ABANT IZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY

FUNCTIONAL AND STRUCTURAL ANALYSIS OF RECOMBINANT MUTANT BILE SALT HYDROLASES (BSHS) FROM *LACTOBACILLUS PLANTARUM* B14

MASTER OF SCIENCE

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APPROVAL OF THE THESIS

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FUNCTIONAL AND STRUCTURAL ANALYSIS OF RECOMBINANT MUTANT BILE SALT HYDROLASES (BSHS) FROM *LACTOBACILLUS PLANTARUM* B14 submitted by **ZEKİYE KILIÇSAYMAZ** in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Biology, The Graduate School of Natural and Applied Sciences of ABANT IZZET BAYSAL UNIVERSITY in 29/12/2017** by

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Zekiye KILIÇSAYMAZ

ABSTRACT

FUNCTIONAL AND STRUCTURAL ANALYSIS OF RECOMBINANT MUTANT BILE SALT HYDROLASES (BSHS) FROM *LACTOBACILLUS PLANTARUM* B14
MSC THESIS
ZEKİYE KILIÇSAYMAZ
ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: ASSOC. PROF. DR. MEHMET ÖZTÜRK)

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Bile salt hydrolase (BSH), synthesized by some intestinal bacteria, catalyzes the hydrolysis of taurine or glycine-linked bile acid into the amino acid and deconjugated bile acid in human intestine. Deconjugated bile acids potentially play an important role in the reduction of blood cholesterol level and formation of some gastrointestinal diseases such as cholestasis, gallstone formation and colon cancer. Because of the link between BSH and human health, the structural and biochemical features of BSHs necessitate an intense study to better understand their role in regulating bacterial and host metabolism. BSHs exhibit higher variations in their catalytic activities and substrate specificities. However, six amino acids, supposed to be responsible from catalytic activity, are totaly conserved in all members of the BSH family. In order to analyze the correlation between two of these strictly conserved amino acids and catalytic activity of BSH, the asparagine-170 (N170) and arginine-223 (R223) amino acids were substituted for the valine-170 (V170) and phenylalanine-223 (F223) amino acids respectively by PCR-based site directed mutagenesis. The mutant recombinant BSHs (rBSHs) were expressed in *E. coli* BLR(DE3) strain. While the effect of the mutations on catalytic activity was detected by direct plate and ninhidrin assays, the effect of the mutations on formation of the BSHs was observed by SDS-PAGE analysis. It was found that V170 and F223 mutations resulted in prevention of BSH formation. This is the first experimental work showing that the N170 and R223 could be critical amino acids for folding or stability of the BSH, but not for the catalytic activity of this enzyme as predicted by in silico analysis of BSH related data in literature.

KEYWORDS: Probiotics, Bile Salt Hydrolase (BSH), Site-Directed Mutagenesis.

ÖZET

LACTOBACILLUS PLANTARUM B14'DEN ELDE EDİLEN REKOMBINANT MUTANT SAFRA TUZU HİDROZ (STH) ENZİMLERİNİN FONKSİYONEL VE YAPISAL ANALİZİ
YÜKSEK LİSANS TEZİ
ZEKİYE KILIÇSAYMAZ
ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
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Bazı bağırsak bakterileri tarafından sentezlenen safra tuzu hidrolaz (STH), insan bağırsağında taurin veya glisin bağlı safra asitlerini aminoasit ve dekonjüge safra asitlerine hidrolizini katalize etmekedirler. Dekonjüge safra asitleri kandaki kolesterol düzeyinin azalmasında ve kolestaz, safra taşı oluşumu ve kolon kanseri gibi bazı gastrointestinal hastalıkların oluşumunda potansiyel olarak önemli bir rol oynamaktadır. STH ve insan sağlığı arasındaki ilşkiden dolayı, STH'lerin bakteriyel ve konakçı metabolizmasındaki düzenleyici rölünü daha iyi anlamak için STH'lerin yapısal ve biyokimyasal özellikleri yoğun bir çalışma yapmayı gerektirmektedir. STH'ler, katalitik aktivitelerinde ve sübstrat özelliklerinde yüksek varyasyonlar göstermektedirler. Ancak, katalitik aktiviteden sorumlu olduğu varsayılan altı aminoasit, STH ailesinin tüm üyelerinde tamamen korunmuş durumdadır. STH'ın tamamen korunmuş aminoasitlerinin ikisi arasındaki ilişkiyi ve katalitik aktivitelerini analiz etmek için, asparajin-170 (N170) ve arginin-223 (R223) aminoasitleri sırasıyla PZR temelli yönlendirilmiş mutagenez ile sırası ile valin-170 (V170) ve fenilalanin-223 (F223) aminoasitlerine dönüştürülmüştür. Mutant rekombinant STH'ler (rSTHs) *E. coli* BLR(DE3) suşunda ifade edildi. Mutasyonların katalitik aktivite üzerindeki etkisi direkt besiyeri ve ninhidrin analizleriyle saptanırken, mutasyonların STH'lerin oluşumuna etkisi ise SDS-PAGE analizi ile gözlenmiştir. V170 ve F223 mutasyonlarının STH oluşumunu engellediği sonucu bulunmuştur. Bu çalışma, N170 ve R223 aminoasitlerinin literatürdeki STH verilerinin siliko analizlerinden tahmin edildiği gibi STH enziminin katalitik aktivitesinden sorumlu olmadığı fakat STH'ın katlanmasından ya da kararlılığında rol alan kritik aminoasitler olabileceğini gösteren ilk deneysel çalışmadır.

ANAHTAR KELİMELER: Probiyotik, Safra Tuzu Hidrolaz (STH), Yönlendirilmiş Mutagenez

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LIST OF ABBREVIATIONS AND SYMBOLS

bp : Base pair

BSA : Bovine Serum Albumin

BSH : Bile Salt Hydrolase

CHD : Coronary Heart Disease

DCA : Deoxycholic Acid

DTT : Dithiothreitol

FDA : Food and Drug Administration

GDCA : Glycodeoxycholic Acid

GIT : Gastrointestinal Tract

GRAS : Generally Recognized As Safe

HDL : High-Density Lipoproteins

IBD : Inflammatory Bowel Disease

IBS : Irritable Bowel Syndrome

IPTG : Isopropyl β-D-1-thiogalactopyranoside

kbp : Kilo base pair

Lb : *Lactobacillus*

LB : Luria-Bertani -Broth

LCA : Lithocholic Acid

LDL : Low-Density Lipoproteins

M : Molar

OD : Optic Density

PAGE : Polyacrylamide Gel Electrophoresis

PCR : Polymerase Chain Reaction

rpm : Rotation per minute

SIB : Swiss Institute of Bioinformatics

TCA : Trichloroacetic Acid

TDCA : Taurodeoxycholic Acid

Tween-20 : Polyethylene Glycol Sorbitan Monolaurate

µl : Microliter

µM : Micromolar

WHO : World Health Organization

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1. INTRODUCTION

A great number of bacteria are normally found within the human body and are involved in many tissues like the skin, the respiratory tract, the genital tract and the gastro intestinal track (GIT). The GIT have diverse and concentrated microbial population and one of the key organs of the human body. Colonisation of the GIT of human starts with contamination from birth canal and microflora formed completely in two years as to that of adults (Bullen et al. 1977). It is estimated that 300 – 500 different species live in human GIT (Guarner and Malagelada, 2003; O'Hara and Shanahan 2006). The gut microflora has a number of physiological functions associated to overall health. Therefore, bacteria may be detrimental if disturbance occurs in ecological balance of GIT (Holzapfel et al. 1998). These days, people can live many health problems because of the lifestyles. Cardiovascular diseases (CDVs) are one of the major causes of death globally. Colorectal cancer along with many other diseases like type I and II diabetes, Inflammatory bowel disease (IBD) and Irritable bowel syndrome (IBS) have been linked with an altered GIT microbiota (Sanders, 2010). World Health Organization (WHO) has estimated that 8.2 million people died from coronary heart disease (CHD) and cancers in 2012 (World Cancer Report, 2014). Therefore, potential microorganisms can be used to solve or at least alleviate the symptoms by the dietary intake of probiotics. The most commonly used probiotics are strains of lactic acid bacteria, e.g., *Bifidobacterium, Streptococcus* and *Lactobacillus.* Most of the *Lactobacillus* species, resist gastric acid, bile salts and pancreatic enzymes, considered important components of the gastrointestinal flora and relatively harmless. Furthermore, lactic acid bacteria have been demonstrated to inhibit the in vitro growth of many enteric pathogens including *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Clostridium difficle* and *Clostridium perfringens* (Meurman et.al., 1995).

1.1. Probiotics

Probiotic is derived from the Greek word probiosis that means "for life" opposed to "antibiotics" that means "against life". A probiotic is a live microorganism and indicates beneficial effects on the host's health beyond inherent fundamental nutrition (Sultan et al., 2006). The history of probiotics began with consuming of

fermented foods by human (Guarner et al., 2005). Metchnikoff (1908) discovered the therapeutic effects of probiotics in the early twentieth century. He hypothesized that Bulgarians are long-lived and healthy people because of the consumption of fermented milk products that consists of *Lactobacillus* spp. He also demonstrated that consuming lactic acid bacteria could result not only in the prolongation of the life of the host but also in the reduction of toxin-producing bacteria in the gut.

Probiotics can be defined as many different definitions. Although, the term "probiotics" was first used by Lilly and Stillwell (1965) and was defined as "substances secreted by one microorganism that stimulate the growth of another", this term was described as a "substances and organisms which contribute to intestinal microbial balance" by Parker in 1974. Today the universal meaning the term "probiotic" was defined by the WHO and the Food and Agriculture Organization of the United States as a "live microorganisms which is administered in adequate amounts, have a beneficial effect on health of the host organism" (Vasiljevic and Shah 2008). However, the most preferred definition is Fuller's (1992) definition, which is "a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance".

A great number of microorganisms are found within the different tissues of human body (Figure 1.1). These microorganisms are most often lactic acid bacteria such as *Lactobacilli*, *Bifidobacteria*, and *Streptococci* in the GIT of the human. In addition to lactic acid bacteria, GIT contains some species of yeast like *Saccharomyces boulardii* and non-lactic acid bacteria like *Bacillus*. These microorganisms are found throughout the entire GI tract, but the majority of them residing in the colon. Most of these bacteria are regarded as beneficial bacteria because they fight harmful bacteria and improve the immune function. These bacteria in the GIT are either transient microbes that are just fleeting, or a commensal that are colonizing microorganisms. Probiotics are considered as a transient microorganism although some of them belong to commensal species. Some of the probiotics are able to replicate and persist in the gut for a few days and then they disappear after cessation of their intake.

Prebiotics is another term less known by the public. The prebiotics are specific plant fiber that nourishes the beneficial bacteria in GIT. Prebiotics act as a fertilizer of the beneficial bacteria present in GIT. Since prebiotics can not be digested in GIT, they are only used to augment the growth of the beneficial microbes in the GIT. As result, these will turn in to provide the health benefits of the host. Probiotics and prebiotics work in parallel to form symbiosis even though probiotics have demonstrated their effectiveness in managing certain gastrointestinal diseases. The separate effects synergistically boost overall health of the host's GIT although the combination operates independently throughout the small and large intestine (Pineiro et al., 2008).

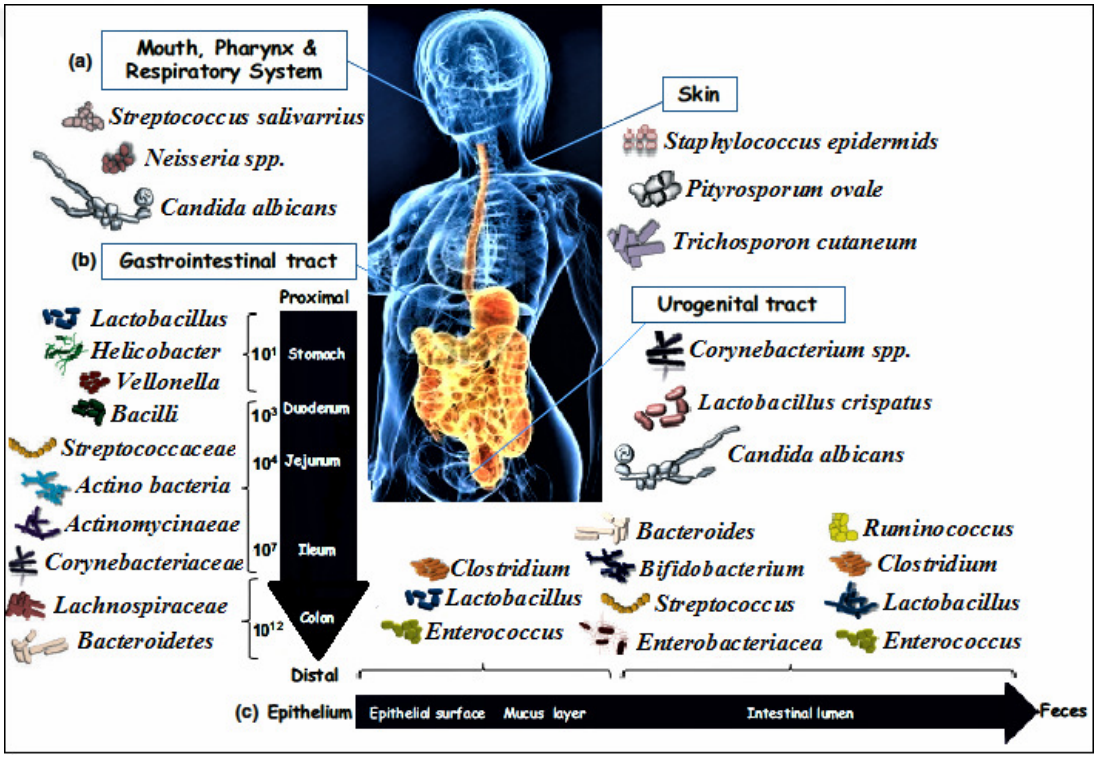

Figure 1.1 The microorganisms of human body (Adapted from https://esalvucci.wordpress.com/2016/08/05/el-microbioma-humano/)

1.2. The Selection Criteria for Probiotics

Probiotics varies from other types of bacteria in that they fit certain criteria. In order to be able to exert beneficial effects of probiotics, a successful potential probiotic strain is expected to have some desirable properties. They must not be

harmful meaning they should fit the Food and Drug Administration (FDA) criteria for specific uses or be considered as Generally Recognized As Safe (GRAS). The important criteria used for selecting probiotic strains includes that the organism should be: nonpathogenic and non-toxic (Singh et al. 2011); isolated from the same species as its intended host (Maurya et al. 2014); able to adhere and colonize the intestinal epithelium (Kechagia et al. 2013; Maurya et al. 2014); able to produce antimicrobial substances like the bacteriocins against the pathogens (Kral et al. 2012); able to stabilize normal intestinal microflora (Parvez et al. 2006); having a demonstrated beneficial effect on the host (Fontana et al. 2013); durable enough to withstand the duress of commercial manufacturing processing and distribution (Khan and Naz 2013); able to survive during transit through the gastrointestinal tract by being acid and bile resistant (Khan and Naz 2013; Maurya et al. 2014) and also having good sensory characteristics by not providing unpleasant flavors or textures (Parracho et al. 2007; Mitropoulou et al. 2013). In order for the probiotics to exert their effects, they need to survive the journey through the GIT and should be capable of sustaining the bile salts and severe acidic condition of the stomach (pH between 1.5 and 3.0). Therefore having a BSH responsible from bile resistance is the main remarks of probiotic selection criteria. The BSH activity provide two benefits to bacteria which are increasing their resistance to conjugated bile salts and enhancing their survival in the gastrointestinal tract for colonization (Ridlon et al., 2006; Jones et al., 2008). There are several genera of microorganisms which have these characteristics and lactobacilli are one of the most popular genera that have attracted much attention with noticeable probiotic features (Collins and Gibson, 1999).

1.3. The Genus *Lactobacillus*

Lactobacilli are gram-positive and non-spore-forming microorganisms producing lactic acid by fermentation (Kandler and Weiss, 1986). They are fermentative, microaerophylic and chemo-organotrophic microorganisms, requiring rich media to grow. Lactobacilli can be divided into three groups based on fermentation characteristics: facultatively heterofermentative, obligately heterofermentative and obligately homofermentative (Pot et al., 1994; Hammes and Vogel, 1995). According to Taxonomic Outline of the Prokaryotes (Garrity et al., 2004), the *Lactobacillus* genus belongs to the phylum *Firmicutes*, class *Bacilli*, order

Lactobacillales, family *Lactobacillaceae* and its closest relatives, being grouped within the same family.

The *Lactobacillus* genus is a group of gram-positive bacteria that are commensal residents of the human and animal GIT, as well as human mouth and genital tract and in sewage and plant material (Felis and Dellaglio, 2007). The *Lactobacillus* are characterized by their ability to produce lactic acid as a by-product of carbohydrate metabolism and by a genomic GC content lower than 54 mol. When glucose is the main source of carbon, many Lactobacilli degrade it using either heterofermentative or homofermentative pathway depending on the organism. The homofermentative bacteria such as *Lb. casei*, *Lb. acidophilus,* and *Lb. plantarum* yield lactate as at least 85% of their end metabolic products, whereas the heterofermentative ones like *Lb. fermentum* and *Lb. brevis* produce ethanol, lactate and carbon dioxide (Kuipers et al., 2000). *Lactobacillus* are also part of the microbiota found on mucosal membranes, such as skin, mouth, intestines, genital organs and urinary track of both humans and animals (Daoud Harzallah, 2013). This remarkable ability to colonize such places testifies to the impressive metabolic versatility of these bacteria (Soccol et al., 2010). Seven species of *Lactobacillus* are associated with human GIT most of which are common inhabitants of oral cavity as well as GIT (Walter, 2005). Colonizing human gut -specifically in intestine- increases the popularity of lactobacilli as a probiotic.

Lactobacilli can exert health benefits when applied under various conditions. Several meta-analyses have proved the efficacy of some lactobacilli in the prevention of antibiotic-associated diarrhea and in acute infectious (Sazawal et al., 2006). Some lactobacilli may prevent necrotizing enterocolitis in preterm neonates (Deshpande et al., 2007) and reduce the re-occurrence of *Clostridium difficile* associated diarrhea (Pillai and Nelson, 2008). Some promising results indicated that certain Lactobacilli can be prevented of colorectal cancer (Rafter et al., 2007) and can be treated the irritable bowel syndrome (Camilleri, 2006). Health promoting capacities of the probiotic Lactobacilli overlap with three mechanisms: (i) enhancement of epithelial barrier function, (ii) pathogen inhibition and restoration of microbial homeostasis through microbe-microbe interactions and (iii) modulation of immune responses (Boirivant and Strober, 2007; Fedorak and Madsen, 2004; Marco et al., 2006).

1.4. Gastrointestinal Tract Environment

The digestive system is made up of the GIT, pancreas, liver, and gallbladder. In parts of the gastrointestinal tract, different enzymes, pH values (Kararli, 1995) and different group of the bacteria can be observed. The human GIT contains tens of trillions of microorganisms (Gareau et al., 2010). The microbial flora of GIT must resist to changing in nutrient limitation, elevated osmolarity and pH generate potential handicaps to survival. In addition to these harsh conditions of the GIT, liver and gallbladder, named as accessory organs of the digestive system, contain some materials such as cholesterol, bile salts and bile acids, have antimicrobial activity and constitute a toxic environment to bacteria. Health benefit bacteria (probiotics) with bile salt hydrolase enzyme (*bsh*) activity can eliminate this adverse condition (De Boever and Verstraete, 1999). Some studies indicated that *bsh* enzyme activity play an important role in the colonization of the lactobacilli in human GIT (Kumar et al., 2012).

1.5. Physiology of Bile Production

1.5.1. Bile

Bile is a water-soluble yellow greenish substance produced in the liver using cholesterol as a precursor. The main components of the bile are conjugated bile acids, cholesterol, phospholipids bile pigments and electrolytes (Hoffman, 1994). Bile is synthesized in the liver by hepatocytes and then it is secreted into the gall bladder and stored in there. Gallbladder has two functions on bile: 1) acidification via exchanging Na^+ / H^+ ions 2) concentration via removing water and electrolytes. The gallbladder is stimulated by the hormone cholecystokinin and bile releases into the duodenum when the ingested foods get in the intestinal tract (Rehfeld, 2004). Bile has many important functions in the body. The main function is solubilizing and emulsifying fats as a biological detergent, which provides antimicrobial properties to bile to support the defense mechanism of body. For instance, excess amount of the cholesterol is excreted from body via bile circulation (Hofmann, 1999).

1.5.2. Bile Acids

Bile acids are amphipathic molecules containing both a hydrophilic and a hydrophobic ends so they can act as emulsifying agents in the intestine. The bile acids are composed of 2- or 3-hydroxyl group, 24 carbons and a side chain terminated in a carboxyl group in the bile duct. Bile acids are synthesized in liver from cholesterol by a multistep process. The synthesis of bile acids starts with synthesizing of the primary bile acids, cholic acid and chenodeoxycholic, from cholesterol by some midification (Chiang, 2009). In these modifying steps, the hydrocarbon chain is shortened by three carbons, the double bond of the cholesterol B ring is reduced, hydroxyl groups are inserted at 3, 7, 12 positions in the cholic acid and 3,7 positions in the chenodeoxycholic acid.

The essential functions of bile acids are especially lipid metabolism. They improve the solubility and digestion of triacylglycerol by lipases, liberating the free fatty acids in the small intestine. Emulsification increases the surface area of fat and then lipase can access the inside of fat droplets and lipids are digested effectively. Emulsification function of bile acid provides transportation of lipid molecules in an aqueous environment (Hofmann, 1994).

1.5.3. Bile Salts

Bile acids are further metabolized by the liver. They are conjugated to an amino acid molecule ‘‘taurine or glycine’’ by an amide bond between the amino group of the added compound and the carboxyl group of the bile acid. Chenodeoxycholic and cholic acids, primary bile acids, are synthesized from cholesterol (Chiang, 2009). These lipid-soluble bile acids, conjugated to either taurine molecules or glycine to form water-soluble primary conjugated bile acids by addition of an amide bond between the amino group of the added compound in the liver and the carboxyl group of the bile acid, usually called bile salts. In human bile, the ratio of glycoconjugates to tauroconjugates is 3:1 but this ratio depends on the diet and it can be changed according to the diet type. The amount of tauroconjugated bile salts increases in gallbladder of the human if the people who have a diet in animal red meat. Conjugation reaction of bile acids with the amino acids causes

decreasing the pKa value of bile salts that provide solubilization over a wide range of ionic strengths and pH values (Hofmann and Mysels, 1992). Bile salts are hydrophilic on one side and hydrophobic on the other; therefore, they tend to gather around the small drops of lipids to form micelles, with their hydrophobic sides facing the fat molecule and their hydrophilic sides facing outwards. The negative charges on the hydrophilic sides prevent the re-aggregation of fat droplets. On the other hand, conjugated bile salts are converted to free amino acids and bile acids by the bile-salt hydrolase enzyme of the probiotics. For this reason, bile salts do not have to be in the same conditions, although they are completely ionized at physiological pH (6.5-7.5). This result provides increasing concentration of bile that promotes excretion of lipids such as cholesterol.

1.5.4. Cholesterol

Cholesterol is an organic modified steroid molecule synthesized by all animal cells. It is a very hydrophobic compound consisting of four fused hydrocarbon rings that are called as “steroid nucleus”. Cholesterol has an eight-carbon that branched hydrocarbon chain attached to the D ring C-17. Ring A has a hydroxyl group at C-3 and ring B has a double bond between C-5 and C-6. Cholesterol is required to build and maintain the structural integrity and modulates the fluidity of the animal cell membrane (Hanukoglu, 1992). The cholesterol within the cells is a precursor molecule for the synthesis of bile acids and is used in several biochemical pathways. Furthermore, cholesterol is required for the synthesis of vitamin D and all steroid hormones like progesterone, testosterone, cortisol, aldosterone, estrogens, and many others. For this reason, presence of an appropriate supply of cholesterol is important for all body cells. A series of biosynthesis, transportation and regulation mechanisms have been evolved biosynthesis. Two forms of lipoproteins, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) carry the cholesterol. LDL cholesterol is referred to as “bad” cholesterol and high levels of which can cause cholesterol to build up in your arteries. LDL is very dangerous since those arteries are responsible for carrying the blood from our heart to the other components of body providing them oxygen and nutrients. HDL cholesterol on the other hand is called “good” cholesterol since it carries the cholesterol from other parts of your body back to the liver. The liver is the main organ to regulate the cholesterol homeostasis of the body. The cholesterol in the

human body can participate in the endothelial linings of blood vessels. Because of these events, lipid deposition causes a plaque formation and blood vessels narrows and risk of coronary artery disease increases. Therefore, having healthy levels of both types of lipoproteins is important and lowering our cholesterol level might reduce or even stop the cholesterol plaque buildup in our arteries (Gary, 2013).

1.6 Enterohepatic Circulation

Primary bile salts, CA and CDCA, are synthesized from cholesterol by hepatocytes and are conjugated with taurine and glycine in liver, then deposited in gall bladder. After a meal, cholecystokinin is secreted from the duodenum to cause contraction of the gallbladder and bile is then released into the duodenum (Hofmann, 1999). When bile salts entered the bloodstream, they are integrated to plasma proteins and are returned to the liver. The majority of conjugated bile salts (95%) is actively reabsorbed from intestinal wall and is transported to liver by portal circulation. When bile salts reached to the liver, they are cleared efficiently from the circulation by active transporters and then rapidly secreted into bile (Vlahcevic et al., 1996). This process is termed enterohepatic circulation that was shown in Figure 1.2. A small part of primary, conjugated bile salts is deconjugated into free bile salts and amino acids by BSH activity of intestinal microbiota and may be dehydroxylated into secondary bile salts (DCA and LCA). Secondary bile salts are passively absorbed from large intestine and 400 mg of them is excreted with feces daily. Intestinal bacteria can convert some of the primary bile acids into “secondary” bile acids by removing a 7-hydroxyl group. Because of this reaction, toxic deoxycholic and lithocholic acids are produced from cholic and chenodeoxycholic acids respectively by the activity of microorganisms in the human GIT. Therefore, bile salt hydrolase enzyme has an important role in host health whose function on the producer bacteria is still under discussion.

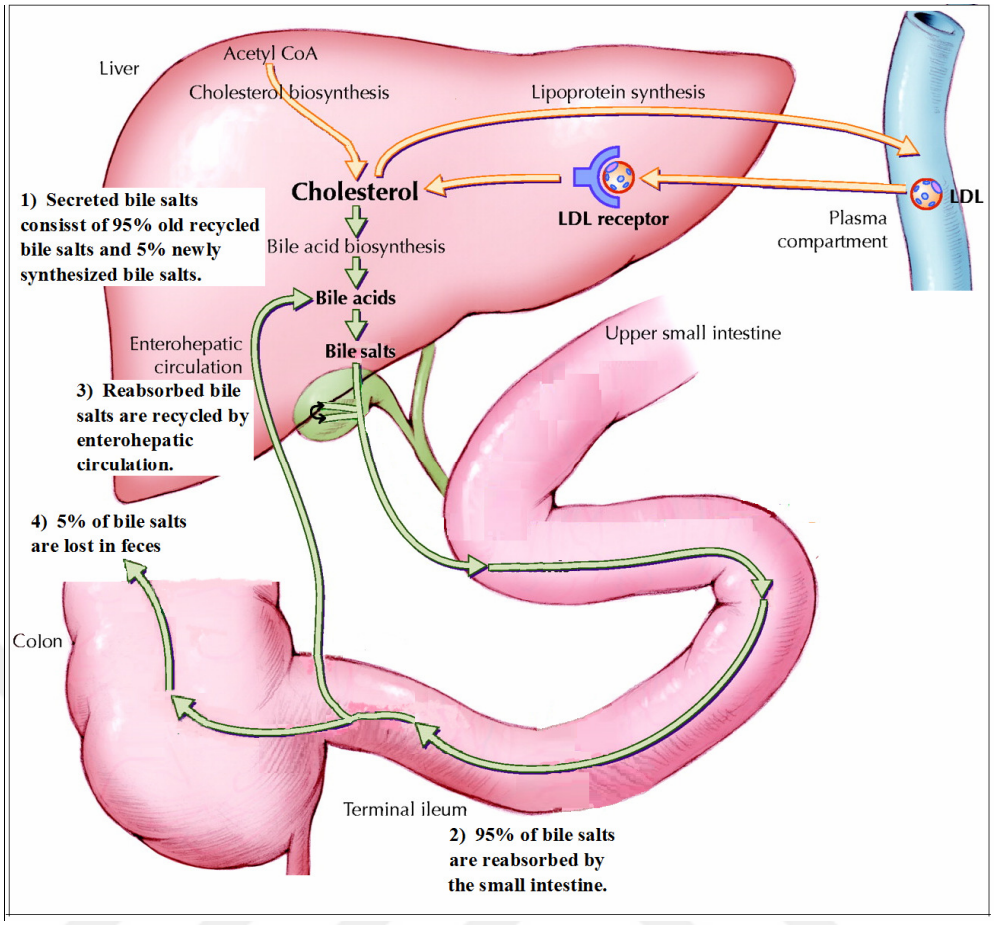

Figure 1.2. Enterohepatic circulation of bile salts (adapted from https://humananatomyly.com with some modification)

1.7 Bile Salt Hydrolase

Bacterial BSH is a member of the choloylglycine hydrolase family and is associated with gastrointestinal bacteria of both animals and humans. Additionally, it is named as an N-terminal nucleophilic hydrolase that can recognize either steroid nucleus or conjugated amino acid (Patel et al., 2010). When bile salts enter the terminal ileum and the proximal colon, they are rapidly deconjugated by prokaryotic enzymes microorganisms, bile salt hydrolases (BSH) (Ridlon et al., 2006, Begley et al., 2005). BSH have different affinities for taurine or glycine conjugates (Jones et al., 2008). BSHs are wide spread enzymes among prokaryote taxa. Functional metagenomic screening demonstrated that BSH located in the three major bacterial divisions Firmicutes (30%), Bacteroidetes (14.4%) and Actinobacteria (8.9%) (Jones et al., 2008). *Methanobrevibacter smithii* and *Methanosphaera stadmanae*, the only two archaeal species known to inhabit the human gut possess BSH (Jones et al.,

2008). BSH is particularly abundant in lactic acid fermenting probiotic strains like *Bifidobacteria* and *Lactobacilli* (Begley et al., 2006). BSH functions largely as a detoxification mechanism to reduce the levels of bile salts in the colonic environment (Begley et al., 2005). An expression of the functional bile salt hydrolase allows the bacteria to grove and to colonize in the intestinal system where at least six different bile salts were found. For this reason, having the expression of functional bile salt hydrolase ability is one of the criteria for the selection of the candidate probiotic strains.

1.7.1. Positive Effects of Bile Salt Hydrolase

The primary bile acids in human, mostly chenodeoxycholic acid (CDCA) and cholic acid (CA), are synthesized from hepatic cholesterol followed by conjugation with either taurine or glycine (Peraira and Gibson., 2002; Begley et al., 2004) and stored in the gall bladder in the liver, then secreted into the small intestine. Nearly 95% of primary BSs are re-absorbed in the distal ileum by enterohepatic circulation (McAuliffe et al., 2005; Ridlon et al., 2006). However, remaining of the primary bile acids (5 %) was metabolized in the small intestine by BSH enzymes of the gut microbiota those transform a conjugated bile salt into a deconjugated bile salt. In this situation, since deconjugated BSs are less water soluble, they are excreted through the feces. Increased levels of free bile acids lost following their hydrolysis trigger cholesterol consumption, resulting in further synthesis of bile salts with a consequential lowering of the cholesterol levels (Kim and Lee., 2005; Nguyen et al., 2007). In addition to this, recent findings suggest that the metabolites that were produced by modification and degradation of intestinal BAs by BSH of the gut microbiota have been found to influence Fernasyl X receptor (FXR) signaling and to mediate host metabolism (Sayin et al., 2013). The link between host metabolism and BSH capacity of gut microbiota has important implications for the development of the probiotics for the weight control in humans. Analysis of bile acid metabolites in rats revealed their widespread distribution in diverse indicating that BAs act as mediators of host signaling in compartments of multiple tissues (Swann et al., 2011).

1.7.2. Side Effect of Bile Salt Hydrolase

The further major bile salt modifications in the human GIT can also occur through bacterial actions including oxidation of hydroxyl groups at C-3, C-7 and C-12, and epimerization (Bortolini et al., 1997). Some members of the microbiota of human GIT play an important role in the conversion of the deconjugated form of the primary bile salts, cholic acid (CA) and chenodeoxycholic acid (CDCA), to secondary bile salts, deoxycholic acid (DCA) lithocholic acids (LCA) in the human intestine (Ridlon et al., 2006; Ohara et al., 2010). The main secondary bile acids, lithocholic acid and deoxycholic acid, are generated by 7α-dehydroxylation (Ridlon et al., 2006). The 7α-dehydroxylase enzyme can convert the deconjugated primary bile acids to secondary bile acids. The genes that are responsible for this conversion have been investigated in *Clostridium scindens* (Ridlon et al., 2012), but they seem to be less widespread than bile salt hydrolases in the microbiota of human GIT. Some of the secondary bile acids, such as DCA, cause a DNA damage of intestinal epithelium that might lead to apoptosis in a p53-independent manner (Powolny et al., 2001). Secondary bile acids may also influence colorectal cancer (CRC) by mediating interactions with important secondary messenger signaling systems (Radley et al., 1992). Many studies have indicated that high concentrations of secondary bile acids in feces, blood, and bile have been linked to formation the gall stone formation (Mamianett et al., 1999; Thomas et al., 2000) and colon cancer (McGarr et al., 2005; Bernstein et al., 2005, Kostic et al., 2011; Marchesi et al., 2011). The molecular mechanisms that mediate the bile acids cytotoxicity are more complex (Barrasa et al., 2013). Since secondary bile acids are more hydrophobic, they are more potent at disrupting cell membranes. This is likely to lead to the ROS generation via the activation of membrane-associated proteins such as phospholipase A2 and NAD(P)H oxidases (Májer et al., 2014). Besides these, secondary bile acids can interact with nuclear receptors and can activate cellular signaling pathways that induce apoptosis. However continuous exposure of bile acids to high levels can lead to apoptotic resistance (Barrasa et al., 2013)

1.7.3 Characteristics of Bile Salt Hydrolase Enzyme

Although several *bsh* genes have been cloned and characterized from various lactic acid bacteria, the precise function of BSH enzymes has not been yet fully understood. Until now, many *BSH* related genes were identified in the strains of different *Lb. plantarum* such as *Lb. plantarum* WCFS (Lambert et al., 2008), *Lb. plantarum* ST-III (Ren et al., 2011), *Lb. plantarum* BBE7 (Dong et al., 2012), *BSH Lb. plantarum* CGMCC No. 8198 (Gu et al., 2014), and *Lb. plantarum* M1-UVS29 strains (Chang-ging and Rong, 2015). In addition to *Lb. plantarum* strains, more *bsh* related genes were also identified in some other strains of *Lactobacillus* species such as *Lb. acidophilus* NCFM (McAuliffe et al., 2005), LA11 and LA4 (Jiang et al., 2010) strains, *Lb. johnsonii* 100-100 (Elkins et al., 2001) and PF01 (Chae et al., 2012) strains. Despite the isolation and characterization of BSH related enzymes from various lactic acid bacteria, the function and mechanism of BSH isozymes are still ambiguous.

Although BSHs differ in kinetic properties, subunit size and composition, gene organization, optimum pH, substrate specificity and regulation, they share in strictly conserved amino acids in their active sites. The in silico analysis in the literature showed that at least six strictly conserved amino acid residues, Cys-2, Arg16, Asp-19, Ans79, Asn-170 and Arg-223 (*Lb. plantarum* numbering) involved in the catalytic activities of the bile salt hydrolase enzymes (Begley et al., 2006). However, the identification of the catalytic activity-determining residues of BSH is not well known because there is no much experimental research on catalytic key residues of the substrate-binding pocket obtained from crystal structures. Therefore, to confirm the structure–activity relationship, the construction of BSH mutants on the catalytic binding pocket of BSH and the structural studies may be necessary.

2. AIM AND SCOPE OF THE STUDY

Conjugated bile acids (BAs) are a diverse group of amphipathic steroids that are required for digestion of lipids in mammals. The primary bile acids in human, mostly cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from hepatic cholesterol followed by conjugation with either taurine or glycine and stored in the gall bladder of liver, then secreted into the small intestine. Following secretion of the BAs into the intestinal lumen, BAs are deconjugated into taurine or glycine amino acids and unconjugated bile salts by BSH, expressed in many bacterial genera residing in the human gastro intestinal track (GIT). An expression of the functional BSHs plays a vital role in their survival and colonization in the human GIT. For this reason, the presence of an active BSH has been considered an important criterion for selection of the candidate bacteria strain that would be used as a probiotics.

The implications of BSH on human health make BSH the potential candidate for the development of the probiotics. The unconjugated BAs have significant impacts on its host physiology because of their endocrine functions and fat metabolism alterations. Therefore, BSH plays an important role on host lipid metabolism, body weigh, cholesterol level. On the other hand, there is also strong relationship between BSH activity and some human diseases such as bowl syndrome, gall stone formation and colon cancer. Considering all of these implications between BSH and negative or positive health consequences, further experimental functional studies of BSH and site directed mutagenesis of some strictly or partially conserved amino acids are required for better understanding the structural and biochemical features of BSH.

Although the crystal structures of BSHs are available from *B. longum*, *C. perfringens* and *L. salivarius*, to date, structural bases of such an important BSH's function is unknown. In silico analysis of the BSH related proteins have suggested that they were grouped into the penicillin V acylase (PVA) and the N-terminal nucleophilic (Ntn) hydrolase superfamily. Despite a vast diversity in amino acid sequence, both superfamily members have some conserved resides in the active site of BSHs (Cys2, Arg16, Asp19, Asn170 and Arg223). It was predicted that these five

strictly conserved amino acids might be responsible for the catalytic activity of the BSH using in silico analysis such as x-ray crystallography, 3D structure and amino acid alignments of all of the known BSHs. However, there is no experimental investigation supporting this assumption in the literature except Cys2. To date, Cys2 is the only amino acid that has been mutated by site directed mutagenesis and been validated for its essential role of it in BSH activity. It was found that Cys2 is important for catalysis and serves as a nucleophile molecule and proton donor. The function of the four remaining strictly conserved amino acids of BSH is unknown. This study aims to better understand the biochemical and structural function of the strictly conserved N170 and R223 amino acids of BSH enzyme from *Lactobacillus plantarum* B14 strain.

3. MATERIALS AND METHODS

3.1 Reagents and preparation of standards

All enzymes and other chemicals used in this study were purchased from Thermo Scientific (Fermantas, Europe) and Sigma-Aldrich (St. Louis, MO) respectively and they were used according to supplier's instructions. Bile acid standards, glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA) were obtained from Sigma-Aldrich and Calbiochem (San Diego, CA).

3.2 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmid DNAs used in this study were listed in Table 1. The pCON1 construct, used as template DNA that contains the *bsh* gene of the *L. plantarum* B14, was prepared in previous studies (Unpublised data). Cultures were inoculated in sterile Luria-Bertani (LB) broth from 30% frozen glycerol stocks and were incubated aerobically for two consecutive passages. The cultures of transgenic *E. coli* XL1-Blue and BLR(DE3) strains harboring pBluescript (Stratagene, USA) and pET22b (Novagen, USA), plasmids were inoculated in liquid LB medium or solid LB medium supplemented with ampicillin (100 µg/ml) and incubated at 37 °C with 175 RPM shaking agitation. Solid LB medium was solidified with with 1.5 % (w/v) agar.

Table 3.1. Bacterial strains and plasmids

Strain	Genotype – Origin	Phenotype	Reference
Lb.plantarum B14	Human origin		Previous study
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	Previous study
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	Previous study
pCON1N170V	Substitution of asparagine for valine in pCON1	Amp^r	This work
pCON1R223F	Substitution of arginine for phenylelanine in pCON1	Amp^r	This work
pZEK170V	Substitution of asparagine for valine in pET22b(+)	Amp^r	This work
pZEK223F	Substitution of arginine for phenylalanine in pET22b(+)	Amp^r	This work

3.3 Sequence Analysis of Wild-type BSH Enzyme

The *bsh* gene sequence of the *Lb. plantarum* B14 was aligned by the Clustal W program. The theoretical molecular mass of BSH was predicted by the ExPASY program that is available at http://web.expasy.org/compute_pi/. The BSH amino acid sequences were determined and compared with the amino acids sequences of BSH genes available in GenBank (NCBI) using Jalview 2.8 program. The complete monomeric unit of the BSH enzyme generated and the positions of the target amino acids were detected by PyMOL program (PyMOL Executable Build is Copyright(C) 2006 DeLano Scientific LLC, South San Francisco, California, USA).

3.4 Construction of pCON1N170V and pCON1R223F Mutant in *E. coli* XL-1 Blue Cells

3.4.1. Preparation of Desired Mutants by Site-directed Mutagenesis

The “AAC” and “AGA” codons of recombinant bile salt hydrolase gene (*rbsh*), coding asparagine-170 (N170) and arginine-223 (R223) amino acids, were substituted for the “GTT” and “TTT” codons, coding valine-170 (V170) and phenylalanine-223 (F223) amino acids respectively using pCON1 construct as a

template DNA. These mutations were generated by PCR based site-directed mutagenesis using appropriate primers in Table 3.2. The reactions were carried out in a 50 µl volume containing $MgCl_2$ (25mM), dNTP mix (5mM), forward and reverse primers (25µM each), plasmid DNA as a template and the Pfu DNA polymerase enzyme (Fermentas, Europe) with reaction buffer (10X) (Table 3.3 and 3.4). PCR amplicons were obtained using Techne 3000 PCR System (Barloworld Scientific) under the conditions descirbed in Table 3.5. The PCR amplicons were digested with *Dpn*I (Fermentas, Thermo Scientific) to eliminate methylated pCON1 template DNA at the conditions in Table 3.5.

Table 3.2. Forward and reverse primers used for site-directed mutagenesis

Primers name	Primer Sequences (5' to 3')	Tm
N170VF	5'- cagtaggtgtgttaaca**GTT**aatcctaattttgac -3'	60°C
N170VR	5'- gtcaaaattaggatt**AAC**tgttaacacacctactg -3'	60°C
R223FF	5'- cttgtcctcaatgtct**TTT**tttgtcagagccgc -3'	69°C
R223FR	5'- gcggctctgacaaa**AAA**agacattgaggacaag -3'	69°C

Table 3.3. PCR Reactants for N170V and R223F 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 3.4. PCR Reaction conditions

Cycles	Temperatures		Time
	N170V	R223F	
Initial denaturation	95°C	95°C	30 sec
Denaturation	95°C	95°C	1 min
Annealing	57°C	64°C	1 min
Extension	72°C	72°C	9 min
Final extension	72°C	72°C	5 min
Number of cycles		20	

Table 3.5. Reaction conditions for 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**	

3.4.2 Purification of the PCR Products

The PCR producs of pCON1*N170V and pCON1*R223F (* represents the desired mutations) were loaded in 1% agarose gel (Sigma, USA) and separated by electrophoresis at 75 volt for 1 hour using 1X TAE buffer and almost 1 kb PCR products were purified individually from agarose gels by DNA recovery kit (Vivantis, Selangor, Malasia) according to manufacturer's protocol.

3.4.3 Preparation of the XL1-Blue and BLR(DE3) Competent Cells

For the transformation process, *E. coli* XL1-Blue and BLR(DE3) competent cells were prepared in several steps. The *E. coli* XL1-Blue (Stratagene, USA) and BLR(DE3) (Novagen, USA) strains were streaked on solid LB medium and incubated at 37°C for overnight. A single colony of XL1-Blue and BLR(DE3) were inoculated separately in a 3 ml LB broth medium and incubated at 37°C with 175 rpm shaking for nearly 14-16 hours. Then 1% of this culture was transferred into a 50 ml LB medium and re-incubated at 37°C with shaking 125 rpm for 2-2.5 hours until the concentration of culture would reach OD_{600} 0.5. When the growth of the culture medium was reached OD_{600} 0.5, culture medium was separated into 2 sterile falcon tubes of 20 ml each. Cells were harvested by centrifugation at 4000 rpm and 4°C for 5 minutes. Then the cell pellets were resuspended in 10 ml of $CaCl_2$ (100mM, pH: 7.0) and centrifuged again at 4000 rpm and 4°C for 10 minutes. Finaly, the pellets were resuspended in 1 ml of $CaCl_2$ solution containing 10% glycerol and were alloquated into 100 µl of culture for each eppendorf tubes and stored at -80 °C freezer.

3.4.4 Transformation of pCON1*N170V and pCON1*R223F into *E.coli* XL1-Blue Strains

Ten µl of the gel purified PCR products of pCON1*N170V and pCON1*R223F were transferred individually into 100 µl of XL1-Blue competent cells. Fist, the samples were incubated on ice for 45 minutes and then they were incubated at 37°C for 5 minutes before transformation of them to ice for the heat shock treatment. After heat shock process, the samples were added in 1 ml of LB and

they were grown in shaking incubator at 37°C and 175 rpm for 1 hour. The cells were harvested by centrifugation at 6000 rpm for 3 minutes and the pellet was re-suspended in 100 µl LB. The host cell suspension was spread on LB agar plate with ampicillin (100µg/ml) and was incubated overnight at 37°C with a shaking 175 rpm for 16-18 hours. After four random colonies were picked from the LB plates containing transformats, they were subcultured into 5 ml LB medium with ampicillin. Plasmid DNAs were isolated from the transformed cell pellets using Plasmid Miniprep Kit (Thermo Scientific GeneJET, Lithuania, EU). Finaly to confirm presence of the inserted *bsh* genes, the DNAs of the randomly selected clones were cut with *Xho*I restriction enzyme and loaded on agarose gel before sequencing of them. Finaly, the gel purified PCR products were transformed into the *E. coli* XL-Blue competent cells.

3.4.5 DNA Sequencing and Sequence Analysis

Almost 1 kb nucleotide residues of the *bsh* genes from pCON1/N170V and pCON1/R223F constructs were 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/N170V and pCON1/R223F constructs were confirmed by the BioEdit Sequence Alignment Program.

3.4.6 Stock Preparation

For the long term storage of the clones, the pCON1N170V and pCON1R223F clones that had the desired N170V and R223F mutations were grown in 5ml LB liquid medium supplemented with ampicillin (100µg/ml) at 37°C with a shaking 175 rpm for nearly 16-18 hours. Then, the cell cultures were centrifuged at 4000 rpm and 25°C for 4 minutes. The obtained pellets were resuspended in 4 ml of LB broth with 30% glycerol and allocated into 2 different cryogenic tubes and then stored at -80°C freezer.

3.5 Preparation of pZEKN170V and pZEKR223F Constructs

3.5.1 pET22b Plasmid DNA Isolation and Preparation of It

Since the pET derivative expression vectors are low coppy plasmid vectors, the *E. coli* BLR(DE3) strain containing pET22b expression vectors was propagated in a 10 ml LB broth with ampicillin (100µg/ml) and incubated at 37°C with 175 rpm shaking for overnight. After washing the overnight grown cell cultures with 1 ml of LB medium they were centrifuged at 4000 rpm and 25°C for 4 minutes and then they were collected by centrifugation at 6000 rpm for 3 minutes. Plasmid DNAs were extracted from the pellet using Plasmid Miniprep Kit (Thermo Scientific GeneJET, Lithuania, EU) according to protocol of manufectures. To clone mutant and wild-type *bsh* genes of *Lb. plantarum* B14 into pET22b expression vector, pET22b plasmid DNA was restricted with *EcoR*I and *Not*I restriction enzymes (Thermo Scientific) at 37°C for 45 minute in the reaction conditions in Table 3.Then, digested pET22b expression vector was purified from 1% agarose gel by Gel DNA Recovery Kit (Vivantis, Selangor, Malasia).

Table 3.6 Digestion condition of the pET22b DNA by *Not*I and *EcoR*I restriction enzymes

Reactants	Quantities	
Up H_2O	20 µl	at 37 °C
pET22b (>500 ng)	30 µl	
Fast Digest Buffer (10X)	6 µl	for 45 min
*EcoR*I enzyme (Fast Digest)	2 µl	
*Not*I enzyme (Fast Digest)	2 µl	
TOTAL VOLUME	**60 µl**	

3.5.2 Digestion of pCON1N170V and pCON1R223F DNAs by *EcoR*I and *Not*I Restriction Enzymes

To obtain the N170V and R223F mutant *bsh* genes of *Lb. plantarum* B14 from pCON1N170V and pCON1R223F clones, the pCON1N170V and pCON1R223F plasmid DNAs were digested individually with *EcoR*I and *Not*I restriction enzymes (Thermo Scientific, Lithuania, EU) at 37°C for 2 hours (Table 3.7). Digested DNA samples were loaded on 1% agarose gel and separated by electrophorases. The obtained mutant N170V and R223F *bsh* gene fragments were

purified from 1% agarose gel using the Vivantis GF-1 Gel DNA Recovery Kit (Vivantis, Selangor, Malasia) according to the manufacturer's instructions.

Table 3.7. Digestion of pCON1N170V and pCON1R223F plasmid DNAs by *EcoR*I and *Not*I restriction enzymes

Reactants	Quantities	
Up H_2O	18 µl	at 37 °C
pCON1N170V or pCON1R223F Plasmid DNA (>500 ng)	40 µl	
Fast Digest Buffer	7 µl	for 2 h.
*EcoR*I Enzyme (Fast Digest)	2.5 µl	
*Not*I Enzyme (Fast Digest)	2.5 µl	
TOTAL VOLUME	**70 µl**	

3.5.3 Insertion of mutant N170V and R223F *bsh* genes into pET22b Expression Vector

The *EcoR*I/*Not*I cut and gel purified *bsh* DNA fragments form mutant N170V and R223F were ligated individually into the expression vector, pET22b cleaved with the same restriction enzymes by T4 DNA ligase enzyme (Thermo Scientific) at 22°C for 1 hour (Table 3.8). The new constructs were named as pZEKN170V and pZEKR223F. For an efficient ligation reaction, the appropriate quantities of insert and vector DNA were determined by the following formula:

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

Table 3.8. Ligation reaction condition for mutant N170V and R223F bsh genes

Reactants	Quantities	
Up H_2O	11.2 µl	at 22 °C
pET22b Vector (*EcoR*I/*Not*I ended) (>500 ng)	4 µl	
*EcoR*I/*Not*I cut N170V or R223F *bsh* DNA	1.8 µl	for 1 h.
T4 10X Ligase Buffer	2 µl	
T4 DNA Ligase Enzyme (5 U/µl)	1 µl	
TOTAL VOLUME	**20 µl**	

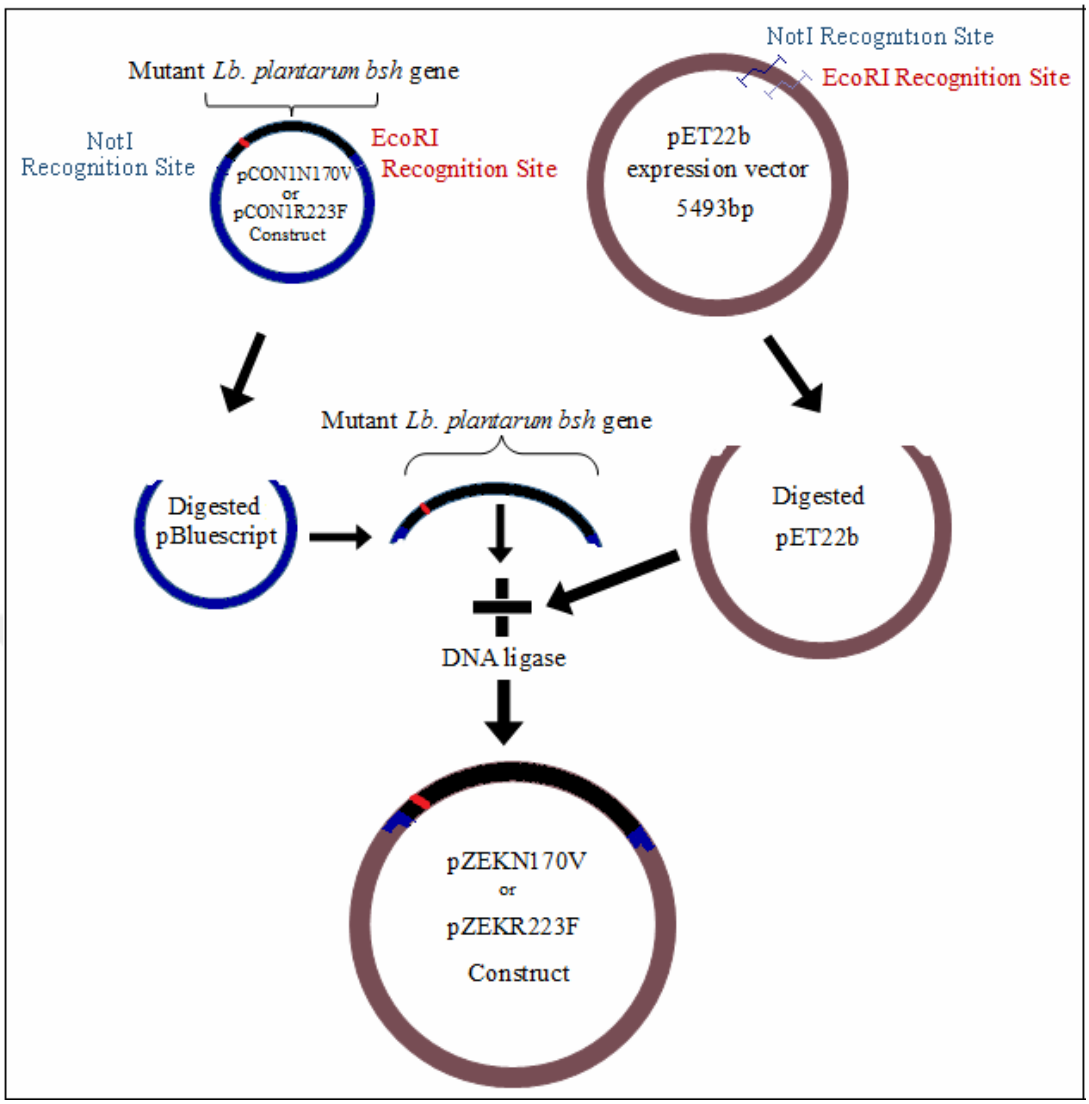

Figure 3.1 Preparation of pZEKN170V and pZEKR223F constructs

3.5.4 Transformation of pZEKN170V and pZEKR223F into *E. coli* BLR(DE3) Strains

10 µl of the pZEKN170V or pZEKR223F ligation products were transferred individually into 100 µl BLR(DE3) competent cells. For heat shock treatment, the samples were kept on ice for 45 minutes then incubated at 37°C for 5 minutes and finally kept on ice for 5 minutes. The ligation samples were mixed with 1 ml of LB broth medium and grown in shaking incubator at 37°C and 175 rpm for 1 hour. The cells were collected by centrifugation at 6000 rpm for 3 minutes and the pellet was re-suspended in 100 µl LB. The suspension was spread to LB agar plate with ampicillin (100µg/ml) and incubated overnight at 37°C and 175 rpm for about 16-18

hours. Four colonies were selected randomly from the LB plates containing transformant and propagated at 37°C in 10 ml LB medium suppemented with ampicillin (100µg/ml) at 175 rpm in the shaker. The transformed cell pellets were obtained for isolation of plasmid DNAs.

3.5.5 Detection of Right-oriented pZEKN170V and pZEKR223F Constructs

The pZEKN170V and pZEKR223F plasmid DNAs were isolated individually from *E.coli* BLR(DE3) clones using Thermo Scientific GeneJET Plasmid Miniprep Kit (Lithuania, EU) and then they were digested with *EcoR*I and *Not*I restriction enzymes (Thermo Scientific)) at 37°C for 30 minute (Table 3.9 and Table 3.10). To confirm the presence of mutant *bsh* gene fragments in the pZEKN170V and pZEKR223F constructs, *EcoR*I/*Not*I digested samples of pZEKN170V and pZEKR223F DNAs were loaded on 1% agarose gel and DNA fragmnets were separated by electrophorases for 1 hour. After verification of pZEKN170V and pZEKR223F constructs, the pZEKN170V and pZEKR223F transformants that had the right orientation of mutant N170V and R223F *bsh* gene were incubated overnight in 10 ml LB liquid medium with ampicillin (100µg/ml) at 37°C and 175 rpm in the shaker for 16-18 hours. The cell cultures were centrifuged at 4000 rpm and 25°C for 4 minutes and the pellets obtained were resuspended in 4 ml of LB broth with 30% glycerol and allocated into two cryogenic tubes and stored at -80°C.

Table 3.9. Digestion condition of pZEKN170V plasmid DNA with *EcoR*I/*Not*I restriction enzymes

Reactants	Quantities	
Up H_2O	5.5 µl	at 37 °C
pZEKN170V plasmid DNA (>500 ng)	3 µl	
Fast Digest Buffer (10X)	1 µl	for 30 min
*EcoR*I Enzyme (Fast digest)	0.25 µl	
*Not*I Enzyme (Fast digest)	0.25 µl	
TOTAL VOLUME	**10 µl**	

Table 3.10. Digestion condition of pZEKR223F plasmid DNA with *EcoR*I/*Not*I restriction enzymes

Reactants	Quantities	
Up H_2O	5.5 µl	at 37 °C
pZEKR223F plasmid DNA (□500 ng)	3 µl	
Fast Digest Buffer (10X)	1 µl	for 30 min
*EcoR*I Enzyme (Fast digest)	0.25 µl	
*Not*I Enzyme (Fast digest)	0.25 µl	
TOTAL VOLUME	**10 µl**	

3.6 Determination of BSH Activity

3.6.1 Direct Plate Assay

The activity of recombinant BSH and mutant rekombinat BSHs (mrBSH/N170V and mrBSH/R223F) were determined qualitatively by direct plate assay (Christiaens et al., 1992) with some modification. To determine *bsh* activity of recombinant and mutant BSH enzymes, LB agar plates with ampicillin (100µg/ml), GDCA (2 mM) or TDCA (5 g/lt), IPTG (0.1 mmol), $CaCl_2$ (0.35 g/L) were used. Then the plates that were streaked with positive control (pCON2/BLR(DE3)), negative control (pET22b/BLR(DE3)) and either mutant transformant (pZEKV170V/BLR(DE3)) or (pZEKR223F/BLR(DE3)) were incubated at 37°C for 72 hours and samples were compared with their opaque appearance. If there is a bright opaque halo formation due to precipitated bile acid, it was considered a positive reaction for BSH activity.

3.6.2 Overexpression of rBSH and mrBSHs Enzymes

After the successful transformation of pZEKN170V and pZEKR223F into the *E. coli* BLR(DE3) competent cells, the pZEKN170V, pZEKR223F and pCON2 (positive control; pET22b/*bsh*) transformants were incubated individually in a 10 ml LB broth medium with ampicillin (100µg/ml) at 37°C and 175 rpm in the shaker for 16-18 hours. After 5 ml (2% of total volume) of these overnight cultures were transferred into a 250 ml LB broth containing ampicillin (100µg/ml) and 4% Glycerol, they were grown at 37°C and 175 rpm until the optical density of the cultures reached OD_{600}: 0.45-0.6. Protein expressions of rBSH and mrBSHs were induced with 0.3 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and then the

cultures were re-incubated at 37°C, and 175 rpm until the OD_{600} values of the cultures reached 1.5 - 3.0. When the final concentration of cells reached OD_{600}: 1.5-3.0, the cultures were divided into two falcon tubes and centrifuged at 4˚C and 8000g (Rotina R28 Centrifuge, Hettich) for 15 minutes. The pellets were resuspended with 2 ml cold binding buffer (pH: 7.8) containing 20 mM sodium phosphate and 500 mM NaCl. Then lysozyme (1mg/ml) was added into the cell suspension and incubated on a rocking platform at 4°C for 10 minutes. After the incubation completed, Tween-20 (1%), DNase and RNase (5µg/ml) were added in the mixture respectively and were incubated on a rocking platform at 4°C for 10 minutes. The cells-debris was removed from the soluble fraction by centrifugation at 4°C and 14000 g for 20 min. The supernatant was collected through a 0.45µm filter into clean polystyrene tubes. The prepared cell extracts were used for Ninhydrin assay and purification of rBSH and mrBSH proteins. The total protein concentration of the cell extract was measured by the Lowry method (Lowry, 1950) with Bovine Serum Albumin (BSA) as a standard protein in the ninhyrin assay.

3.6.3 Ninhydrin Assay

The catalytic activities of the rBSH and mrBSH enzymes were detected by ninhydrin assay with some modification from Tanaka et al. (1999). This assay based on the interaction of the amino acids released from hydrolysis of the conjugated bile salts with ninhydrin reagent and the detection of this reaction with colorimetric assay. The total protein concentration of cell extracts were measured by Lowry assay (Lowry et al., 1950) and normalized according to the lowest value of 10 µl sample with appropriate dilution before starting ninhydrin assay. The substrate specificities and catalytic activities of rBSH and mrBSH enzymes against the two different human bile acids, taurodeoxycholic acid (TDCA) and glycodeoxycholic acid (GDCA) were determined at 37 ºC and pH 6 by ninhydrin assay. Since wild-type BSH had highest activity for GDCA, and TDCA at optimum pH (5) and temperature (37ºC) in the previous studies, two major human bile salts, GDCA and TDCA, were selected. For this assay, 10 µl of the partially purified cell extract was added to the mixture containing 100 µl of 0.1M sodium phosphate buffer (pH:6.0), 50 µl of 40 mM Dithiothreitol (DTT) and 50 µl of 40 mM GDCA or TDCA. The final mixture was incubated at 37°C for 30 minutes. Then, 210 µl of Trichloroacetic acid (TCA)

was added to terminate the enzymatic reaction and the mixture was centrifuged at 14000 g for 15 minutes. 20 µl of the upper solution was transferred into the sterile glass tube including 1.9 ml of ninhydrin reagent (0.5 ml of 1% ninhydrin in 0.5M sodium citrate buffer pH:5.5, 1.2 ml glycerol and 0.2ml of 0.5M sodium citrate buffer pH:5.5) and 80 µl of UP water. The sample was vortexed and boiled for 15 minutes. After cooling the tubes in tap water, the absorbance of the samples were measured at 570 nm with glycine as a standard curve by the U-1900 spectrophotometer (HITACHI, Japan). All experiments were performed in triplicate.

3.7 Partially Purification of the rBSH and mrBSHs Enzymes and SDS PAGE Analysis

Following the overexpression and cell extraction of rBSH and mrBSHs, the His-tagged rBSH and mrBSHs were partially purified from cell extracts of *E. coli* BLR(DE3) hosts, pCON2/BLR(DE3), pZEKV170V/BLR(DE3) and pZEKR223F/BLR(DE3) using the ÄKTAprime plus™ chromatography system (GE Healthcare Bio-sciences, Uppsala, Sweden) with a Nickel chelated column according to the manufacturer's instruction. The soluble fraction of cell extracts, containing the rBSH and mrBSHs, were loaded into His Trap™ HP column. The protein bound column was washed twice with 5 bed volumes of wash elution buffer (sodium phosphate buffer (20 mM), NaCl (500 mM), imidazole (50 mM) at pH: 6). The bound proteins then were eluted from the His Trap™ HP column by elution buffer (sodium phosphate buffer (20 mM) supplemented with imidazole (50 mM), NaCl (500 mM) at pH: 7.8). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 0.1% (w/v) SDS and a 12% (w/v) polyacrylamide separating gel as described by Laemmli (1970) was performed to monitor production of the rBSH and mrBSHs.

4. RESULTS

4.1 Sequence Analysis of Wild-type BSH Enzyme

The 324 amino acids residues of BSH from *Lactobacillus plantarum* B14 was aligned with the amino acid sequence of BSHs from different *Lactobacillus* species such as *Lb. plantarum* Y1, *Lb. reuteri, Lb. salivarus, Lb. acidophilus, Lb. gasseri* and *Clostridium perfringens* and the penicillin V acylase (PVA) of *Bacillus sphaericus* using Jalview 2.8.0b1 program. The alignment indicated that C2, R16, D19, N170 and R223 amino acids (*Lb. plantarum* numbering) are totaly conserved and amino acid motifs around these amino acids are also conserved except for PVA of *Bacillus sphaericus* (Figure 4.1).

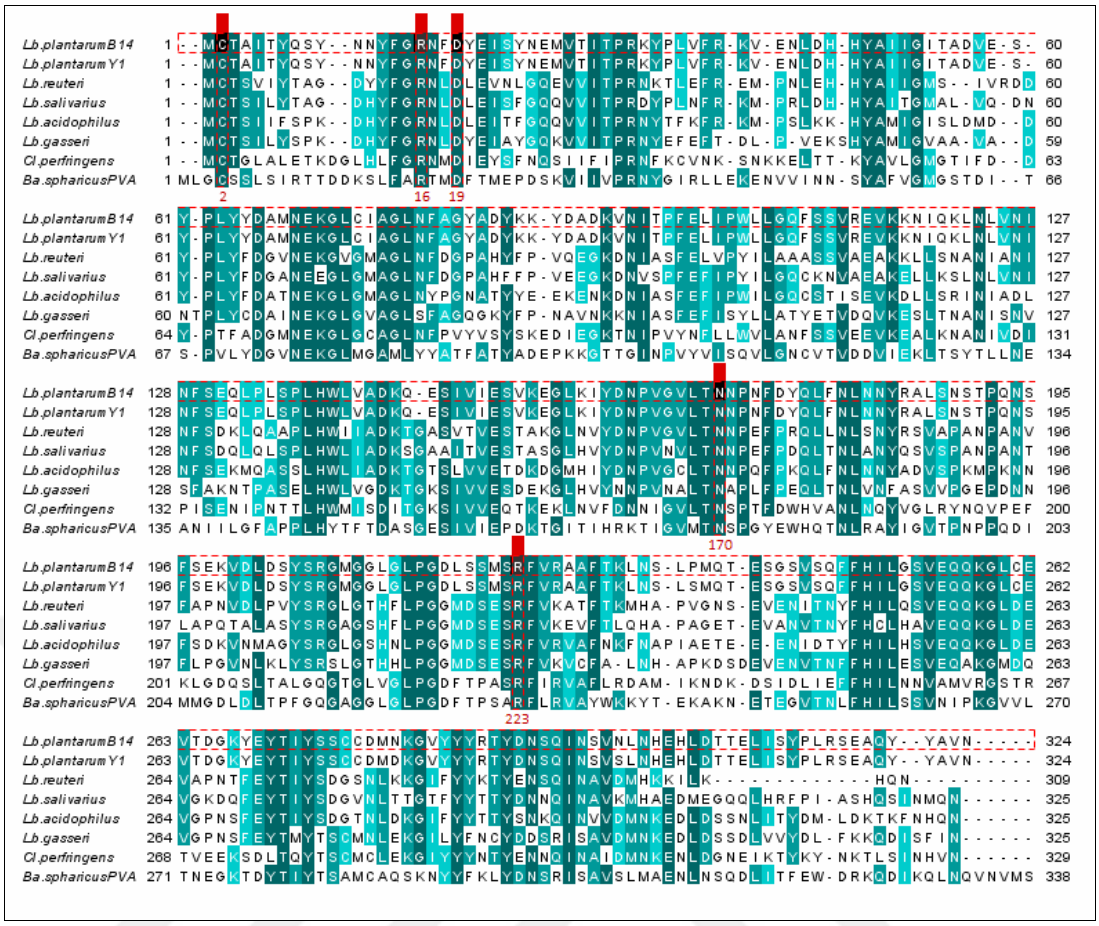

Figure 4.1. The multiple sequence alignment of BSH proteins from *Lb. plantarum* B14 (AN: KY080706), *Lb. plantarum* Y1 (AN: HM163165), *Lb. reuteri* (AN: FJ006722), *Lb. salivarus* (AN: FJ591087), *Lb. acidophilus* (AN: FJ439780), *Lb. gasseri* (AN: FJ439777) and conjugated bile acid (CBAH) of *Clostridium perfringens* species (AN: AAC43454) and penicillin acylase of *Bacillus sphaericus* (AN: P12256) by Jalview 2.8.0b1. Arrows represent conserved amino acids and their amino acid motifs around active sites: C, R, D, N and R.

4.2 Phylogeny of BSH from *Lactobacillus plantarum* B14

Analysis of the amino acid residues of BSH related enzymes, obtained from *Lb. plantarum* Y1, *Lb. reuteri, Lb. salivarus, Lb. acidophilus, Lb. gasseri* and *Clostridium perfringens* and the penicillin V acylase (PVA) of *Bacillus sphaericu*were, clearly divided organisms into two clusters. These are PVA cluster containing penicillin V acylase related proteins and Ntn hydrolase superfamily

cluster contain BSH enzymes (Figure 4.2). 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 BSH of *Lb. plantarum* Y1, whereas BSH of *Lb. plantarum* B14 showed 34% similarity to penicillin acylase (PVA) of *Bacillus sphaericus* (Figure 4.3).

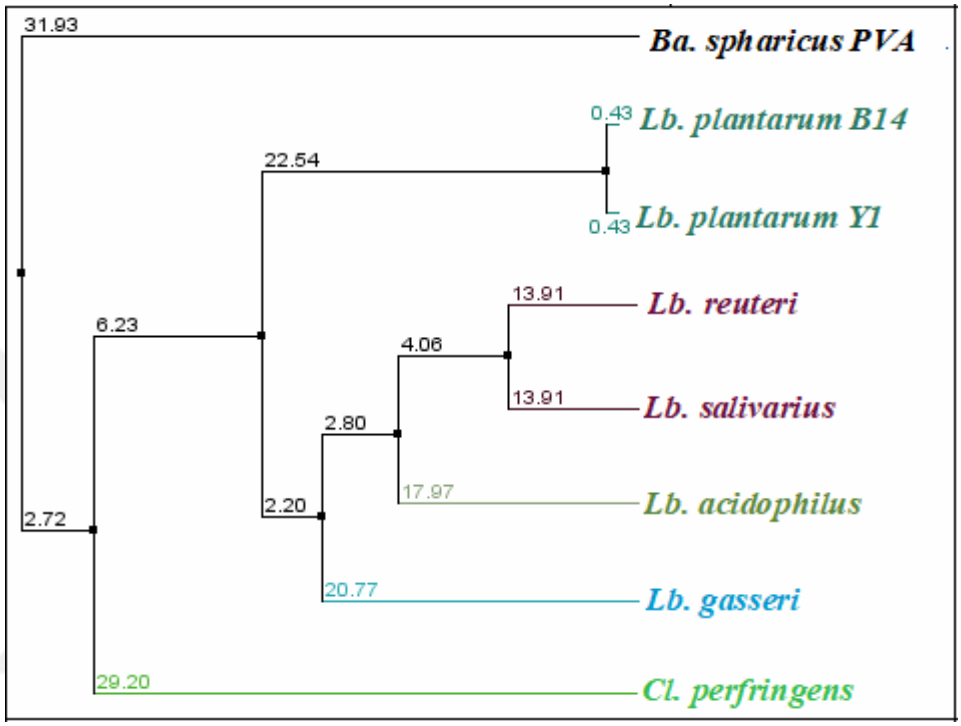

Figure 4.2. The Phylogenetic of *Lb. plantarum* B14 BSH enzyme (AN: KY080706) was represented in a tree structure containing other species; *Lb. plantarum* Y1 (AN: HM163165), *Lb. reuteri* (AN: FJ006722), *Lb. salivarus* (AN: FJ591087), *Lb. acidophilus* (AN: FJ439780), *Lb. gasseri* (AN: FJ439777) and conjugated bile acid (CBAH) of *Clostridium perfringens* species (AN: AAC43454) and penicillin acylase of *Bacillus sphaericus* (AN: P12256) by Jalview 2.8.0b1. The Phylogenetic tree indicated that the *Lb. plantarum* B14 BSH enzyme related with BSH enzyme of *Lb. plantarum* Y1.

	Lb. plantarum B14	*Lb. plantarum* Y1	*Lb. reuteri*	*Lb. salivarus*	*Lb. acidophilus*	*Lb. gasseri*	*Cl. perfringens*	*Ba. Sphaericus* PVA
Lb. plantarum B14	100%	99%	51%	51%	52%	46%	40%	34%
Lb. plantarum Y1	99%	100%	51%	51%	52%	45%	40%	34%
Lb. reuteri	51%	51%	100%	69%	63%	57%	38%	30%
Lb. salivarus	51%	51%	69%	100%	59%	52%	35%	29%
Lb. acidophilus	52%	52%	63%	59%	100%	58%	39%	31%
Lb. gasseri	46%	45%	57%	52%	58%	100%	36%	33%
Cl. perfringens	40%	40%	38%	35%	39%	36%	100%	36%
Ba. Sphaericus PVA	34%	34%	30%	29%	31%	33%	36%	100%

Figure 4.3. The BLAST identity percentage analysis of amino acids sequences of BSH from *Lb. plantarum* B14 and multiple other sequences by the Standard Nucleotide BLAST tool available at blast.ncbi.nlm.nih.gov.

4.3 3D Model Structure and Catalytic Site for BSH Enzyme

The overall 3D monomer structure of BSH was detected by PyMOL program (PyMOL Executable Build is Copyright$^{(C)}$ 2006 DeLano Scientific LLC, South San Francisco, California, USA) and the location of the strictly conserved target amino acids, Asn170 and Arg223, were shown in this structure (Fig. 4.1). The 3D structure of BSH indicated that the Asn-170 in the loop connecting β13 and α3 and the Arg223 on α5 were close to the catalytic Cys2. Although interaction of these two amino acids with Cys2 seems critical, it is not clear whether this interaction is necessary for catalytic function or stability of the active site geometry of BSH from this 3D structure. The 3D structure of the BSH revealed that Asn170 and Arg223 side chains interacted within a distance of 2.8 A (Fig. 4.4).

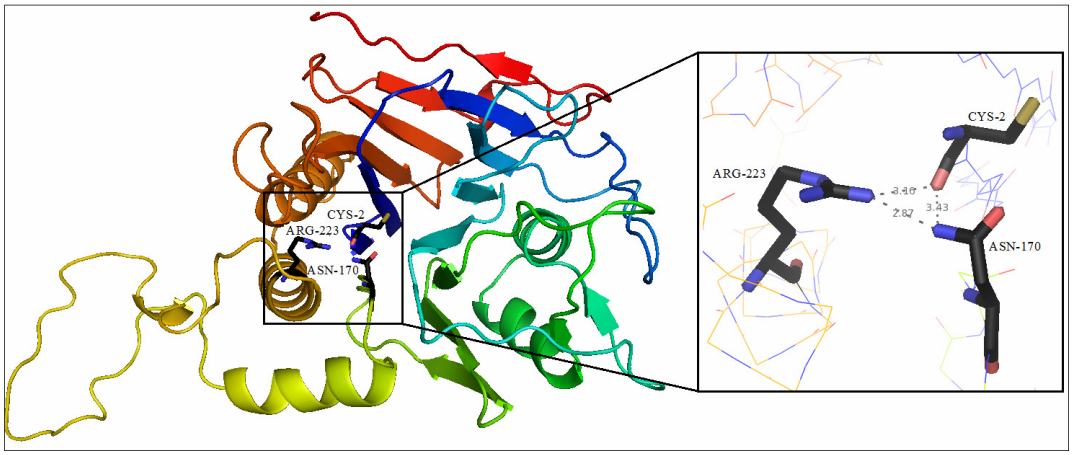

Figure 4.4. Three-dimentional structure of *Lb. plantarum* monomer of BSH protein. Catalytic site of BSH of *Lb. plantarum* B14 shows strictly conserve amino acids; cysteine 2, asparagine 170 and arginine 223. This figure was created by PyMOL programe (PyMOL Executable Build is Copyright (C) 2006 DeLano Scientific LLC, South Sanfrancisco, California, USA).

4.4 Preparation of pCON1N170V and pCON1R223F Constructs

4.4.1 PCR- Based Site Directed Mutagenesis of N170 and R223 Aminoacids

To substitute N170 and R223 amino acids for V170 and F223 amino acids by PCR based site direct mutagenesis, pCON1 DNAs were used as a template. For this purposes, first pCON1 DNA was isolated from *E. coli* XL-1 Blue cells (Lanes 1 in Figure 4.5 and Figure 4.6). N170 and R223 amino acids were substituted for V170 and F223 amino acids by PCR based site direct mutagenesis acid using appropriate primers listed in Table 4.1 as described in materials and methods section. The resulting approximately 4000 bp recombinant pCON1 (pBluescript/*bsh*; *Lb. plantarum* B14 (AN: KY080706) *bsh* gene (975bp) and pBluescript (2961bp) template DNA and the mutant N170V and R223F PCR products were resolved by agarose gel electrophoresis to verify their integrity (Lanes 2, 3 and 4 in Figure 4.5 and Figure 4.6).

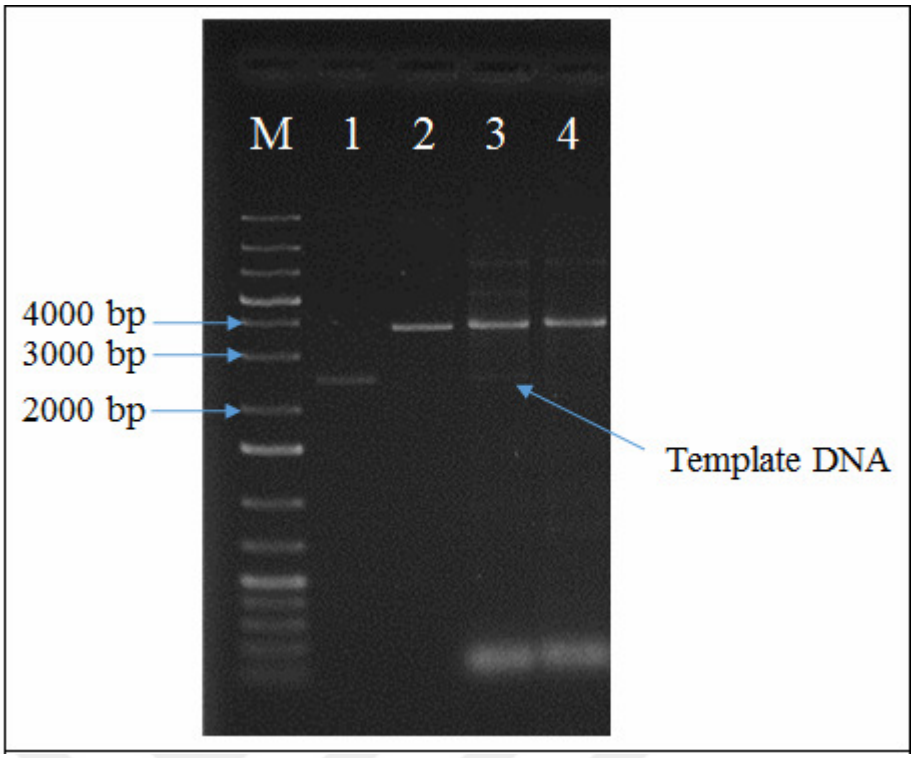

Figure 4.5. The site-directed mutagenesis of pCON1*N170V (* represents designed mutation) by PCR. M, 1 kb plus DNA ladder; 1, uncut pCON1 template DNA (1/5 diluted); 2, *Xho*I/ cut pCON1 template DNA; 3, pCON1*N170V PCR product; 4, pCON1*N170V PCR product digested with *Dpn*I Restriction Enzyme.

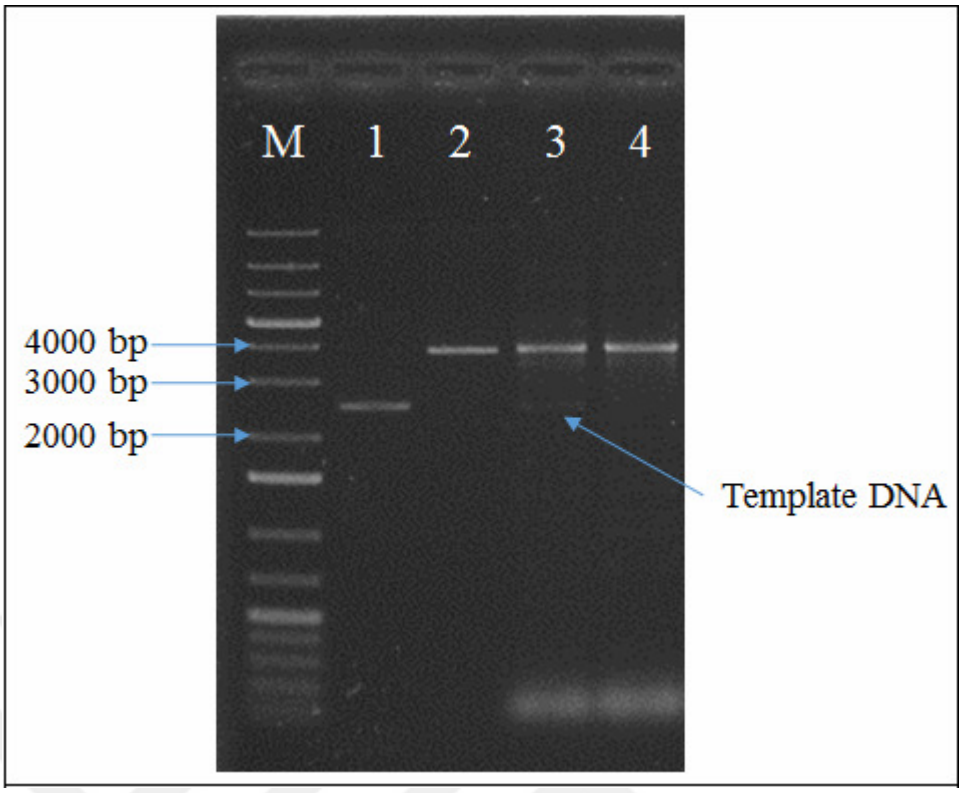

Figure 4.6. The site-directed mutagenesis of pCON1*R223F (* represents designed mutation) by PCR. M, 1 kb plus DNA ladder; 1, uncut pCON1 template DNA (1/5 diluted); 2, *Xho*I/ cut pCON1 template DNA; 3, pCON1*R223F PCR product; 4, pCON1*R223F PCR product digested with *Dpn*I Restriction Enzyme.

4.4.2 Purification of the PCR Products

To eliminate nospecific PCR products and undigested methylated template DNA, the mutant pCON1*N170V and pCON1*R223F PCR products were purified individually from 1% agarose gels (Figure 4.7 and Figure 4.8).

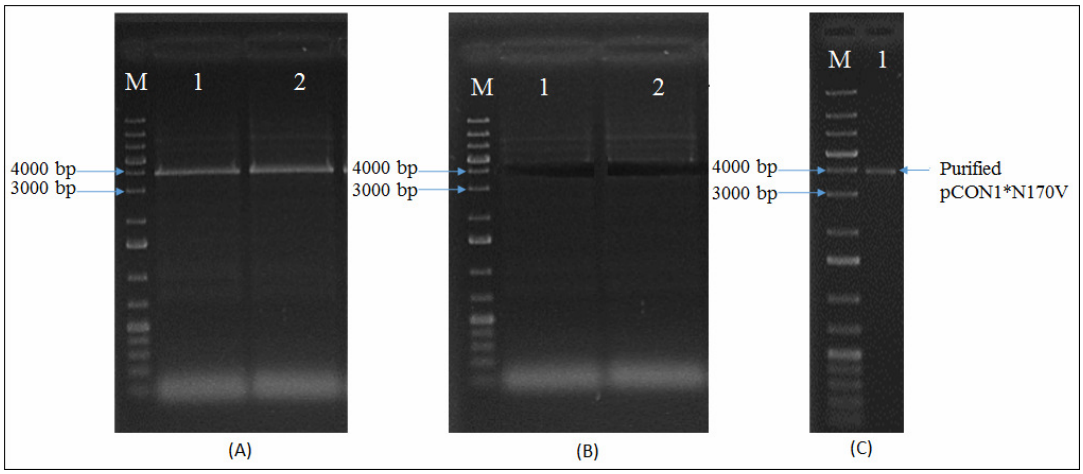

Figure 4.7. Purification of the pCON1*N170V PCR products from 1% agarose gel. M, 1 kb plus DNA ladder. A, pCON1*N170V PCR products; B, sliced pCON1*N170V PCR products; C, purified pCON1*N170V (4.0 kb)

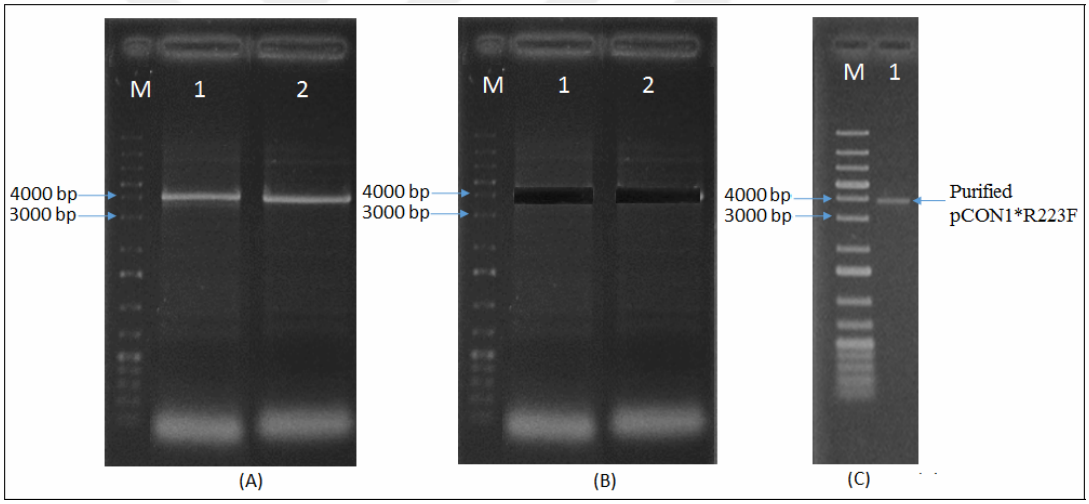

Figure 4.8. Purification of the pCON1*R223F PCR products. M, 1 kb plus DNA ladder; A, pCON1*R223F PCR products; B, sliced pCON1*R223F PCR products; C, purified pCON1*R223F (4.0 kb)

4.4.3 Transformation of pCON1*N170V and pCON1*R223F Constructs into *E.coli* XL1-Blue strains

The gel purified recombinant plasmid DNAs, pCON1*N170V and pCON1*R223F, were transferred individually into calcium chloride ($CaCl_2$) treated

E. coli XL1-Blue competent cells. The recombinant pCON1*N170V and pCON1*R223F plasmid DNAs size of which are the same (nearly 4.0 kb) with that of recombinant plasmid pCON1 were isoalted from the 1% agarose gel (Figure 4.9 and 4.10).

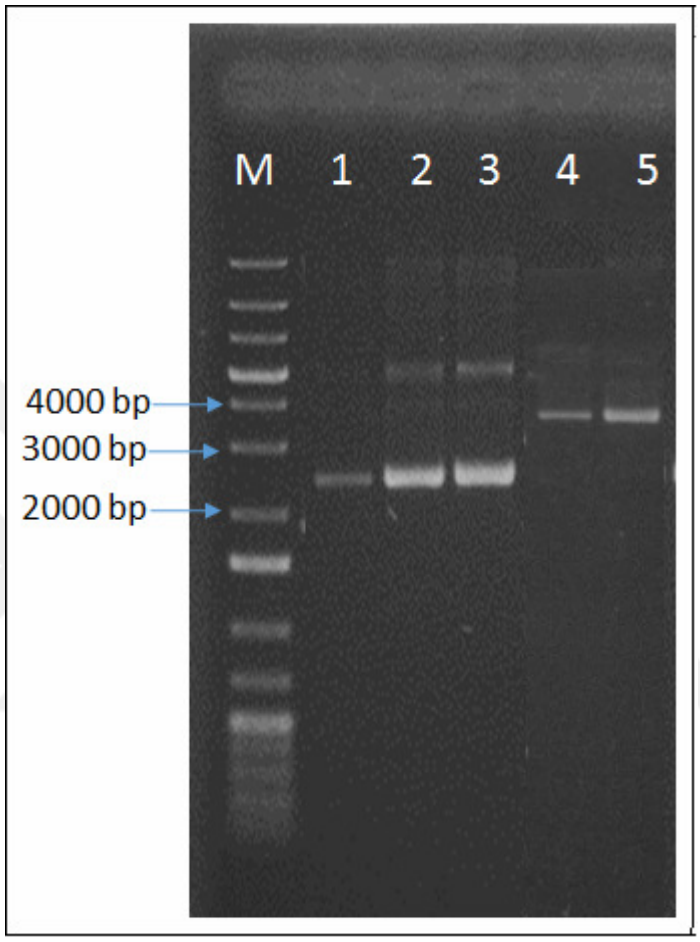

Figure 4.9. Transformation of pCON1*N170V into *E. coli* XL1-Blue strains. M, 1 kb plus DNA ladder; 1, uncut pCON1 DNA; 2 and 3, pCON1*N170V DNAs from first and second clones respectively; 4, *Xho*I/ cut pCON1 DNA; 5, *Xho*I/ cut pCON1*N170V DNA.

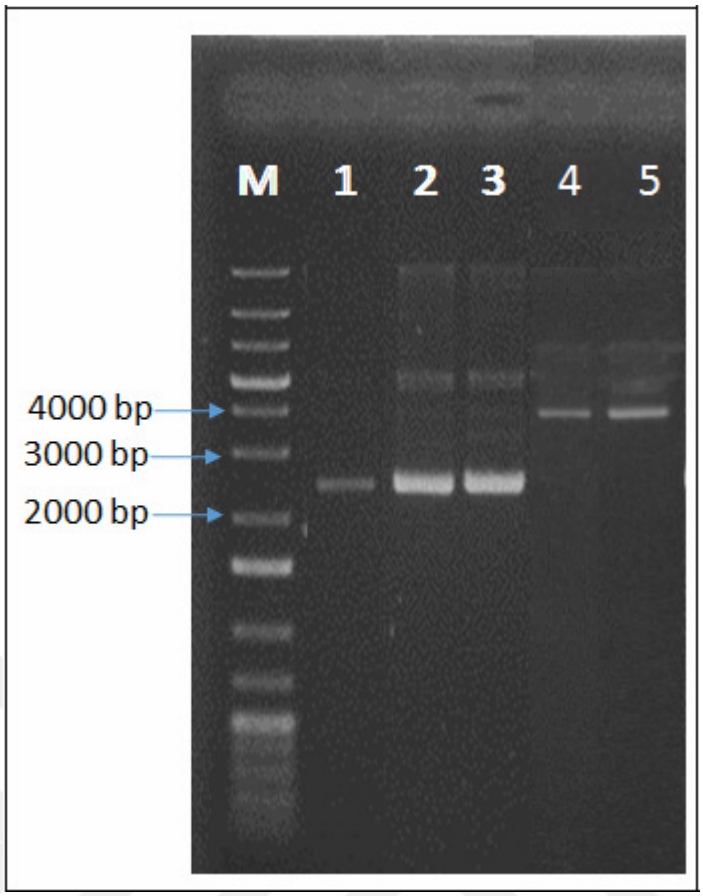

Figure 4.10. Transformation of pCON1*R223F into *E. coli* XL1-Blue strains. M, 1 kb plus DNA ladder; 1, uncut pCON1 DNA; 2 and 3, pCON1*R223F DNAs from first and second clones respectively; 4, *Xho*I/ cut pCON1 DNA; 5, *Xho*I/ cut pCON1*R223F DNA.

4.4.4 DNA Sequencing of pCON1*N170V and pCON1*R223F

The N170V and R223F mutations of *bsh* genes on pCON1 construct were detected by DNA sequencing, Macrogen (Seoul, Korea) with the universal primers, M13/pUC Forward (5'-GTAAAACGACGGCCAGT-3') and M13/pUC Reverse (5'-CAGGAAACAGCTATGAC-3'). Obtained DNA sequences were compared with the nucleotide residues of the wild-type *bsh* gene obtained from pCON1 constract to see desired mutations. Sequencing indicated that AAC and AGA codons coding asparagine 170 and arginine 223 amino acid were converted to GTT and TTT codons coding valine 170 and phenylalanine 223 amino acids respectively (Figure 4.11 and Figure 4.12). It was found that there was no mutation rather than desired one. The

clones that have desired mutations were named as pCON1N170V and pCON1R223F. The clones that had only desired mutations in pCON1N170V and pCONR223F were clarified 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 4.13 and Figure 4.14).

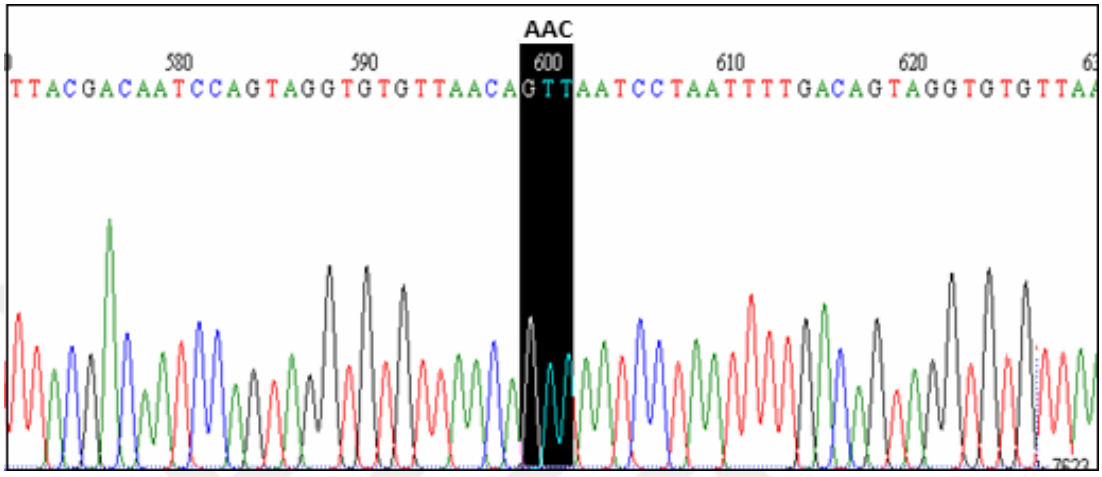

Figure 4.11. Partial sequence of pCON1N170V transformant. AAC codon coding asparagine amino acid was substituent for GTT codon coding valine amino acid.

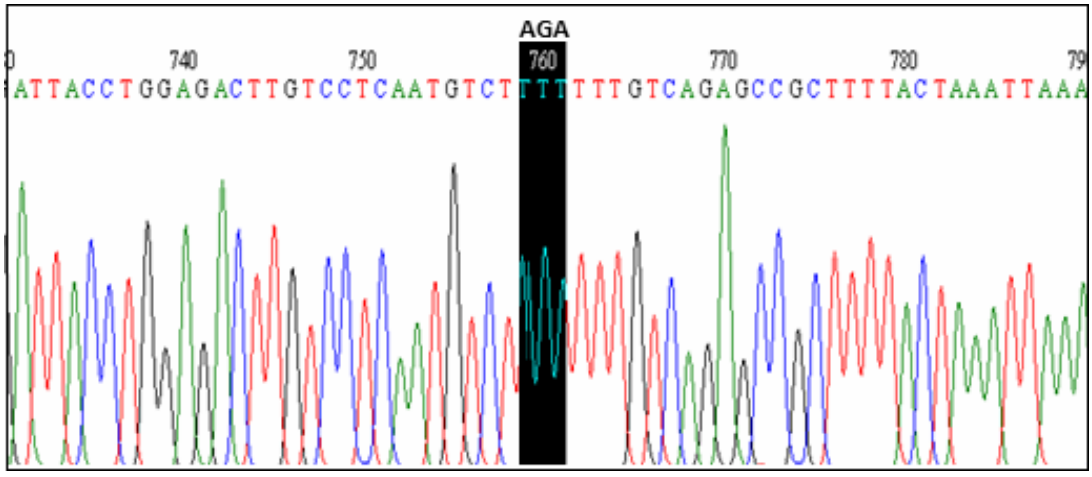

Figure 4.12. Partial sequence of pCON1R223F transformant. AGA codon coding arginine amino acid was substituent for TTT codon coding pheylalanine amino acid.

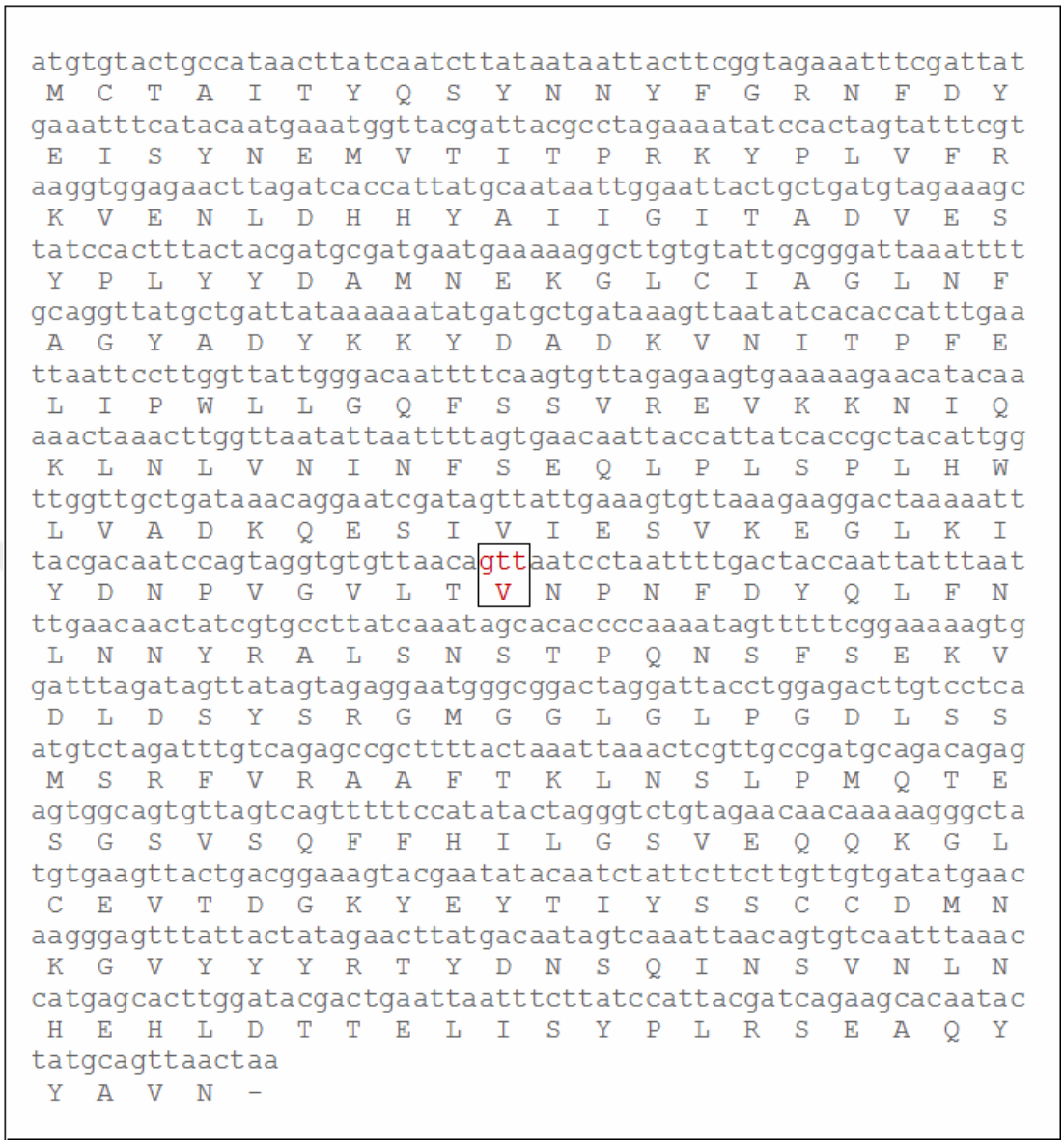

Figure 4.13. The 975 bp nucleotides and 324 amino acid residues of the mutant N170V *bsh* gene obtained by using the SIB (Swiss Institute of Bioinformatics) ExPASy sequence translate tool (http://web.expasy.org/translate/).

Figure 4.14. The 975 bp nucleotides and 324 amino acid residues of the mutant R223F *bsh* gene obtained by using the SIB (Swiss Institute of Bioinformatics) ExPASy sequence translate tool (http://web.expasy.org/translate/).

4.5 Preparation of pZEKN170V and pZEKR223F Constructs

4.5.1 Preparation of the pET22b Expression Vector

Approximately 5.5 kbp low copy pET22b expression vector DNA was isolated and digested with *Eco*RI and *Not*I restriction enzymes then it was purified from 1% agarose gels (Figure 4. 15).

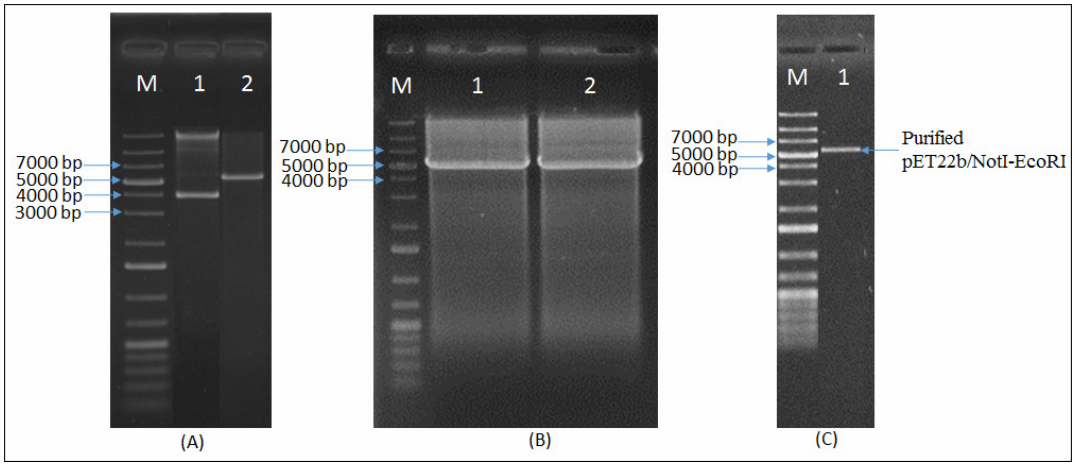

Figure 4.15. Preparation of pET22b expression vector DNA. M, 1 kb plus DNA ladder; A, 1: uncut pET22b DNA; 2: *Not*I/*Eco*RI digested pET22b DNA; B, *Not*I/*Eco*RI digested pET22b linear DNA before gel purification; C, gel purified linear pET22b DNA.

4.5.2 Digestion of pCON1N170V and pCON1R223F DNAs by *Eco*RI and *Not*I Restriction Enzymes

The pCON1N170V and pCON1R223F plasmid DNAs were cut with *Eco*RI and *Not*I restriction enzymes then the resulting approximately 1000 bp mutant *bsh* DNA fragments were purified from 1% agarose gels (Figure 4.16 and Figure 4.17).

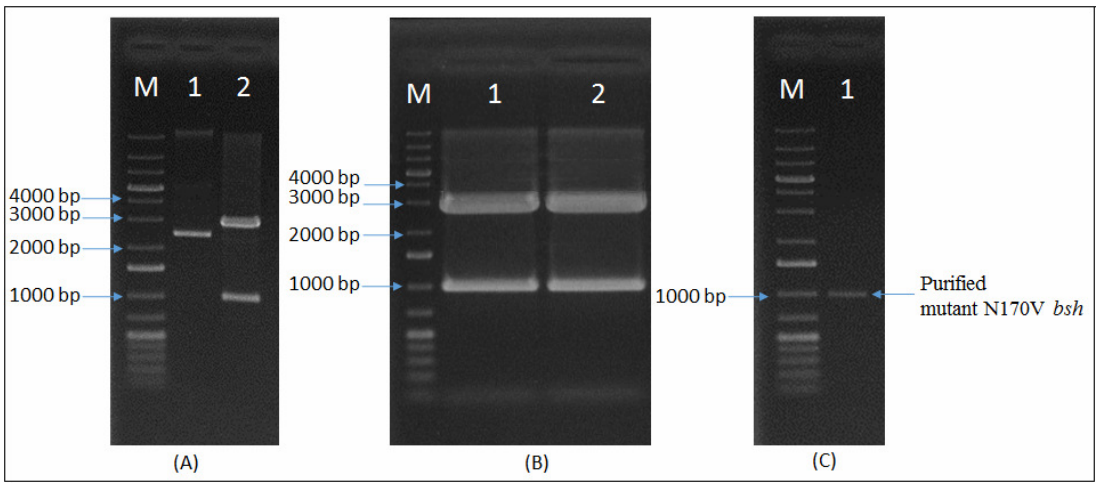

Figure 4.16. Digestion of pCON1N170V plasmid DNA by *Not*I and *EcoR*I REs. M, 1 kb plus DNA ladder; A, 1: uncut pCON1N170V DNA; 2: *Not*I/*EcoR*I cut pCON1N170V DNA; B, Large volume of linear pCON1N170V DNA before gel purification; C, gel purified N170V *bsh* DNA fragment from pCON1N170V DNA.

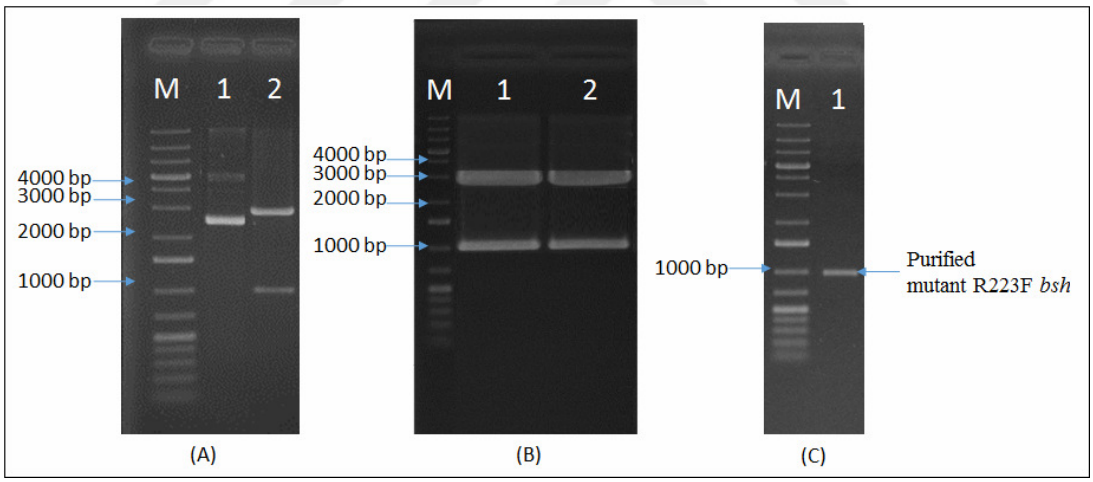

Figure 4.17. Digestion of pCON1R223F plasmid DNA by *Not*I and *EcoR*I REs. M, 1 kb plus DNA ladder; A, 1: uncut pCON1N170V DNA; 2: *Not*I/*EcoR*I cut pCON1R223F DNA; B, Large volume of linear pCON1R223F DNA before gel purification; C, gel purified R223F *bsh* DNA fragment from pCON1R223F DNA.

4.5.3 Preparation and Analysis of pZEKN170V and pZEKR223F Constructs

Approximately 1000 bp DNA fragments containind the desired mutations, pZEKN170V and pZEKR223F, and 5.5 kbp linear pET22b were ligated and transformed to *E. coli* BLR(DE3) competent cells. Four single colonies were selected randomly from each tarnsformat plates. DNAs isolated from selected clones were digested with *EcoR* I and *Not* I restriction enzymes to evaluate whether they received mutat BSH DNA (Figure 4.18 and Figure 4.19). The clones containing desired mutants were named as as pZEKN170V and pZEKR223F.

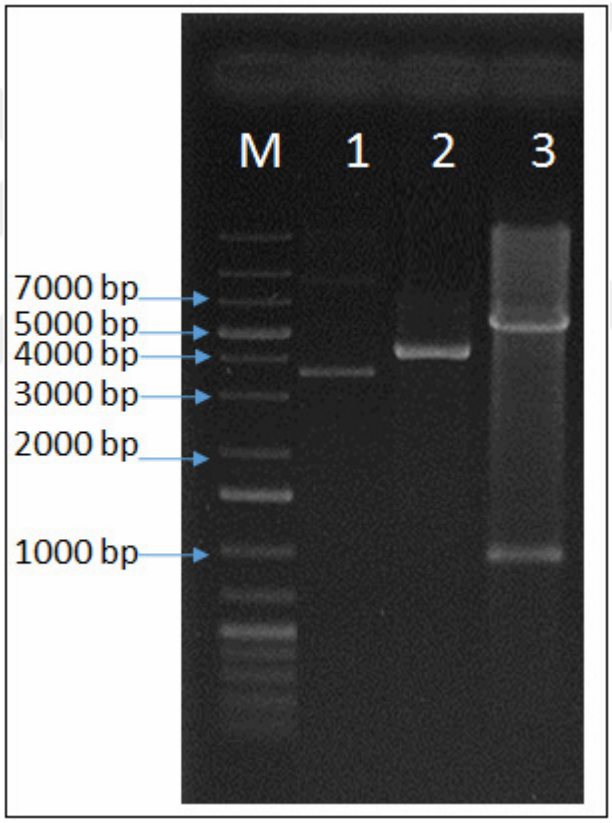

Figure 4.18. Preparation and verification of pZEKN170V. M, 1 kb plus DNA ladder; 1, circular pET22b plasmid DNA (negative control); 2, circular pZEKN170V DNA; 3, *Not*I/*EcoR*I digested of pZEKN170V DNA.

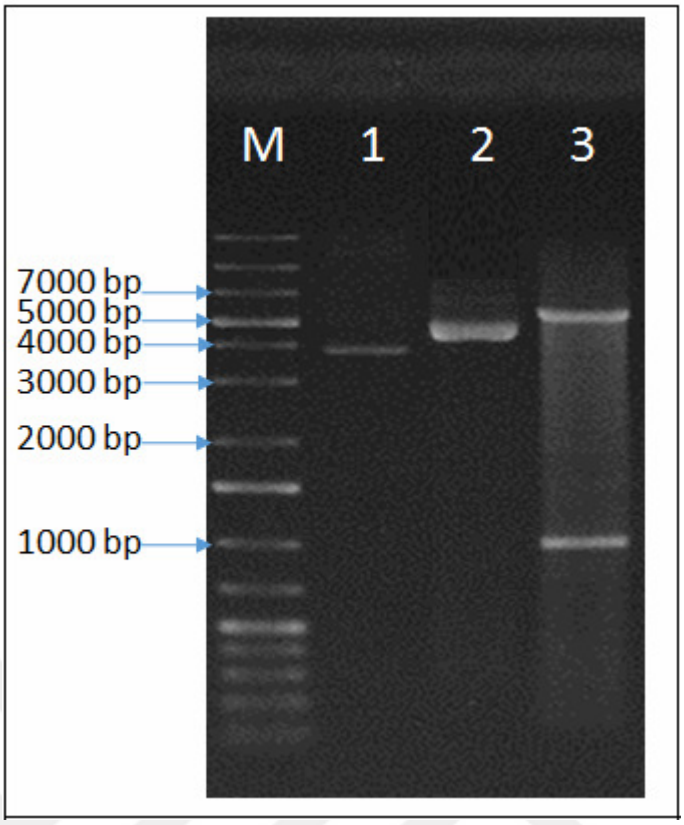

Figure 4.19. Preparation and verification of pZEKR223F. M, 1 kb plus DNA ladder; 1, circular pET22b plasmid DNA (negative control); 2, circular pZEKR223F DNA; 3, *Not*I/*Eco*RI digested of pZEKR223F DNA.

4.6 Determination of the BSH Activity

4.6.1 Detection of mrBSHs Activities by Direct Plate Assay

The activity of the rBSH of the mrBSHs and rBSH were determined by direct plate assay to compare the activity of mrBSH/N170V and mrBSH/R223F mutants with the rBSH enzyme as described in Dashkevicz et al. [1989]. In this assay, BSH activity was indicated by white colonies with surrounding precipitation zones, in the case of high activity, as shown in Fig. 4.20. Direct plate assay results revealed that the *E. coli* BLRDE3 strains with rBSH from *L. plantarum* B14 exhibited high BSH activity against GDCA (Fig. 4.20). On the other hand, the same strain failed to demonstrate convincing activity against TDCA in the same assay (Fig. 4.20). However, mrBSH/N170V and mrBSH/R223F appeared to have lost the ability to

hydrolyze both salts, as indicated by the lack of precipitation in the LB agar (Fig. 4.20).

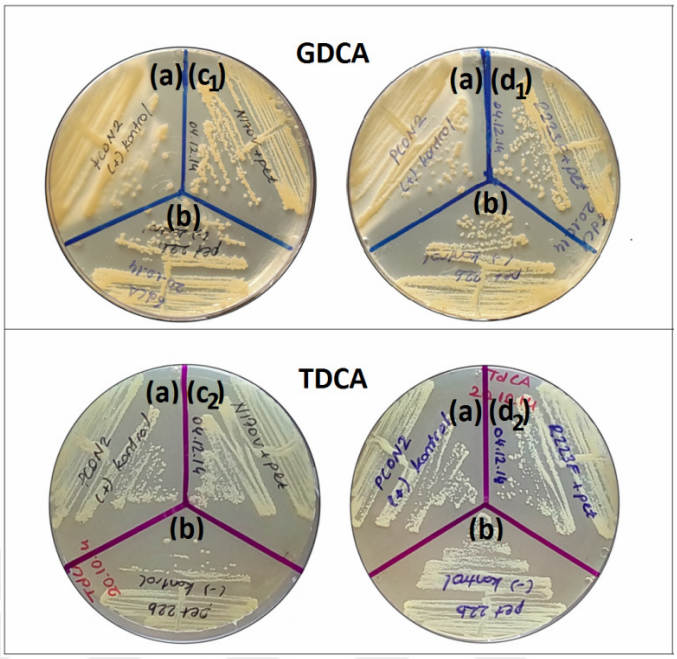

Figure 4.20. Detection of the mrBSHs activities by direct plate assay. The letters (a) in all plates represent phenotypes of positive controls (pCON2/BLR(DE3)); all regions labeled as (b) letters represent phenotypes of negative controls (pET22b /BLR(DE3)); the (c1) and (d1) leters on the LB-plates containing GDCA represent phenotypes of pZEKN170V/BLR(DE3) and pZEKR223F/BLR(DE3) mutants respectively; the (c2) and (d2) in the LB-plate containing TDCA represent phenotypes of pZEKN170V/BLR(DE3) and pZEKR223F/BLR(DE3) mutants respectively. BSH active colonies have a halo of precipitated cholic acid phenotypes.

4.6.2 Detection of mrBSHs Activities by Ninhydrin Assay

The catalytic activities of the rBSH and mrBSHs were determined by ninhidrin assay using partially purified cell extracts from the clones that are expressing rBSH and mBSHs. Two major human bile salts, GDCA and TDCA, were

used as a substrate for this assay. Overall, the rBSH enzyme of *L. plantarum* B14 strain displayed a broad substrate affinity and was able to hydrolyze both the glycine and taurine conjugates. However, the rBSH exhibited higher preference for glycine-conjugated bile acid than for taurine-conjugated bile acids (Fig. 4 21 and Fig. 4.22). The highest hydrolysis activity occurred when GDCA (defined as 100% activity) was used as the substrate at 37°C and pH 6.0. Catalytic activity of the rBSH for TDCA was 18.4% of the activity toward the GDCA. However, both mrBSHs appeared to have lost the ability to hydrolyze both salts (Fig. 4.21 and Fig. 4.22).

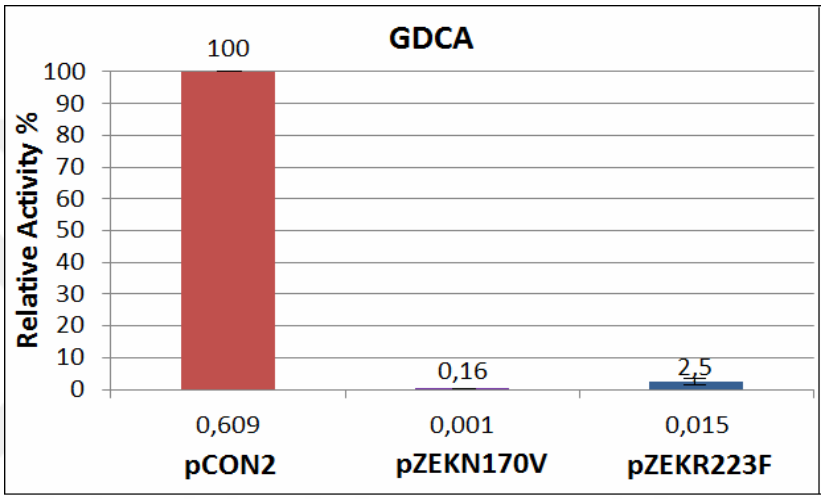

Figure 4.21. Determination of the catalytic activities of rBSH and mrBSH enzymes by ninhydrin assay in the presence of GDCA. The rBSH and mrBSH enzyme activities were measured by determining the amount of amino acids liberated from glycodeoxycholic acid (GDCA). Relative activity was defined as the measured 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. While pCON2 reflects rBSH activity, pZEKN170V and pZEKR223F reflects mBSH activities in against to GDCA.

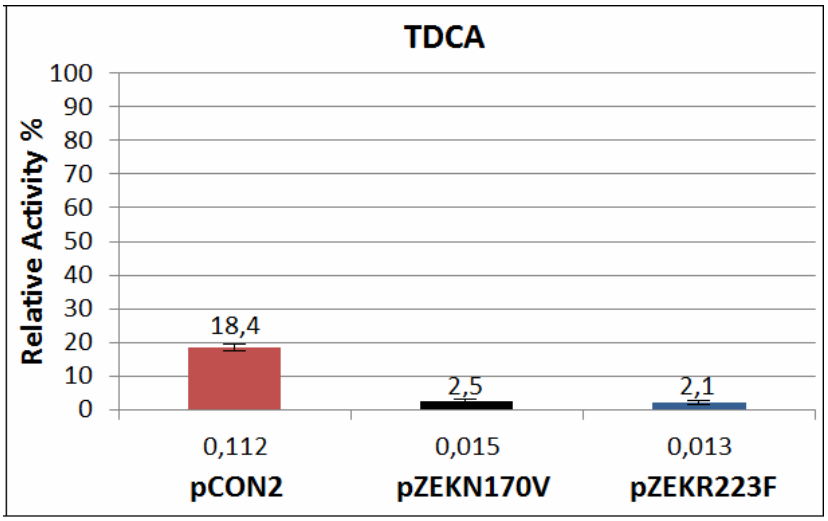

Figure 4.22. Determination of the catalytic activities of rBSH and mrBSH enzymes by ninhydrin assay in the presence of TDCA. The rBSH and mrBSH enzyme activities were measured by determining the amount of amino acids liberated from taurodeoxycholic acid (TDCA). Relative activity was defined as the measured 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. While pCON2 reflects rBSH activity, pZEKN170V and pZEKR223F reflects mBSH activities in against to TDCA.

4.7 SDS-PAGE Analysis of the Partially Purified mrBSHs Enzymes

Partially purified rBSH and mrBSHs were loaded on 12% Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE) and rBSH with molecular mass of 37 kDa was observed on SDS-PAGE. The theoretical molecular mass of rBSH, obtained as 36.7 kDa using BSH amino acid residues by ExPASy program (httweb.expasy.org/compute.pi/.), is in agreement with that of our experimental results (Fig. 4.23). The replacement of the N170 and R223 amino acids with V170 and F223 amino acids resulted in loss of activity of BSH since mutations completely disrupted either synthesis or formation of the BSHs. Although the strictly conserved N170 and R223 amino acids were predicted to be essential for the catalytic activity of the BSH obtained by previous in silico analysis, our results indicated that they are critical amino acids for the stability of the BSHs but not catalysis shown in Fig. 4.24

and Fig. 25 lanes 3 and 4 respectively. This result suggests that N170 and R223 amino acids are probably important for folding or maintaining of the stability of the BSH.

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

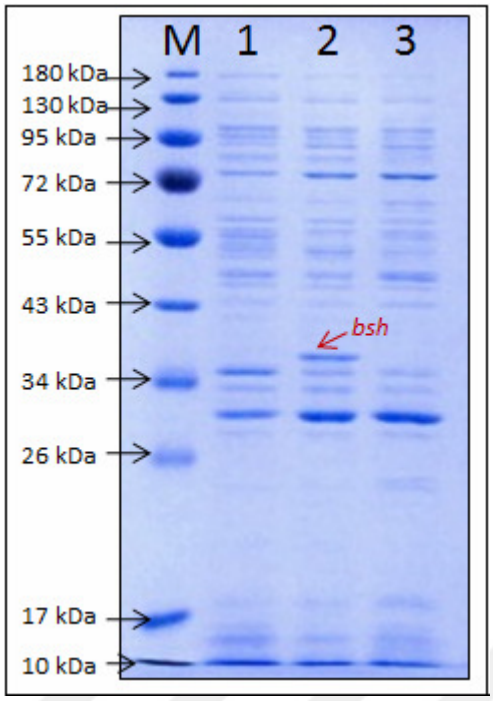

Figure 4.24. SDS-PAGE profile of partially purified mrBSH/N170V. M, Protein ladder; lane 1, pET22b (empty vector for negative control); lane 2, the partially purified *Lb. plantarum* BSH protein (positive control); lane 3, partially purified recombinant BSH/N170V mutant protein.

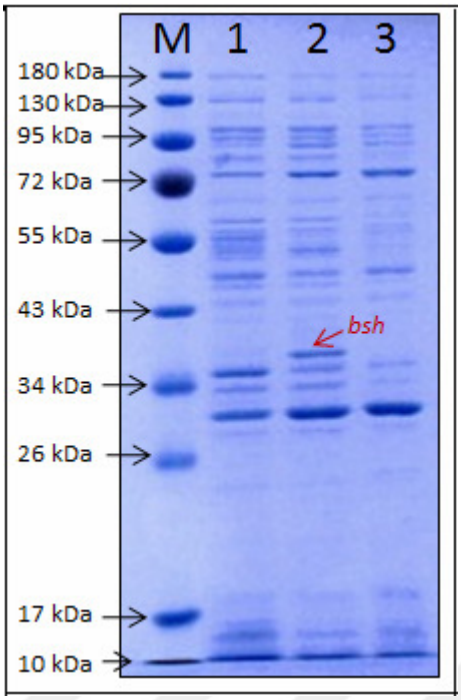

Figure 4.25. SDS-PAGE profile of partially purified mrBSH/R223F. M, Protein ladder; lane 1, pET22b (empty vector for negative control); lane 2, the partially purified rBSH protein (positive control); lane 3, the partially purified mrBSH/R223F protein.

5. DISCUSSIONS

One of the specific criteria for probiotic strain selection is bile salts hydrolyse ability of probiyotic strains. The biotransformation of bile salt fulfilled by intestinal bacteria is an considerable reaction in the intestinal tract. BSH producing probiotics provides curative effects, which reduce serum cholesterol level and play a important role in reducing the risk of stroke, atherosclerosis and coronary heart disease caused by hypercholesterolemia (Maria et al., 2006; Park et al., 2008; Sridevi et al., 2009; Jones et al., 2012; Guo et al., 2014). On the other hand, bacterial BSH activity has also been considered to be potentially detrimental to the host physiology due to the dehydroxylation and xidation steps of primary bile salts into secondary bile salts by intestinal bacteria. Especially, the production of secondary bile salts have been linked to cholesterol gallstone disease, colon cancer and various intestinal diseases (Thomas et al., 2000; McGarr et al., 2005; Ridlon et al., 2006; Bernstein et al., 2011). Whether BSH activity is actually a desirable feature in a probiotic bacteria is not completely clear because of the role of BSH in positive and negative biological processes in the host. Taking into account the biological implications of BSH activity, it is crucial to ensure an accurate description of the BSH genes in bacterial DNA sequences. We can do this by studying the catalytic activity and substrate preference mechanisms of the BSH enzyme. Based on the biological effects of BSH activity, it is crucial to provide an accurate description of the BSH genes in bacterial DNA sequences. We can do this by studying the catalytic activity and substrate preference mechanisms of the BSH enzyme.

The BLAST search analysis and the CLUSTAL multiple sequence alignment indicated that the sequence annotated as BSH in *Lactobacillus plantarum* B14 were compared with different *Lactobacillus* species, penicillin V acylase (PVA) of *Bacillus sphaericus* (Kim et al., 2004) and conjugated bile acid (CBAH) of *Clostridium perfringens* species (Rossocha et al., 2005) were members of the N-terminal nucleophile (Ntn) hydrolase superfamily, having an N-terminal catalytic nucleophile which cleaves an amide bond (Oinonen et al., 2000). Based on the relationship between the proteins, the phylogenetic tree divided into two main groups, a BSH branch with BSH enzymes and a PVA branch that includes penicillin

V acylase related proteins. Although the BSHs from various bacterial species of the Ntn hydrolase superfamily showed significant variation of sequence, multiple sequence alignment demonstrated that these BSH enzymes include all conserved, catalytically substantial amino acid residues in the proposed active site of BSH (Tanaka et al., 2000; Kim et al., 2005; Rossocha et al., 2005; Begley et al., 2006; Oh et al., 2008; Kumar et al., 2010). The five predicted catalytic sites, identified as Cys-2, Arg-16, Asp19, Asn-170 and Arg 223 in *Lb. plantarum* B14 were exactly conserved in all bile salt hydrolase enzymes of *Lactobacillus* species, *Bacillus sphaericus* PVA and conjugated bile acid (CBAH) *Clostridium perfringens* species.

Qualitative BSH activities of rBSH enzyme of *Lb. plantarum* B14 strain, obtained by direct plate assay, showed good amount of enzymatic activity on the plates with GDCA but not with TDCA. In addition, although similar results were obtained from different strains of *Lb. plantarum* BBE7 (Dong et al., 2012), high substrate spesificity for TDCA was detected in strains of *Lb. plantarum* MBUL90, Lp10, Lp11, Lp21, Lp90, Lp91, NCDO82 strains (Kumar et al., 2010, 2011, 2012). The catalytic activity of BSH enzyme was also determined quantitively by ninhydrin assay. Our ninhidrin assay result indicated that BSH-positive control were more effective in hydrolyzing GDCA compared to TDCA substrate (Fig. 4.21 and Fig. 4.22).

Multiple sequence alignment of the Ntn hydrolase family amino acid residues have revealed that the C2, D16, N18, N170 and R223 were strictly conserved amino acids on the active sites of all BSH (Ridlon et al., 2005). Therefore, the structural-based computational works and theoretical approaches were emphasized that especially construction of BSH mutants in the critical sites may provide prediction of the structures of BSH enzymes and facilitate more exact identification of the components that determine their substrate specificities Comprehensive amino acid substitution mutagenesis were advised by recent articles in orther to discover residues critical in catalysis, understand their function and catalytic–binding pocket relationships (Yip et al., 2011; Chae et al., 2012; Geng et al., 2017). However, there is no experimental research on these strictly and partially conserved amino acids except Cys2. Tanaka and his colleagues (Tanaka et al., 2000) found that the first N-terminal amino acid residue of the purified mature BSH was cysteine amino acid

because of the formylmethionin processing. The important role of Cys2 was confirmed by changing of Cys2 to alanine 2 (Ala2) and Cys2Ala mutant led to complete inactivation of BSH (Tanaka et al., 2000). The more conservative substitutions, Cys2Ser and Cys2Thr, inactivated the BSH from *Bifidobacterium bifidum* (Kim et al., 2004). It was reported that Cys2 plays an important role in the activity of BSH (Rossocha et al., 2005; Kumar et al., 2006; Xu et al., 2016). In order to confirm the structure–activity relationship of other strictly conserved amino acids, further construction of BSH mutants and structural analysis are necessary.

6. CONCLUSIONS

In this study, we have endeavored to better understand the structure and catalytic activty of *Lb. Plantarum* B14 *bsh* gene belong to CBAH superfamily members by utilizing site-directed mutagenesis. Briefly, we have shown that the previous predications from a large scale of in silico studies are not consistent with our mutagenesis results. Although strictly conserved Asn170 and Arg2123 amino acids were predicted to be responsible from catalytic activity of the Ntn hydrolase superfamily members, our experimental result showed that these amino acids had a critical role for folding or stability of the BSH enzyme. Therefore, N170 and R223 amino acids are not essential beterbe stability of the active site geometry and catalytic chemistry of BSH but they are crucial for stability or folding of the BSHs. This is the first experimental report indicating the N170 and R223 amino acids are critical for the folding or stability of BSH enzyme. However, further biochemical investigations and more site-directed mutagenesis studies are required for better understanding the exact functions of the N170, R223 and other remaining strictly conserved amino acids.

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APPENDICES

8. APPENDICES

Appendix A

BACTERIAL GROWTH MEDIA

- **Liquid LB (Luria Bertani) Medium**

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

These contents are then dissolved in 900 ml UpH_2O and the pH is adjusted to 7.5 with NaOH.

The final volume is then completed to 1 L with UpH_2O and sterilized at 121°C for 15-20 minutes.

- **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.

- **Ampicillin**

✓ 0.25 g Ampicillin Sodium Salt

✓ 10 ml UpH_2O

The Final solution is then sterilized by using a 0.22 μm _itler and stored at -20°C.

- **LB Plate with ampicillin**

To make an LB plate with ampicillin, 4 ml of ampicillin (25mg/ml) is added for 1 L of sterilized liquid LB medium.

Appendix B

SOLUTIONS AND BUFFERS

- **For primer resuspension**

- **TE Buffer**

✓ 10 Mm Tris (Ph:8.0)
✓ 1 Mm EDTA (Ph:8.0)

- **For Electrophoresis**

- **10X TAE Buffer**

Contents	For 1L
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

- **For Competent Cell Preparation**

- **0.1 M $CaCl_2$**

✓ 1.48g $CaCl_2$

✓ 100ml UpH_20

The Ph is adjusted to 7.0.

- **0.1 M $CaCl_2$ with 10% glycerol**

✓ 9 ml lof 0.1 M $CaCl_2$

✓ 1 ml of glycerol

- **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 buffer solution was then sterilized at 121°C for 15-20 minutes.

- **1M Na_2HPO_4 Buffer**

14.2 g of dibasic sodium phosphate (Na_2HPO_4) are dissolved in 100 ml ddH_2O. The buffer solution was then sterilized at 121°C for 15-20 minutes.

- **0.1M 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.

- **Binding Buffer (Ph: 7.8)**

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

- **For Lowry Assay**

- **0.1M NaOH**
- ✓ 0.4 g NaOH
- ✓ 100 ml UpH_2O

- **1% SDS in 0.1M NaOH**
- ✓ 0.05 g Sodium Dodecyl Sulfate
- ✓ 5 ml of 0.1 M NaOH

- **Reagent A**
- ✓ 1 g Na_2CO_3 (Sodium carbohydrate)
- ✓ 50 ml of 0.1 M NaOH

- **Reagent B**
- ✓ 5 mg $CuSO_4.5H_2O$ (Cupper II sulphate pentahydrate)
- ✓ 0.5 ml of UpH_2O

- **Reagent C**
- ✓ 10 g $C_4H_4KnaO_6.4H_2O$ (Potassium sodium tartarate)
- ✓ 0.5 ml of UpH_2O

- **Reagent D**
- ✓ 500 µl Reagent B
- ✓ 500 µl Reagent C

- **Reagent E**
- ✓ 50 ml Reagent A
- ✓ 1 ml Reagent D

- **1mg/Ml BSA**
- ✓ 0.00 1g BSA (Bovine Serum Albumin)
- ✓ 1 ml of UpH_2O

- **For Ninhydrine Assay**

- **0.2M Glycine Solution**
- ✓ 0.7507 g H_2NCH_2COOH (Glycine)
- ✓ 50 ml of UpH_2O

- **1M DTT**
- ✓ 0.154 g $C_4H_{10}O_2S_2$ (Sodium Dithiothreitol)
- ✓ 1 ml of H_2O

- **0.5 M Sodium Citrate Buffer**
- ✓ 7.353 g $C_6H_5Na_3O_7.2H_2O$ (tri-Sodium Citrate dihydrate)
- ✓ 50 ml of UpH_2O

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

- **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

- **0.1M Sodium Phosphate Buffer (Ph: 6.0)**

The mixture of 1M monobasic and dibasic sodium phosphate were used to obtaine the Ph: 6.0. The buffer solution was then sterilized by filtration.

- **15% Trichloroacetic Acid (TCA)**
- ✓ 7.5 g Trichloroacetic acid
- ✓ 50 ml of UpH_2O

The solution was then sterilized by filtration.

- **For Purification of Rbsh and mrBSH Enzymes**

- **Binding Buffer Ph: 7.8**

Contents	**For 1L**
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**

- **%30 Acrylamide-Bis Acrylamide Mixture**

✓ 14.6 g Acrylamide

✓ 0.4 g Bis Acrylamide

✓ 50 ml of UpH_2O

- **4x Separating Gel Buffer (Ph: 8.8)**

✓ 9.1 g Trizma base

✓ 50 ml of UpH_2O

- **4x Stacking Gel Buffer (Ph: 6.8)**

✓ 3.05 g Trizma base

✓ 50 ml of UpH_2O

- **10% SDS**

✓ 10 g Sodium Dodecyl Sulfate

✓ 100 ml UpH_2O

- **10% APS**

✓ 0.1 g Ammonium persulfate

✓ 1 ml UpH_2O

- **10X Electrode Buffer Ph: 8.3**

Contents	**For 1L**
0,25 M Tris	30 g
1.92 M Glycine	144 g

These mixture is dissolved in UpH_2O for a final volume of 1 L. The Ph is adjusted with HCl.

- **1X Electrode Buffer**

✓ 100 ml 10X Electrode buffer

✓ 1 g SDS

✓ 900 ml UpH_2O

- **Loading Dye**

Contents	For 1ml
10% SDS	400 µl
Glycerol	120 µl
0.5 M Tris/HCl (Stacking buffer) (Ph: 6.8)	100 µl
0.5% Bromophenol Blue	40 µl
UpH_2O	340 µl

Comassie Stain Solution

Contents	For 1L
Comassie Brillant G Blue	1 g
Glacial acetic acid	100 ml
Methanol	400 ml
UpH_2O	500ml

De-Stain for Comassie

Contents	For 1L
Glacial acetic acid	100 ml
Methanol	100 ml
UpH_2O	800 ml

- **12% Separating Gel**

Contents	For 15 ml
UpH_2O	5. 1 ml
30% Arcy/Bis.	6 ml
Separating Gel Buffer (Ph: 8.8)	3.75 ml
10% SDS	150 µl
TEMED	7. 5 µl
10% APS	75 µl

- **4% Stacking Gel**

Contents	For 10 ml
UpH_2O	6. 2 ml
30% Arcy/Bis.	1.3 ml
Stacking Gel Buffer (Ph: 6.8)	2.5 ml
TEMED	10 µl
10% APS	50 µl

Appendix C

CHEMICAL

Acrylamide (Sigma)

Agar (Merck)

Agarose (Sigma)

Ammonium persulfate [APS] (Sigma)

Ampicillin (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)

Ethanol (Merck)

Glacial acetic acid (Carlo Erba)

Glycerol, cell culture tested (Sigma)

Glycine (Merck)

Glycodeoxycholic Acid, Sodium Salt (Calbiochem)

Imidazole (Alfa Aesar)

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

N,N'-Methyenebisacrylamide (Sigma)

Methanol (Merck)

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)

Taurodeoxycholic Acid, Sodium Salt (Calbiochem)

TEMED (Sigma)

Trichloroacetic Acid (Merck)

Trizma Base (Sigma)

Trizma Hydrochloride (Sigma)

Yeast Extract Granulated (Merck)

Tween® 20 (Sigma)

Appendix D

ENZYMES AND OTHER CHEMICALS

Dnase (Sigma)

Dntp Mix (Fermentas)

*Dpn*I (Thermo Scientific)

*EcoR*I Fast Digest (Thermo Scientific)

1 kb Plus DNA Ladder (Fermentas)

6X Loading Dye Solution (Fermentas)

Lysozyme (Fluka)

*Not*I Fast Digest (Thermo Scientific)

Page Ruller Prestain Protein Ladder (Thermo Scientific)

Pfu DNA Polymerase (Fermentas)

Rnase (Thermo Scientific)

T4 DNA Ligase (Fermentas)

*Xho*I (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)

+4°C refrigerators (Arçelik)

AKTAprime^{TM} plus (GE Healthcare)

Autoclave (Hirayana)

Centrifuge (Hettich Rotina 38R)

Desktop centrifuge (Hettich Micro 120)

Digital and Analog Heatblock (VWR)

Electrophoresis system (Thermo Scientific)

Imaging system (UVP Photo Doc-It TM)

Micropipettes (Thermo Scientific)

PCR (Techne, TC 3000)

Ph meter (Thermo Scientific)

Power supply (Thermo EC 250-90)

Spektrophotometer (HITACHI U-1900)

Thermoshaker (Gerhardt)

UV Transalluminator (UVP)

Vortex (Yellowline TTS2)

Water Purification System (Human Corporation)

9. CURRICULFUM VITAE

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Kaya Y, Hacıbeyoğlu K, Kılıçsaymaz Z, Öztürk M (2015) "Molecular Cloning, Expression and Characterization of Bile salt hydrolase Enzyme from *Lactobacillus rhamnosus* KPB7 and E9", 4th National Molecular Biology and Biotechnology Congress, August 21-24, Afyonkarahisar, TURKEY.

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