from agarose gel by Gene JET Gel Extraction Kit (#K0691, Thermo Scientific) according to the manufacturer's instructions.

Table 3.2. PCR reactants for amplification of *bsh* gene

Reactants	Concentrations	Quantities
UP (ultra pure) H_2O	-	32 µl
Genomic DNA (50-100 ng)	100.0 ng/µl	2 µl
$MgCl_2$ (25 mM)	25.0 mM	4 µl
dNTP mix (5 mM)	5.0 mM	2 µl
Forward Primer (25 µM)	25.0 µM	2 µl
Reverse Primer (25 µM)	25.0 µM	2 µl
Pfu DNA Polymerase Buffer (10X)	1.0 X	5 µl
Pfu DNA Pol. Enzyme (2.5 U/µl)	2.5 u/µl	1 µl
Total Volume	-	**50 µl**

Table 3.3. PCR reaction conditions for amplification of *bsh* gene

Cycles	Temperatures	Time
Initial denaturation	95°C	30 sec
Denaturation	95°C	1 min
Annealing	40°C	1 min
Extension	72°C	9 min
Final extension	72°C	5 min
Number of cycles	**30**	

3.2.3 *Sma*I Digestion of pBluescript

pBluescript was digested with *Sma*I enzyme and incubated for 4 hours at 30ºC (Table 3.4) and then the digested vector was seperated and visualized on 0.7% Agarose gel for purification using GeneJET Gel Extraction Kit (#K0691, Thermo Scientific)

Table 3.4. Reaction conditions for pBluescript digestion by *Sma*I

Reactants	Quantities	
Up H_2O	11 µl	at 30 °C
pBluescript (>100 ng)	40 µl	
Tango Buffer (10X)	6 µl	for 4 hours
*Sma*I enzyme (10U/µl)	3 µl	
Total Volume	**60 µl**	

3.2.4 Ligation of *bsh* Gene into pBluescript SKII $^{+}$ *Vector*

T4 Ligase (Fermentas-Thermo Scientific) was used for the ligation reactions. The linear vector pBluescript DNAs were dephosphorylated with Shrimp Alkaline Phosphatase (SAP) (Fermentas-Thermo Scientific). 100 ng vector and 3/1 insert-vector ratio were used for the ligation reactions (Table 3.5). The ligation mixture was incubated in a thermal cycler at 22°C for 1 hour. The amount of insert DNA to be used for ligation reaction was calculated by the following equation:

$$\text{Insert Mass (ng)} = \text{Ration x} \left[\frac{\text{Insert Length (kb)}}{\text{Vector Length (kb)}} \right] \text{x Vector Mass (ng)}$$

Table 3.5. Reaction condition of pBluescript and *bsh*1 genes ligation

Reactants	Quantities	
Up H_2O	7.5 µl	at 22 °C
pBluescript Vector (*SmaI* cut) (>500 ng)	0.5 µl	
*bsh*1 (PCR product)	7 µl	for 1 h
T4 10X Ligase Buffer	2 µl	
T4 DNA Ligase Enzyme (5 U/µl)	1 µl	
PEG (4000U)	2 µl	
Total Volume	**20 µl**	

3.2.5 Preparation of $CaCl_2$ Competent Cells of *E. coli* XL1 Blue and BLR(DE3)

Day I: *E. coli* XL1 Blue and BLR(DE3) cells (from stock -80˚C) were streaked out onto LB plates and incubated at 37˚C for overnight. Day II: 5 ml LB medium was inoculated with single colony from the plate and agitated at 175 rpm at 37˚C for overnight. Day III: 50 ml LB medium in a 250 ml flask was inoculated with 500 µl overnight culture of *E. coli* XL1-Blue and BLR(DE3) strains. The inoculated cultures were incubated at 37°C with vigorous shaking about 2 hours until OD_{600} 0.4 - 0.5, then cells were transferred in to ice. The grown cultures were centrifuged for 10 minutes at 5000 rpm and 4°C. Supernatants were discarded and pellets were resuspended in 20 ml (1/2 of the culture volume) of ice cold 100 mM $CaCl_2$ and incubated on ice for 30 minutes. Cells were precipitated by centrifugation for 10 minutes at 5000 rpm and 4°C. Supernatants were discarded and pellets were resuspended in 2 ml (1/20 of the culture volume) ice cold 100 mM $CaCl_2$ and containing 10% glycerol. Competent cells were divided into 100 µl aliquots and stored at -80°C until use.

3.2.6 Transformation of pBluescript SKII $^+$ *bsh* Constructs into *E. coli* XL1-Blue Strains

Ten µl ligation reaction mixture was added into 100 µl competent cells (*E. coli* XL1 Blue) and incubated on ice for 45 minutes. The cells were heat shocked to at 37°C for 5 minutes, taken on ice and incubated for 5 more minutes. 1 ml LB was added to the samples and cells were incubated for 1 h at 37°C with vigorous shaking. After incubation, cells were centrifuged at 6000 rpm for 3 minutes and

the pellets were resuspended in 100 µl LB. Cells were streaked on IPTG-X-gal-Amp plates for selection and incubated at 37°C for 16-18 hours (Davis et. al 1988). Four transformants were selected by using Blue/White Colony Assay in the presence of ampicillin (100 µg/ml). To determine the correctly-oriented insert, plasmid DNAs were isolated from transformants and digested with *Xba*I (Fermentas) at 37°C for one hour (Table 3.7). When the insert is correctly oriented, 700 bp and 3000 bp fragments were produced. On the other hand when insert was mis-oriented, 268 bp, 700 kbp and 3000 kbp fragments were produced following digestion. The correct clone having properly oriented insert DNA (*bsh* gene) was designated as pCON1.

Table 3.6. Digestion reaction conditions of selected clones

Reactants	Quantities	
Up H_2O	6.5 µl	at 37 °C
pCON1 Plasmid DNA (>500 ng)	2 µl	
Tango Buffer (10X)	1 µl	
*Xba*I enzyme (10U/µl)	0.5 µl	for 1 hours
Total Volume	**10 µl**	

3.2.7 Stock Preparation

For the storage of the pCON1 clone, 5ml LB liquid medium containing ampicillin (100µg/ml) was inoculated with *E. coli* XLI-Blue cells harboring pCON1 plasmid and incubated overnight at 37°C and 175 rpm for nearly 16-18 hours. The cells were harvested by centrifugation at 25°C for 4 minutes at 4000 rpm. The cell pellet was re-suspended in 4 ml of LB broth containing with 30% glycerol and divided into two cryogenic tubes and then stored at -80°C freezer.

3.2.8 DNA Sequencing and Sequence Analysis

The *bsh* gene sequence containing 975 bp from pCON1 was obtained with DNA sequencing by Macrogen Inc (Seoul, Korea) using the universal primers, M13/pUC forward and M13/pUC reverse. The desired mutations on the pCON1/F18L, pCON1/Y24F, pCON1Y24L, pCON1/N79V, pCON1/L138E and

pCON1/N180H constructs were confirmed by the BioEdit Sequence Alignment Program.

3.3 Construction of pCON1 Mutants in *E. coli* XL1-Blue

3.3.1. PCR Based Site-directed Mutagenesis of The Target Codons

Five amino acids predicted to be important for the substrate specificity of the BSH activity of *Lb. plantarum,* were changed to the amino acids characteristic of which are different than those of PCR based site directed mutagenesis using appropriate primers in Table 3.7. The Quick Change Site-Directed Mutagenesis (Stratagene) Protocol was used to substitute target codons for the desired codons. The pCON1 DNA (pBluescript/*bsh*1) was used as the template for the F18L (substitution of Phenyalaline for Leucine), Y24F (substitution of Tyrosine for Phenyalaline), Y24L (substitution of Tyrosine for Leucine), N79V (substitution of Asparagine for Valine), L138E (substitution of Leucine for Glutamic acid) and N180H (substitution of Asparagine for Histide) substitutions. The *Pfu* DNA polymerase was prefered having lower error rate compared to the other polymerases and avoiding second site-mutations (Flaman et al., 1994; Cline et al., 1996). PCR reactions were performed by thermal temperature cycler (Techne TC-3000) (Table 3.9 and 3.10).

Table 3.7. Forward and reverse primers used for site-directed mutagenesis

Primers name	Primer Sequences (5' to 3')	Tm
F18LF	5'- CTTCGGTAGAAAT**CTA**GATTATGAAATTTC-3'	55°C
F18LR	5'-GAAATTTCATAATC**TAG**ATTTCTACCGAAG-3'	55°C
Y24FF	5'-GATTATGAAATTTCA**TTT**AATGAAATGGTTACG-3'	54°C
Y24FR	5'- CGTAACCATTTCATT**AAA**TGAAATTTCATAATC-3'	54°C
Y24FR	5'- CGTAACCATTTCATT**AAA**TGAAATTTCATAATC-3'	54°C
Y24LF	5'-GATTATGAAATTTCA**CTG**AATGAAATGGTTACG-3'	61°C
Y24LR	5'- CGTAACCATTTCATT**CAG**TGAAATTTCATAATC-3'	61°C
N79VF	5'-GTATTGCGGGATTA**GTT**TTTGCAGGTTATGCTG-3'	62°C
N79VR	5'-CAGCATAACCTGCAAA**AAC**TAATCCCGCAATAC-3'	62°C
L138EF	5'-CAATTACCATTATCACCG**GAA**CATTGGTTGGTTGC-3'	63°C
L138ER	5'-GCAACCAACCAATG**TTC**CGGTGATAATGGTAATTG-3'	63°C
N180HF	5'-CTACCAATTATTT**CAT**TTGAACAACTATC-3'	53°C
N180HR	5'-GATAGTTGTTCAA**ATG**AAATAATTGGTAG-3'	53°C

Table 3.8. PCR Reactants for F18L, Y24F, Y24L, N79V, L138E and N180H mutagenesis

Reactants	Concentrations	Quantities
UP (ultra pure) H_2O	-	33.25 µl
Template DNA (100 ng)	135 ng/µl	0.75µl
$MgCl_2$ (25 mM)	25 mM	4 µl
dNTP mix (5 mM)	5 mM	2 µl
Forward Primer (25 µM)	25 µM	2 µl
Reverse Primer (25 µM)	25 µM	2 µl
Pfu DNA Polymerase Buffer (10X)	10X	5 µl
Pfu DNA Pol. Enzyme (2.5 U/µl)	2.5 u/µl	1 µl
Total Volume	-	**50 µl**

Table 3.9. PCR Reaction conditions

Cycles	Temperatures						Time
	F18L	**Y24F**	**Y24L**	**N79V**	**L138E**	**N180H**	
Initial denaturation	95°C	95°C	95°C	95°C	95°C	95°C	30 sec
Denaturation	95°C	95°C	95°C	95°C	95°C	95°C	1 min
Annealing	50°C	49°C	56°C	57°C	58°C	48°C	1 min
Extension	72°C	72°C	72°C	72°C	72°C	72°C	9 min
Final extension	72°C	72°C	72°C	72°C	72°C	72°C	5 min
Number of cycles							**20**

At the end of the PCR, products were treated with *Dpn*I (Fermentas) for 2 hours at 37°C to digest the template DNA under the reaction condition defined in Table 3.11. After PCR product digestion, *Dpn*I treated and untreated PCR products were loaded on to 0.8 % agarose gel and electrophoresed for 1 hour. After degradation of the template DNA, PCR products were transformed into component XL1-Blue cells. Finally, to find out transformants with desired mutations, four transformants for each mutation were randomly selected and DNA isolation was performed for sequencing.

Table 3.10. Reaction conditions for pCON1* Mutant PCR products digestion by *Dpn*I

Reactants	Quantities	
Up H_2O	4 µl	at 37 °C
PCR product (>500 ng)	40 µl	
Tango Buffer (10X)	5 µl	for 2 hours
*Dpn*I enzyme (10U/µl)	1 µl	
Total Volume	**50 µl**	

3.3.2 Purification of the pCON1* Mutant PCR Products

The pCON1* Mutant PCR amplicons (* represents the desired mutations) were loaded and separated from unwanted products on a 1% agarose gel (Sigma,

USA) for extraction and purification. After being separated by electrophoresis at 75 volt for 1 hour, the PCR products were purified using the protocol described in the Thermo Scientific GeneJET Gel Extraction Kit (Massachusetts, USA).

3.3.3 Transformation of pCON1* Mutants into *E. coli* XL1-Blue Strains

Ten µl of each gel-purified PCR product of pCON1* expectedto carry the desired mutations, F18L, Y24F, Y24L, N79V, L138E and N180H, were transferred individually into 100 µl of XL1-Blue competent cells. The samples have undergone a heat shock treatment of 45 minutes of incubation on ice then 5 minutes incubation at 37°C and finally 5 minutes incubation on ice. Then, the samples were added in 1 ml of LB and incubated at 37°C and 175 rpm for 1 hour. Later, the samples were centrifuged at 6000 rpm for 3 minutes and the pellet were re-suspended in 100 µl LB. The re-suspended cells were spread individually on LB agar plates containing ampicillin (100 µg/ml). The plates were incubated overnight at 37°C for nearly 16-18 hours. Four random colonies were selected from transformant LB plates and grown in an overnight culture of 5 ml LB with ampicillin. The cultures were used for DNA isolation using the protocol prescribed on the Vivantis plasmid DNA Isolation Kit (California, United States).

3.3.4 DNA Sequencing and Detection the Desired Mutations

DNA of the selected clones of pCON1* were isolated and sequenced at MacroGen (Korea) by using universal primers, M13-PUC 5-GTAAAACGACGGCCAGT-3 CAGTATCGACAAAGGAC-3 and M13R-PUC, 5’-CAGGAAACAGCTATGAC-3’ (17-bp), to confirm target mutation.

Obtained sequences compared with wild type *bsh* gene sequence from gene data bank. After verification the mutants by the BioEdit Sequence Alignment Program, the transformants containing desired mutations were designated as

pCON1F18L, pCON1Y24F, pCON1Y24L, pCON1N79V, pCON1L138E and pCON1N180H and stored at -80°C.

3.4 Subcloning of theWild Type (pCON2) and the Mutant *bsh* (pCON2/*bsh**) into pET22b Expression Vector

3.4.1 Plasmid DNA Isolation of pET22b

The low copy plasmid pET22b expression vectors was grown in a 10 ml LB broth containing ampicillin (100 µg/ml) at 37°C and 175 rpm for nearly 14-16 hours. The cells were harvested by centrifugation at 4000 rpm at 25°C for 4 minutes and then resuspended pellet in 1 ml of LB medium was centrifugated at 6000 rpm for 3 minutes. pET22b plasmid DNA were isolated from the pellet using the protocol of Plasmid Miniprep Kit (Thermo Scientific GeneJET, Lithuania, EU).

3.4.2 Digestion of pCON1, Mutant pCON2/*bsh** and pET22b DNAs by *EcoR*I and *Not*I

For cloning of *Lb. plantarum* B14 wild-type and mutant *bsh** genes into pET22b expression vector, pET22b plasmid DNA, pCON1 and pCON1/*bsh** mutants (pCON1F18L, pCON1Y24F, pCON1Y24L, pCON1N79V, pCON1L138E and pCON1N180H) were digested individually with *EcoR*I and *Not*I (Thermo Scientific) at 37°C for 45 minutes. Reaction conditions defined in Table 3.11 and 3.12. Digested samples were purified from 1% agarose gel by Gel DNA Recovery Kit (Vivantis, Selangor, Malaysia).

Table 3.11 Digestion condition of the pET22b DNA by *Not*I and *EcoR*I restriction enzymes

Reactants	Quantities	
Up H_2O	20.0 µl	at 37 °C
pET22b (>500 ng)	30.0 µl	
Fast Digest Buffer (10X)	6.0 µl	for 45 min
*EcoR*I enzyme (Fast Digest)	2.0 µl	
*Not*I enzyme (Fast Digest)	2.0 µl	
Total Volume	**60.0 µl**	

Table 3.12. Digestion of pCON1 and pCON1/*bsh** Mutant plasmid DNAs by *EcoR*I and *Not*I restriction enzymes

Reactants	Quantities	
Up H_2O	18.0 µl	at 37 °C
pCON1 and pCON1/*bsh** DNAs (>500 ng)	40.0 µl	
Fast Digest Buffer	7.0 µl	for 2 h.
*EcoR*I Enzyme (Fast Digest)	2.5 µl	
*Not*I Enzyme (Fast Digest)	2.5 µl	
Total Volume	**70.0 µl**	

3.4.3 Ligation of the Wild-type and the Mutant *bsh* genes into pET22b Expression Vector

The wild-type and the mutant *bsh* genes were ligated individually into the *EcoR*I/*Not*I digested pET22b expression vector by T4 DNA ligase (Thermo Scientific) at 22°C for 1 hour (Table 3.13). For ligation reaction, the suitable amount of insert and vector DNA were calculated by the equation;

Insert mass (ng) = ratio x (insert length in bp (ng) /vector length in bp) x vector mass (ng)

Table 3.13. Ligation of wild-type and mutant *bsh* DNAs to pET22b vector

Reactants	Quantities	
Up H_2O	11.2 µl	at 22 °C
pET22b Vector (*EcoR*I/*Not*I digested) (>500 ng)	4.0 µl	
Wild-type or mutant *bsh* DNAs (*EcoR*I/*Not*I cut)	1.8 µl	for 1 h.
T4 10X Ligase Buffer	2.0 µl	
T4 DNA Ligase Enzyme (5 U/µl)	1.0 µl	
Total Volume	**20.0 µl**	

3.4.4 Transformation of pCON2 and pCON2/*bsh** Mutants into *E. coli* BLR(DE3) Strains

Ten µl of the each ligation mixture was added to 100 µl *E. coli* BLR(DE3) competent cells and incubated on ice for 45 minutes. Then, heat shock was applied to the cells at 37°C for 5 minutes. Samples were taken on ice and incubated for 5 minutes. 1 ml LB was added to the samples and cells were incubated for 1 h at 37°C with vigorous shaking. After incubation, cells were centrifuged at 6000 rpm for 3 minutes and pellets were resuspended in 100 µl LB. Cells were spread on LB-Amp (100 µg/ml) plates and incubated at 37°C for 16-18 hours. After transformations, four random colonies were selected and incubated in LB medium for overnight at 37°C in 10 ml LB medium containing ampicillin (100 µg/ml) at 175 rpm as well as on an ampicillin containing LB Agar plate. Then the pellets of overnight culture were prepared for exraction of plasmid DNAs.

3.4.5 Detection of the Transformants those had Desired Insert

For the detection of the transformants having desired insert, the pCON2, pCON2F18L, pCON2Y24F, pCON2Y24L, pCON2N79V, pCON2L138E and pCON2N180H plasmid DNAs were exracted individually from *E.coli* BLR(DE3) transformants via Thermo Scientific GeneJET Plasmid Miniprep Kit (Lithuania, EU). Then, plasmid DNAs were digested with *EcoR*I and *Not*I (Thermo Scientific) to verify the structure of the wild-type and mutant *bsh* gene in constructs. The reaction conditions were indicated in Table 3.14. The clones having following constructs; pCON2, pCON2F18L, pCON2Y24F, pCON2Y24L, pCON2N79V, pCON2L138E and pCON2N180H, were stored in LB broth with 30% glycerol into cryogenic tubes at -80°C.

Table 3.14. Digestion condition of pCON2 and pCON2 mutant DNAs with *EcoR*I/*Not*I restriction enzymes

Reactants	Quantities	
Up H_2O	5.5 µl	at 37 °C
pCON2 or pCON2 mutant DNAs (>500 ng)	3.0 µl	
Fast Digest Buffer (10X)	1.0 µl	for 30 min
*EcoR*I Enzyme (Fast digest)	0.25 µl	
*Not*I Enzyme (Fast digest)	0.25 µl	
Total Volume	**10.0 µl**	

3.5 Determination of BSH Activity and Substrate Specifity

3.5.1 Direct Plate Assay of pCON2 and Mutant pCON2/*bsh** Constructs

BSH activity was measured using a modified plate assay method (Fang, 2009). For measuring BSH activity of *E. coli* BL(DE3) derivatives, an optimized LB medium (including 0.3% taurodeoxycholic acid or 0.3% glycodeoxycholic acid, 0.1 mmol/l isopropylthiolgalactopyranoside (IPTG), 0.35 g $CaCl_2 \cdot 2H_2O$ and 10 g glucose) was used. The clones that have pCON2/*bsh** constructs; (pCON2F18L, pCON2Y24F, pCON2Y24L, pCON2N79V, pCON2L138E or pCON2N180H) were incubated at 37°C for 72 hours. pCON2 (the wild Bile salt hydrolase transformant) represents the positive control, pET22b represents the negative control and BSH active colonies have a halo of precipitated cholic acid.

3.5.2 Expression of Wild-type and Mutant *bsh* Genes in *E. coli* using IPTG-inducible Promoters

3.5.2.1 Cell Growth and Extraction

Wild and mutant type *bsh* harboring transformants were inoculated into 100 ml LB broth containing ampicillin (100 µg ml-1) and 2% Glycerol and incubated on a shaker at 37°C at 175 rpm until OD_{600} of 0.45 – 0.6 were reached. The expressions were induced by the addition of 0.3 mM IPTG and growth

continued until OD_{600} reached to 1.5 – 3.0. Cells were harvested by centrifugation at 6,000 rpm for 15 minutes by Rotina 38R, centrifuge (Hettich, Germany).

The chemical lysis method modified from Molecular cloning : a laboratory manual (Green and Sambrook, 2012). Cell pellets were suspended with 4 ml binding buffer (pH:7.8) per 100 ml of cell culture. Lysozyme was added to a final concentration of 1mg/ml and the cell suspension was incubateted on ice for 30 minutes. Then, the mixture incubated on rocking platform for 10 minutes at 4°C. After the incubation of suspention with DNAse (1%), RNAse (5 µg/ml) and Tween-20 (5 µg/ml), they were incubated with rocking platform for another 10 minutes at 4°C. To remove insoluble debris, mixture was cenrifugated at 11,000 rpm for a 20 minutes at 4°C. The supernatant (cell lysate) was filtred by using 45 µm membrane filter and collected in a fresh tube.

3.5.2.2 Lowry Assay

Lowry Protein Assay was used to measure protein concentration of the cell lysates with Colorimetric methods. The method includes the reactions of copper ions to the peptide bonds under alkaline context (the Biuret test) with the oxidation of aromatic protein residues. The appropriate protein concentrations of 0.01–1.0 mg/ml. It is based on the reaction of Cu+, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent (Lowry et al., 1951). Bio-Rad Protein assay standard II bovine serum Albumin (BSA) were dissolved in dH_2O and diluted to 1.0 mg/ml and six different dilutions were used to create standard curve (Table 3.15). 50 µl each cell lysates and 150 µl SDS/0.1M NaOH were used for a samples. After addition of 1 ml reagent E (see Appendix B) to samples, they were vortexed and incubated for 5 minutes at room temperature. At the end of first incubation 100µl 1N folin reagent (Merck) was added to each tube and they were vortexed and incubated for 30 minutes at room temperature. Each sample was measured at OD_{660} nm by using a UV-1601PC spectrophotometer (Hitachi, Japan).

Table 3.15. BSA standards of Lowry Assay

BSA µg/ml	µlof 1mg/1ml BSA	µl %1 SDS/0.1M NaOH
0	0	200 µl
20 µg/ml	4 µl	196 µl
40 µg/ml	8 µl	192 µl
60 µg/ml	12 µl	188 µl
80 µg/ml	16 µl	184 µl
100 µg/ml	20 µl	180 µl

3.5.3 Determinations of Temperature and pH Optima

Ninhydrin (2,2-Dihydroxyindane-1,3-dione) is a chemical used to detect ammonia or primary and secondary amines. When reacting with these free amines, a deep blue or purple color known as Ruhemann's purple is produced. For the determination of optimum temperature of BSH enzyme activity, the Ninhydrin assay was performed using the standard procedure with glycodeoxycholic acid (GDCA) as the substrate at various temperatures ranging from 25°C to 65°C and pH values ranging from 4 to 6.5 using various 0.1 M sodium phosphate buffer of different pH (4 to 6.5).

3.5.4 Substrate Specifity Test for BSH Enzyme by Ninhydrine Assay

Ninhydrin assay method was used to determine the substrate specifity of BSH enzyme against six of bile salts, taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GA), glycodeoxycholic acid (GDCA) and glycochenodeoxycholic acid (GCDCA) modified from Tanaka et al, (1999), Fang et al, (1999) and Chae et al, (2012). Based on this assay, standart dilutions were made from (40 mmol/L glycine stock so that 0, 4, 8, 12, 16 and 20 mmol 1^{-1} range was obtained. The solutions also continued 10 mmol/L of dithiotreitol (DTT) and 100 mmol 1^{-1} sodium phosphate (pH 6.0). For each of the reaction tubes, 10 µl of BSH enzyme was added to 190 µl of reaction mixtures consist of (100 mmol 1^{-1} sodium phosphate (pH 6.0), 10 m mol 1^{-1} conjugated bile acids (glyco- or tauro-conjugated bile salts) and 10 mmol 1^{-1} of dithiotreitol (DTT) incubated at 37°C for 30 minutes. Each reaction was stopped using 15% trichloroacetic acid (Merck). The mixture was centrifuged at 14.000 rpm for 15 minutes (Micro120, Hettich, Germany) to obtain and recover

the reaction samples from precipitates. The Ninhydrin protein assay was applied to measure the amount of released amino acids using Ninhydrin reagent. Standard series and reaction samples were boiled for 15 minutes and then cooled to room temperature. Spectrophotometric analysis was performed at OD_{570} and enzyme activities was calculated. The highest value designated as 100%. Substrate specificity and activity assays were performed in triplicate in individual experiments to produce standard deviations.

3.6 Partial Purification of the Wild type and Mutant BSH Enzymes with Colon Chromotograpy

After preparation of cell free extracts from wild type and mutant BSH expressing cells, BSH proteins were purified by using immobilized metal ion affinity chromatography (IMAC). Histrap HP, Ni Sepharose, media precharged with nickel ions (Ni^{2+}) column was used. All protein purifications were done with ÄKTAprime plus chromatography system that performed simple purifications of tagged and untagged proteins (GE Healthcare). In the chromatography system, proteins were bounded to His Trap^{TM} HP column and eluted by elution buffer containing 50 mM imidazole, 20 mM sodium phosphate buffer, 500 mM NaCl, at pH: 6.

3.7 SDS-PAGE Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed using 12% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS, as described by Laemmli (1993), to confirm the production of the wild type and mutant BSH enzymes. Protein bands were detected using Coomassie Brilliant Blue R250 staining. The protein concentrations were measured by the Lowry assay (Lowry et al., 1951), using bovine serum albumin as the standard. Page Ruller Prestained Protein Ladder (Thermo Scientific) was used as a marker.

3.8 3D Structure Modelling of BSH

The overall 3D monomer structure of BSH was visualized by PyMOL program (PyMOL Executable Build is Copyright$^{(C)}$ 2006 DeLano Scientific LLC, South San Francisco, California, USA). *Enterococcus faecalis* (AN: 4WL3_A), was used as the template and homology between *Lb. plantarum* B14 BSH (AN: KY080706), and the template was 70.44% sequence identity and 0.52 sequence similarity.

3.9 Sequence Alignment and Phylogenetic Tree Analysis of BSH

The 975 bp nucleotides and 324 amino acid residues of the wild type *bsh* gene obtained by using the SIB (Swiss Institute of Bioinformatics) ExPASy sequence translate tool (http://web.expasy.org/translate/). Phylogenetic tree analaysis were prepared with the Jalview 2.8.0b1, a multiple alignment program, by using FASTA protein databases files from BLAST (https://blast.ncbi.nlm. nih.gov).

4. RESULTS

4.1 pCON1 Construction

The bile salt hydrolase (*bsh*) gene (aproximately 1.0 kb) has been cloned to pBluescrip KS$^+$ (2.9 kb) cloning vector and named as pCON1 (4.0 kbp). *Bsh* gene was amplified by PCR using species specific primers and pBluescript DNA was digested with *Sma*I restriction enzyme (Figures 4.1-3). The resulting 2.9 kbp and 1 kb DNA fragments were used as vector and insert respectively during ligation process. 10 µl of ligation mixture was transformed into XL1-Blue cells. Transformants were selected on the basis of ampicillin resistance and also IPTG and X-Gal (Figure 4.4). The clones were tested with *Xba*I (Figure 4.5).

4.1.1 Genomic DNA Isolation and Amplification of *bsh* gene

For preparation of the insert *bsh* DNA, *Lb. plantarum* B14 genomic DNAs were isolated than *bsh*1 gene was amplified by PCR using species specific primers and aproximately 1.0 kbp DNA band was detected on agarose gel electrophoresis (Figure 4.1). Then PCR products of *bsh* genes were purified from 1% agarose gel (Figure 4.2).

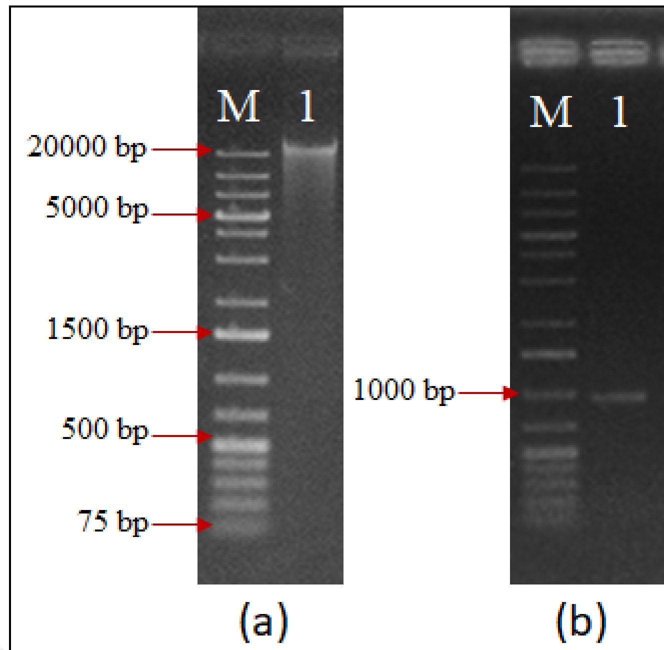

Figure 4.1. Genomic DNA isolation and PCR product of *Lb. plantarum* B14. M, 1 kb plus DNA ladder; a, genomic DNA of *Lb. plantarum* B14; b, PCR product of *bsh* gene from *Lb. plantarum* B14.

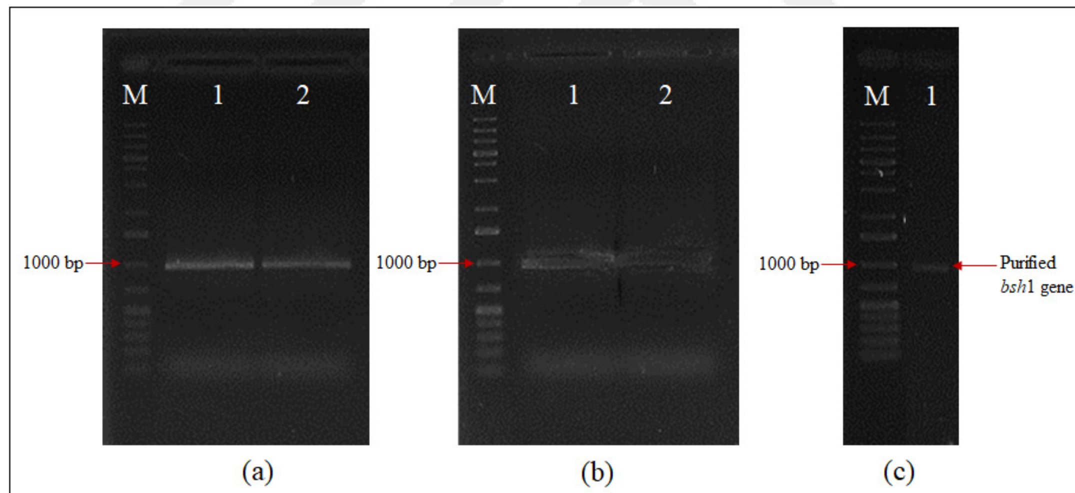

Figure 4.2. Purification of the *Lb. plantarum* B14 *bsh* gene PCR products from 1% agarose gel. M, 1 kb plus DNA ladder. a, *Lb. plantarum* B14 *bsh* gene PCR products; b, sliced *Lb. plantarum* B14 *bsh* gene PCR products; c, purified *bsh*1 gene (aproximately 1.0 kb) from *Lb. plantarum* B14.

4.1.2 Preparation of the pBluescript KS$^+$ Cloning Vector

For preparing cloning vectors, pBluescript KS$^+$ (2.9 kbp) was isolated from *Ecoli* XL1 stain and digested with *SmaI*. Then digested pBluescript DNAs were purified from 1% agarose gel electrophoresis (Figure 4.3).

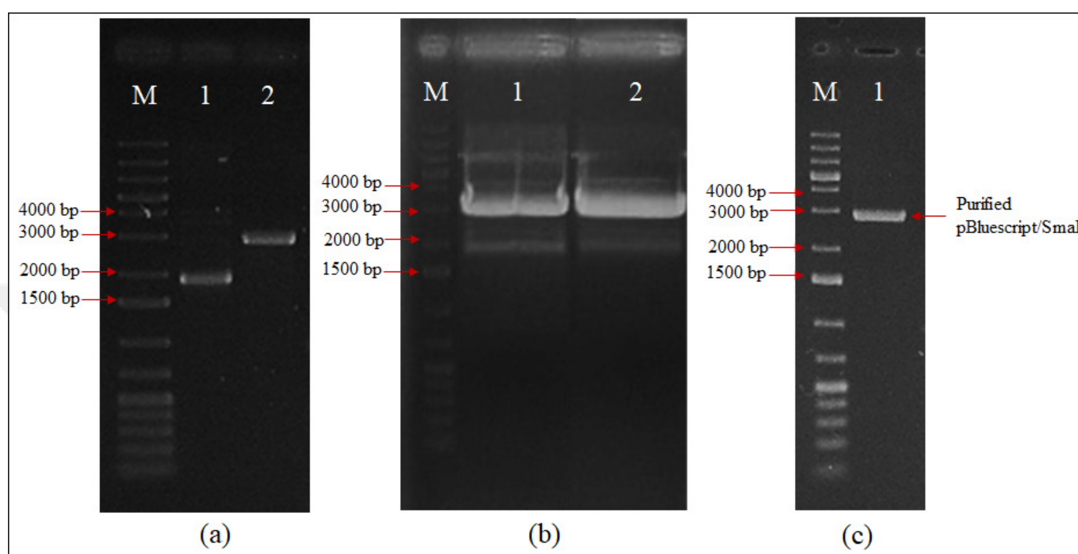

Figure 4.3. Preparation of pBluescript cloning vector DNA. M, 1 kb plus DNA ladder; a, 1: uncut pBluescript DNA; 2: *Sma*I digested pBluescript DNA; b, *Sma*I digested pBluescript linear DNA before gel purification; c, gel purified linear pBluescript DNA.

4.1.3 Preparation of the pCON1

After ligation and transformation procedures, white colonies were selected from the LB plate supplemented with ampicillin, IPTG and X-Gal. Selected white colonies were grown and inoculated on LB plates and in LB liquid medium for plasmid isolation (Figure 4.4). The ligation product, pBluescript/*bsh* was detected on gel electrophoresis. Orientation of the insert in the clone was verified by *Xba*I restriction enzyme. Aproximately 3.6 kbp and 0.3 kbp DNA bands were detected on an agarose gel indicating the presence of the right oriented clones (Figure 4.5). The new contruct was named pCON1.

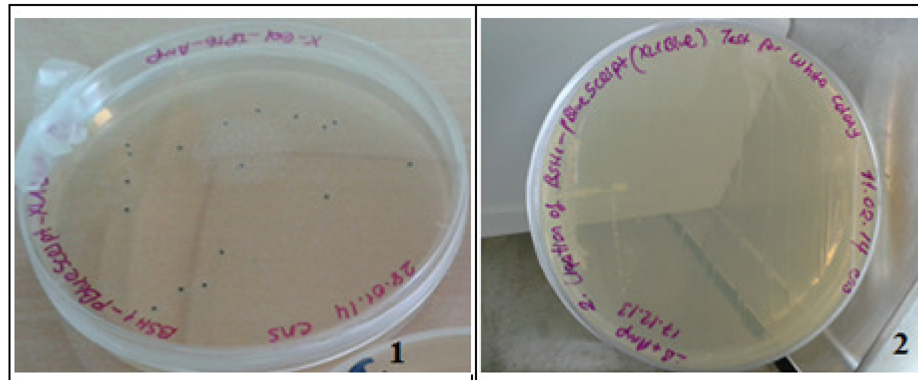

Figure 4.4. Selection of the white colonies on LB plates containing ampicillin, IPTG and X-Gal. 1: Transformants on LB plate supplemented with ampicillin, IPTG and X-Gal; 2: pBluescript/*bsh*1 on LB plate supplemented with ampicillin.

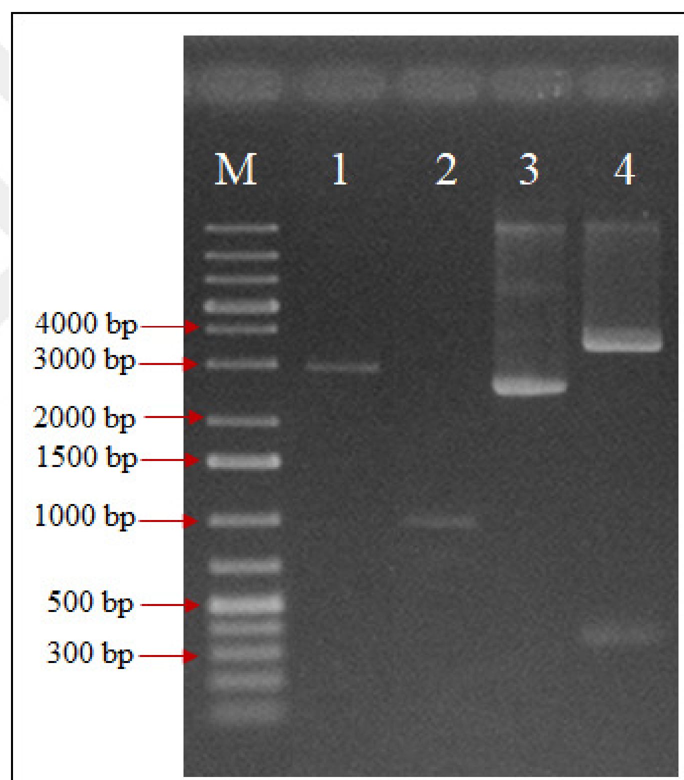

Figure 4.5. Preparation of the pCON1 construct. M, 1 kb plus DNA ladder; 1, gel purified linear pBluescript DNA; 2, gel purified *bsh*1 gene; 3, uncut pCON1 DNA; 4, *Xba*I cut pCON1 DNA.

4.2 Sequence Analysis of Wild-type BSH Enzyme

The *bsh* gene on pCON1 construct was sequenced at Macrogen (Seoul, Korea) by using universal primers, M13-PUC 5-GTAAAACGACGGCCAGT-3 and M13R-PUC, 5’-CAGGAAACAGCTATGAC-3’. The clone that have wild

type *bsh* genes in pCON1 was visulised by the BioEdit Sequence Alignment program and visulised by the SIB (Swiss Institute of Bioinformatics) ExPASy sequence translate tool (http://web.expasy.org/translate/) (Figure 4.6).

Figure 4.6. The amino acid residues of the wild type *bsh* gene. Frames represent target amino acids for site directed mutagenesis.

```
Bifidobacterium adolescentis ATCC bsh                     1 -MCTGVRFSDEEGNMYFGRNLDWSFSY---------GESILATPRGYHYDN-VFGAER-KATPNAVIGVGVVMADRPMYFDCANEHGLA 77
Bifidobacterium longum bsh                                1 -MCTGVRFSDDEGNTYFGRNLDWSFSY---------GETILVTPRGYHYDT-VFGAGG-KAKPNAVIGVGVVMADRPMYFDCANEHGLA 77
Bifidobacterium bifidum bsh                               1 -MCTGVRFSDDEGNMYFGRNLDWSFSY---------GETILVTPRGYQYDY-VYGAEG-KSEPNAVIGVGVVMVDRPMYFDCANEHGLA 77
Bifidobacterium breve DSM bsh                             1 -MCTGVRFSDDEGNTYFGRNLDWSFSY---------GETILVTPRGYRYDY-AYGAKG-KSEPNAVIGVGVVMADRPMYFDCANEHGLA 77
Bifidobacterium animalis subsp. lactis KL612 bsh          1 -MCTAVRFDDGQNNMYFGRNLDWSEDY---------GEKIVFAPHDYHY---APAFNA-EDKNHPVIGIGIIVEDTPLYFDCMNDAGLA 75
Lactobacillus_johnsonii pf01 conjbsh                      1 -MCTGLRFTDDQGNLYFGRNLDVGQDY---------GEGVIITPRNYPL-P-YKFLDN-TTTKKAVIGMGIVVDGYPSYFDCYNEDGLG 76
Enterococcus faecium bsh                                  1 -MCTSITYVTS--DHYFGRNFDYEISY---------NEVVTITPRNYKL-N-FRKVND-LDTHYAMIGIAAGIADYPLYYDATNEKGLS 74
Enterococcus durans bsh                                   1 -MCTSITYVTS--DHYFGRNFDYEISY---------NEVVTVTPRNYKL-N-FRKVND-LDNHYAMIGIAAGIADYPLYYDATNEKGLS 74
Lactobacillus delbrueckii ACNH                            1 -MCTAITFATN--DHYIGRNLDLDFSY---------NETVTITPRNYVFPM--RKVPD-LNSHYAIIGMATVVGDYPLYYDAVNEKGLG 74
Lactobacillus acidophilus bsha                            1 -MCTSIIFSPK--DHYFGRNLDLEITF---------GQQVVITPRNYTF-K-FRKMPS-LKKHYAMIGISLDMDDYPLYFDATNEKGLG 74
Streptococcus pasteurianus ATCC ch                        1 -MCTAITYQTK--DNYFGRNLDLDFSY---------HEEVTICPRNYPL-S-FKYETK-QDTHLAIIGMATVVGDYPLFYDATNEKGLS 74
Lactobacillus ruminis S23 ch                              1 -MCTAATYTGK--DHYFGRNLDLELSY---------NESVTVTPRKFPL-K-FHQVHD-MNEHFAIIGMATVVADYPLYYDATNEKGLS 74
Listeria monocytogenes bsh                                1 -MCTSITYTTK--DHYFGRNFDYELSY---------KEVVVVTPKNYPF-H-FRKVED-IEKHYALIGIAAVMENYPLYYDATNEKGLS 74
Lactobacillus gasseri ATCC bsh                            1 -MCTSILYSPK--DHYFGRNLDYEIAY---------GQKVVITPRNYEF-K-FANLPA-EKSHYAMIGIAAVANNTPLYCDAINEKGLG 74
Lactobacillus_johnsonii pf01 bshA                         1 -MCTSIVYSSN-NHHYFGRNLDLEISF---------GEHPVITPRNYEF-Q-YRKLPN-KKAKYAMVGMAIVEDNYPLYFDASNEEGLG 75
Clostridium perfringens 13 CBH                            1 -MCTGLALETKDGLHLFGRNMDIEYSF---------NQSIIFIPRNFKCVN-KSNKKE-LTTKYAVLGMGTIFDDYPTFADGMNEKGLG 77
Lactobacillus hominis DSM pbsh                            1 -MCTSILYSPK--DHYFGRNLDYEIAY---------GQKVVITPRNYEF-E-FTDLPA-EKSHYAMIGVAAVADNTPLYCDAINEKGLG 74
Streptococcus infantarius subsp. infantarius CJ18 cbsh    1 -MCTAITYTTK--DSYFGRNLDLDFSY---------NETVTICPRTYPF-S-FKHKGE-NKDHYAMIGMATVVADYPLFYEAVNEKGVG 74
Lactobacillus plantarum B14 bsh                           1 -MCTAITYQSY--NNYFGRNFDYEISY---------NEMVTITPRKYPL-V-FRKVEN-LDHHYAIIGITADVESYPLYYDAMNEKGLC 74
Lactobacillus plantarum WCFS1 CBAH                         1 -MCTAITYQSY--NNYFGRNFDYEISY---------NEMVTITPRKYPL-V-FRKVEN-LDHHYAIIGITADVESYPLYYDAMNEKGLC 74
Listeria ivanovii subsp. ivanovii PAM 55 pcbah            1 -MCTSITYTTR--DHYFGRNFDYELSY---------NEVLVVTPKNFPF-H-FRKVEN-MKQHYALIGIASVMENYPLYYDATNEKGLS 74
Lactobacillus coryniformis bsh                            1 -MCTAITYQSY--NNYFGRNFDYEISY---------NEMVTITPRKYPL-V-FRKVEN-LDHHYAIIGITADVESYPLYYDAMNEKGLC 74
Lactobacillus crispatus CNH                               1 -MCTSIIFSPQ--DHYFGRNLDLEITF---------GQQVVVTPRNYAF-N-FRKMPE-MKHHYAMVGIALDAGNYPLYFDAANEKSLG 74
Lactobacillus reuteri bsh                                 1 -MCTSVIYTAG--DYYFGRNLDLEVNL---------GQEVVITPRNKTL-E-FREMPN-LEHHYAIIGMSIVRDDYPLYFDGVNEKGVG 74
Lactobacillus salivarius ACNH                             1 -MCTAITLNGN--NNYFGRNLDLDFSY---------GEQVIITPAEYEF-K-FRKEKA-IKNHKSLIGVGIVANDYPLYFDAINEDGLG 74
Streptococcus lutetiensis cbsh                            1 -MCTAITYTTK--DNYFGRNLDLDFSY---------NETVTICPRNYPF-S-FRHQGE-NNSHFAMIGMATVVADYPLYYEAVNEKGLG 74
Lactobacillus acidophilus bshb                            1 -MCTSICYNPN--DHYFGRNLDYEIAY---------GQKVVIVPRNYEF-K-YREMPS-QKMHYAFIGVSVVNDDYPLLCDAINEKGLG 74
Lactobacillus antri ACNH                                  1 -MCTSILYTAG--DCYFGRNLDLEVSF---------GQEVVITPRDYRF-N-FRQMPA-LDHHYAIIGMALVQDNYPLYFDGANEEGLG 74
Methanobrevibacter smithii CBAH                           1 -MCTAANYLTK--CHYFGRNFDYEISY---------NERVTITPRNYPL-I-FRDTDD-IENHYGIIGIAAGIDEYPLYYDACNEKGLA 74
Streptococcus pneumoniae ch                               1 -MCTSLTLQTKNFQHLFARTMDFTLDM---------NQEVIIIPRRYQW-N-NITGET-IRTKKAVVGMGINFGGRVMMADGVNEAGMT 76
Eubacterium cylindroides ATCC pch                         1 -MCTSFTYYNN--DFYLGRNLDLDCGF---------GEEVVITPREYRF-Q-FRHEPT-IEKHSAMIGMATIVEGYPLYAEAINEHGLG 74
Bacillus cereus group ACNH                                1 -MCTSLTLQTKNGQHLFARTMDFTLDM---------NQEVIIIPRHYQW-N-NITGEI-INTKHATVGMGINHQGRIIMADGVNEAGMT 76
Bacillus mycoides ACNH                                    1 -MCTSLTLQTKSGKHLFARTMDFTLDF---------NQEVIIVPRSYQW-N-NITGET-IEAKQAVVGMGINHQGRIILADGVNESGMT 76
Bacillus anthracis ACNH                                   1 -MCTSLTLETKNGQHLFARTMDFTLDM---------NQEVIIIPRHYQW-N-NITGEI-INTKHATVGMGINHQGRIIMADGVNEAGMT 76
Bacillus thuringiensis ACNH                               1 -MCTSLTLQTKSGQHLFARTMDFALDF---------NQEVIIVPRRYQW-N-NITGET-IKAKQAVVGMGINDQGRIILADGVNESGMT 76
Lactobacillus buchneri CD034 ch                           1 -MCTSVTFLSETGDNFLARTMDFAFEL---------DGRPIVIPRKQHF-D-SDAAEAGYDTTYGFVGAGRNLGHY-ILVDGVNEKGLS 76
Carnobacterium maltaromaticum ACNH                        1 MMCTSLIYQSNDQSNFLSRTMDFAFEL---------DASPVYLPRGYVFKSNVVSNVT-YKSKYAFLGAGRKLEDY-FFADGLNEKGLS 78
Staphylococcus haemolyticus ACNH                          1 -MCTGFTFQAKNGDVILGRTMDYDYPL---------TGHPAVQPRHYYW-E-SRVDYK-GTTTYGFTGAGSDMEGF-IFGDGVNEHGLA 75
Lactobacillus salivarius bsh                              1 -MCTSVSVISEDGTHVMGRTMDW-YDL---------YVKPMYIPRGYQWKS-AFDNKK-YTNKYAIVGGGFQDNNYIDLSDGVNECGLM 76
Staphylococcus epidermidis ACNH                           1 -MCTAISLYTKQRYHYLARTMDFAFEF---------NGIPTIVPRHYHY-Q-FDLDSD-MRLEYGFVGTNLKVGRY-RFGDGINEKGLA 75
Lactobacillus terrae ACNH                                 1 -MCTSLSYEALDGTKFLARTMDFAFEL---------NGKPTFLPRGYNWLS-SLDNKT-YNSSYAILGTGAKYGHNYMVADGFNEHGLA 77
Lactobacillus rhamnosus bsh                               1 -MCSSMTIKSLQGDIFWGRTMDYNTSFFHESPAGGVPGKIVSLPANQTLPA---QTAT-WKTKYAAVGVG--VDQSVALFDGVNSEGLA 82
Lactobacillus fermentum NCDO bsh                          1 -MCTSINVIAQDGYHVLGRTMDWD-DL---------LVSPIFTPRHYQLASVFDHRVH--ENPYAIIGGGSITERRTDVSDGVNEFGLM 76
Staphylococcus aureus CC5 ch                              1 -MCTGFTIQTLNNQVLLGRTMDYDYPL---------DGSPAVTPRNYRW-T-SRTGTT-GQTQYGFIGTGTDMEGF-IYGDGVNEHGVA 75
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Figure 4.7. The multiple sequence alignment of BSH, PVA and CBAH proteins by Jalview 2.8.0b1. (a, 1-74 aminoacid sequence, b, 75-156 aminoacid sequence, c, 157-241 aminoacid sequence, d, 242-324 aminoacid sequence for *Lb. plantarum*).

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Bifidobacterium adolescentis ATCC bsh                78 IAGLNFPG--YAEFVHEPVEGTDNVATFEFPLWVARNFDSVDEVEEALKNVTLVSQIVP---GQQESLLHWIIGDSE-RSIVVEQM-AD 159
Bifidobacterium longum bsh                           78 IAGLNFPG--YASFVHEPVEGTENVATFEFPLWVARNFDSVDEVEETLRNVTLVSQIVP---GQQESLLHWFIGDGK-RSIVVEQM-AD 159
Bifidobacterium bifidum bsh                          78 IAGLNFPG--YASFAHEPVEGTENVATFEFPLWVARNFDSVDEVEEALKNVTLVSQVVP---GQQESLLHWFIGDGT-RSIVVEQM-AD 159
Bifidobacterium breve DSM bsh                        78 IAGLNFPG--YASFAHEPVEGTENVATFEFPLWVARNFDSVDEVEEALKDVTLVSQVVP---GQQESLLHWFIGDGT-RSIVIEQM-AD 159
Bifidobacterium animalis subsp. lactis KL612 bsh     76 VAGLNFAK--YCKYATEAVNFTTNVAAYEFPLWVTRNFTSVDDVQEALKNVTIVGKPIN--DRFPVATLHWIIADNT-RSIVVECT-ED 158
Lactobacillus johnsonii pf01 conjbsh                 77 IAGLNFPH--FAKFSDGPIDGKINLASYEIMLWVTQNFTHVSEVKEALKNVNLVNEAIN--TSFAVAPLHWIISDSD-EAIIVEVSKQY 160
Enterococcus faecium bsh                             75 MAGLNFSG--YADYKE-IQEGKDNVSPFEFIPWILGQCSTVGEAKKLLKNINLANINYS--DELPLSPLHWLLADKE-KSIVIESM-KD 156
Enterococcus durans bsh                              75 MAGLNFSG--YADYKE-IQEGKDNVSPFEFIPWILGQCSTVGEAKKLLKNINLANINYS--DELPLSPLHWLLADKE-KSIVIESM-KD 156
Lactobacillus delbrueckii ACNH                       75 MAGLNFPG--NAHYFP-DQEGKDNIASFEFIPYILGTCKSVAEAKEALKKISISAEDFS--KDFPTSTLHWIIADKD-SSIVVESM-ED 156
Lactobacillus acidophilus bsha                       75 MAGLNYPG--NATYYE-EKENKDNIASFEFIPWILGQCSTISEVKDLLSRINIADLNFS--EKMQASSLHWLIADKTGTSLVVETD-KD 157
Streptococcus pasteurianus ATCC ch                   75 MAGLNFPE--NADFKPAKD-GKTNVASFEFMLWILSQCESVSEVRQQCENLNLTDDAFS--SDYPVSPLHWMISDSK-ESIVVEPV-KD 156
Lactobacillus ruminis S23 ch                         75 MAGLNFPG--NADYKEPAE-DVDNVASFEFIPWILGQCETVADVRKLLAKINITNVEFS--EQFPPSPLHWMISDKN-ESITVEQT-KA 156
Listeria monocytogenes bsh                           75 MAGLNFSG--NADYKD-FAEGKDNVTPFEFIPWILGQCATVKEARRLLQRINLVNISFS--ENLPLSPLHWLMADQT-ESIVVECV-KD 156
Lactobacillus gasseri ATCC bsh                       75 VAGLSFAG--QGKYFP-VVEDKKNIASFEFISYILATYETVDQVKENLTDVNISDVSFS--KNTPASELHWLVGDKTGKSIVVESD-EK 157
Lactobacillus johnsonii pf01 bshA                    76 IAGLNFDG--PCHYFP-EVSGKNNVTPFELIPYLLSQYTTVAEVKEALKSVNLVKINFS--EKLQLSPLHWLMADKTGESIVVEST-LS 158
Clostridium perfringens 13 CBH                       78 CAGLNFPV--YVSYSKEDIEGKTNIPVYNFLLWVLANFSSVEEVKEALKNANIVDIPIS--ENIPNTTLHWMISDITGKSIVVEQT-KE 161
Lactobacillus hominis DSM pbsh                       75 VAGLSFAG--QGKYFP-NAADKKNIASFEFISYLLATYETVDQVKESLTNANISNVSFA--KNTPASELHWLVGDKTGKSIVVESD-EK 157
Streptococcus infantarius subsp. infantarius CJ18 cbsh 75 MAGLNFPE--NADYKEVAK-GKDNVASFELIPWLLSQCDSVAQVKELCQNLNVTNEAFS--DDFQPSPLHWLIADRN-ESIVLESV-AS 156
Lactobacillus plantarum B14 bsh                      75 IAGLNFAG--YADYKK-YDADKVNITPFELIPWLLGQFSSVREVKKNIQKLNLVNINFS--EQLPLSPLHWLVADKQ-ESIVIESV-KE 156
Lactobacillus plantarum WCFS1 CBAH                    75 IAGLNFAG--YADYKK-YDADKVNITPFELIPWLLGQFSSVREVKKNIQKLNLVNINFS--EQLPLSPLHWLVADKQ-ESIVIESV-KE 156
Listeria ivanovii subsp. ivanovii PAM 55 pcbah       75 MAGLNFSG--NADYKE-MVEGKDNVTPFEFIPWILGQCATVKEARVLLEKINLVNISFS--DNLPLASLHWLMADQT-ESIVIESV-KD 156
Lactobacillus coryniformis bsh                       75 IAGLNFAG--YADYKK-YDADKVNITPFEIIPWLLGQFSSVREVKKNIQKLNLVNINFS--EQLPLSPLHWLVADKQ-ESIVIESV-KE 156
Lactobacillus crispatus CNH                          75 MAGLNYPD--NATYYD-EVANKDNIASFEFIPWILGQCATVADAKVLLKKINIVNLNFS--DKMQASPLHWLIADKTGVSIVVETD-KD 157
Lactobacillus reuteri bsh                            75 MAGLNFDG--PAHYFPVQE-GKDNIASFELVPYILAAASSVAEAKKLLSNANIANINFS--DKLQAAPLHWIIADKTGASVTVEST-AK 157
Lactobacillus salivarius ACNH                        75 MAGLNFPG--NAYYSDALENDKDNITPFEFIPWILGQCSDVNEARNLVERINLINLSFS--EQLPLAGLHWLIADRE-KSIVVEVT-KS 157
Streptococcus lutetiensis cbsh                       75 MAGLNFPE--NADYKEVAE-GKDNLASFELIPWFLSQCQSVAEVKKLCQNLNVTNEAFS--DDFQPSPLHWLIADRN-EAIVLESV-AS 156
Lactobacillus acidophilus bshb                       75 IAGLNFQG--PNHYFP-KIEGKKNIASFELMPYLLSNCENTDDVKEILDNANILNISFS--ANYPAADLHWILSDKAGKSIAVEST-NS 157
Lactobacillus antri ACNH                             75 MAGLNFAG--PAHYFPVDD-HKDNVSPFEFIPYILGQCKNVVEAKQLLKKLNLVKINFS--DHLQLSPLHWLIADRSGKSIVVEST-VS 157
Methanobrevibacter smithii CBAH                      75 MGGLNFPD--YCDYKPLDK-SKVNIASFEIIPYILSQAKTISDAERLLENLNISDEKFS--AQLPPSPLHWIISDRN-ASIVVEVV-EE 156
Streptococcus pneumoniae ch                          77 CATLYFPG--FATYSSHVDSNKTNVAPFDFVTWSLTQFNSVEELRKSIDSIAFIDVPLP--ILGVTPPLHWILADKSGECIVLEPT-AD 160
Eubacterium cylindroides ATCC pch                   75 ICGLEFKG--NAKYFD-LMDGKDNIAPFEIIPWILSNCVTAKEARSYFEKMNIIDEDFA--PNLPLSPLHWIIADKD-DCFVVEST-KD 156
Bacillus cereus group ACNH                           77 CATLYFPG--FATYSQSIDDNTTNLAPFDFVTWSLTQFNSVKELKKSVDSITFLDIPLP--DLGLTPPLHWILADKWGDCIVLEPT-SE 160
Bacillus mycoides ACNH                               77 CATLYFPG--FASYSNSIDENKTNLAPFDFVAWSLTQFNSVEELKNSVDSISFLDVPLP--VLGVTPPLHWILADKWGDCIVLEPT-TE 160
Bacillus anthracis ACNH                              77 CATLYFPG--FATYSQSIDDNTTNLAPFDFVTWSLTQFNSVKELKKSVDSITFLDIPLP--DLGLTPPLHWILADKWGDCIVLDPT-SE 160
Bacillus thuringiensis ACNH                          77 CATLYFPG--FATYSNSIDDNKTNLAPFDFVAWSLTQFNSVEELKKSVDSISFLDVPLS--VLGVIPPLHWILADKWGECIVLEPT-IE 160
Lactobacillus buchneri CD034 ch                      77 AAALYFSG--EATYAKTAKTNRVNLAPHEVLNWLLGNASDCEDLGRKVDQLNIIDAPVK--ILGKATPLHWIISDRSGACYVLEML-AD 160
Carnobacterium maltaromaticum ACNH                   79 VCALYFSD--YAEYNPSQEPGKVNIAPHELVSWLLGNIASIDELKKEASNINVVSAKNN--LLDVIVPLHWIVADQTGSSIIIEIT-KS 162
Staphylococcus haemolyticus ACNH                     76 ISNQYDRG--YASYANKIHDGYINISQTEVLTWVLGYNKTIEELIEQADQVNVVAHTLN--DINEAPPLHYHVSDATGRTAEITFV-EG 159
Lactobacillus salivarius bsh                         77 AQKLTFSN--GAQLVDDKHDDKIQLEAYEFVTYILGNFSSVTEVEENIEKIELMSNVIN-NTKHGGSELHFSLSDESGRNIIVEPS-QH 161
Staphylococcus epidermidis ACNH                      76 ISNHYFTG--EASYSTHKRYGYFNLAPEEFIVWVLGFNKSISELKQKVKKINIMNEKNT--TLNIVPPLHFMVTDETGHTVAIEPH-NG 159
Lactobacillus terrae ACNH                            78 CAELYFDH--EAVYEAAPEEGKINLVSEEFILWVLGHNKSIAELRKNLEDVRIIDSDAG--VMGANQPLHWIVTDRSGATYVIEPR-GQ 161
Lactobacillus rhamnosus bsh                          83 GDLQVLVECSWASAESLAKRNLKPIKGEEFVTLALTTCKNVAEVRALASQYGLLDEPFQFGGQGVKIPLHYTFVDPSGAGLVIELT-GH 170
Lactobacillus fermentum NCDO bsh                     77 AQKLTFKN--GARLVDERHPDKVQLAAFELIFYLLGHFKSVADVAAHLDQIELMNDVNA-DVPFGYSEQHFVLSDPTGRCVVIEPS-EH 161
Staphylococcus aureus CC5 ch                         76 ISTQYFRG--YSSYGSTHKADAMNITQNEIVTWILGYTTSIEDMKQQASQIHVVAVYLN--DIGEVPPLHYHVSDATGHSVEVSFK-EG 159
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Figure 4.7. (cont'd)

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Bifidobacterium adolescentis ATCC bsh                        160 GMHVHHDDVDVLTNQPTFGFHMENLRNYMCVGNEM-AEPATWGKASLSA--WGA-GVSMHGIPGDVSSPSRFVRVAYANTHYPQQEGEA 244
Bifidobacterium longum bsh                                   160 GMHVHHDDVDVLTNQPTFDFHMENLRNYMCVSNEM-AEPTSWGKASLTA--WGA-GVGMHGIPGDVSSPSRFVRVAYTNAHYPQQNDEA 244
Bifidobacterium bifidum bsh                                  160 GMHVHHDDVDVLTNQPTFDFHMENLRNYMCVSNEM-AEPTTWGKAELSA--WGA-GVSMHGIPGDVSSPSRFVRVAYTNTHYPQQNNEA 244
Bifidobacterium breve DSM bsh                                160 GMHVHHDDVDVLTNQPTFDFHMENLRNYMCVSNEM-AEPTTWGKAELSA--WGA-GVSMHGIPGDVSSPSRFVRVAYTNTHYPQQNNES 244
Bifidobacterium animalis subsp. lactis KL612 bsh             159 GMHVYDDDVDVLTNQPPFPQQIEHLDNYAYVSPRT-GKSVKWGSSELET--NQD-SNSSQGLPGGYGSMARFVRAAYNNTHYPTQSGEN 243
Lactobacillus johnsonii pf01 conjbsh                         161 GMKVFDDKVGVLTNSPDFNWHLTNLGNYTGLNPHD-ATAQSWNGQKVAP--WGV-GTGSLGLPGDSIPADRFVKAAYLNVNYPTAKGEK 245
Enterococcus faecium bsh                                     157 GLHIYDNPVGVLTNNPSFDYQLFNLNNYRVLSSET-PKNNFSNQISLNA--YSR-GMGGIGLPGDLSSVSRFVKATFTKLNSVSGDSES 241
Enterococcus durans bsh                                      157 GLHIYDNPVGVLTNNPSFDYQLFNLNNYRVLSSET-PKNNFSNQISLNA--YSR-GMGGIGLPGDLSSVSRFVKATFTKLNSVSGDSES 241
Lactobacillus delbrueckii ACNH                               157 GLHVYDNPVGVMTNNPPFPIMKFALNDYYALSAHC-LDHKFADGVELTE--YSR-GMSSIGLPGDLSSKSRFVRCVFTKYNSLCDKDEA 241
Lactobacillus acidophilus bsha                               158 GMHIYDNPVGCLTNNPQFPKQLFNLNNYADVSPKM-PKNNFSDKVNMAG--YSR-GLGSHNLPGGMDSESRFVRVAFNKFNAPIAETEE 242
Streptococcus pasteurianus ATCC ch                           157 KVAIYDNPIGVLTNNPTFDKQLFNLNNYRHLSPKV-SDNLFSKVLDLDV--YSR-GMGGIGLPGDLSSMSRFVKVAFTKLNAVADCSEA 241
Lactobacillus ruminis S23 ch                                 157 GLNVYDNPVGIMTNNPEFPFQMFTLNNYRRVSPKP-VASTFADGLELDE--YTR-GMGSMGLPGDLSSNSRFVKATFTKLNAPKMADEN 241
Listeria monocytogenes bsh                                   157 GLHIYDNPVGVLTNNPTFDYQLFNLNNYRVLSSET-PENNFSKEIDLDA--YSR-GMGGIGLPGDLSSMSRFVKATFTKLNSVSGDSES 241
Lactobacillus gasseri ATCC bsh                               158 GLHVYDNPVNALTNAPLFPQQLTNLANYAAVVPGQ-PNNDFLPGVDLKM--YSR-SLGTHHLPGGMDSESRFVKVCFALNHAPKDSDEV 242
Lactobacillus johnsonii pf01 bshA                            159 GLHVYDNPVHVLTNNPEFPGQLSNLANYSNIAPSQ-PKNTLVPGVDLNL--YSR-GLGTHFLPGGMDSASRFVKVAFVRAHSPQGNNEL 243
Clostridium perfringens 13 CBH                               162 KLNVFDNNIGVLTNSPTFDWHVANLNQYVGLRYNQ-VPEFKLGDQSLTA--LGQ-GTGLVGLPGDFTPASRFIRVAFLRDAMIKNDKDS 246
Lactobacillus hominis DSM pbsh                               158 GLHVYDNPVNALTNAPLFPEQLTNLANYASVVPGE-PDNNFLPGVNLKL--YSR-SLGTHHLPGGMDSESRFVKVCFALNHAPKDSNEV 242
Streptococcus infantarius subsp. infantarius CJ18 cbsh       157 GLKVYDNPTGVLTNNPTFDKQLFNLNNYRHLSPKV-SDNLFSTEIQLDT--YSR-GMGGLGLPGDLSSMSRYVKVAFTKLNSVAEDTEA 241
Lactobacillus plantarum B14 bsh                              157 GLKIYDNPVGVLTNNPNFDYQLFNLNNYRALSNST-PQNSFSEKVDLDS--YSR-GMGGLGLPGDLSSMSRFVRAAFTKLNSLPMQTES 241
Lactobacillus plantarum WCFS1 CBAH                           157 GLKIYDNPVGVLTNNPNFDYQLFNLNNYRALSNST-PQNSFSEKVDLDS--YSR-GMGGLGLPGDLSSMSRFVRAAFTKLNSLSMQTES 241
Listeria ivanovii subsp. ivanovii PAM 55 pcbah               157 GLHIYDNPVGVLTNNPPFNYQLFNLNNYRVLSSKT-PENHFSKKLELDP--YSR-GMGGMGLPGDLSSMSRFVKVAFTKLNSISGNSES 241
Lactobacillus coryniformis bsh                               157 GLKIYDNPVGVLTNNPNFDYQLFNLNNYRALSNST-PQNSFSEKVDLDS--YSR-GMGGLGLPGDLSSMSRFVRAAFTKLNSLPMQTES 241
Lactobacillus crispatus CNH                                  158 GMHVYDNPVGCLTNNPQFSCQLFNLNNYADVSPAM-PKNNFSKEVNMNG--YSR-GLGSRNLPGGMDSESRFVRVAFNKFNAPVGKSEE 242
Lactobacillus reuteri bsh                                    158 GLNVYDNPVGVLTNNPEFPRQLLNLSNYRSVAPAN-PANVFAPNVDLPV--YSR-GLGTHFLPGGMDSESRFVKATFTKMHAPVGNSEV 242
Lactobacillus salivarius ACNH                                158 GVHIYDNPIGVLTNNPEFNYQMYNLNKYRNLSIST-PQNTFSDSVDLKV--DGT-GFGGIGLPGDVSPESRFVRAAFSKLNSSKGTTVE 242
Streptococcus lutetiensis cbsh                               157 GLKVYDNPTGVLTNNPTFDKQLFNLNNYRHVSPKV-SDNLFSTEITLDT--YSR-GMGGLGLPGDLSSMSRYVKAAFTKLNSVAEDTEV 241
Lactobacillus acidophilus bshb                               158 GLHIYDNPVNVLTNNPEFPDQLIKLSDYADVTPHN-PKNTLVPNVDLNL--YSR-GLGTHHLPGGMDSSSRFVKVAFVLAHTPQGKNEV 242
Lactobacillus antri ACNH                                     158 GLHVYDNSVNVLTNNPEFPGQLTNLANYQSISPEQ-PTNGLAPNIKIGF--YSR-GLGSRMLPGGMDSQSRFVKEVFTLQHAPAGASEV 242
Methanobrevibacter smithii CBAH                              157 GLDIYDNPVGVLTNNPPFDKQLFNLNNYMALSNRT-PENTFGGNLDLAT--YSR-GMGSIGLPGDVSSQSRFVKAAFVKENSVSGDSEK 241
Streptococcus pneumoniae ch                                  161 GIKVYDNPIGVMTNSPEFSWHLQNLRQYIGLKSQP-FAPTEWGDVPLSA--FGQ-GSGSMGLPGDFTPPSRFVRAAYGKQNIQSIENEE 245
Eubacterium cylindroides ATCC pch                            157 GLMIYDNIFGTLTNNPPFPYHLSNMINYLNLTPKY-PENRFSKRLDLEP--FAN-GMGALGLPGDASSPSRFVKTVFLKENMTSSGTEE 241
Bacillus cereus group ACNH                                   161 GLKLYDNSLGVMTNSPEFNWHLQNLRQYIGLKSQP-FAPTEWSNLPLSA--FGQ-GSGSMGLPGDFTPPSRFVRAAYGKQNIQGIDNEE 245
Bacillus mycoides ACNH                                       161 GLKTYDNPLGVMTNSPEFNWHLQNLRQYIGLKSQP-YAPTQWGDVPLSA--FGQ-GSGSMGLPGDFTPPSRFVRAAYGKQNIQGLDNEE 245
Bacillus anthracis ACNH                                      161 GLKLYDNPLGVMTNSPEFNWHLQNLRQYIGLKSQP-FAPTEWSNLPLSA--FGQ-GSGSMGLPGDFTPPSRFVRAAYGKQNIQGIDSEE 245
Bacillus thuringiensis ACNH                                  161 GLKMYDNPLGVMTNSPEFNWHLQNLRQYVGLKSQP-YAPTQWGDVPLSA--FGQ-GSGSMGLPGDFTPPSRFVRAAYSKQNIQGVDNEE 245
Lactobacillus buchneri CD034 ch                              161 GVHYQKNPVGVMTNSPDFGWHLKNLSNYTELKPSP-HPARSYNGYEITS--FGP-GSGALGMPGDYTSVSRFIRTVFMREYADQVPT-D 244
Carnobacterium maltaromaticum ACNH                           163 GMSIYDNKVRVMTNSPDYPWHLANLNHYSFLSNQL-KPASHFYKFKPNS---GELGNGAFGMPGDYTSESRFIRTVFNAEFTEPGKDKI 247
Staphylococcus haemolyticus ACNH                             160 RIVLHDNPVGVLTNSPDLNWHYENLKNYANVTPYK-PQYKRYKNLY-----IGN-ESGTSGLPGGYTSAERYVRAAYLVSNML-PDDGD 240
Lactobacillus salivarius bsh                                 162 PMRIIDNPLGVVTNMPKFERQLAKLENYMEFTDEF-KENSIKYGKFHVT--TGKLG-GKKTPPGSYSPSQRFIRASYLKELVDKPKTRD 246
Staphylococcus epidermidis ACNH                              160 LLIVKDNYVHTLTNEPKLDWHLSNLRNYAFLTPQK-STNQLIGKVLVRS--MGC-EAGTNGLPGGYTSTDRFIRATYLRHQLRCSHNED 244
Lactobacillus terrae ACNH                                    162 GLELEEDKVGVMTNTPDYQWHKTNLSNYLGVQTTN-FQGMRFGNQEVEK--LGQ--NGTFRLPGGFTAVDRFVRESFVHEVVQKPNTAS 245
Lactobacillus rhamnosus bsh                                  171 GAFKLYDSVGAMTNSPEYGWHTTNLRNYVSLNDRDYPEGADLGDQHLEPIELGT-GYGMFGLPGDYTSPSRFVRAMFVSRNLDPFNS-N 257
Lactobacillus fermentum NCDO bsh                             162 PLKLIDNPLGIMTNMPKFDHQLERLQDYLDFTPDF-LNGTLAPNTFHVT--TGK-LSGKKTPPGAYTPKGRYVRAAYMKELADQPASKD 246
Staphylococcus aureus CC5 ch                                 160 EVVIKDNPIGVLTNHPDLDWHYSNLRQYINISPYP-ATAKLLEGVTIEP--LGN-EAGTFGLPGGFTSTERFVRMAFMKANIAQNNDKE 244
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Figure 4.7. (cont'd)

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Bifidobacterium adolescentis ATCC bsh                        245 ANVSRLFHTLGSVQMVDGM-AKMS-NGQFERTLFTSGYSSKTNTYYMNTYDDPAIRSYAMADFDMDSS-ELITAA-------------- 316
Bifidobacterium longum bsh                                   245 ANVSRLFHTLGSVQMVDGM-AKMG-DGQFERTLFTSGYSSKTNTYYMNTYDDPAIRSYAMADYDMDSS-ELISVAR------------- 317
Bifidobacterium bifidum bsh                                  245 ANVSRLFHTLVSVQMVDGM-SKMG-NGQFERTLFTSGYSGKTNTYYMNTYEDPAIRSFAMSDFDMDSS-ELITAD-------------- 316
Bifidobacterium breve DSM bsh                                245 ANVSRLFHTLASVQMVEGM-SKMG-NGQFERTLFTSGYSGKTNTYYMNTYEDPAIRSFAMSDFDMDSS-ELITAD-------------- 316
Bifidobacterium animalis subsp. lactis KL612 bsh             244 ANVNRLFKTLSTAAVIEGT-AISA-NAEFEKTLFSDCYSTATQTVYLKKYDDMAVHSYAVKDFDASSN-QLQSK--------------- 314
Lactobacillus johnsonii pf01 conjbsh                         246 ANVAKFFNILKSVAMIKGS-VVND-QGKDEYTVYTACYSSGSKTYYCNFEDDFELKTYKLDDHTMNST-SLVTY--------------- 316
Enterococcus faecium bsh                                     242 ESISQFFHILGSVEQQKGL-CDVG-DGKYEYTIYSSCCNVDKGIYYYRTYEDSQITAIDMNKEDLDSH-KLISYPIIEKQQIKYIN--- 324
Enterococcus durans bsh                                      242 ESISQFFHILGSVEQQKGL-CDVG-DGKYEYTIYSSCCNVDKGIYYYRTYEDSQITAIDMNKEDLDSH-KLISYPIIEKQQIKYIIS-- 325
Lactobacillus delbrueckii ACNH                               242 SSVNQFFKILGSVEQVKGL-CEVT-PGEYEYTIYSSCMNQEKGIYYYTTYGNPEVHAVDMHKTDLDGK-ELTSYKLQKDLQFHFDN--- 324
Lactobacillus acidophilus bsha                               243 ENIDTYFHILHSVEQQKGL-DEVG-PNSFEYTIYSDGTNLDKGIFYYTTYSNKQINVVDMNKEDLDSS-NLITYDMLDKTKFNHQN--- 325
Streptococcus pasteurianus ATCC ch                           242 SSVNQFFHILKSVEQQKGL-CYVDESNGYEYTIYSSCVNVDKGIYYYTTYENSQITAIDMHQENLDSS-RLIRYPLQKEWNVLYQNR-- 326
Lactobacillus ruminis S23 ch                                 242 TSVSQFFHILGSVEQQKGC-CDVG-NGKFEFTIYSSCCNVDRGIYYYKTYDNSQITAVDMHKEDLDSA-ALKSYKLIEEQQIFAQN--- 324
Listeria monocytogenes bsh                                   242 ESISQFFHILGSVEQQKGL-CDVG-GGKYEHTIYSSCCNIDKGIYYYRTYGNSQITGVDMHQEDLESK-ELAIYPLVNEQRLKIVNK-- 325
Lactobacillus gasseri ATCC bsh                               243 ESVTNFFHILQSVEQVKGM-DEVG-PNIFEYTMYTSCMNLEKGILYFNCYDDSRISAVDMNKEDLSSS-DLIVFDLFKKQDISFIN--- 325
Lactobacillus johnsonii pf01 bshA                            244 SSVTNYFHILHSVEQPKGT-DEVG-PNSYEYTIYSDGTNLETGTFYYTNYENNQINAIELNKENLNGD-ELIDYKLIEKQTINYQN--- 326
Clostridium perfringens 13 CBH                               247 IDLIEFFHILNNVAMVRGS-TRTV-EEKSDLTQYTSCMCLEKGIYYYNTYENNQINAIDMNKENLDGN-EIKTYKYNKTLSINHVN--- 329
Lactobacillus hominis DSM pbsh                               243 ENVTNFFHILESVEQAKGM-DQVG-PNSFEYTMYTSCMNLEKGILYFNCYDDSRISAVDMNKEDLDSS-DLVVYDLFKKQDINFVN--- 325
Streptococcus infantarius subsp. infantarius CJ18 cbsh       242 SSVNQFFHILKSVEQQKGL-CYVDESGKYEYTIYSSCINADKGIYYYTTYDNSQITAVDMHKENLDDS-KLVTYPLVKDWQVNYQN--- 325
Lactobacillus plantarum B14 bsh                              242 GSVSQFFHILGSVEQQKGL-CEVT-DGKYEYTIYSSCCDMNKGVYYYRTYDNSQINSVNLNHEHLDTT-ELISYPLRSEAQYYAVN--- 324
Lactobacillus plantarum WCFS1 CBAH                           242 GSVSQFFHILGSVEQQKGL-CEVT-DGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVSLNHEHLDTT-ELISYPLRSEAQYYAVN--- 324
Listeria ivanovii subsp. ivanovii PAM 55 pcbah               242 ESISQFFHILGSVEQQKGL-CDVG-GEKYEHTIYSSCCNMDKGIYYYKTYDNSQITGIDMHRENLESE-ELVIYPLINSQQVNWVNK-- 325
Lactobacillus coryniformis bsh                               242 GSVSQFFHILGSVEQQKGL-CEVT-DGKYEYTIYSSCCDMDKGVYYYRTYENSQINSVSLNHEHLDTT-ELISYPLRSEAQYYAVN--- 324
Lactobacillus crispatus CNH                                  243 ENVDNYFHILHSVEQQKGL-DQVG-PNSFEYTIYSDGTNLDKGIFYYTTYTNQQINVVDINKEDLDAK-ELIKYDMLTKPTFNHQN--- 325
Lactobacillus reuteri bsh                                    243 ENITNYFHILQSVEQQKGL-DEVA-PNTFEYTIYSDGSNLKKGIFYYKTYENSQINAVDMHKKILKH-----------------QN--- 309
Lactobacillus salivarius ACNH                                243 EDITQFFHILGTVEQIKGV-NKTE-SGKEEYTVYSNCYDLDNKTLYYTTYENRQIVAVTLN-EDKNGN-RLIAYPFERKQVINKLN--- 324
Streptococcus lutetiensis cbsh                               242 SSVNQFFHILKSVEQQKGL-CYVDESGKYEYTIYSSCINTEKGIYYYTTYDNSQITAVDMHKENLDNN-KLVTYPLIKDWQVNYQN--- 325
Lactobacillus acidophilus bshb                               243 ENVTNYFHILHSVEQPDGL-DEVE-DNRYEYTMYTDCMNLDKGILYFTTYDNNRINAVDMHKADLGSE-DLICYDLFKKQDIEYMN--- 325
Lactobacillus antri ACNH                                     243 ENVTNYFHCLHAVEQQKGL-DEVA-PNTFEHTIYSDGINLSTGTFYYTTYANHQINAVSLQHADLNNN-TLAPFPLREQQSINMQN--- 325
Methanobrevibacter smithii CBAH                              242 ESVSQFFHILASVEQQKGCTLVEEPD-KFEYTIYSDCYNTDKGILYYKTYDGPQTSVNIHDEDLETNQ-LIN-----------FELVD- 316
Streptococcus pneumoniae ch                                  246 EGISAIFHILSNCEVPKGA-VITE-DGILDNTIYTSAMCMESGTYYYHTYDCRQIIAIHLFNEDLDTA-EIKTYPFQRKQKIFYEN--- 328
Eubacterium cylindroides ATCC pch                            242 VNVQQYFRVLNQVSMIKGS--VITKENREDYTVYSACMNATKGIYYYQTYENMHINSVCMHDENLDSS-VLIAYKLDKTINFLKSNSKA 327
Bacillus cereus group ACNH                                   246 EGVSALFHILSNCEVPKGG-VITE-EGTLDNTIYTSVMCMESGTYYYHTYDCRQITAIHLFHENLDTD-EIKAYPFQRKQKIFYEN--- 328
Bacillus mycoides ACNH                                       246 EGISAIFHILSNCEVPKGG-VITE-EGTLDNTIYTSAMCMESGTYYYHTYDCRQIIAIHLFHENLDTD-EIKTYPFQRKHKILYEN--- 328
Bacillus anthracis ACNH                                      246 EGVSALFHILSNCEVPKGG-VITE-EGALDNTIYTSVMCMESGTYYYHTYDCRQIIAVHLFHENLDTD-EIKAYPFQRKQKIFYEN--- 328
Bacillus thuringiensis ACNH                                  246 EGVSAVFHILSNCELPKGG-VITE-EGTIDNTIYTSAMCMESGIYYYHTYDCRQIIAIHLFNENLDTY-EIKTYPFQRKQKIYYQN--- 328
Lactobacillus buchneri CD034 ch                              245 QTVNELSHILNSVEIPKG--VKLKQDGSEDYTQYRGYMDMKNCAYYMQPYDNQTIYKVSLSEELLNKK-APSEFP-IKHQQVYETLR-- 327
Carnobacterium maltaromaticum ACNH                           248 SAINSLFHILSSVSIPKGV--KVKEDGTLDYTQYTAIMDVTNLDYYMSSYEDTYPLKISLNSKLISQT-EPIVFESSKVQQFNVLTP-- 331
Staphylococcus haemolyticus ACNH                             241 DAVLDAFRILDSVSIPKG--AVRPSEDEFHYTLYQTVFNLTSRTLYVKYYNSNRIAELQLTEELLNSD-DLVIFDPIPQGLSTDKVN-- 324
Lactobacillus salivarius bsh                                 247 EAASLAFGILDTVSIPKS-----K-AHRPTYTVYRAVTVSEDRTYYYQANGRATVSGIKLDEELMQNT-EPIVFNVSNIWNPQMLS--- 325
Staphylococcus epidermidis ACNH                              245 ENLMNCFKVLESVSIPQG--AVIDAN-KIHYTQYQLVMESKERSYYIKPYFSNQIFKIKLTEDLLSKN-EMT-FLPINHEL--KITSIQ 326
Lactobacillus terrae ACNH                                    246 EAVNTIMHMFDTVRIPKGV--NVKDNNTISYTQYEAVLDITNCVMYYTPYSNRTVYKIELTPEMINSGIEPKEFSVDFEQQVKSLN--- 329
Lactobacillus rhamnosus bsh                                  258 EGIRVLYNAFKTVLIPQGLGRDPKHSILTDYTQYWSGYDLSKRAIFVQDANTLTMTTKTLDPNLTEVT----YDDLVKTEQFNQL---- 338
Lactobacillus fermentum NCDO bsh                             247 EALATTWHLLDSVTVPKSK------AHRPTFSVYRAATVAEDRTYYFQSYHQAQVTSVKLTDDLLKRA-TPLVFDTADVWAPVKLN--- 325
Staphylococcus aureus CC5 ch                                 245 MDLMNAFYLLDAVNIPIG--IVRPHDADNHYTMYQTVINLTTRTLYIKYYGSNEIVALKLTDDLMNRK-DMTIFKPEKHITIRKLNDNQ 330
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Figure 4.7. (cont'd)

4.3 Phylogenetic analysis of BSH from *Lb. plantarum* B14

Sequence analysis and of the amino acid sequences homologue BSH sequences retrieved for, *Lb. plantarum* WCFS1*, Lb. reuteri, Lb. salivarus, Lb. acidophilus, Lb. gasseri* and *Clostridium perfringens* and other Ntn-hydrolase superfamily cluster contain BSH enzymes (Figure 4.7) and the phylogenetic tree was represented in a tree structure (Figure 4.8). Identity percentage analysis revealed the relationship between members of the Ntn-hydrolase superfamily. It was found that *Lb. plantarum* B14 BSH showed 99% similarity with BSHs of *Lb. coryniformis*, *Lb. plantarum* WCSF1. On the other hand, BSH of *Lb. plantarum* B14 showed 60-69% similarity with *Enterococcus* and *Streptococcus* genus whereas 30-39% similarity to *Bacillus* and *Bifidobacterium genus* (Table 4.1).

Table 4.1. BSH and BSH related enzyme including strains

Strain	Protein	Identity	AC Number	Referance
Bifidobacterium adolescentis ATCC 15705	BSH	36	AAX86039.1	Kim et al., 2005 PUBMED 16086241
Bifidobacterium breve DSM 20213 = JCM 1192	BSH	36	ABC26911.1	Goswami et al., Unpublished
Bifidobacterium bifidum	BSH	36	ABC26910.1	Goswami et al., Unpublished
Bifidobacterium longum SBT2928	BSH	38	AAF67801.1	Tanaka et al., 2000 PUBMED 10831430
Lactobacillus salivarius	Linear amide C-N hydrolase	55	WP_101898179.1	ncbi
Lactobacillus salivarius	BSH		AFP87506.1	Wang et al., 2012 PUBMED 23064348
Lactobacillus acidophilus	BSHb	46	ACL98176.1	Jiang et al., 2010
Lactobacillus acidophilus	BSHa	52	ACL98175.1	Jiang et al., 2010
Lactobacillus gasseri ATCC 33323 = JCM 1131	BSH	48	ARB50240.1	Ba and Öztürk, 2018
Clostridium perfringens str. 13	CBH	40	P54965	Coleman and Hudson,1995 PUBMED 7618863
Bifidobacterium animalis subsp. *lactis* KL612	BSH	32	AAS98803.1	Kim and Lee, 2008 PUBMED 18444998
Lactobacillus fermentum NCDO 394	BSH	30	AEZ06356.1	Kumar et al., 2012 PUBMED 23673477
Enterococcus faecium FAIR-E 345	BSH	69	AAP20760.1	Wijaya et al., 2003 Unpublished
Lactobacillus reuteri	BSH	51	ACH81023.1	Bustos et al., 2008 Unpublished
Listeria monocytogenes	BSH	67	AKI55714.1	Bergholz et al., 2014 Unpublished
Lactobacillus crispatus	C-N hydrolase	51	WP_101887068.1	ncbi
Lactobacillus delbrueckii	Linear amide C-N hydrolase	55	WP_013439461.1	ncbi
Lactobacillus plantarum B14	BSH	100	AQU14359.1	Öztürk et al., 2018 unpublished
Lactobacillus plantarum WCFS1	CBAH	99	Q06115.4	Christiaens et al., 1992 PUBMED 1476424
Lactobacillus johnsonii pf01	Choloylglycine hydrolase-BSHA	53	EGP12224.1	Lee et al., 2011 PUBMED 21742886
Lactobacillus johnsonii pf01	Choloylglycine hydrolase-BSHA	38	EGP13287.1	Lee et al., 2011 PUBMED 21742886
Lactobacillus ruminis S23	Choloylglycine hydrolase	58	KLA46842.1	O'Donnell et al., 2015 PUBMED 25879663
Methanobrevibacter smithii CAG:186	CBAH	60	CDF29476.1	Rasmussen et al., 2012 ,unpublished
Staphylococcus aureus CC5	Choloylglycine hydrolase	31	AVU05845.1	Bothello et al., 2017 ,unpublished
Lactobacillus hominis DSM 23910 = CRBIP 24.179	PBSH	47	CCI81875.1	Cousin et al., 2012 unpublished

Lactobacillus antri	**Linear amide C-N hydrolase**	**50**	**WP_007123019.1**	**ncbi**
Bacillus anthracis	Linear amide C-N hydrolase		ASE28680.1	Duncan et al., 2017 unpublished
Bacillus cereus	Linear amide C-N hydrolase	37	WP_098922295.1	ncbi
Lactobacillus terrae	Linear amide C-N hydrolase	28	WP_099973971.1	ncbi
Staphylococcus epidermidis	Linear amide C-N hydrolase	31	PJM54063.1	Sacchi et al., 2017 unpublished
Staphylococcus haemolyticus	Linear amide C-N hydrolase	30	AVH46281.1	Premkrishnan et al., 2018 PUBMED 29748397
Bacillus thuringiensis	Linear amide C-N hydrolase	38	WP_061667227.1	ncbi
Streptococcus lutetiensis	bile salt (choloylglycine) hydrolase	62	SQF41913.1	Doyle, 2018 unpublished
Bacillus mycoides	Linear amide C-N hydrolase	38	WP_002169387.1	ncbi
Carnobacterium maltaromaticum	Linear amide C-N hydrolases	36	CRH18292.1	Cailliez-grimal, 2015 unpublished
Lactobacillus coryniformis	Bile salt hydrolase	99	APV46192.1	Ding et al., 2016 unpublished
Streptococcus pasteurianus ATCC 43144	Choloylglycine hydrolase	59	BAK29772.1	Lin et al., 2011 PUBMED 21633709
Listeria ivanovii subsp. ivanovii PAM 55	Putative conjugated bile acid hydrolase		CBW86536.1	Buchrieser et al., 2011 PUBMED 22072644
Lactobacillus buchneri CD034	Choloylglycine hydrolase	33	AFR99367.1	Heinl et al., 2012 PUBMED 22465289
Streptococcus pneumoniae	Choloylglycine hydrolase	37	CGG00948.1	ncbi
Enterococcus durans	Bile salt hydrolase	69	AHH25138.1	Guo and Huo, 2013 Direct Submission
Streptococcus infantarius subsp. infantarius CJ18	Bile salt (choloylglycine) hydrolase	62	AEZ61987.1	Jans et al., 2012 PUBMED 22461547
Eubacterium cylindroides ATCC 27803	Putative choloylglycine hydrolase	45	ERK44633.1	Weinstock et al., 2013 Direct Submission
Lactobacillus rhamnosus	Bile salt hydrolase	26	AEP69108.1	Kumar et al., 2011 Direct Submission
Bifidobacterium longum	BSH	38	Q9KK62	Kumar et al., 2006

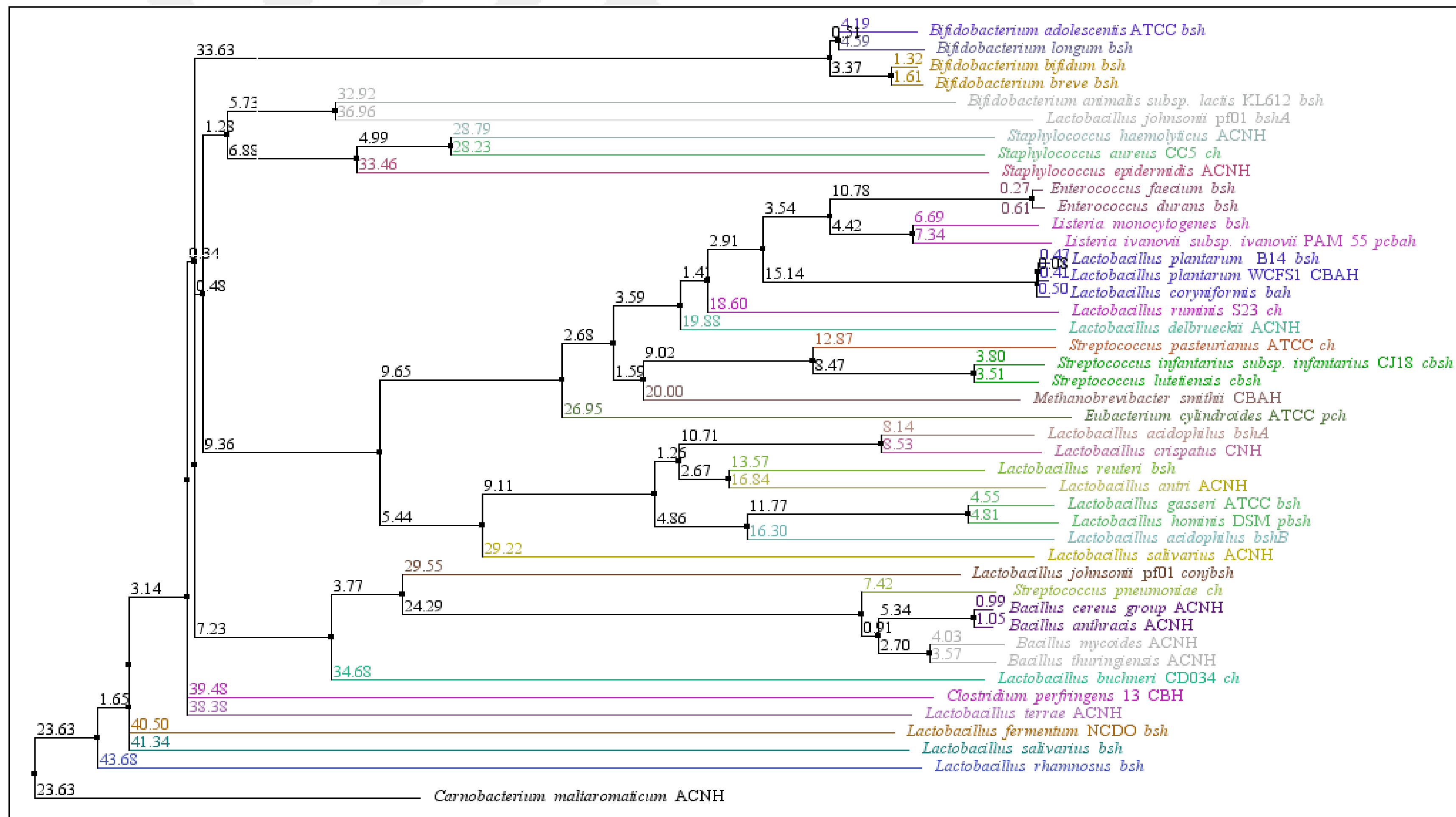

Figure 4.8. The Phylogenetic tree indicated of *Lb. plantarum* B14 BSH enzyme (AN: AQU14359.1) and BSH related enzymes. Blue color represented in close relatives with; *Lb. plantarum* WCSF1 (AN: Q06115.4) and *Lb. coryniformis* (AN: APV46192.1).

4.4 3D Model Structure and Substrate Specifity Sites for BSH Enzyme

The location of the partially conserved target amino acids, Phe18, Tyr20, Asn79, Leu138 and Asn180, were depicted in Figure 4.9. These the partially conserved target amino acids compared with other strictly conserved amino acids were showed in Fig. 4.10-13.

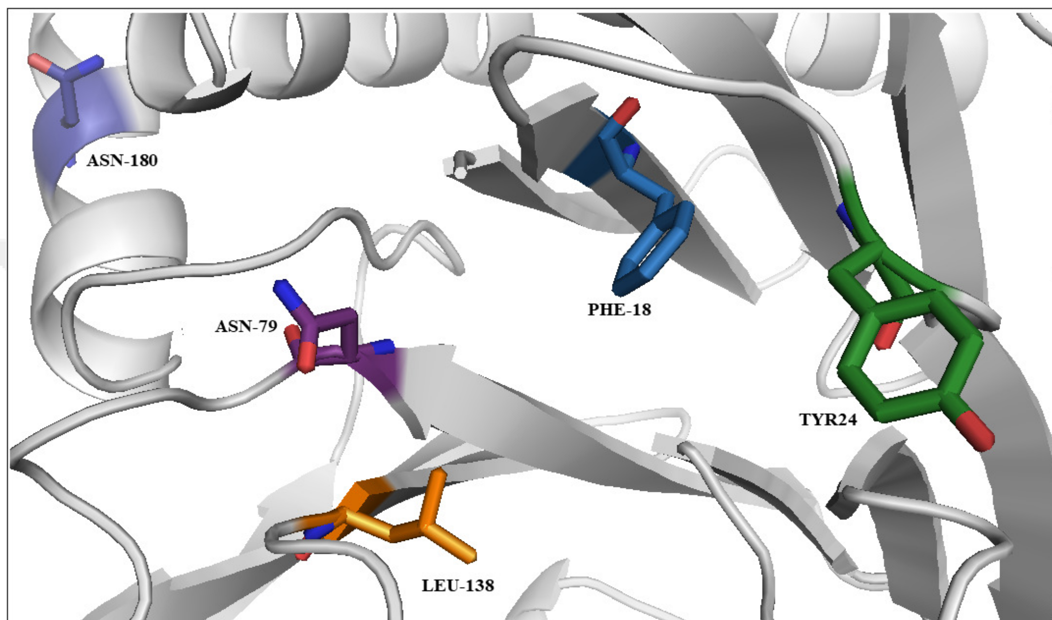

Figure 4.9. Three-dimentional structure of *Lb. plantarum* monomer of BSH protein. The target amino acids of BSH of *Lb. plantarum* B14 shows partially conserved amino acids; phenylalaline 18, tyrosine 20, asparagine 79, leucine 138 and arginine 180.

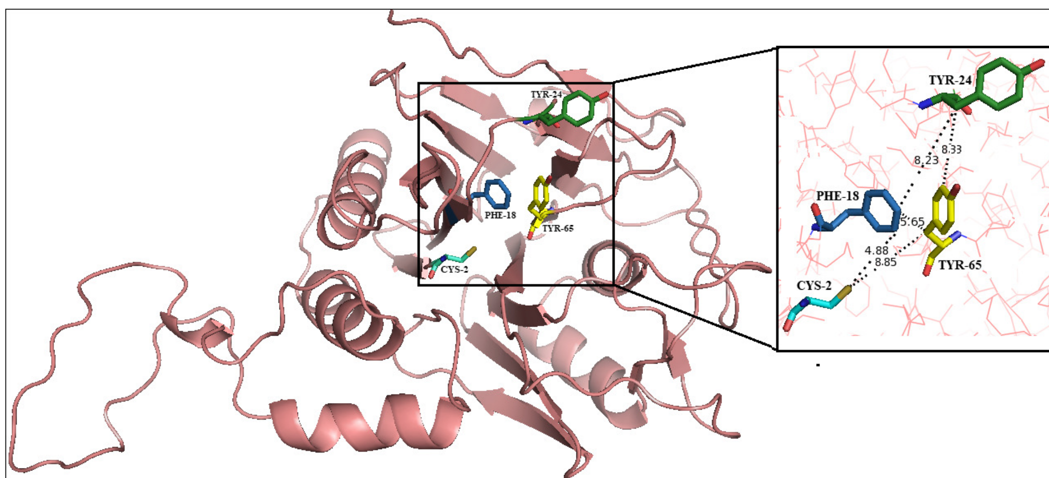

Figure 4.10. Three-dimentional structure of *Lb. plantarum* monomer of BSH protein. BSH of *Lb. plantarum* B14 shows strictly conserved amino acid; cysteine 2 partially conserved aminoacids; phenylalaline 18, thyrosine 24 and tyrosine 65.

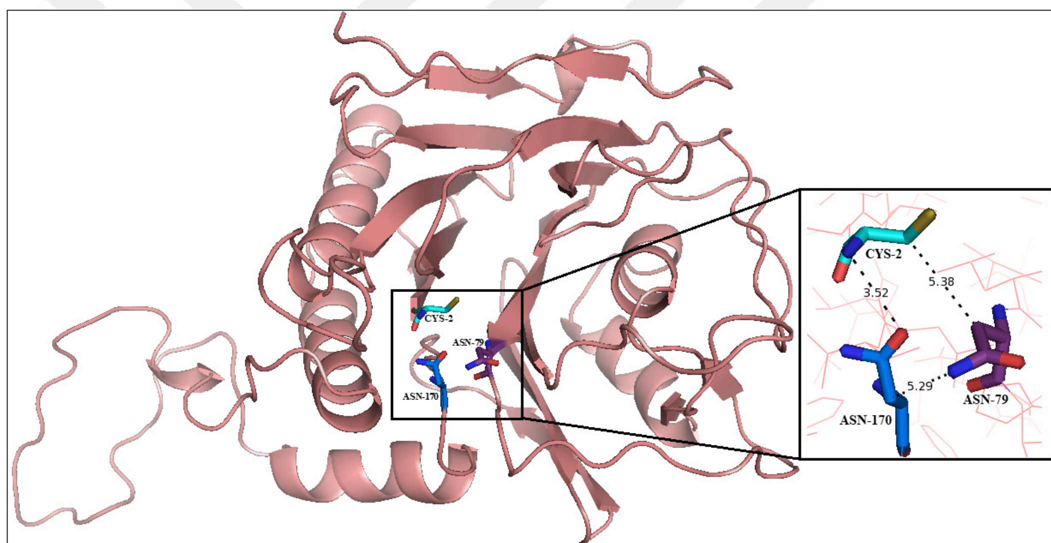

Figure 4.11. Three-dimentional structure of *Lb. plantarum* monomer of BSH protein. BSH of *Lb. plantarum* B14 shows strictly conserved amino acid; cysteine 2 and partially conserved aminoacids; asparagine 79 and asparagine 170.

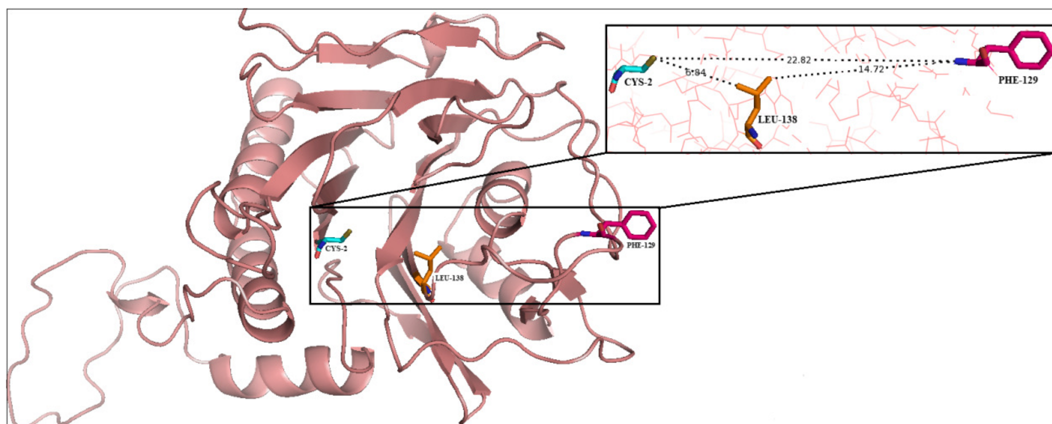

Figure 4.12. Three-dimentional structure of *Lb. plantarum* monomer of BSH protein. BSH of *Lb. plantarum* B14 shows strictly conserved amino acid; cysteine 2 and partially conserved aminoacids; phenylalaline 129 and leucine 138.

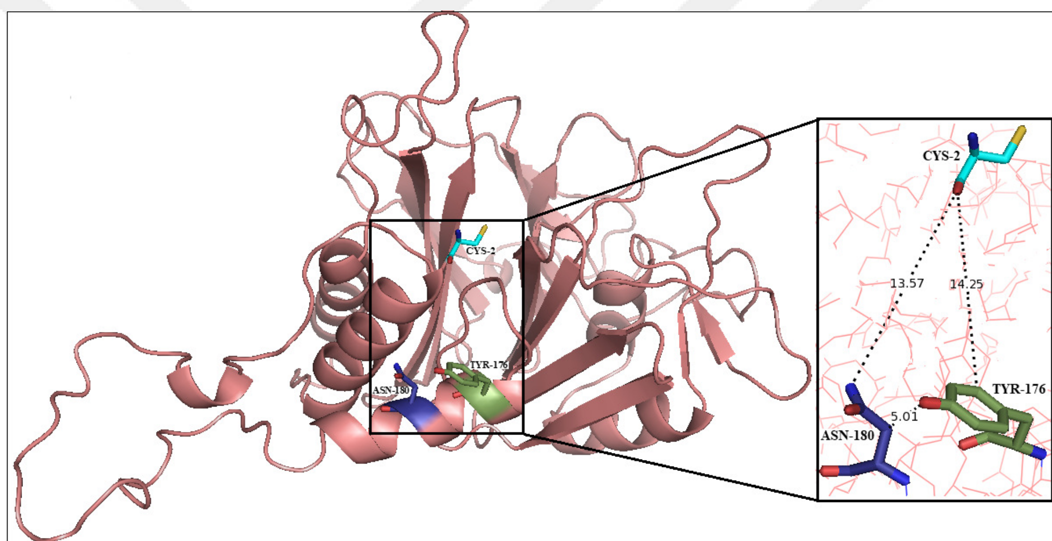

Figure 4.13. Three-dimentional structure of *Lb. plantarum* monomer of BSH protein. BSH of *Lb. plantarum* B14 shows strictly conserved amino acid; cysteine 2 and partially conserved aminoacids; thyrosine 176 and asparagine 180.

4.5 Preparation of Mutant pCON1 Constructs

4.5.1 PCR- Based Site Directed Mutagenesis of Target Aminoacids

pCON1 was used as a template DNA for the F18L, Y24F, Y24L, N79V, L138E and N180H substitutions. PCR products were treated with *Dpn*I and loaded on agarose gel to degrade template DNAs (Figure 4.8).

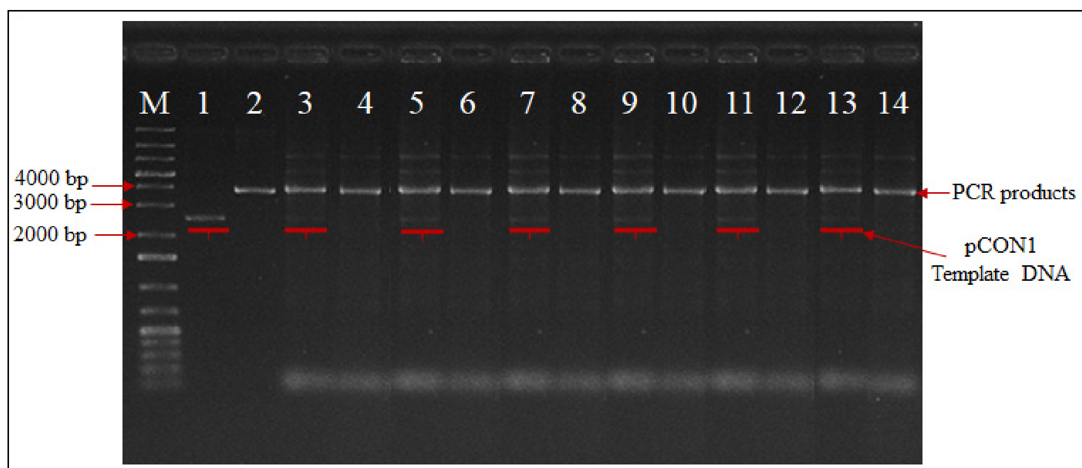

Figure 4.14. Site-directed mutagenesis of pCON1* (* represents desired mutation) by PCR. M, 1 kb plus DNA ladder; 1, uncut pCON1 template DNA (1/5 diluted); 2, *Xho*I cut pCON1 template DNA; 3, pCON1*F18L PCR product; 4, pCON1*F18L PCR product digested with *Dpn*I restriction enzyme; 5, pCON1*Y24F PCR product; 6, pCON1*Y24F PCR product digested with *Dpn*I restriction enzyme; 7, pCON1*Y24L PCR product; 8, pCON1*Y24L PCR product digested with *Dpn*I restriction enzyme; 9, pCON1*N79V PCR product; 10, pCON1*N79V PCR product digested with *Dpn*I restriction enzyme; 11, pCON1*L138E PCR product; 12, pCON1*L138E PCR product digested with *Dpn*I restriction enzyme; 13, pCON1*N180H PCR product; 14, pCON1*N180H PCR product digested with *Dpn*I restriction enzyme.

4.5.2 Purification of the pCON1* Mutant PCR Products

The mutant pCON1*F18L, pCON1*Y24F, pCON1*Y24L, pCON1*N79V, pCON1*L138E and pCON1*N180H PCR products were purified individually from 1% agarose gels to remove nospecific PCR products (Figure 4.15).

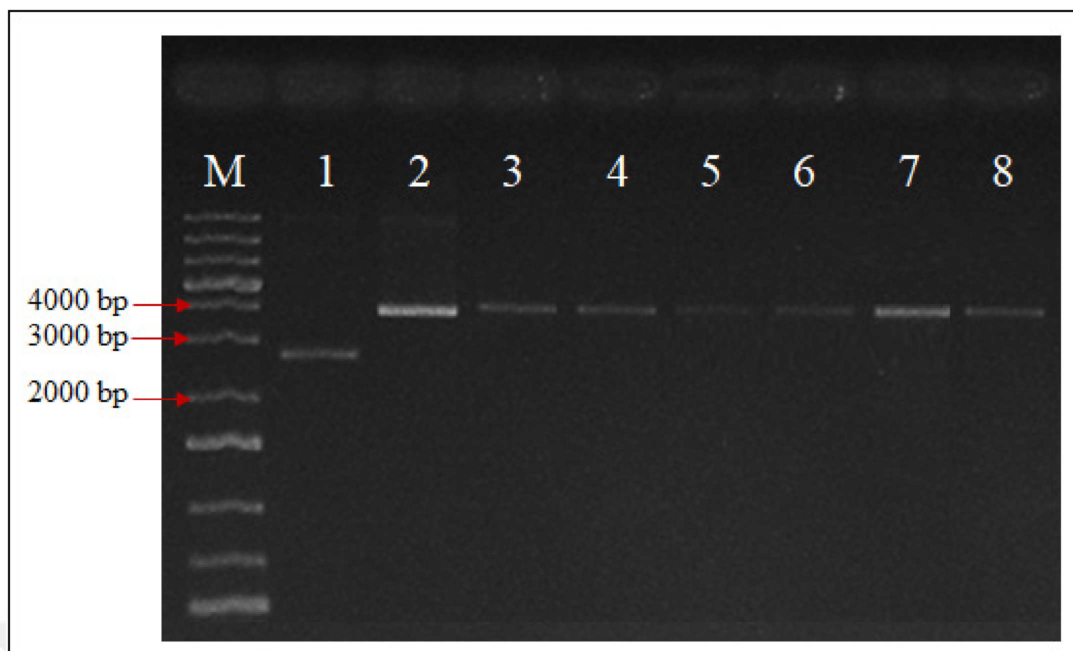

Figure 4.15. Purified PCR products of pCON1* Mutants. M, 1 kb plus DNA ladder; 1, uncut pCON1 template DNA (1/5 diluted); 2, *Xho*I cut pCON1 template DNA (4.0 kb); 3, purified pCON1*F18L (4.0 kb); 4, purified pCON1*Y24F (4.0 kb); 5, purified pCON1*Y24L (4.0 kb); 6, purified pCON1*N79V (4.0 kb); 7, purified pCON1*L138E (4.0 kb); 8, purified pCON1*N180H (4.0 kb)

4.5.3 Transformation of pCON1* Mutant Constructs into *E. coli* XL1-Blue strains

100 ng DNA of purified PCR products were transformed to XL1 Blue cells. To detect transformants with the desired mutations, 4 transformants for each mutation were randomly selected and pCON1* DNA isolation was performed for sequencing. All DNAs from transformatants and pCON1DNA were digested with *Xho*I RE to produce linear DNA. Aproximately 4.0 kbp (3936 bp) DNA bands were detected from all transformatants; pCON1*F18L, pCON1*Y24F, pCON1*Y24L, pCON1*N79V, pCON1*L138E and pCON1*N180H (Figures 4.16-20).

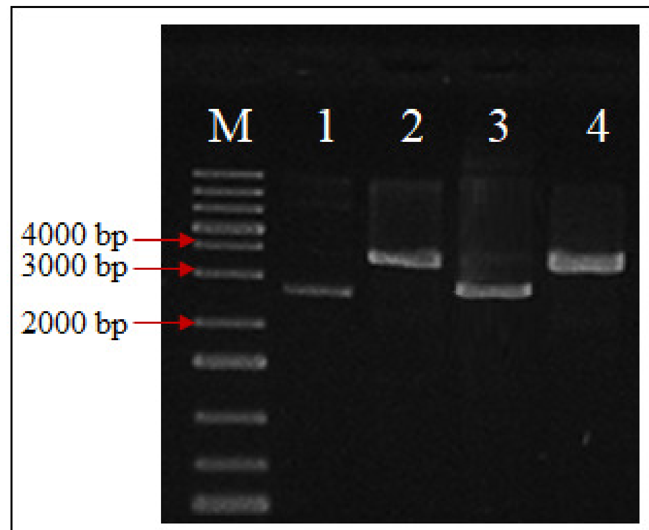

Figure 4.16. Transformation of pCON1*F18L into *E. coli* XL1-Blue strains. M, 1 kb plus DNA ladder; 1, uncut pCON1 DNA; 2 *Xho*I cut pCON1 DNA; 3, pCON1*F18L DNA; 4, *Xho*I cut pCON1*F18L DNA.

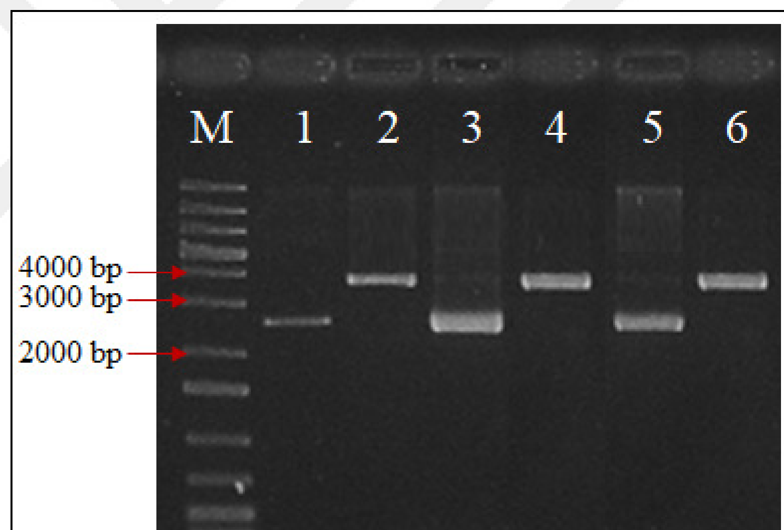

Figure 4.17. Transformation of pCON1*Y24F and pCON1*Y24L into *E. coli* XL1-Blue strains. M, 1 kb plus DNA ladder; 1, uncut pCON1 DNA; 2 *Xho*I cut pCON1 DNA; 3, pCON1*Y24F DNA; 4, *Xho*I cut pCON1*Y24F DNA; 5, pCON1*Y24L DNA; 6, *Xho*I cut pCON1*Y24L DNA.

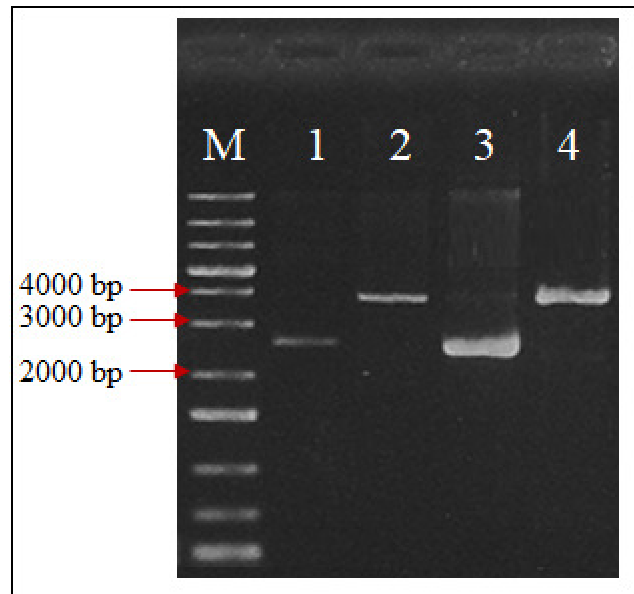

Figure 4.18. Transformation of pCON1*N79V into *E. coli* XL1-Blue strains. M, 1 kb plus DNA ladder; 1, uncut pCON1 DNA; 2 *Xho*I cut pCON1 DNA; 3, pCON1*N79V DNA; 4, *Xho*I cut pCON1*N79V DNA.

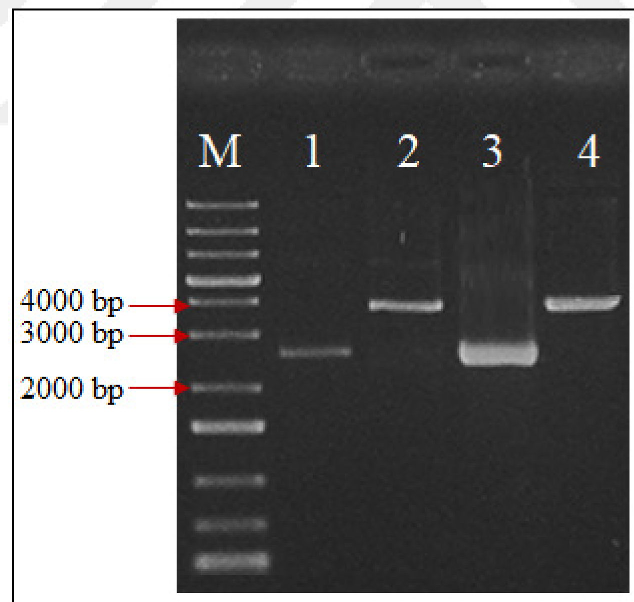

Figure 4.19. Transformation of pCON1*L138E into *E. coli* XL1-Blue strains. M, 1 kb plus DNA ladder; 1, uncut pCON1 DNA; 2 *Xho*I cut pCON1 DNA; 3, pCON1*L138E DNA; 4, *Xho*I cut pCON1*L138E DNA.

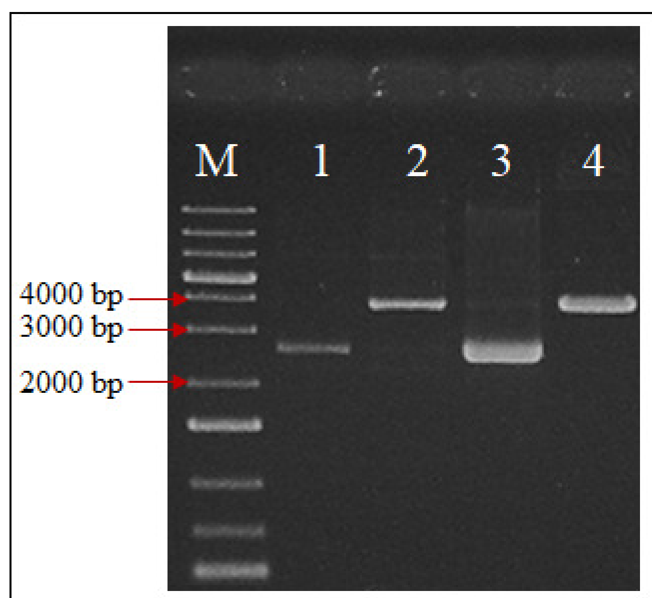

Figure 4.20. Transformation of pCON1*N180H into *E. coli* XL1-Blue strains. M, 1 kb plus DNA ladder; 1, uncut pCON1 DNA; 2 *Xho*I cut pCON1 DNA; 3, pCON1*N180H DNA; 4, *Xho*I cut pCON1*N180H DNA.

4.5.4 DNA Sequencing of pCON1* Mutants

Selected pCON1*F18L, pCON1*Y24F, pCON1*Y24L, pCON1*N79V, pCON1*L138E and pCON1*N180H constructs were sequenced by Macrogen (Seoul, Korea) using universal primers, M13/pUC-F and M13/pUC-R and they were visualized by the BioEdit Sequence Alignment program. It was found that pCON1F18L, pCON1Y24F, pCON1Y24L, pCON1N79V, pCON1L138E and pCON1N180H constructs harbored the targeted mutations, CTA in Figure 4.21, TTT in Figure 4.22, CTG in Figure 4.23, GTT in Figure 4.24, GAA in Figure 4.25 and CAT in Figure 4.26 respectively. Multiple sequence alignment of the mutated sequences against wild type BSH gene was performed using Jalview to show amino acids positions (Figure 4.27).

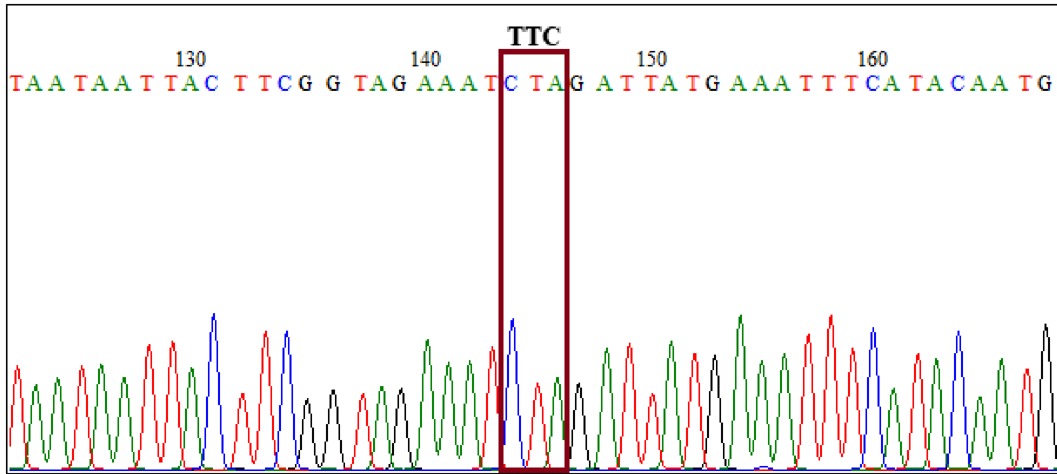

Figure 4.21. The partial nucleotide sequence of pCON1F18L transformant. Pheylalanine (F) codon, TTC (UUC), was converted to Leucine (L) codon, CTA (CUA), labelled with a frame.

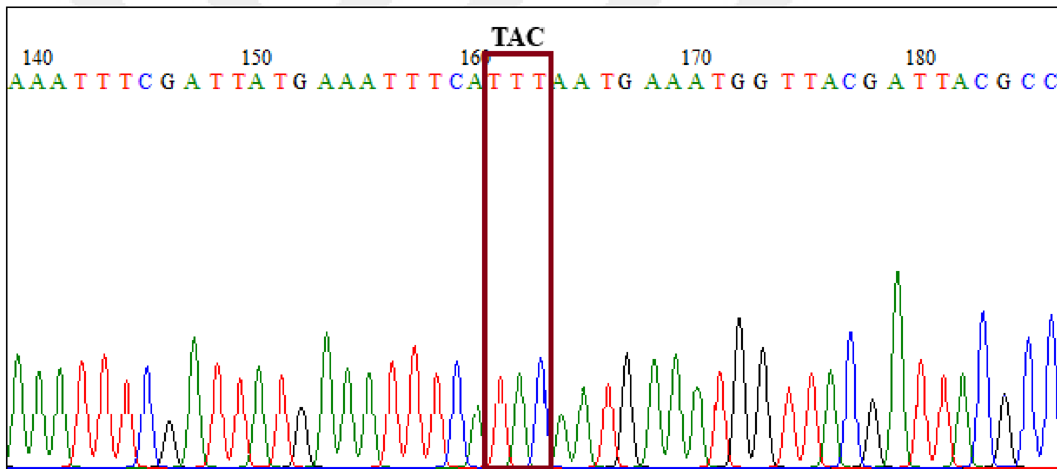

Figure 4.22. The partial nucleotide sequence of pCON1Y24F transformant. Tyrosine (Y) codon, TAC (UAC), was converted to Pheylalanine (F) codon, TTT (UUU), labelled with a frame.

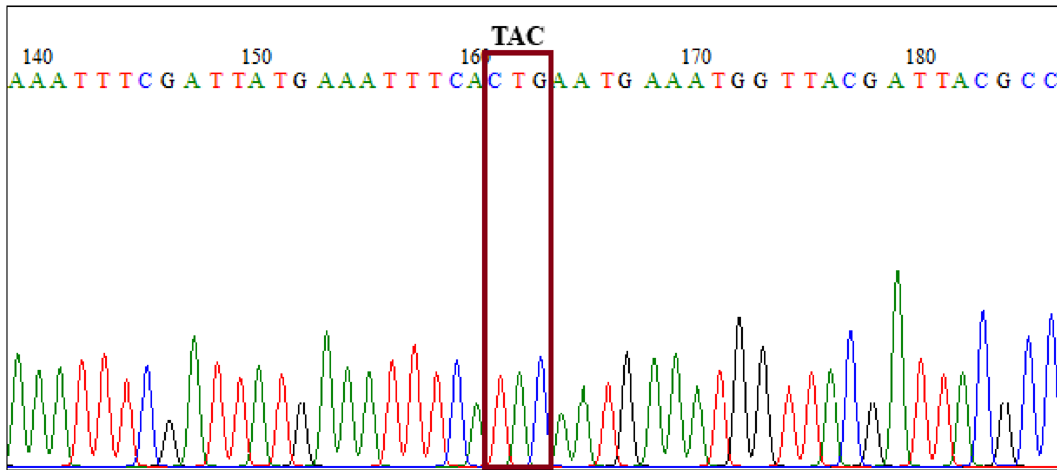

Figure 4.23. The partial nucleotide sequence of pCON1Y24L transformant. Tyrosine (Y) codon, TAC (UAC), was converted to Leucine (L) codon, CTG (CUG), labelled with a frame.

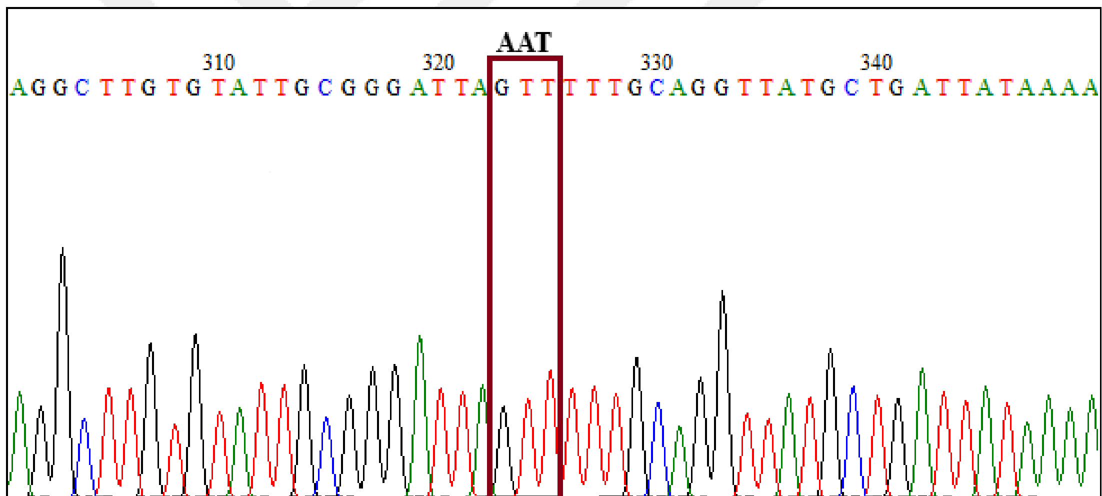

Figure 4.24. The partial nucleotide sequence of pCON1N79V transformant. Asparagine (N) codon, AAT (AAU), was converted to Valine (V) codon, GTT (GUU), labelled with a frame.

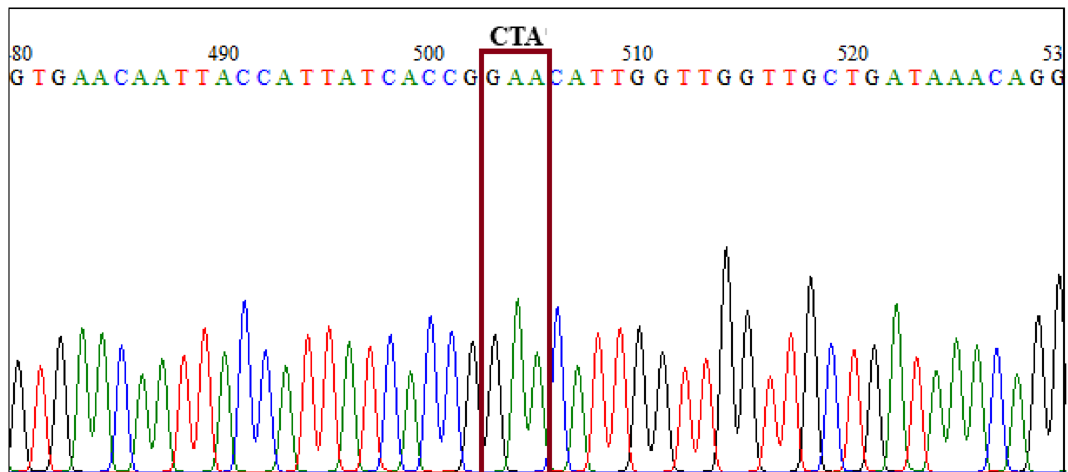

Figure 4.25. The partial nucleotide sequence of pCON1L138E transformant. Leucine (L) codon, CTA (CUA), was converted to Glutamic acid (E) codon, GAA, labelled with a frame.

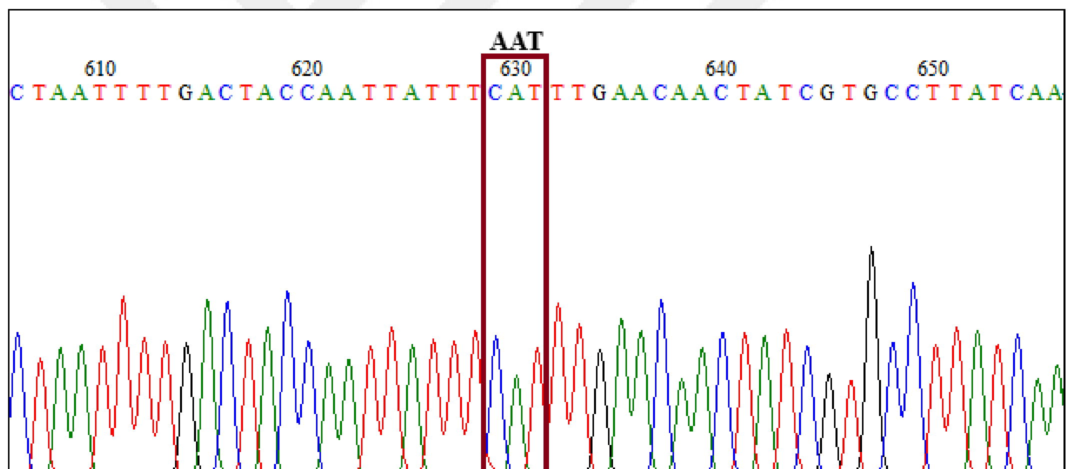

Figure 4.26. The partial nucleotide sequence of pCON1N180H transformant. Asparagine (N) codon, AAT (AAU), was converted to Histidine (H) codon, CAT (CAU), labelled with a frame.

```
wpCON1          1 MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGI 54
pCON1F18L       1 MCTAITYQSYNNYFGRNLDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGI 54
pCON1Y24F       1 MCTAITYQSYNNYFGRNFDYEISFNEMVTITPRKYPLVFRKVENLDHHYAIIGI 54
pCON1Y24L       1 MCTAITYQSYNNYFGRNFDYEISLNEMVTITPRKYPLVFRKVENLDHHYAIIGI 54
pCON1N79V       1 MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGI 54
pCON1L138E      1 MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGI 54
pCON1N180H      1 MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYAIIGI 54
                                   18    24

wpCON1         55 TADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQ 108
pCON1F18L      55 TADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQ 108
pCON1Y24F      55 TADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQ 108
pCON1Y24L      55 TADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQ 108
pCON1N79V      55 TADVESYPLYYDAMNEKGLCIAGLVFAGYADYKKYDADKVNITPFELIPWLLGQ 108
pCON1L138E     55 TADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQ 108
pCON1N180H     55 TADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFELIPWLLGQ 108
                                          79

wpCON1        109 FSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYD 162
pCON1F18L     109 FSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYD 162
pCON1Y24F     109 FSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYD 162
pCON1Y24L     109 FSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYD 162
pCON1N79V     109 FSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYD 162
pCON1L138E    109 FSSVREVKKNIQKLNLVNINFSEQLPLSPEHWLVADKQESIVIESVKEGLKIYD 162
pCON1N180H    109 FSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIVIESVKEGLKIYD 162
                                               138

wpCON1        163 NPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPG 216
pCON1F18L     163 NPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPG 216
pCON1Y24F     163 NPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPG 216
pCON1Y24L     163 NPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPG 216
pCON1N79V     163 NPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPG 216
pCON1L138E    163 NPVGVLTNNPNFDYQLFNLNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPG 216
pCON1N180H    163 NPVGVLTNNPNFDYQLFHLNNYRALSNSTPQNSFSEKVDLDSYSRGMGGLGLPG 216
                                   180

wpCON1        217 DLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLCEVTDGKYEY 270
pCON1F18L     217 DLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLCEVTDGKYEY 270
pCON1Y24F     217 DLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLCEVTDGKYEY 270
pCON1Y24L     217 DLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLCEVTDGKYEY 270
pCON1N79V     217 DLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLCEVTDGKYEY 270
pCON1L138E    217 DLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLCEVTDGKYEY 270
pCON1N180H    217 DLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHILGSVEQQKGLCEVTDGKYEY 270

wpCON1        271 TIYSSCCDMNKGVYYYRTYDNSQINSVNLNHEHLDTTELISYPLRSEAQYYAVN 324
pCON1F18L     271 TIYSSCCDMNKGVYYYRTYDNSQINSVNLNHEHLDTTELISYPLRSEAQYYAVN 324
pCON1Y24F     271 TIYSSCCDMNKGVYYYRTYDNSQINSVNLNHEHLDTTELISYPLRSEAQYYAVN 324
pCON1Y24L     271 TIYSSCCDMNKGVYYYRTYDNSQINSVNLNHEHLDTTELISYPLRSEAQYYAVN 324
pCON1N79V     271 TIYSSCCDMNKGVYYYRTYDNSQINSVNLNHEHLDTTELISYPLRSEAQYYAVN 324
pCON1L138E    271 TIYSSCCDMNKGVYYYRTYDNSQINSVNLNHEHLDTTELISYPLRSEAQYYAVN 324
pCON1N180H    271 TIYSSCCDMNKGVYYYRTYDNSQINSVNLNHEHLDTTELISYPLRSEAQYYAVN 324
```

Figure 4.27. The 324 amino acid residues of the wild type *bsh* gene (wpCON1) was aligned with mutant F18L, Y24F, Y24L, N79V, L138E and N180H *bsh* genes from pCON1F18L, pCON1Y24F, pCON1Y24L, pCON1N79V, pCON1L138E and pCON1N180H constructs by Jalview 2.8.0b1. Labelled amino acids represent the target mutant amino acids of *Lb. plantarum* B14.

4.6 Preparation of pCON2 and pCON2* Mutant Constructs

4.6.1 Digestion of pCON1, pCON1* Mutants and pET22b Expression Vector DNAs by *Eco*RI and *Not*I

After verification of the desired mutations by sequencing of the insert DNAs, wild-type pCON1 and mutant pCON1* plasmid DNAs, pCON1F18L, pCON1Y24F, pCON1Y24L, pCON1N79V, pCON1L138E and pCON1N180H, were isolated and digested with *Not*I and *Eco*RI restriction enzymes (Fermentas-Thermo Scientific) (Figure 4.28. (a)). Meanwhile pET22b vector also was digested with same enzymes (Figure 4.28. (a)). *Eco*RI/*Not*I digested vector (pET22b), pCON1 and mutant pCON1 plasmid DNAs were loaded on 1% agarose gel and then approximately 5467 bp vector and 975 bp mutant *bsh*1 genes were purified from agarose gel by using GeneJET Gel Extraction Kit #K0691 (Thermo Scientific in Figures, 4.28-35; b and c).

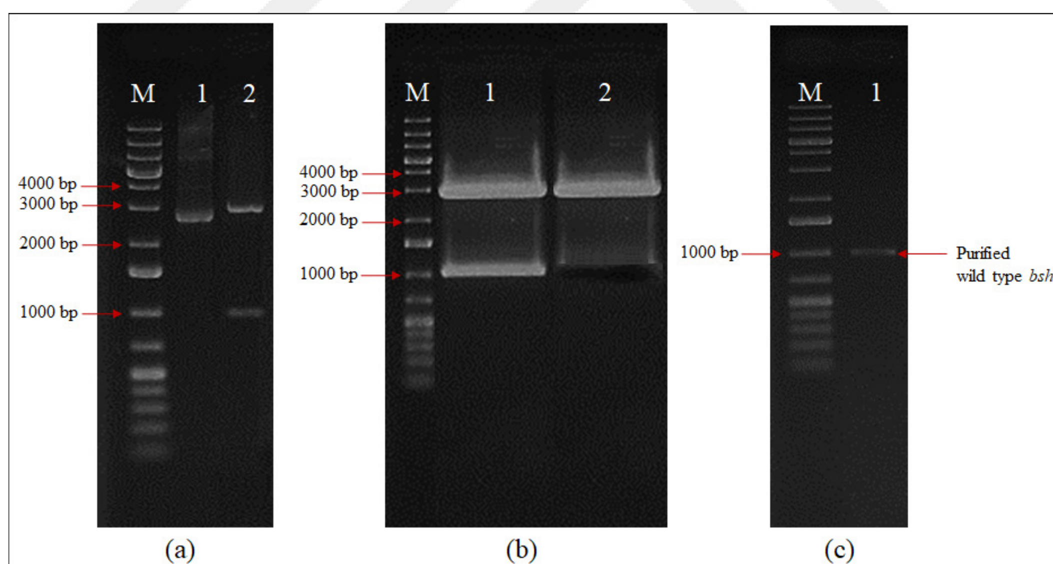

Figure 4.28. Digestion of pCON1 plasmid DNA by *Not*I and *Eco*RI. M, 1 kb plus DNA ladder; a, 1: uncut pCON1 DNA; 2: *Not*I/*Eco*RI cut pCON1 DNA; b, 1: Large volume of linear pCON1 DNA before gel purification; 2: sliced wild type *bsh* DNA fragment from pCON1 DNA. C, gel purified wild type *bsh* DNA fragment from pCON1 DNA.

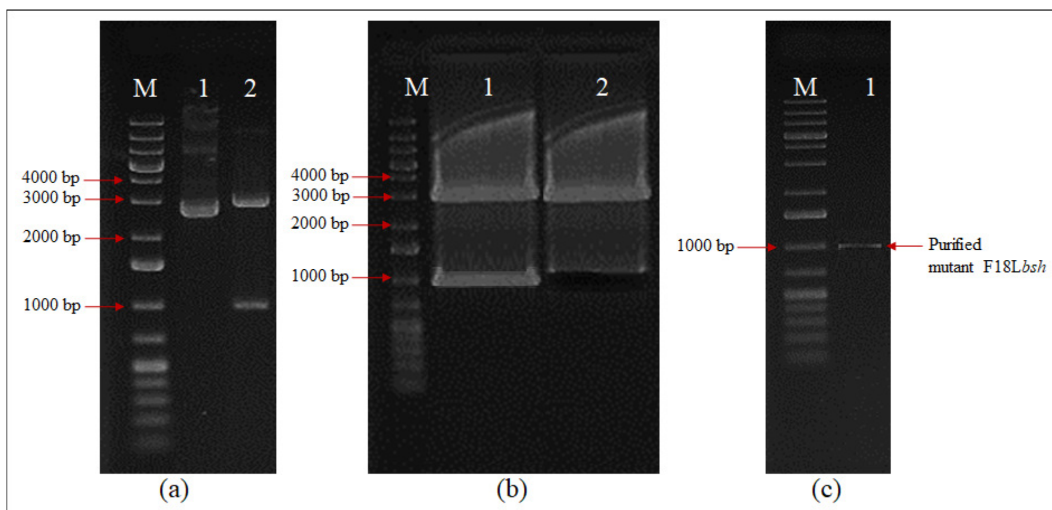

Figure 4.29. Digestion of pCON1F18L plasmid DNA by *Not*I and *Eco*RI. M, 1 kb plus DNA ladder; a, 1: uncut pCON1F18L DNA; 2: *Not*I/*Eco*RI cut pCON1F18L DNA; b, 1: Large volume of linear pCON1F18L DNA before gel purification; 2: sliced F18L *bsh* DNA fragment from pCON1F18L DNA. c, gel purified F18L *bsh* DNA fragment from pCON1F18L DNA.

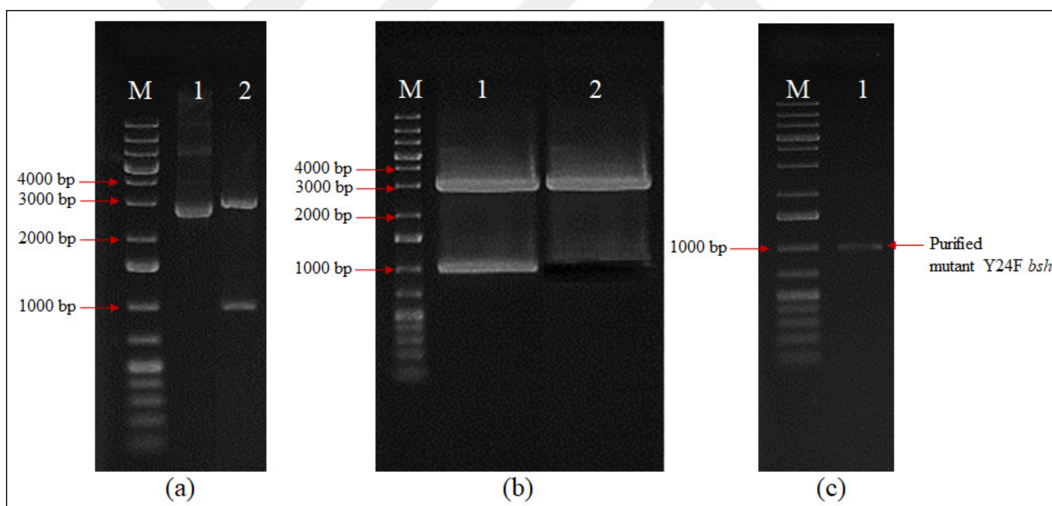

Figure 4.30. Digestion of pCON1Y24F plasmid DNA by *Not*I and *Eco*RI. M, 1 kb plus DNA ladder; a, 1: uncut pCON1Y24F DNA; 2: *Not*I/*Eco*RI cut pCON1Y24F L DNA; b, 1: Large volume of linear pCON1Y24F DNA before gel purification; 2: sliced Y24F *bsh* DNA fragment from pCON1Y24F DNA. c, gel purified Y24F *bsh* DNA fragment from pCON1Y24F DNA.

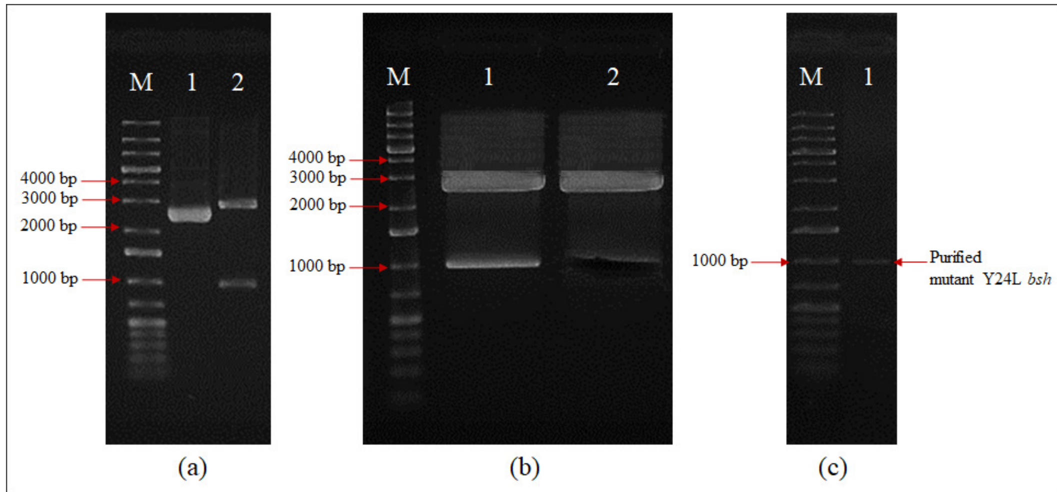

Figure 4.31. Digestion of pCON1Y24L plasmid DNA by *Not*I and *Eco*RI. M, 1 kb plus DNA ladder; a, 1: uncut pCON1Y24L DNA; 2: *Not*I/*Eco*RI cut pCON1Y24L DNA; b, 1: Large volume of linear pCON1Y24L DNA before gel purification; 2: sliced Y24L *bsh* DNA fragment from pCON1Y24L DNA. c, gel purified Y24L *bsh* DNA fragment from pCON1Y24L DNA.

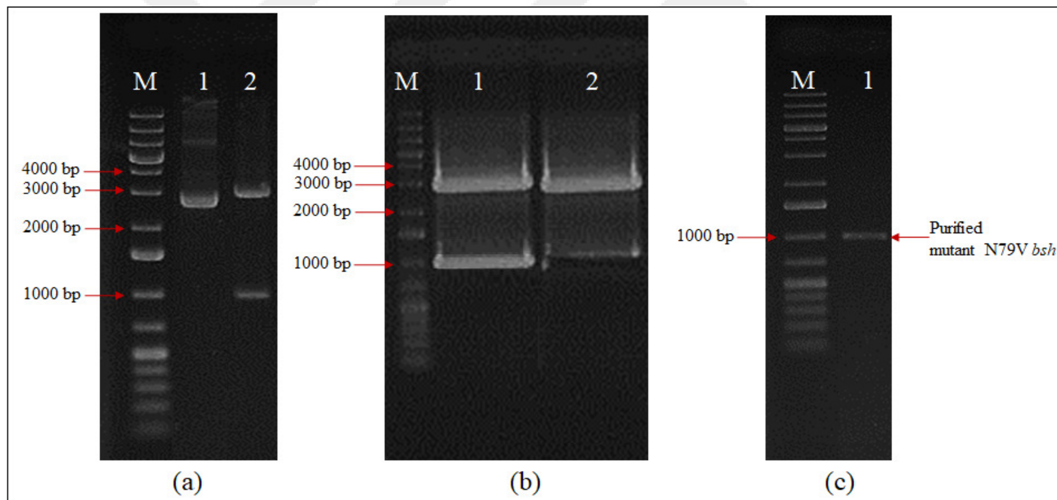

Figure 4.32. Digestion of pCON1N79V plasmid DNA by *Not*I and *Eco*RI. M, 1 kb plus DNA ladder; a, 1: uncut pCON1N79V DNA; 2: *Not*I/*Eco*RI cut pCON1N79V DNA; b, 1: Large volume of linear pCON1N79V DNA before gel purification; 2: sliced N79V *bsh* DNA fragment from pCON1N79V DNA. c, gel purified N79V *bsh* DNA fragment from pCON1N79V DNA.

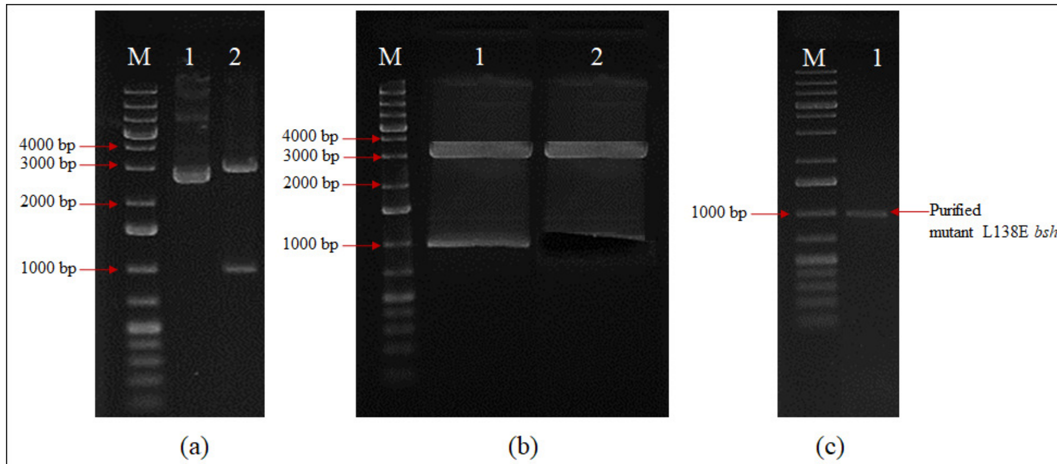

Figure 4.33. Digestion of pCON1L138E plasmid DNA by *Not*I and *Eco*RI. M, 1 kb plus DNA ladder; a, 1: uncut pCON1L138E DNA; 2: *Not*I/*Eco*RI cut pCON1L138E DNA; b, 1: Large volume of linear pCON1L138E DNA before gel purification; 2: sliced L138E *bsh* DNA fragment from pCON1L138E DNA. c, gel purified L138E *bsh* DNA fragment from pCON1L138E DNA.

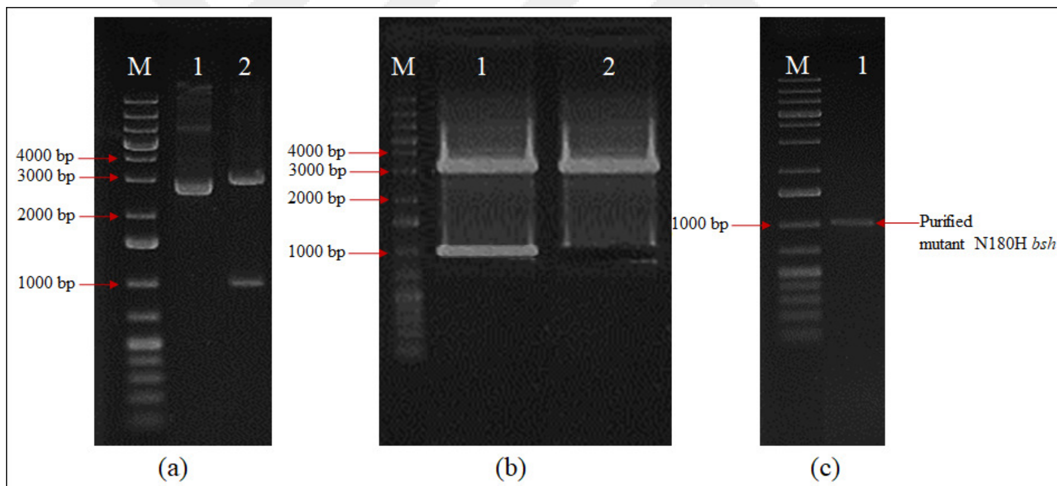

Figure 4.34. Digestion of pCON1N180H plasmid DNA by *Not*I and *Eco*RI. M, 1 kb plus DNA ladder; a, 1: uncut pCON1N180H DNA; 2: *Not*I/*Eco*RI cut pCON1N180H DNA; b, 1: Large volume of linear pCON1N180H DNA before gel purification; 2: sliced N180H *bsh* DNA fragment from pCON1N180H DNA. c, gel purified N180H *bsh* DNA fragment from pCON1N180H DNA.

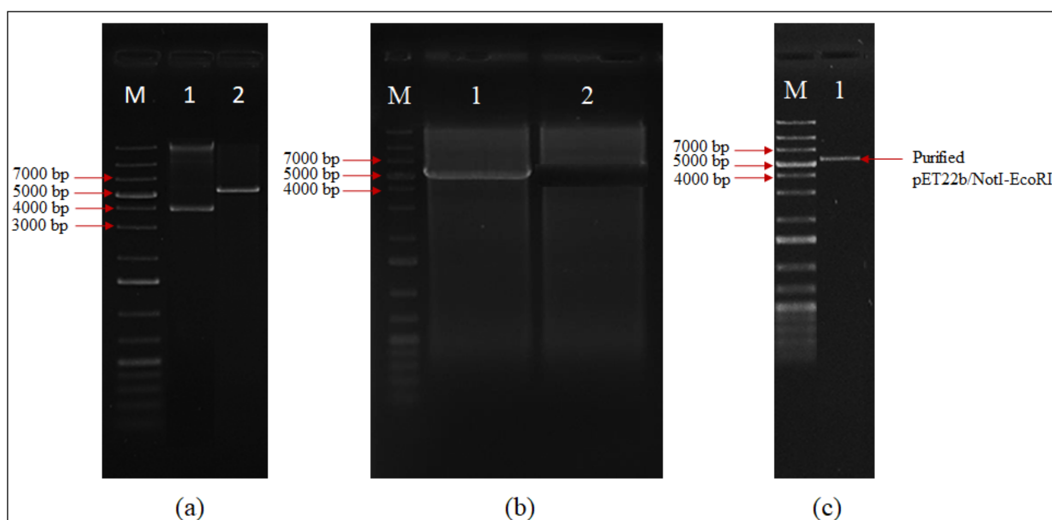

Figure 4.35. Preparation of pET22b expression vector DNA. M, 1 kb plus DNA ladder; a, 1: uncut pET22b DNA; 2: *Not*I/*Eco*RI digested pET22b DNA; b, *Not*I/*Eco*RI digested pET22b linear DNA before gel purification; 2: sliced linear pET22b DNA; c, gel purified linear pET22b DNA.

4.6.2 Transformation of Wild-type and Mutant *bsh* Genes to pET22b Expression Vector and Analysis of pCON2 and pCON2 Mutant Constructs

Approximately 1.0 kbp wild type and desired mutat *bsh* genes were inserted into pET22b vector by using T4 Ligase enzyme and ligation products were transformed into $CaCl_2$ treated *E. coli* BLR(DE3) competent cells. Plasmid DNAs were isolated from transformants that were selected on the basis of ampicillin resistance. For confirmation of *bsh* insert genes in pCON2 and pCON2 mutant clones, the DNAs obtained from clones were digested with *Eco*RI and *Not*I restriction enzymes. New transformants were designated as pCON2, pCON2F18L, pCON2Y24F, pCON2Y24L, pCON2N79V, pCON2L138E and pCON2N180H (Figure 4.36) and stored at -80 °C.

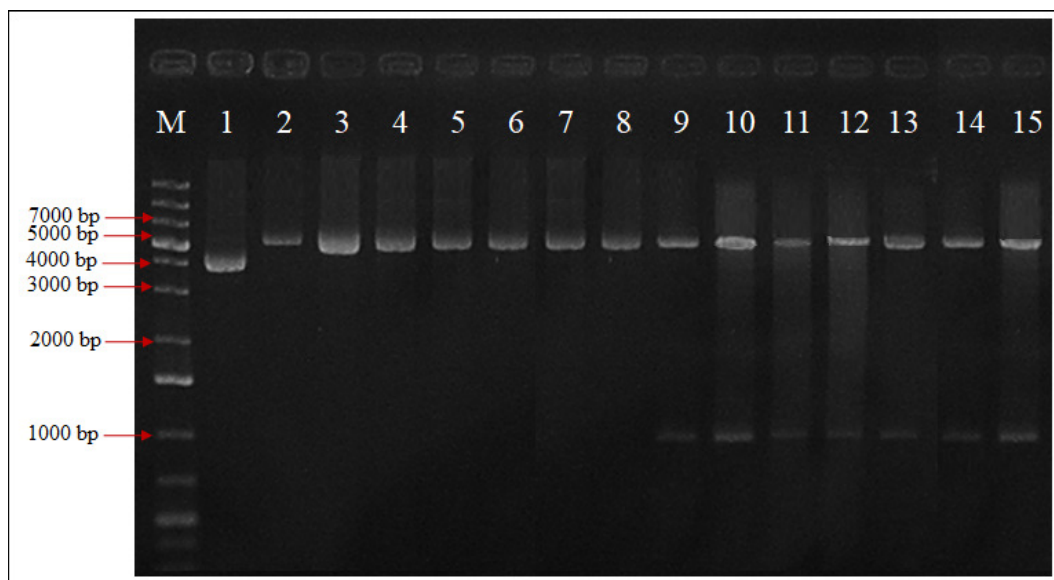

Figure 4.36. Preparation and confirmation of wild type and mutant *bsh* genes in pCON2 and mutant pCON2 clones. M, 1 kb plus DNA ladder; 1-8, represent circular DNAs of pET22b, pCON2, pCON2F18L, pCON2Y24F, pCON2Y24L, pCON2N79V, pCON2L138E and pCON2N180H; 9-15, represent *Not*I/*Eco*RI digested DNAs of pCON2, pCON2F18L, pCON2Y24F, pCON2Y24L, pCON2N79V, pCON2L138E and pCON2N180H.

4.7 Catalytic Activity of rBSH and Mutant rBSHs

4.7.1 Qualitative Activity of pCON2 and Mutant pCON2 Constructs

BSH activity was detected using a Direct Plate Assay method. Activity results of the pET22b (negative control), pCON2/BLR(DE3) (positive control) and mutant pCON2F18L/BLR(DE3), pCON2Y24F/BLR(DE3), pCON2N79V/BLR(DE3), pCON2L138E/BLR(DE3) and pCON2N180H/BLR(DE3) against GDCA and TDCA were depicted in Figures 4.37-41. The high BSH activity of pCON2/BLR(DE3) against GDCA with halo of precipitated cholic acid, showed no activity against TDCA. Moreover pCON2F18L, pCON2Y24F, pCON2N79V, pCON2L138E and pCON2N180H exhibited BSH activity against GDCA and almost no activity against TDCA (Figures 4.37-41 $c_{1,2,3,4,5}$ and $d_{1,2,3,4,5}$).

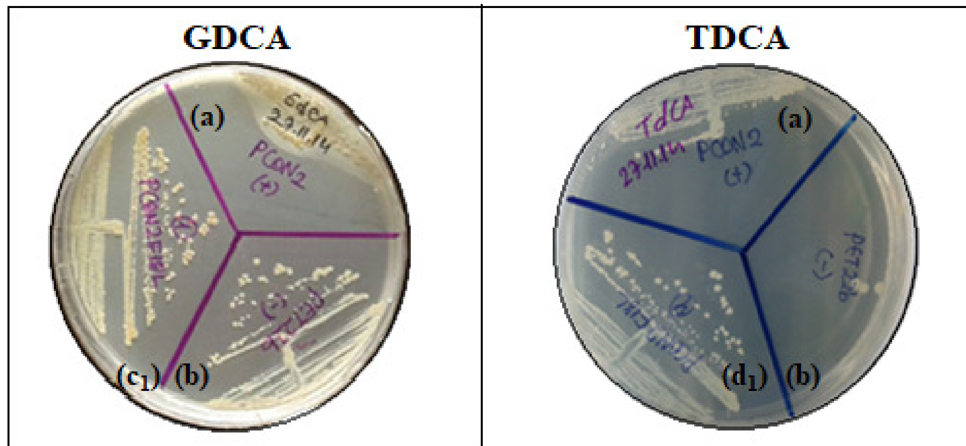

Figure 4.37. Detection of the pCON2F18L activity by direct plate assay. (a), positive controls (pCON2/BLR(DE3); (b), negative controls (pET22b/BLR(DE3); (c_1), BSH activity of pCON2F18L/BLR(DE3) in LB-plate with GDCA ; (d_1), BSH activity of pCON2F18L/BLR(DE3) in LB-plate with TDCA.

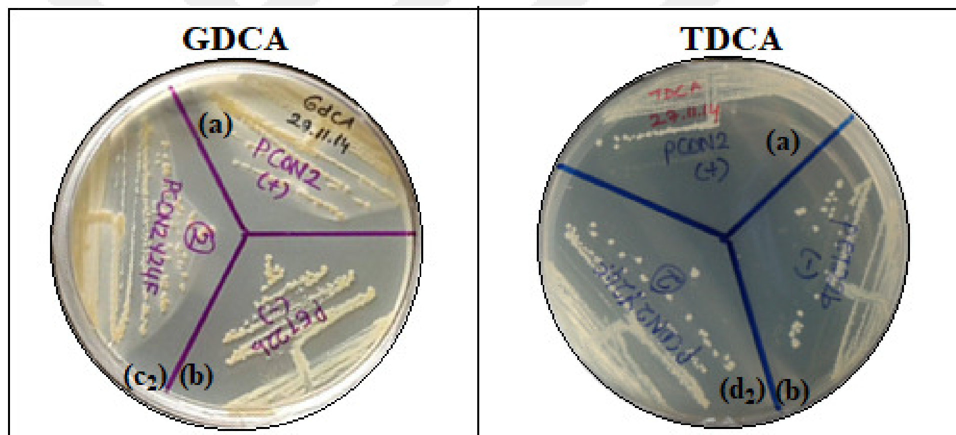

Figure 4.38. Detection of the pCON2Y24F activity by direct plate assay. (a), positive controls (pCON2/BLR(DE3); (b), negative controls (pET22b/BLR(DE3); (c_2), BSH activity of pCON2Y24F/BLR(DE3) in LB-plate with GDCA ; (d_2), BSH activity of pCON2Y24F/BLR(DE3) in LB-plate with TDCA.

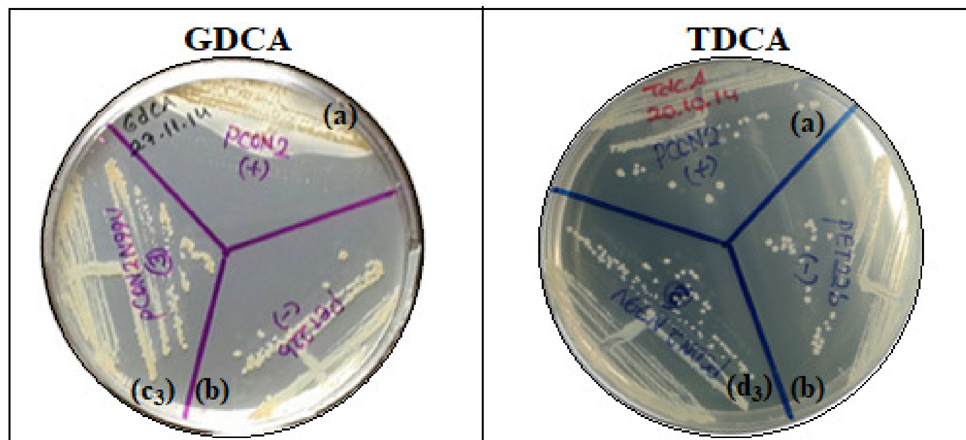

Figure 4.39. Detection of the pCON2N79V activity by direct plate assay. (a), positive controls (pCON2/BLR(DE3); (b), negative controls (pET22b/BLR(DE3); (c_3), BSH activity of pCON2N79V/BLR(DE3) in LB-plate with GDCA ; (d_3), BSH activity of pCON2N79V/BLR(DE3) in LB-plate with TDCA.

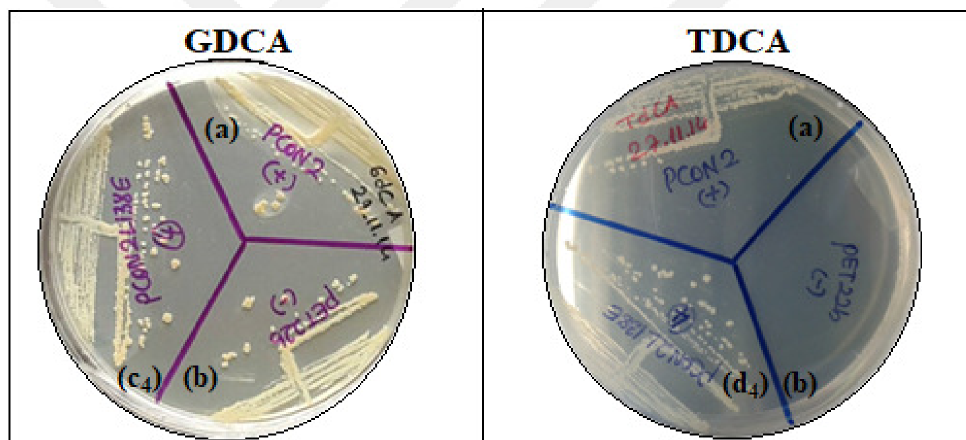

Figure 4.40. Detection of the pCON2L138E activity by direct plate assay. (a), positive controls (pCON2/BLR(DE3); (b), negative controls (pET22b/BLR(DE3); (c_4), BSH activity of pCON2L138E/BLR(DE3) in LB-plate with GDCA ; (d_4), BSH activity of pCON2L138E/BLR(DE3) in LB-plate with TDCA.

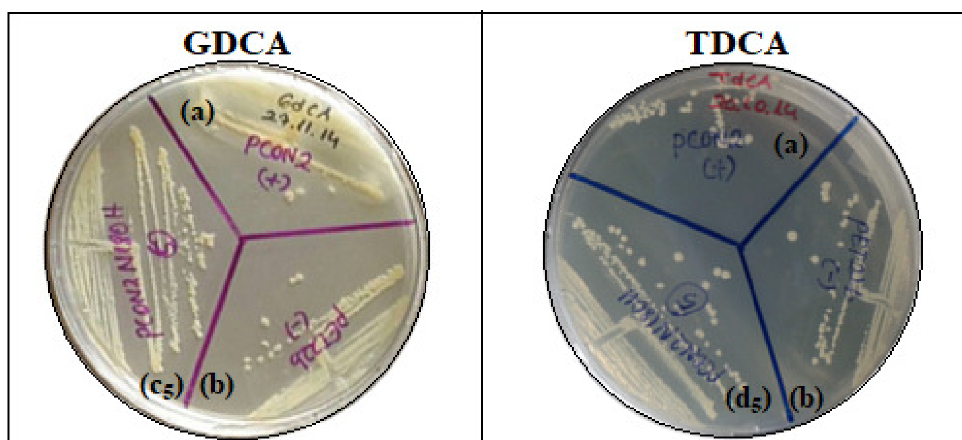

Figure 4.1. Detection of the pCON2N180H activity by direct plate assay. (a), positive controls (pCON2/BLR(DE3); (b), negative controls (pET22b/BLR(DE3); (c_5), BSH activity of pCON2N180H/BLR(DE3) in LB-plate with GDCA ; (d_5), BSH activity of pCON2N180H/BLR(DE3) in LB-plate with TDCA.

4.7.2 Optimum pH and Temperature of the wild-type recombinant BSH Enzyme

The optimum temperature and pH of the wild-type recombinant BSH enzymes (wrBSHs) were determined by ninhydrin assay. The effects of various temperatures (from 25°C to 65°C) and pH (from 4 to 6.5) values on its relative activities were determined using the common human bile salts, glycodeoxycholicacid (GDCA). The maximum hydrolysis activity of wrBSH enzyme occurred at 37°C (Figure 4.42 (c)). The activity of rBSH enzyme peaked at pH 6.0 (Figure 4.42 (a)).

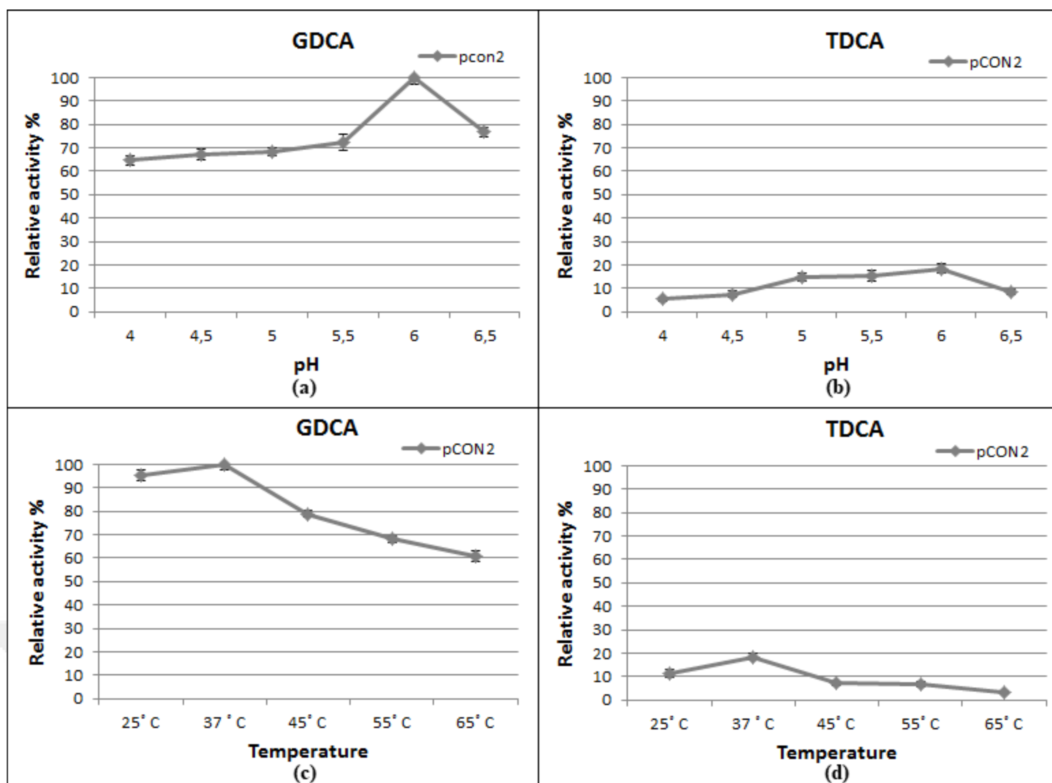

Figure 4.42. Optimum pH and temperature of the wrBSH enzyme. (a), BSH activity of pCON2 at various pH on GDCA; (b), bsh activity of pCON2 at various pH on TDCA; (c), BSH activity of pCON2 at various temperatures on GDCA; (d), BSH activity of pCON2 at various temperatures on TDCA

4.7.3 Substrate Specificity of wrBSHs and mrBSHs

After determination of the wrBSH optimum temperature and pH, hydrolysing capability of BSH of desired mutations were analyzed by ninhidrin assay using their partially purified cell extracts. Six major human bile salts, Glycocholic acid (GC), Glycodeoxycholic acid (GDC), Glycochenodeoxycholic acid (GCDC), Taurocholic acid (TC), Taurodeoxycholic acid (TDC) and Taurochenodeoxycholic acid (TCDC), were used as a substrate for this assay (Figures 4.43-48). The wrBSH enzyme from *Lb. plantarum* B14 strain exhibited the highest affinity for human bile salt glychodeoxycholic acid (GDCA; defined as 100% activity) and higher affinities for glycine-conjugated bile acids than for taurine-conjugated bile acids at 37°C and pH 6.0 (Figure 4.42). The wrBSH and mrBSH enzyme activities were measured by determining the amount of amino acids liberated from deconjugated bile salts. Relative activity was defined as the

measured bile salt hydrolase activity for each substrate compared to the highest activity (taken as %100) in the assay. Values are expressed as the mean of three independent replicates.

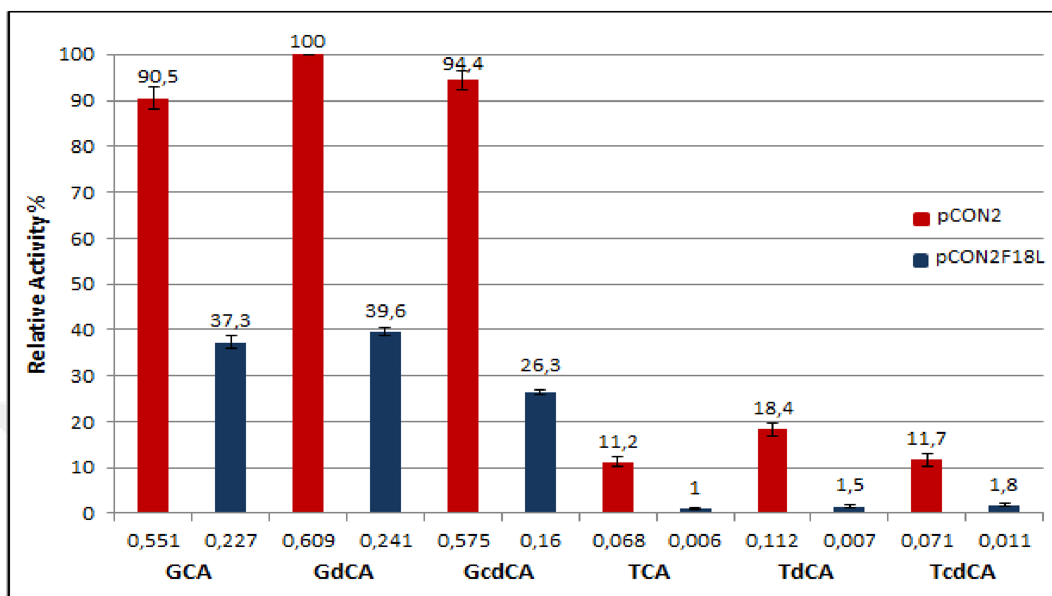

Figure 4.43. Comparison of the substrate specifity of pCON2F18L (mrBSH) with that of pCON2 (wrBSH) enzymes where six major bile salts were used. Relative deconjugation activity was measured for each substrate compared to the highest activity (GdCA taken as %100) in the assay.

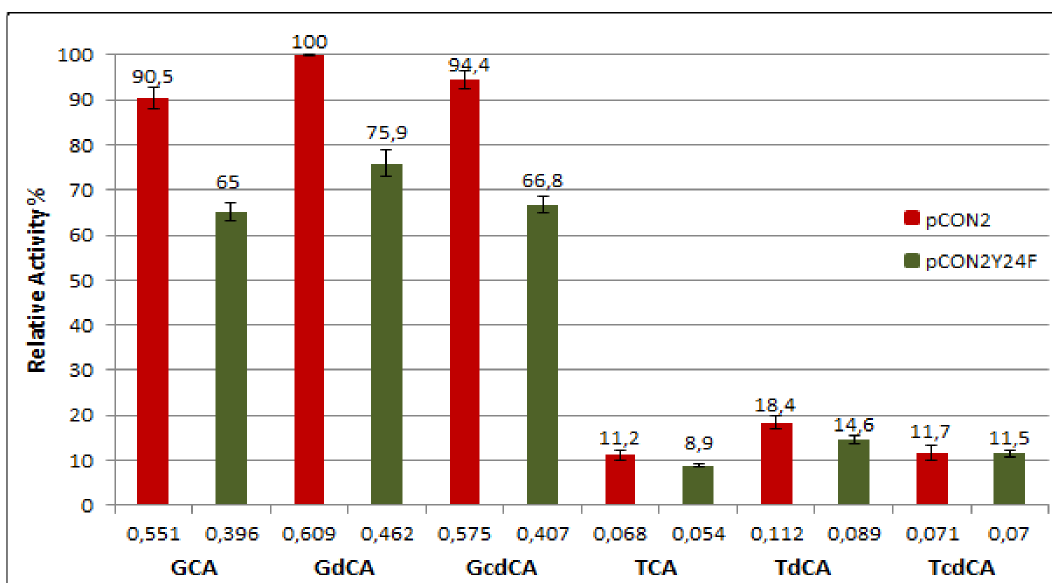

Figure 4.44. Comparison of the substrate specifity of pCON2Y24F (mrBSH) with that of pCON2 (wrBSH) enzymes where six major bile salts were used. Relative deconjugation activity was measured for each substrate compared to the highest activity (GdCA taken as %100) in the assay.

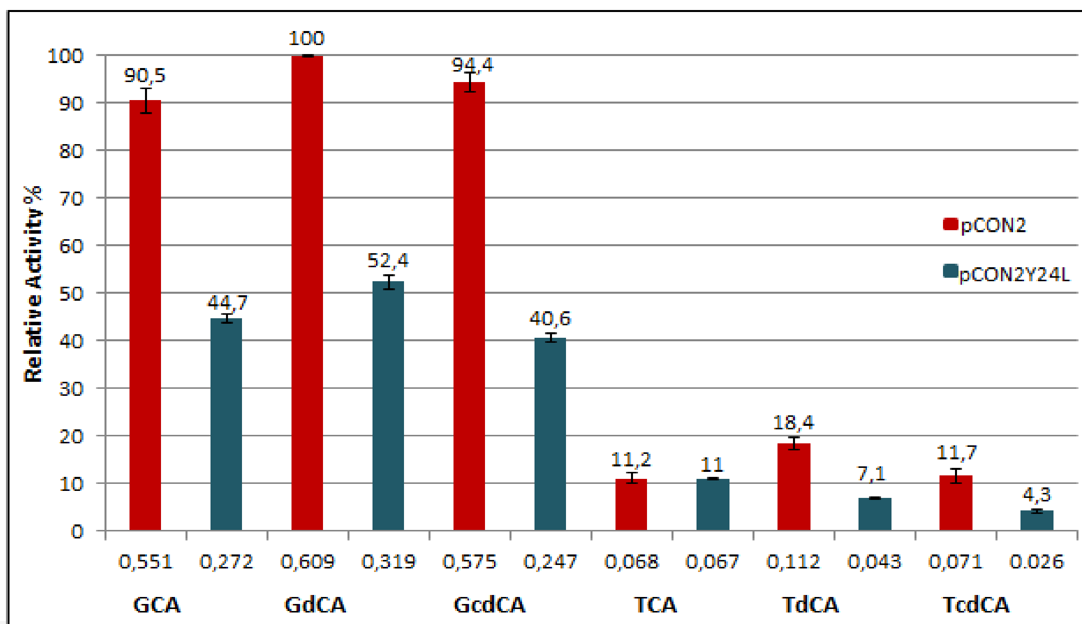

Figure 4.45. Comparison of the substrate specifity of pCON2Y24L (mrBSH) with that of pCON2 (wrBSH) enzymes where six major bile salts were used. Relative deconjugation activity was measured for each substrate compared to the highest activity (GdCA taken as %100) in the assay.

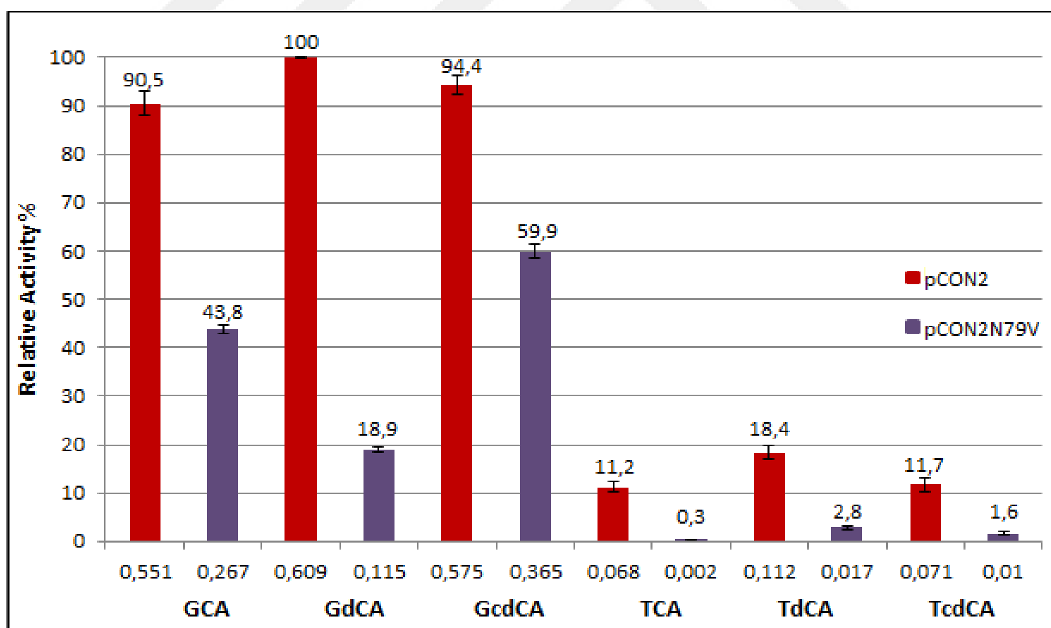

Figure 4.46. Comparison of the substrate specifity of pCON2N79V (mrBSH) with that of pCON2 (wrBSH) enzymes where six major bile salts were used. Relative deconjugation activity was measured for each substrate compared to the highest activity (GdCA taken as %100) in the assay.

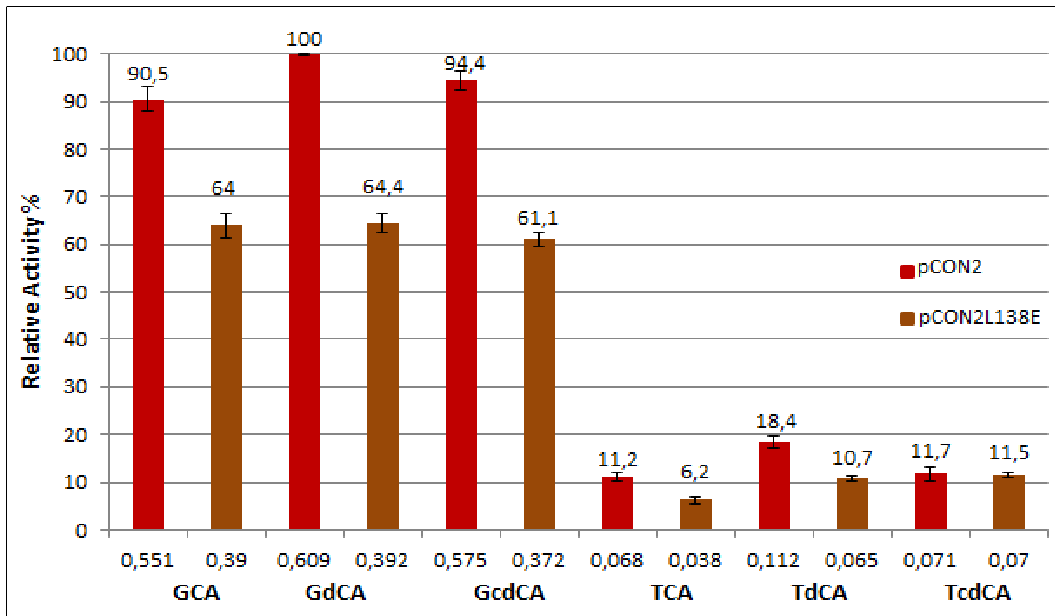

Figure 4.47. Comparison of the substrate specifity of pCON2L138E (mrBSH) with that of pCON2 (wrBSH) enzymes where six major bile salts were used. Relative deconjugation activity was measured for each substrate compared to the highest activity (GdCA taken as %100) in the assay.

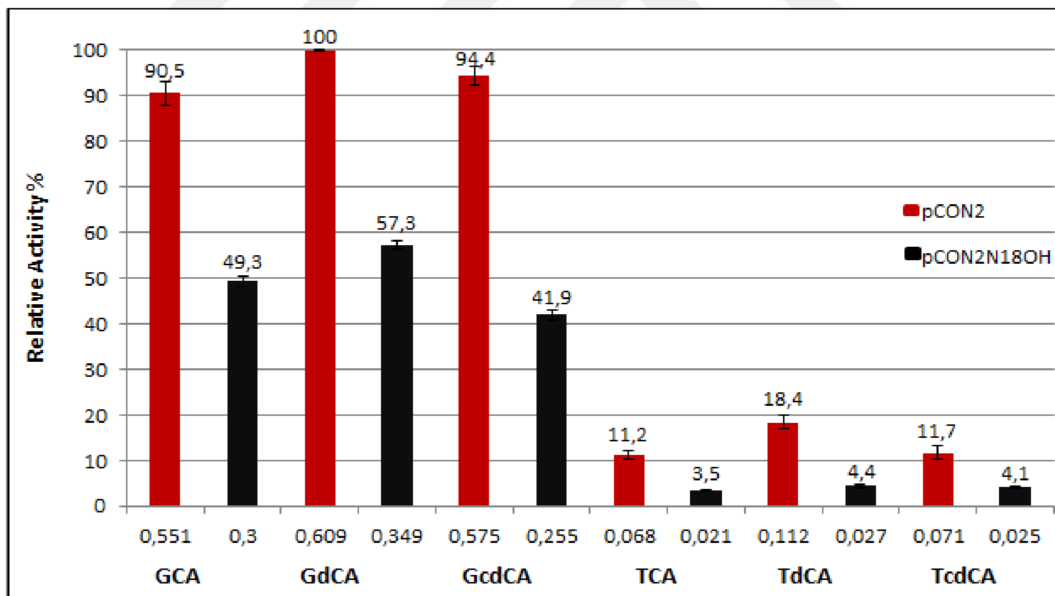

Figure 4.48. Comparison of the substrate specifity of pCON2N180H (mrBSH) with that of pCON2 (wrBSH) enzymes where six major bile salts were used. Relative deconjugation activity was measured for each substrate compared to the highest activity (GdCA taken as %100) in the assay.

4.8 Partial Protein Purification of Wild-type and Mutant BSH Enzymes and SDS PAGE Analysis

Partial protein purification of wrBSH and mrBSH were carried out with ÄKTAprime plus which is a chromatography system that performs simple purifications of tagged and untagged proteins (GE Healthcare). Partially purified wild type and mutant BSH proteins of *Lb. plantarum* B14 strain were visualized by SDS-PAGE (Figures 4.50-55). The 37 kDa molecular mass of BSH enzyme, which is consistent with theoretical molecular mass of wrBSH proteins obtained by ExPASy program (http://web.expasy.org/protparam/), was observed on SDS-PAGE (Figure 4.49).

Number of amino acids: 324
Molecular weight: 37077.69
Theoretical pI: 5.21

Amino acid composition:

Ala (A)	14	4.3%	Lys (K)	17	5.2%
Arg (R)	10	3.1%	Met (M)	7	2.2%
Asn (N)	28	8.6%	Phe (F)	14	4.3%
Asp (D)	17	5.2%	Pro (P)	13	4.0%
Cys (C)	5	1.5%	Ser (S)	29	9.0%
Gln (Q)	13	4.0%	Thr (T)	15	4.6%
Glu (E)	19	5.9%	Trp (W)	2	0.6%
Gly (G)	18	5.6%	Tyr (Y)	27	8.3%
His (H)	6	1.9%	Val (V)	21	6.5%
Ile (I)	18	5.6%	Pyl (O)	0	0.0%
Leu (L)	31	9.6%	Sec (U)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 36
Total number of positively charged residues (Arg + Lys): 27

Figure 4.49. Amino acid composition analysis of the *Lb. plantarum* BSH 14 protein by SIB (Swiss Institute of Bioinformatics) ExPASy ProtParam sequence analysis translate tool (http://web.expasy.org/protparam/).

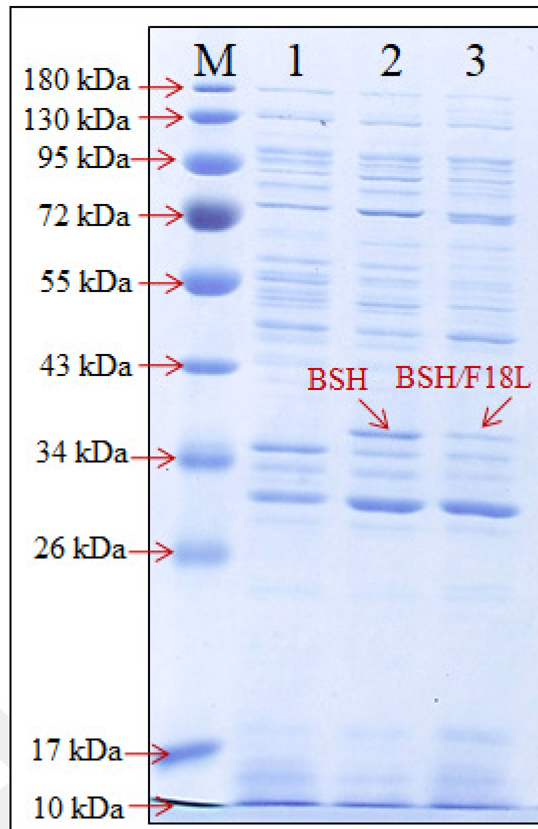

Figure 4.50. SDS-PAGE of partially purified mrBSH/F18L. M, Protein ladder; lane 1, partially purified cell extracts of BLR(DE3) not carrying *bsh* in pET22b expression vector (negative control); lane 2, the partially purified wrBSH protein (positive control); lane 3, partially purified mrBSH/F18L protein.

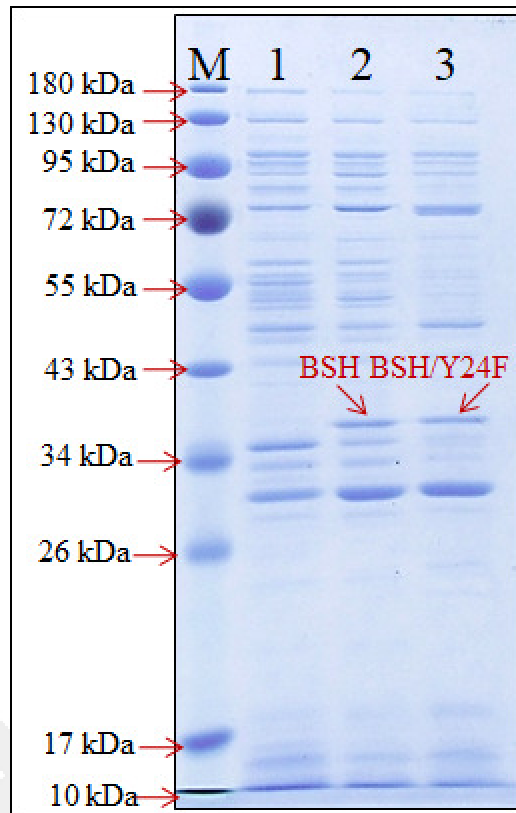

Figure 4.51. SDS-PAGE of partially purified mrBSH/Y24F. M, Protein ladder; lane 1, partially purified cell extracts of BLR(DE3) not carrying *bsh* in pET22b expression vector (negative control); lane 2, the partially purified BSH protein (positive control); lane 3, partially purified mrBSH/Y24F protein.

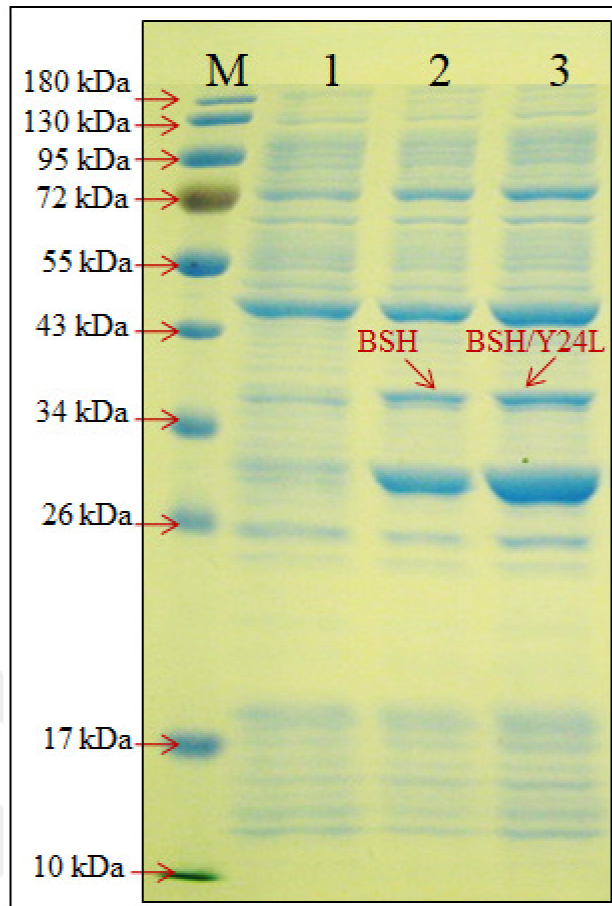

Figure 4.52. SDS-PAGE of partially purified mrBSH/Y24L. M, Protein ladder; lane 1, partially purified cell extracts of BLR(DE3) not carrying *bsh* in pET22b expression vector (negative control); lane 2, the partially purified BSH protein (positive control); lane 3, partially purified mrBSH/Y24L protein.

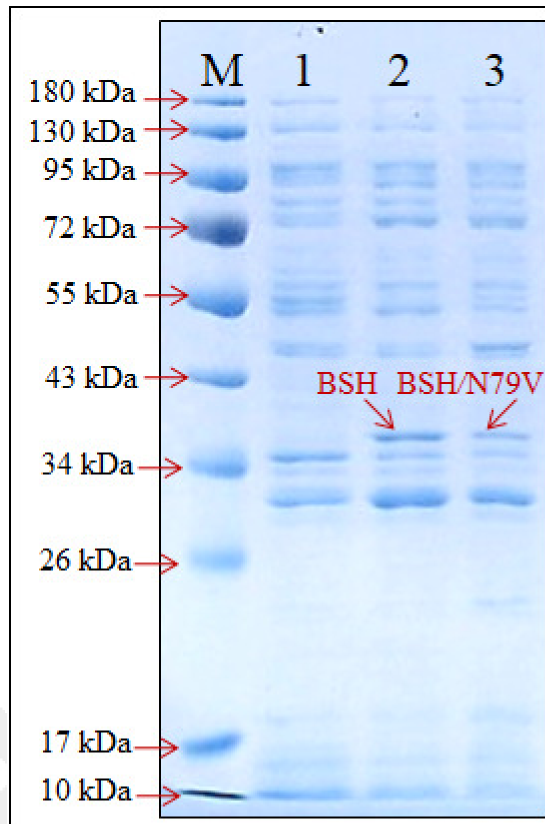

Figure 4.53. SDS-PAGE of partially purified mrBSH/N79V. M, Protein ladder; lane 1, partially purified cell extracts of BLR(DE3) not carrying *bsh* in pET22b expression vector (negative control); lane 2, the partially purified BSH protein (positive control); lane 3, partially purified mrBSH/N79V protein.

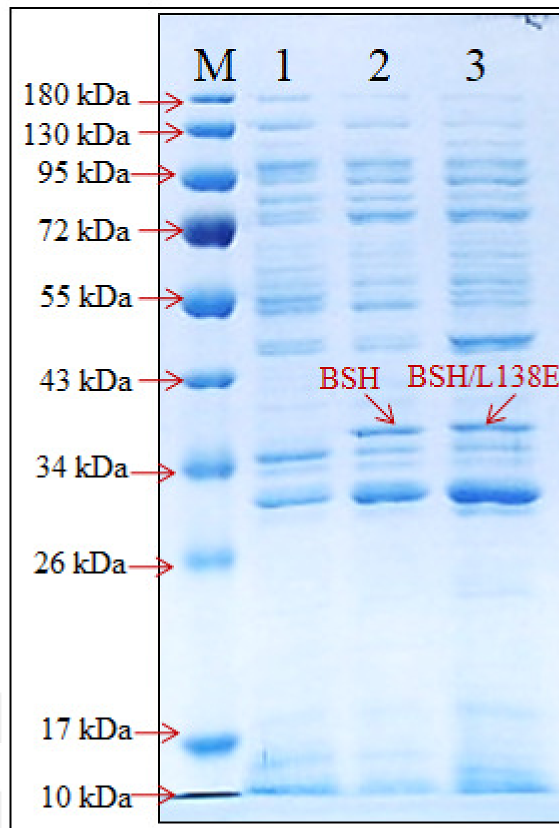

Figure 4.54. SDS-PAGE of partially purified mrBSH/L138E. M, Protein ladder; lane 1, partially purified cell extracts of BLR(DE3) not carrying *bsh* in pET22b expression vector (negative control); lane 2, the partially purified BSH protein (positive control); lane 3, partially purified mrBSH/L138E protein.

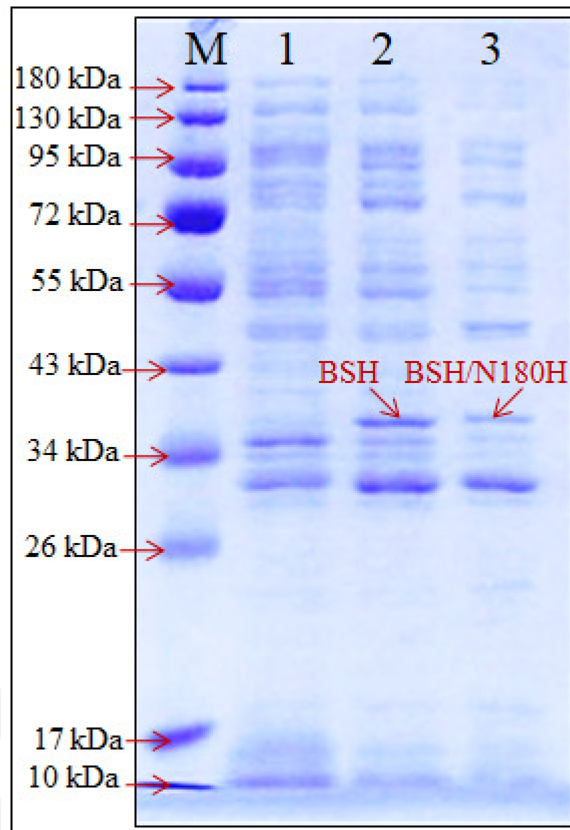

Figure 4.55. SDS-PAGE of partially purified mrBSH/N180H. M, Protein ladder; lane 1, partially purified cell extracts of BLR(DE3) not carrying *bsh* in pET22b expression vector (negative control); lane 2, the partially purified BSH protein (positive control); lane 3, partially purified mrBSH/N180H protein.

5. DISCUSION

Microbiome consists of a very complex ecosystem in which prokaryotic and eukaryotic cells coexist. Diet is the most important factor in the development of microbial composition and is the regulator of mammalian cell physiology and metabolism. In consequence of new "omics" technologies and system biology studies, scientists are beginning to solve complex interactions in the gastrointestinal system, intestinal microbiology, liver and bile acids. Better identification of precursor microorganisms having bile salt hydrolase activity of the intestinal microbiota will enable an understanding of cell signaling pathways and gene expression rates affecting the host. Bile salt hydrolysis is a biologically very important reaction in the gastro-intestinal tract, and is a transitional step in biotransformation of bile salts which carried out by intestinal bacteria (Batta et al., 1990; Jones et al., 2018). The formation and accumulation of seconder bile salts has significant negative effects on various diseases such as gallstone and colon cancer formation (Ajouz et al., 2014), on the physiology of the host, as opposed to the probiotic preference for properties such as lowering blood cholesterol levels (Liong and Shah, 2015), antimicrobial (Harackova et al., 2017) and anti parasitic (Allain et al., 2018) properties and bile tolerance (Ridlon et al., 2006).

Secondary bile salts produced from deconjugated bile salts can not be completely absorbed by terminal ileum and accumulate in the colon. Studies indicate that some of the toxic secondary bile acids such as Lithocholic acid and Deoxycholic acid cause colon cancer by influencing inflammation, DNA damage and apoptosis induction (Korzoni et al., 2000; Barrasa et al., 2011; Ridlon et al., 2016-2). Bile salt hydrolase enzymes play a role in host lipid metabolism. Over the past few decades, probiotic bacteria with BSH activity have been used to decrease cholesterol levels in humans and animals (Jones et al., 2004). In future studies, efforts should be made to control the diversity and metabolic abilities of

the intestinal microbiome like as the bile acid pool combination in order to alleviate pathophysiological effects for the human (Ridlon et al., 2016).

To show the relationship between BSH enzyme and toxic secondary metabolites, structure of the BSH and its specifity must be better understood, although the structure and reaction mechanism of such important BSH enzymes have not known been elucidated in detail. In addition to three dimensional structure studies (Yadav et al., 2107; Liang et al., 2018) of BSH enzyme, site-directed mutagenesis is used to understand structure and function of the enzyme (Chand et al., 2018).

The BSH enzyme catalyzes the deconjugation of both glycoconjugated and tauroconjugated bile acids on the amide bond. For the most part, BHSs of lactic acid bacterial have a higher affinity for glycoconjugate bile salts (Tanaka et al., 2000; Kim et al., 2004). In this study it was shown that *Lb. plantarum* B14-BSH exhibited greater hydrolysis toward glyco-conjugated bile salts compared to tauro-conjugated bile salts properly.

Despite the tremendous diversity of amino acid sequences, all Ntn-hydrolase family members (Figure 5.2), BSH or PVA, share a core structural topology in their active sites (Figure 5.1). Therefore, all these hydrolases exhibit a similar catalytic reaction mechanism (Kumar et al., 2006; Lambert et al., 2018; Chand et al., 2018). The structures of BSHs from *Clostridium perfringens* (Rossacha et al., 2015), *Bifidobacterium longum* (Kumar et al., 2004), *Lb. salivarius* (Xu et al., 2016) and *Enterococcus faecalis* (Chand et al., 2018) and the structures of PVAs from *Bacillus sphaericus* (Suresh et al., 1999), *Bacillus subtilis* (Rathinaswamy et al., 2005), *Bifidobacterium longum* (Kumar et al., 2006), *Pectobacterium atrosepticum* were reported (Avinash et al., 2013). BSH enzyme activity had showed that unique allosteric catalytic behaviour. In addition to this, in many review articles, indicated that the importancy of amino acid residues that responsible for substrate specifity, substrate binding and stability of both Ntn-hydrolases (Avinash et al., 2016; Chand et al., 2018). On the other hand, the alterations in the structure of binding sites effect significiant changes in catalytic specifity of these enzymes (Lambert et al., 2008). PVAs from *Bacillus*

subtilis (Rathinaswamy et al., 2012) and *Petrobacterium atrosepticum* (Avinash et al., 2013) were showed only PVA hydrolase activities but PVA of *Bacillus sphaericus* (Suresh et al., 1999) showed also up to 30% BSH activity. *Bifidobacterium longum* enzyme was showed pure BSH activity (Kumar et al., 2006) while BSH of *Clostridium perfringens* was showed also PVA activity (Rossacha et al., 2005). Hence, comprehensive structural substrate analysis of BSH with site directed mutagenesis of amino acid substitution, would help to reveal the importance of residues in catalysis and to understand why BSH displays a broad substrate specifity and catalytic activity (Geng and Lin, 2017; Xu et al., 2016).

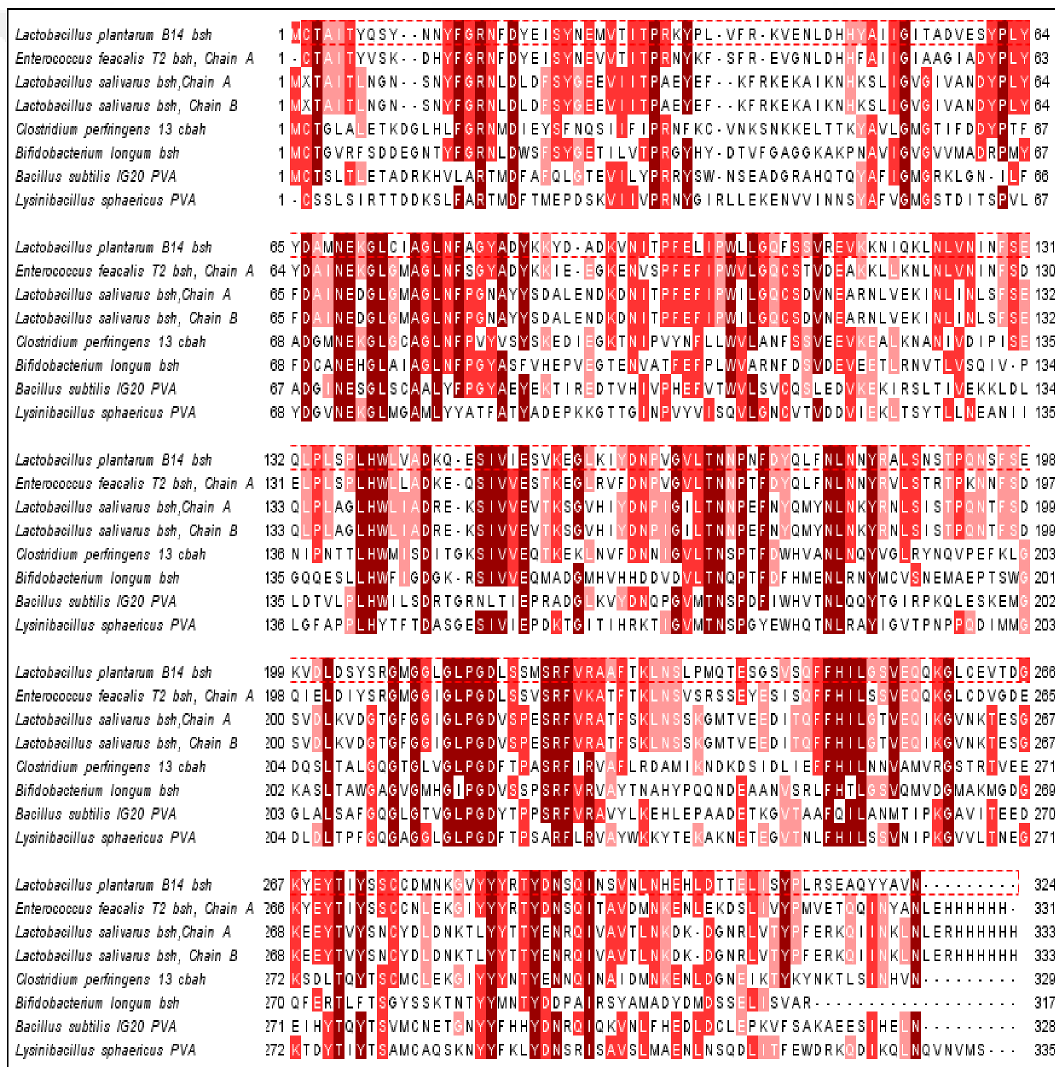

Figure 5.1. The 324 amino acid residues of the wild type *bsh* gene (wpCON1) was aligned with structures determinated BSHs and PVAs by Jalview 2.8.0b1. Labelled amino acids represent the conserved or semiconserved amino acids.

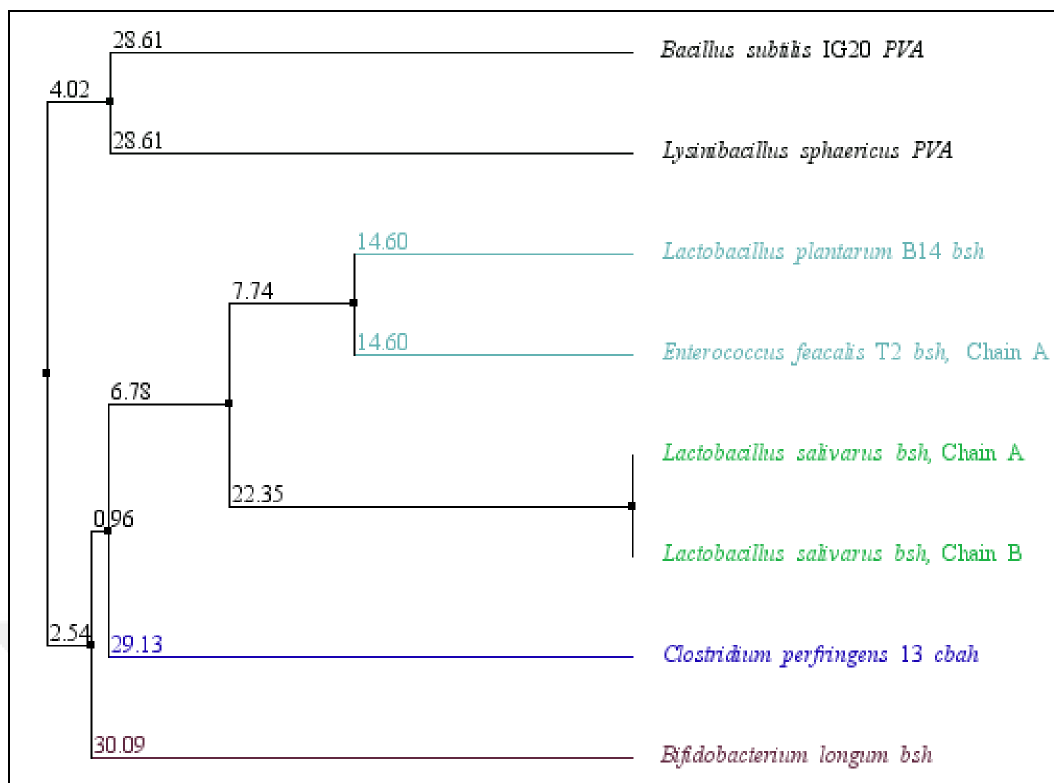

Figure 5.2. *Lb. plantarum* B14 BSH enzyme (AN: KY080706) was represented in a phylogenetic tree structure with containing other BSH species; *Enterococcus feacalis* T2 (AN: 4WL3_A), *Lb. salivarus* (AN: 5HKE_A, 5HKE_B), *Bifidobacterium longum* (AN: Q9KK62), conjugated bile acid (CBAH) of *Clostridium perfringens* species (AN: P54965) and penicillin acylase of *Lysinibacillus sphaericus* (AN: 3PVA_A), *Bacillus subtilis* IG20 (AN: CAJ77223.1) by Jalview 2.8.0b1. The Phylogenetic tree indicated that the *Lb. plantarum* B14 BSH enzyme related with BSH enzyme of *Enterococcus feacalis* T2.

In this study, multiple sequence alignment analysis of the *bsh* gene sequences indicated that BSH of CGH *Lb. plantarum* B14 strain belong to the Ntn- hydrolase super family. All members have both conserved N-terminal cysteine residue (Cys-2) in the active center (Tanaka et al. 2000), which plays role as a nucleophile and a proton donor during of catalysis (Oh et al. 2008; Kumar et al. 2010). Amino acid sequence alignment confirmed that the five residues, Phe-18, Tyr-24, Asn-79, Leu-138 and Asn-180, were substantially conserved in most of BSH enzymes and the amino acid motifs around active sites of BSH proteins (Rani et al., 2017; Chand et al., 2018; Liang et al., 2018) are consistent with BSH of *Lb. plantarum* B14 strain.

Although the BSH of *Lb. plantarum* B14 has similar catalytic domains with those of other *Lactobacillus* species, its substrate preference is different than the others. Phylogenetic analysis suggested that BSH including strains were expanded substrate specificity against conjugated bile acids (Jones et al., 2008). In slico studies by Xu et al. (2016) and Rani et al. (2017) pointed to the fact that substrate preference relates to steroid cholyl or amino acid moieties. Our results showed that mutations on these amino acids altered substrate specifity and created a preferance towards GDCA rather than TDCA. We also found that almost all the mutations we created decreased BSH activity due to substrate specifity shift except Tyr24 mutations. Tyr24Phe mutation displayed the high activity due to *Lb. plantarum* B14 BSH GdCA activity and the substrate specificities slightly decreased towards GCA, GdCA, GcdCA, TCA, TdCA and TcdCA. On the other hand, Tyr24Leu mutation caused 50% decrase in BSH activity. In addition to this, Phe18Leu mutation caused a significiant decrease in the enzyme activity and created relative activities of 37,3%, 39,6% and 26,3% to glycocholic acid, glycodeoxycholic acid and glycochenedeoxycholic acid respectively. It was found that while Asn79Val mutant was significanty decreased BSH enzyme activity against by 73% to glycodeoxycholic acid (GDCA), Leu138Glu mutation decreased specificity of the enzyme against by 22% glycocholic acid (GCA) and Asn180His mutation decreased specificity of the enzyme against by 39% glycochenedeoxycholic acid (GCDCA). PAGE results revealed that all target mutant enzymes were folded and mutations did not affect stability of the enzyme. These results are consistent with results of the previous experiments which indicated that BSH had higher affinity towards glyco-conjugated bile salts (Lambert et al., 2008; Ramasamay et al., 2010). These findings support the idea that BSH primarily recognize substrates with the amino acid residues than the cholate moieties (Rossocha et al., 2005).

Liang et al. (2018) indicated that the residue of 82 (Corresponds to Asn79 in *Lb. plantarum* B14) was considerable affect to the activity of BSH; add to this, Gln82 and Asn82 were supposed to be essential residues for high activity of BSH that in a manner compatible with our work. Studies of the site directed mutantions of Asn79 of *Enterococcus faecalis* BSH by Chand et al. (2018) showed that significant activity reduction was observed at substituted to Trp79 and substituted

to Tyr79 activity while maintaining at a large rate. Similarly, our study showed that substitution of Asn79 to Val79 significantly decreased in BSH of *Lb. plantarum* B14 activitiy. In addition, Jarocki and Targonski (2013) identified that *bsh* and *pva* genes had a significant genetic variation than 16S rRNA sequences determination which a result of the phylogenetic analysis of the sequences. Thus, these different gene sequences encoding bile salt hydrolases may also be useful molecular markers for phylogenetic studies and for the specific identification of human faecal bacterial species.

6. CONCLUSIONS AND RECOMMENDATIONS

In conclusions, BSH enzyme from *Lb. plantarum* B14 was used to substitute partially conserved amino acids, Phe-18, Tyr-24, Asn-79, Leu-138 and Asn-180 supposed to be responsible for substrate specificity, for Leu-18, Phe-24, Leu-24, Val-79, Glu-138 and His-180 amino acids respectively by site directed mutagenesis. *Lb. plantarum* B14 BSH showed that different substrate specifities to six major bile salts; glycocholic acid (GCA), taurocholic acid (TCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDA), and the taurochenodeoxycholic acid (TCDA) The highest BSH activity was observed with glycodeoxycholic acid (determinated as 100% activity) and other glycine conjugated bile acids. *Lb. plantarum* B14 BSH had a maximum activity to GDCA. Direct plate results showed that all mutants had lost the opaque appearance on plate except Phe24Try mutation.

This study also indicated that the enzyme has an optimum activity in range of slightly acidic pH, 2.5-7.0 similar to Tanaka et al., (2001) that they showed that BSH purified from *Bifidobacterium longum*, was stable between 4.0 and 8.0. In addition to optimum temperature of BSH activity was also studied, and it was found that BSH had a maximum activiy at 37°C with the range of 25°C to 60°C. This temperature is also preferred because of its equivalent to human body temperature.

Ninhydrin assay results indicated that all of the mutants BSHs had different alternations of (decreased in different percentances due to different bile salts) the BSH activity against the especially glyco-conjugated bile acids (Table 6.1.).

Table 6.1. The relative activity profiles of rBSH and mrBSHs.

Sample/relative activity (%)	GCA	GdcA	GcdCA	TCA	TdCA	TcdCA
wBSH	90,5	100	94,4	11,2	18,4	11,7
F18L	37,3	39,6	26,3	1	1,5	1,8
Y24F	65	75,9	66,8	8,9	14,6	11,5
Y24L	44,7	52,4	40,6	11	7,1	4,3
N79V	43,8	18,9	59,9	0,3	2,8	1,6
L138E	64	64,4	61,1	6,2	10,7	11,5
N180H	49,3	57,3	41,9	3,5	4,4	4,1

Resuls of this study showed that partially conserved amino acids; Phe-18, Tyr-24, Asn-79, Leu-138 and Asn-180 are responsible for substrate specificity of the BSH enzymes. PAGE results indicated that these amino acids did not affect expression of BSH enzyme. Therefore, our results support the hypothesis that the some partially conserved amino acids are responsible from bile salt substrate specificity of the BSH enzyme. To better understand substrate specificity of BSH enzyme more site-directed mutagenesis, such as double mutated are required on remaining target amino acids such as Val58, Phe129, Gln133 and I136 of BSH from *Lb. plantarum* B14.

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APPENDICES

8. APPENDICES

Appendix A

BACTERIAL GROWTH MEDIA

Liquid MRS (de MAN, ROGOSA and SHARPE) Medium

To prepare a liquid MRS medium, 52.2 g of MRS is dissolved in upH_2O to a final volume of 1 L. MRS broth pH does not require adjusting. The preparation is then sterilized at 121°C for 15-20 minutes.

Liquid MRS with 30% glycerol

To prepare a liquid MRS medium with 30% glycerol, 30 ml of glycerol is dissolved in 100 ml of MRS broth. The preparation is then sterilized at 121°C for 15-20 minutes.

MRS Plate

In order to prepare a MRS plate, 15 g of agar-agar (Merck) is added to 1 L of liquid MRS medium. The mixture is then sterilized at 121°C for 15-20 minutes.

Liquid LB (Luria Bertani) Medium

Contents	For 1 Liter
Tryptone	10 g
Yeast extracts	5 g
NaCl	10 g

All of the above ingredients (Table) were added in 900 ml dH_2O and mixed them thoroughly (with stir bar). The pH was checked with pH meter (Orionstar A111, Thermo Scientific). When pH was adjusted to 7.5 with NaOH, the volume was completed to 1000 ml with dH_2O and it was autoclaved at 121ºC for 15-20 minutes in liquid cycle. Then it was cooled down to 60ºC and antibiotic and glycerol was added if necessary.

LB Plate

In order to prepare an agar plate, the amount of agar used should be 1.5% of the amount of the liquid LB medium.

For instance, in 1 L liquid LB medium, we added 15 agar-agar (Merck) and sterilized the preparation at 121°C for 15-20 minutes.

25mg/ml Ampicillin Stock Solution (for 10 ml)

For 10 ml 25 mg/ml stock solution, 0.25 g Ampicillin Sodium Salt (Sigma) was dissolved in 10 ml dH_2O and sterilized by using 0.22 µm filter, divided per 1000 µl to Appendorf tubes and stored at -20°C. 400µl (25 mg/ml) ampicillin was used for 100 ml medium.

0.1 M IPTG Stock Solution (for 5 ml)

For 5 ml stock solution, 0,119 g (mw: 238.3) was dissolved in up to 5 ml ddH_2O and sterilized by using 0.22 µm filter, divided per 1000 µl to eppendorf tubes and stored at -20°C.

LB Plate with ampicillin

To make an LB plate with ampicillin, 4 ml (0.025 g/ml) of ampicillin is added for 1 L of sterilized liquid LB medium.

LB Plate with IPTG, X-gal and Ampicillin

After sterilizing the 1 L LB plate medium, 1 mM IPTG (Isopropyl-β-D-1 thiogalactopyranoside), 25 μg/ml ampicillin and 40 μg/ml X-gal were added into the medium.

Contents	For 1 Liter
IPTG	3 ml
X-gal	4 ml
Ampicillin	4 ml

Appendix B

SOLUTIONS AND BUFFERS

Solutions and Buffers of Genomic DNA Isolation

20 Mm Tris HCl

1.576 g of Trizma Hydrochloride (Sigma) is dissolved in 10 ml of upH_2O. Then, from that solution, 2 μl is dissolved in 100 ml of upH_2O to get the final concentration of 20 mM.

2 mM EDTA

0.447 g of EDTA is dissolved in 30 ml of upH_2O. Then, 20 μl from that solution is dissolved in 400 μl of upH_2O to get the final concentration of 2 mM. The pH has to be adjusted to 8.0 or else the EDTA will not completely dissolve.

Lysis Buffer

Contents	For 400 μl
20 mM Tris HCl	8.0 μl
2 mM EDTA	20.0 μl
1.2% Tripton X-100	4.8 μl
20 mg/mL Lysozyme	266.7 μl

All this components are added to 100.5 μl of upH_2O for a final buffer volume of 400 μl.

For primer resuspension

TE Buffer

3.634 g of Trizma base
30 ml upH_2O

The pH is adjusted to 8.0.

For Competent Cell Preparation

0.1 M $CaCl_2$

1.48 g of $CaCl_2$

100 ml upH_2O

The pH is adjusted to 7.0.

0.1 M $CaCl_2$ with 10% glycerol

9 ml of $CaCl_2$

1 ml of glycerol

10 X TAE Buffer

Contents	For 1 L
Tris Base	48.50 g
Glacial acetic acid	11.42 g
0.5 M EDTA	20 ml

All these components are dissolved in enough upH_2O to make a final buffer volume of 1 L.

1X TAE Buffer

100 ml of 10X TAE buffer

900 ml upH_2O

Preparation for Cell Extract

1 M NaH_2PO_4 Buffer

12 g of monobasic sodium phosphate (NaH_2PO_4) are dissolved in 100 ml upH_2O. The pH recorded was 3.97. The buffer solution was then sterilized at 121°C for 15-20 minutes.

1 M Na_2HPO_4 Buffer

14.2 g of dibasic sodium phosphate (Na_2HPO_4) are dissolved in 100 ml ddH_2O and pH measured was 10.02. The buffer solution was sterilized with autoclave (121°C for 15-20 minutes).

0.1 M Sodium Phosphate Buffer

For all the 0.1 M sodium phosphate buffer of different pH values, a carefully calibrated mixture of monobasic and dibasic sodium phosphate are used to obtaine the various pH.

Resuspention Buffer (pH: 7.8)

Contents	For 1 L
20 mM NaPi Buffer	20 ml of 1 M NaPi
500 mM NaCl	29.22 g

For Ninhydrine Assay

0.1 M NaOH

0.4 g NAOH
100 ml upH_2O

1% SDS in 0.1 M NaOH

0.05 g Sodium Dodecyl Sulfate
5 ml of 0.1 M NaOH

Preparation of Reagent E for Lowry Assay

***Reagent A*:** 2% (w/v) (Na_2CO_3) Sodium carbonate (Sigma) in 0.1 M Sodium hydroxide (mw: 40.00- Rie-de Haen). 1 g Na2CO3 + 50 ml NaOH (0.1 M)

***Reagent B*:** 1% (w/v) ($CuSO_4 \cdot 5H_2O$) Copper (II) sulfate pentahydrate (mw: 249.68-Sigma) in distilled water. 5mg $CuSO_4 \cdot 5H_2O$ + 50 ml dH_2O

***Reagent C*:** 2% (w/v) ($NaKC_4H_4O_6$) sodium potassium tartrate (mw: 282.23-Sigma) in distilled water. 10mg potassium tartrate + 0.5 ml dH_2O

***Reagent D*:** 1 part B (500 µl) + 1 part C (500 µl)

***Reagent E*:** 1 ml D + 50 ml A.

1 mg/ml BSA

0.001 g BSA (Bovine Serum Albumin)
1 ml of H_2O

0.2 M Glycine Solution

0.7507 g H_2NCH_2COOH (Glycine)
50 ml of H_2O

0.5 M Sodium Citrate Buffer

2.941 g $C_6H_5Na_3O_7.2H_2O$ (Sodium Citrate)

20 ml of H_2O

The pH is adjusted to 5.5 and then buffers are sterilized at 121°C for 15-20 minutes.

15% Trichloroacetic Acid

7.5 g trichloroacetic acid

50 ml of H_2O

The solution was then sterilized by filtration.

1% Ninhydrin Solution

0.1 g ninhydrin

10 ml of Sodium Citrate Buffer

Ninhydrin Reagent

0.5 ml of 1% Ninhydrin Solution

1.2 ml glycerol

0.2 ml of 0.5 M Sodium Citrate Buffer

40 mM DTT (Dithiothreitol) Stock Solution

For 5 ml stock solution, 0.031 g (mw: 154.25g) was dissolved in up to 5 ml ddH_2O and sterilized by using 0.22 µm filter, divided per 1000 µl to eppendorf tubes and stored at -20 °C.

Solutions of bile salts

Bile salt	MW	Gr of used	ddH_2O
GCA	487.6 (Calbiochem)	0.059 g	3000 µl
GDCA	471.6 (Calbiochem)	0.069 g	3000 µl
GCDCA	471.61 (Sigma)	0.025 g	1325 µl
TCA	537.7 (Calbiochem)	0.059 g	3000 µl
TDCA	521.7 (Calbiochem)	0.063 g	3000 µl
TCDCA	521.69 (Sigma)	0.1 g	4792 µl

For Purification of rBSH and mrBSH Enzymes

Binding Buffer pH: 7.8

Contents	For 1 L
20 mM NaPi Buffer	20 ml of 1 M NaPi
500 mM NaCl	29.22 g

These mixture is dissolved in UpH_2O for a final volume of 1 L. The pH is adjusted with phosphoric acid.

Elution Buffer Ph: 6.0

Contents	For 1 L
20 Mm NaPi Buffer	20 ml of 1 M NaPi
500 Mm NaCl	29.22 g
50 Mm Imidazole	3.404 g

These mixture is dissolved in UpH_2O for a final volume of 1 L. The pH is adjusted with phosphoric acid.

For SDS-PAGE Analysis

12% Separating Gel (for 15 ml)

ddH_2O	**5.1 ml**
30% Acrylamide-Bisacrylamide	6.0 ml
Separating Gel Buffer (pH: 8.8)	3.75 ml
10% SDS	0.15 ml
10% APS	75 µl
TEMED	7.5 µl

4% Stacking Gel (for 10 ml)

ddH_2O	**6.2 ml**
30% Acrylamide-Bisacrylamide	1.3 ml
Stacking Gel Buffer (pH:6.8)	2.5 ml
10% APS	50 µl
TEMED	5 µl

Acrylamide-Bisacrylamide (for 50 ml)

Acrylamide	**14.6 g**
Bisacrylamide	0.4 g
ddH_2O	Up to 50 ml

10% SDS (for 10 ml)

1 g SDS was dissolved in up to 10 ml ddH_2O.

4X Stacking Gel Buffer (pH:6.8)

3.05 g Trisma Base (mw: 121.14 g/mol) was dissolved in ddH_2O up to 50 ml. pH adjusted with HCl.

4X Separating Gel Buffer (pH: 8.8)

9.1 g Trisma Base (mw: 121.14 g/mol) was dissolved in ddH_2O up to 50 ml. pH adjusted with HCl.

10% APS (for 500 µl)

0,05 g APS was dissolved in up to 500 µl ddH_2O.

10X Electrode Buffer (for 500 ml)

15 g Trisma Base (mw: 121,14 g/mol) and 72 g Glycine (mw: 75,07 g/mol) was dissolved in ddH_2O up to 500 ml.

Loading Dye for SDS-PAGE (for 1 ml)

ddH_2O	**340 µl**
10% SDS	400 µl
Glycerol	120 µl
0.5 M Tris/HCl (pH:6.8)	100 µl
0.5 % Bromophenol Blue	40 µl

Coomassie Stain Solution (for 1 L)

Coomassie Brillant G Blue	**1 g**
Glacial Acetic Acid	100 ml
Methanol	400 ml
ddH_2O	500 ml

De-Stain Solution for Coomassie (for 1 L)

Methanol	**100 ml**
Glacial Acetic Acid	100 ml
ddH_2O	800 ml

Appendix C

CHEMICALS

Acrylamide (Sigma)

Agar (Merck)

Agarose (Sigma)

Ammonium persulfate [APS] (Sigma)

Ampicillin (Sigma)

Bovine Serum Albumin (Sigma)

Calcium Chloride [minimum 93.0%, granular anhydrous] (Sigma)

Calcium chloride dihdrate [CaCl2] (Sigma)

Comassie Brillant Blue G-250 (Fluka)

Cupper II sulphate pentahydrate (Sigma)

EDTA [Ethylenediaminetetraacetic acid] (Sigma)

EtBr [Ethidium Bromide] (Sigma)

Glycerol, cell culture tested (Sigma)

Glycine (Merck)

IPTG [isopropyl-beta-D-thiogalactopyranoside] (Thermo Scientific)

Man Rogosa Sharpe (MRS) (Sigma)

N,N’-Methyenebisacrylamide (Sigma)

Ninhydrin (Sigma)

Peptone from casein (Merck)

Potassium Sodium Tartarate (Sigma)

Sodium Carbohydrate (Sigma)

Sodium Chloride [NaCl] (Merck)

Sodium Citrate Dihydrate (Merck)

Sodium Dithiothreitol [DTT] (Aplichem)

Sodium dodecyl sulfate [SDS] (Sigma)

Sodium Hydroxide [NaOH] (Merck)

Sodium Phosphate dibasic (Merck)

Sodium Phosphate monobasic (BDLab)

Tetrachloro acetic acid (Sigma)

Trichloroacetic Acid (Merck)

Trizma Base (Sigma)

Trizma Hydrochloride (Sigma)

Yeast Extract Granulated (Merck)

Tween® 20 (Sigma)

Appendix D

ENZYMES AND OTHER CHEMICALS

*Not*I (Fermentas)

*EcoR*I (Fermentas)

Fast Digest Pack (Fermentas)

*Sma*I (Fermentas)

Dnase (Sigma)

Rnase (Sigma)

Lysozyme (Fluka)

Polyethylene Glycol (Fermentas)

T4 DNA Ligase (Fermentas)

Page Ruller Prestain Protein Ladder (Thermo Scientific)

Pfu DNA Polymerase (Fermentas)

dNTP Mix (Fermentas)

1 kb Plus DNA Ladder (Fermentas)

6X Loading Dye Solution (Fermentas)

Glycocholic Acid, Sodium Salt (Calbiochem)

Glycodeoxycholic Acid, Sodium Salt (Calbiochem)

Glycochenodeoxycholic Acid, Sodium Salt (Sigma)

Taurocholic Acid, Sodium Salt (Calbiochem)

Taurodeoxycholic Acid, Sodium Salt (Calbiochem)

Taurochenodeoxycholic Acid, Sodium Salt (Sigma)

X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) (Fermentas)

Appendix E

EQUIPMENTS USED IN THIS STUDY

-80°C deepfreezes (Biolaps) and (Thermo scientific)

-20°C deepfreeze (Arçelik)

34°C and 37°C Incubators (Nuve EN 500, Nuve FN 500)

34°C and 37°C shaker-incubator (Gerhardt)

+4°C refrigerators (Arçelik)

AKTAprime^{TM} plus (GE Healthcare)

Autoclave (Hirayana)

Centrifuge (Hettich Rotina 38R)

Desktop centrifuge (Hettich Micro 120)

Electrophoresis system (Thermo Scientific)

Imaging system (UVP Photo Doc-It TM)

Micropipettes (Finnipipette)

PCR (Techne, TC 3000)

pH meter (HANNA HI 221)

Power supply (Thermo EC 250-90)

Shaker-heater (IKA RCT basic)

Spektrophotometer (HITACHI U-1900)

UV Transalluminator (UVP)

Vortex (Yellowline TTS2)

Water Purification System (Human Corporation)

9. CURRICULUM VITAE

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List of Publications :

a) SCI-

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b) National :

1. Sılan F ve **Zafer C** (2004) “Faktör V Leiden Mutasyonu”, Düzce Tıp Fakültesi Dergisi; 1: 33-36.

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a) International :

1. Öztürk M, **Önal C**, Kılıçsaymaz Z and Ba NM (2016) “Functional Characterization of Val58Met and Phe129Ile Mutants of bile Salt Hydrolase from *Lactobacillus plantarum* in *E. coli* BLR(DE3)”, The FEBS Journal Special Issue Volume 283 Supplement 1 September, 41st Federation of European Biochemical Societies (FEBS) of Molecular and Systems Biology for a Better Life.

b) National :

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1. Silan F, **Zafer C**, Kuru D, Mahmutyazıcıoğlu K and Hacıhanefioğlu S (2007) "Two sibs with currarino syndrome with 7q34 deletion due to maternal t(7;14)(q34;13)" 6th European Cytogenetics Conferance (6th ECC) 7-10 July, Istanbul,Turkey.

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