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

MUTATIONS OF THR169 AND PRO172 AMINO ACIDS OF BSH ENZYME FROM *LACTOBACILLUS PLANTARUM* AND STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE MUTANTS

MASTER OF SCIENCE

FATMA TÜRKER

BOLU, JANUARY 2019

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APPROVAL OF THE THESIS

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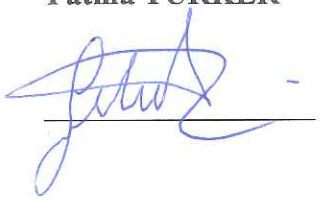
To my lovely family.

DECLARATION

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

Fatma TÜRKER



ABSTRACT

MUTATIONS OF THR169 AND PRO172 AMINO ACIDS OF BSH ENZYME FROM *LACTOBACILLUS PLANTARUM* AND STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE MUTANTS
MSC THESIS
FATMA TÜRKER
BOLU ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: ASSOC. PROF. DR. MEHMET ÖZTÜRK)

BOLU, JANUARY 2019

Bile salt hydrolase (BSH) (EC 3.5.1.24) has a very important role in host metabolism. Glycine and taurine in bile salts can be liberated by BSH which is called deconjugation. This property of BSH exert health benefits to the host such as reduction of blood cholesterol. Therefore BSH active probiotic strains are used in the treatment of hypercholesterolemia related diseases. Moreover, using BSH active strains as an alternative to antibiotic growth promoter (AGP) is under research. However, deconjugation of bile salts may have side effects to the host. Studies showed the relationship between BSH and a couple of diseases; colorectal cancer, cholesterol gallstone and bowel diseases. For this reason, understanding the mechanism of BSH enzyme is crucial. Even though BSH effects on human are well studied, there are not enough information about its mechanism of catalytic activity, substrate preferences and folding. In this study, two conserved amino acids, Thr-169 and Pro-172 from *Lactobacillus plantarum* B14 strain were substituted for valine and histidine respectively in pCON1 construct by site-directed mutagenesis then mutant *bsh* genes were inserted into the pET22b expression vector, and expressed in *Escherichia coli* BRL(DE3) strain. Mutant BSH enzyme activities were determined by ninhydrin assay with six different human bile salts, glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid. The results showed that T169V mutation decreased the activity of BSH. Distinctly; inactive enzyme was obtained from P172H mutant. Meanwhile, stability and folding of the BSH proteins were not affected by these mutations. Our finding demonstrated that these two amino acids might be responsible for catalytic activity of BSH enzyme but not for stability of it.

KEYWORDS: Bile salt hydrolase (BSH), *Lb. plantarum*, Conserved amino acids, Site-directed mutagenesis

ÖZET

LACTOBACILLUS PLANTARUM'UN BSH ENZIMINDE THR169 VE PRO172 AMINOASITLERININ MUTASYONU VE MUTANT ENZIMLERIN YAPISAL VE FONKSIYONEL KARAKTERIZASYONU
YÜKSEK LISANS TEZI
FATMA TÜRKER
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
BIYOLOJI ANABILIM DALI
(TEZ DANIŞMANI: DOÇ. DR. MEHMET ÖZTÜRK)

BOLU, OCAK - 2019

Safra tuzu hidrolaz (STH) (EC 3.5.1.24) enzimi konakçının metabolizması üzerinde çok önemli bir role sahiptir. STH tarafından safra tuzlarında bulunan taurine ve glisin serbest bırakılabilir ki bu durum dekonjugasyon olarak adlandırılır. STH'ın bu özelliğinin sağlığa faydaları vardır örneğin kandaki kolestrolün düşmesini sağlar. Bu yüzden aktif STH içeren probiyotik türler hiperkolestrolemi kaynaklı hastalıkların tedavisinde kullanılmaya başlanmıştır. Buna ek olarak STH aktif türlerin antibiyotik büyüme promotor'e (AGP) alternatif olarak kullanılması araştırılmaktadır. Fakat safra tuzu dekonjugasyonu konakçı üzerinde yan etkileri de görülebilir. Yapılan çalışmalar STH ile kolorektal kanser, safra taşı ve bazı bağırsak hastalıkları arasında ilişki olduğunu göstermektedir. Bu nedenle STH enziminin çalışma mekanizmasını anlamak hayati önem taşımaktadır. BSH'ın insan üzerindeki etkileri iyi çalışılmış olsa da, onun katalitik aktivitesi, substart tercihleri ve katlanması hakkında yeterli bilgi bulunmamaktadır. Bu çalışmada *Lactobacillus plantarum* B14 türünden korunmuş iki amino asit, Thr-169 ve Pro-172, yönlendirilmiş mutagenez kullanılarak pCON1 konstraktı içinde sırasıyla Valin ve Histidin amino asitlerine dönüştürülüp, pET22b ekspresyon vektörüne aktarılmıştır ve *Escherichia coli* BRL(DE3) türünde ifade edilmiştir. Elde edilen mutant BSH enzimlerinin aktiviteleri altı farklı safra tuzu olan glikokolik asit, glikodioksikolik asit, glikokenodioksikolik, taurokolik asit, taurodeoksikolik asit ve taurokenodeoksikolik kullanılarak ninhydrin yöntemi ile belirlenmiştir. Sonuçlar T169V mutasyonunun BSH aktivitesini azalttığını göstermiştir. Bundan farklı olarak P172H mutantından inaktif enzim elde edilmiştir. Bu sırada, BSH proteinin bütünlüğü ve katlanması bu mutasyonlardan etkilenmediği görülmüştür. Bulgularımız bu iki aminoasit BSH enziminin katalitik aktivitesinden sorumlu olabileceğini fakat stabilitesinden sorumlu olamayacağını göstermektedir.

ANAHTAR KELİMELER: : Safra tuzu hidrolaz (STH), *Lb. plantarum*, Korunmuş amino asitler, Yönlendirilmiş mutagenez

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABBREVIATIONS AND SYMBOLS

AGP : Antibiotic Growth Promoter
BS : Bile Salt
BSH : Bile Salt Hydrolase
CA : Cholic Acid
CGs : Cholesterol Gallstones
CHD : Coronary Heart Disease
CRC : Colorectal Cancer
DCA : Deoxycholic Acid
FAO : Food and Agriculture Organization
GI : Gastrointestinal
GRAS : Generally Regarded as Safe
LAB : Lactic Acid Bacteria
WHO : World Health Organization
PCR : Polymerase Chain Reaction
PVA : Penicillin V Acylase
RE : Restriction Enzyme

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1. INTRODUCTION

1.1 Probiotics

Human body contains tenfold more microbial cells (1014) than human cells (Sagave, 1977).These microorganisms find all over the body including oral cavity, skin, urogenital, respiratory and gastrointestinal tract. The gastrointestinal (GI) tract is most densely colonized organ (Figure1.1). The beneficial microflora found in the GI tract was termed as probiotic. According to the World Health Organization (WHO) definition, probiotics are 'live microorganisms' when administered in adequate amount, confer a health benefit on the host (FAO/WHO, 2002). The consent of probiotics evolved early 1900s. Metchinkoff (1908) suggested that people should consume fermented milk products containing lactobacilli to protect the intestine from the damaging effect of other harmful bacteria.

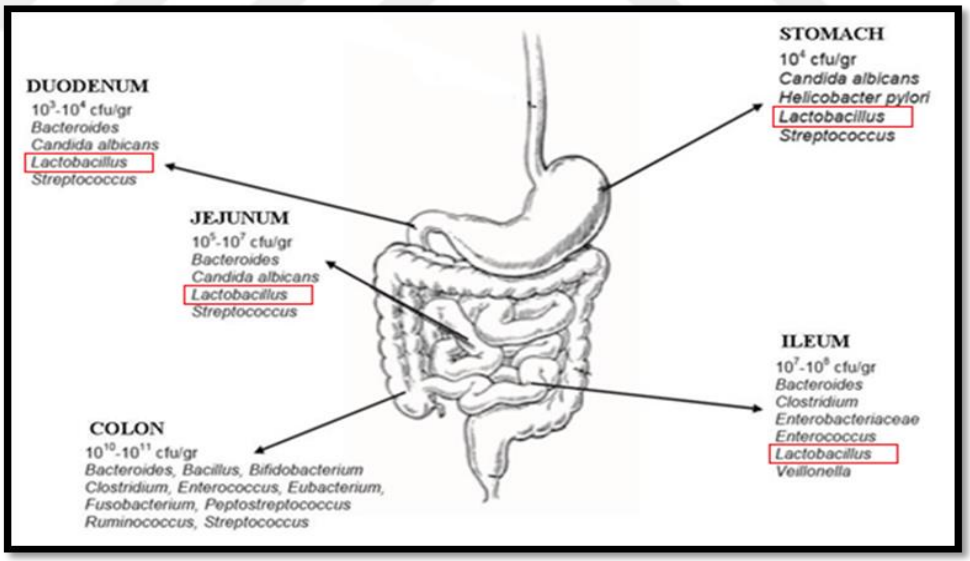


Figure 1.1. Microorganisms found in different part of gastrointestinal tract (modified from Coşkun, 2006).

Probiotics can be bacteria, mold and yeast. However, bacteria are abundant in human body. The major representatives of probiotics are lactic acid bacteria genus;

Lactobacilli, Enterococci and Bifidobacteria (Table 1.1). Lactobacilli are the most extensively studied genus.

Table 1.1. Microorganisms that are used as a probiotics modified from (Goktepe et al., 2006)

Lactic Acid Bacteria	*Lactobacillus* species	*L. acidophilus, L. brevis, L. bulgaricus, L. casei, L. cellobiosus, L. crispatus, L. curvatus, L. delbrueckii, L. fermentum, L. gasseri, L. helveticus, L. johnsonii, L. lactis, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus, L. salivarius, L. sporogenes*
	Pediococcus species	*P. acidilactici, P. cerevisiae, P. pentosaceus*
	Streptococcus species	*S. salivarius, S. cremoris, S. diacetilactis, S. intermedius, S. lactis, S. thermophilus*
	Leuconostoc species	*L. mesenteroides* ssp. *mesenteroides*
	Lactococcus species	*L. lactis*
	Propionibacterium	*P. freudenreichii, P. shermanii*
Other Species	*Bifidobacterium* species	*B. adolescentis, B. animalis, B. bifidum, B. breve, B. infantis, B. lactis, B. longum, B. thermophilum*
	Bacteriodes species	*B. amylophilus, B. capillus, B. ruminicola, B. suis*
	Bacillus species	*B. coagulans, B. lentus, B. licheniformis, B. pumilus, B. subtilis*
	Aspergillus species	*A. niger, A. oryzae*
	Saccharomyces species	*S. cerevisiae, S. boulardii*
	Candida species	*C. torulopsis*

According to Fuller (1989) a good probiotic should be;

1) Beneficial effect on the host.
2) Non-pathogenic and non-toxic.
3) Present as viable cells in large numbers.
4) Remain viable during storage for long time.

An effective probiotic should be capable of surviving and metabolizing in the gut and have good sensory properties (Collins and Gibson, 1999).

Another term, prebiotics were defined as a non-digestible food ingredients that benefit the host organism (Gibson and Roberfroid, 1995). Prebiotics fertilize gut microbiome and augment the growth of the good microbes in the gut. When

probiotics and prebiotics are come together, they produce mixture that is called symbiotic. They form synergism because probiotics cannot augment well in the digestive system.

1.2 Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) are nonsporing, gram positive, fermentative rod or coccus that lack catalase. They have nonmotile cells and producing lactic acid as end product. LAB need to nutritionally rich habitats to grow. They commonly found in fermented meat and fish, pickled vegetables, sourdough, water, sewage and cavities of human and animals. They are major group of healthy microbiota of the human gut (König and Fröhlich, 2009). LABs have been accepted as GRAS (Generally Regarded as Safe).

Lactic acid bacteria have circular and linear plasmids associated with antibiotic resistance mechanisms, bacteriocin production, carbohydrate fermentation, phage defence mechanisms and proteinase activities (Morelli et al., 2012). There are two types of LAB according to end product of carbonhydrate fermentation. Homofermentative LAB (like; *Lactobacillus casei*) produces lactic acid as major end product. Heterofermentative LAB (like; *Lactobacillus brevis*) produces lactic acid, CO_2, acetic acid and ethanol as end products (Nessier, 1994).

LABs consist of many diverse genera such as Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconoctoc, Teragenococcus, Vangococcus and Weissella (Jay, 2008) (Figure 1.2). Same LABs are included in probiotics because of their potential beneficial effect on GI tract of human and animals (Tannock, 2005). The Lactobacillus and Bifidobacterium genus are the most well studied LABs which have potential probiotic properties.

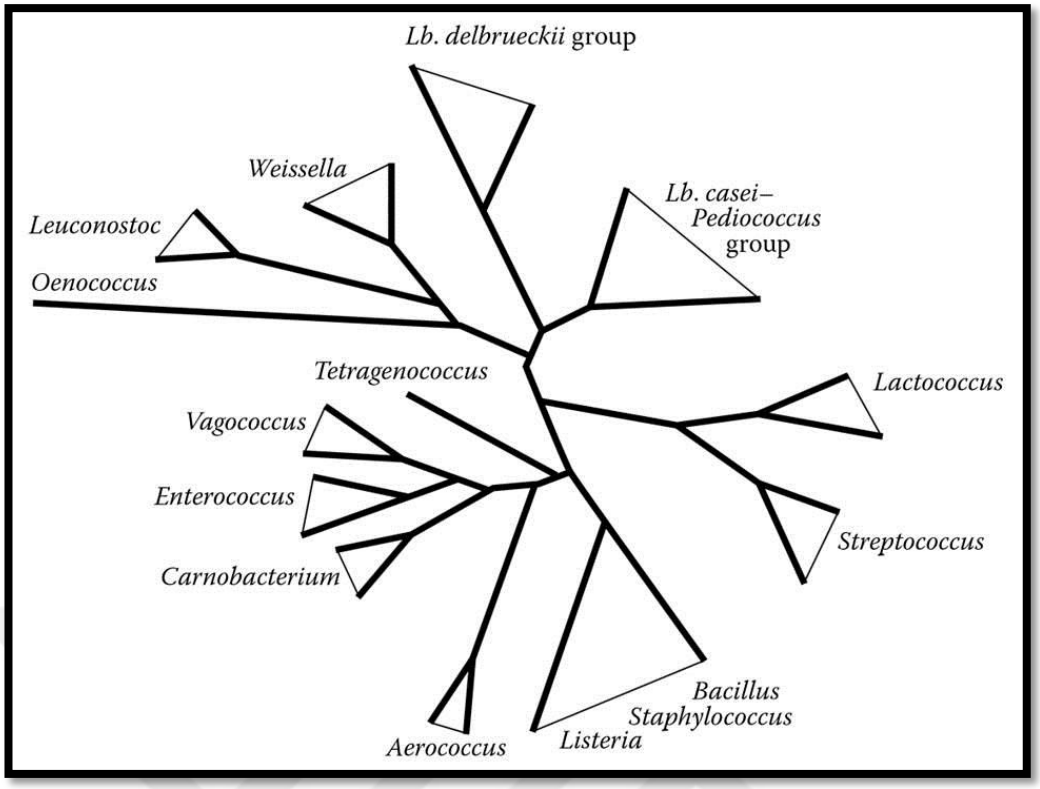


Figure 1. 2. Phylogenetic tree of lactic acid bacteria with related genera (Wright and Axelsson, 2012).

1.2.1 The Lactobacillus Genus

Genus Lactobacillus is lactic acid bacteria which belong to phylum Firmicutes, class Bacilli, and order Lactobacilales and family Lactobacillaceae. Lactobacilli have low G+C content. They are anaerobic, strictly fermentative, gram positive and generally recognized as non-pathogenic (Teuber, 1993). Lactobacilli need nutritionally rich environments to grow like other lactic acid bacteria. They are present as natural microbiota of host and found in various niches such as urogenital tract and oral cavity. They can be found also at ecological niches like soil and plants. Lactobacilli are well adapted in human gastrointestinal tract therefore they are densely colonized in it. Scientists have been hypothesized about beneficial effect of lactobacilli on human according to their research results. For example, lactobacilli prevent antibiotic associated diarrhea (Kale-Pradhan et al., 2010). Moreover, lactobacilli may help to decrease tumor development (Yoon et al., 2000).

Lactobacillus species can be classified into three main groups based of their metabolic products;

1) Obligatory homofermentative species: also called as Thermobacterium. *Lactobacillus acidophilus, L. delbrueckii, L. helveticus, L. delbruckii subsp. bulgaricus, L. delbrueckii subsp. lactis* and *L. helveticus* are belong to this group (Todorov et al., 2010).
2) Obligatory heterofermentative species: also known as Betabacterium. The most important species in this group are *L. brevis, L. fermentum, L. buchneri, L. reuteri and L. kefir* (Kılıç, 2008).
3) Facultative heterofermentative species: also called as Streptobacterium. Some of these group members are *L. plantarum, L. casei* and *L. sakei* (Özgün, 2009).

1.2.2 *Lactobacillus plantarum*

Lactobacillus plantarum is a facultative heterofermentative lactic acid bacterium which grows anaerobically. They can live in diverse niches such as fermented product of meat and vegetables (Gardner et al., 2001) as well as in the gastrointestinal tract of humans and animals (Ahrne et al., 1998). This species have large genomes that encode unique intracellular and extracellular proteins (Boekhorst et al., 2004; Siezen et al., 2010). *L. plantarum* also has high intraspecies genomic conservation. 20% of genetic divergence found in their genome (Molenaar et al., 2005).

L. plantarum is potential probiotic organism. One of the criteria of being probiotic is having beneficial effect on host organisms. *L. plantarum* species produces antifungal (Dal Bello et al., 2007) and antimicrobial (Kaushik et al., 2009) compounds. Bile salt resistance is the other criteria of being probiotic. *L. plantarum* has a four bile salt hydrolase (*bsh*) related genes some of which are able to deconjugated bile salts (Lambert et al., 2008) and bile salt resistance.

1.3 Bile

Bile is yellow-green complex fluid that containing bile acids, biliverdin, cholesterol and phospholipids. It is secreted by liver and stored in the gallbladder. In gallbladder, bile is concentrated almost 10 fold. After food intake, it is excreted into the duodenum. This secretion is controlled by two hormones; secretin and cholecystokinin. Since bile has detergent like property, it helps solubilization of lipids and emulsifies them (Bagley at el., 2006). Bile also has potent antimicrobial activity (Bagley et al., 2005).

1.3.1 Bile Acids and Bile Salts

Bile acids are synthesized from cholesterol in vertebrates by several biosynthetic pathways. Natural pathway is one of the main biosynthetic pathway for human (Monte et al., 2009). Cholic acid (CA) and Chenodeoxycholic acid (CDCA) are mainly synthesized by natural pathway in liver. Bile acids were conjugated with either glycine or taurine. After conjugation, these new components called as bile salts or primary bile acids. The glycoconjugates to tauroconjugates ratio of bile acids are vary from person to person due to their diet. Primary bile acids are fully ionized and highly soluble as a result of conjugation. Since primary bile acids are amphipathic (Figure 1.3.a), they are able to accumulate around the small lipids drops to form micelles (Figure 1.3.b).

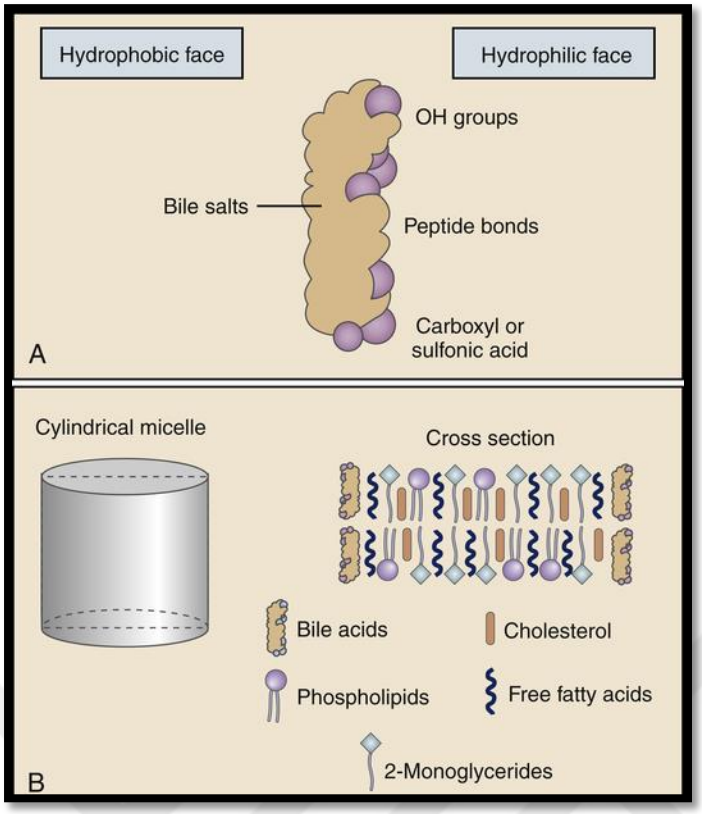


Figure 1.3. Structure of bile acid and micelle. A; amphipathic structure of bile acid. B; structure of a bile salt–lipid mixed micelle (Levy et al., 2006).

The primary bile acids are deconjugated during passage through caecum and colon by intestinal microbiota. Bile salt hydrolases (BSH) enzyme is produced by several intestinal bacteria. This enzyme is liberated glycine and taurine from primary bile acids (Bagley et al., 2004).

Most of conjugated and unconjugated bile salts are returned from intestine to liver by passive and active transport and then secreted again into the bile. This special circulation termed as enterohepatic circulation (Figure 1.4).

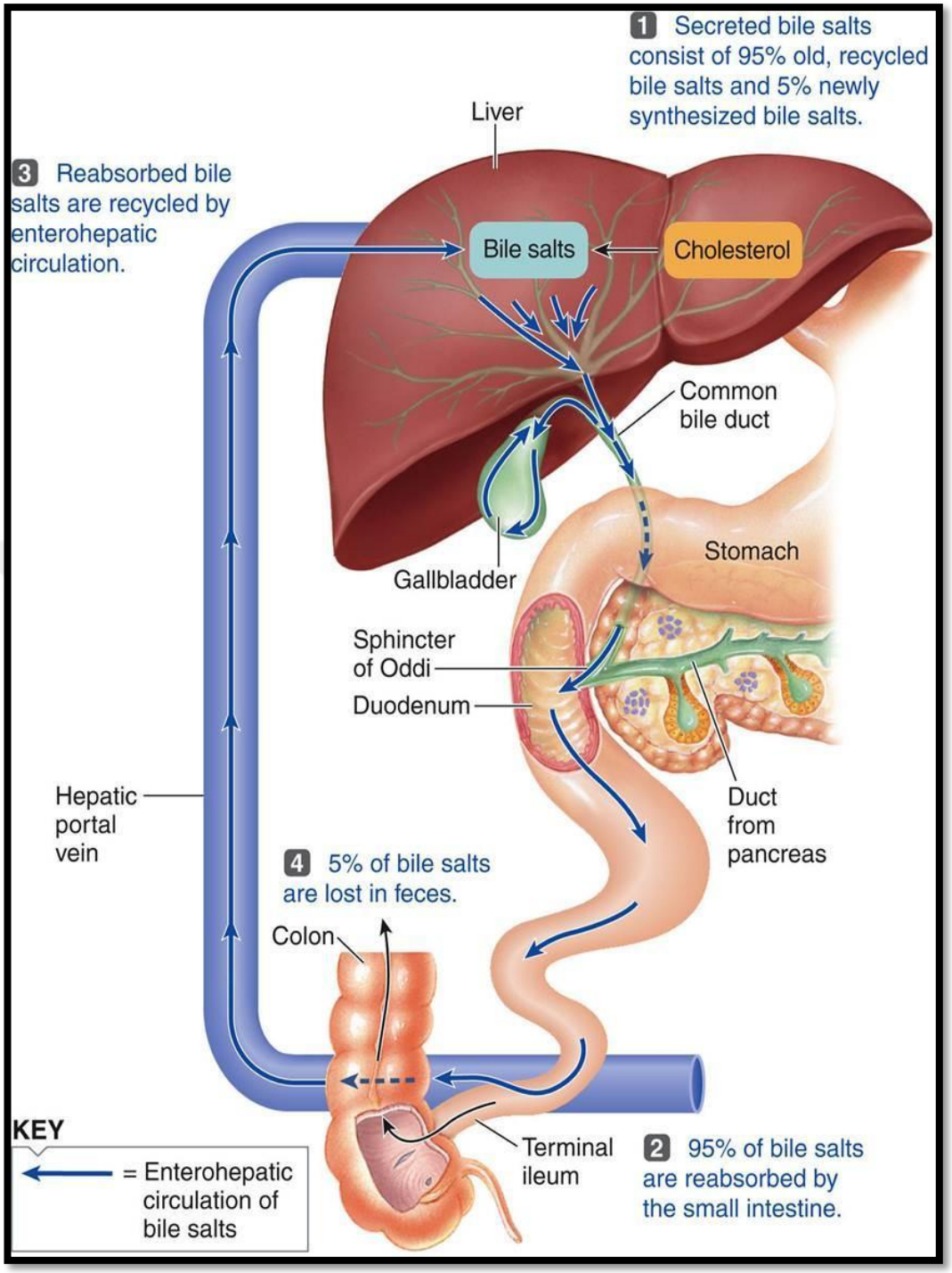


Figure 1.4. Enterohepatic circulation of bile acids (adapted from www.gestaltreality.com).

1.4 Bile Salt Hydrolase (BSH)

Bile salt hydrolase enzyme (EC 3.5.1.24) is belonging to N-terminal nucleophile (Ntn) hydrolase superfamily with penicillin amidase enzyme because of their common residue. Both of them have catalytic N-terminal cysteine residue (Tanaka et al., 2000). BSH are generally located intracellular side of the host. They have slightly acidic pH optima, usually between 5 and 6 and they are sensitive to oxygen.

BSH enzymes hydrolyze the N-acyl bond at C24 position of bile salts and deconjugate bile salts by way of liberating glycine and taurine. This reaction plays important role in host bile acids metabolism in the intestine (Bagley et al., 2006).

Bile salt hydrolase enzyme is produced mostly by gram positive gut bacteria such as *Bifidobacterium* (Jarocki and Targonski, 2013), *Clostridium* (Coleman and Hudson, 1995), *Enterococcus* (Wijaya et al., 2004) and *Lactobacillus* (De Smet et al., 1995). As gram negative bacteria, only Bacteroides genus members had been found (Stellwag and Hylemon, 1976).

Function of BSH enzyme is still examined but there are several hypotheses that have been advanced about it;

1) Source of nutrition

After deconjugation of bile acids by BSH enzyme, certain amino acids are liberated. Ammonia and carbon dioxide can be metabolized from glycine likewise ammonia and carbon dioxide with additional sulfite may be catabolized from taurine. These metabolic products can be used by other bacteria as source of nutrition (Begley et al., 2006). According to Huijghebaert and Eyssen (1982) some *Clostridium* species with BSH activity can be used taurine in Stickland fermentation. Furthermore, resent study on mouse model showed that fed a diet with taurocholic acid caused immediate increase in number of sulfidogenic bacterium (Devkota et al., 2012).

2) Alteration of membrane characteristic

BSHs make easier the cholesterol or bile incorporation into the bacterial membrane (Taranto et al., 2003). As a result of incorporation, tensile strength of the membrane can increase (Boggs, 1987). Besides fluidity or charge of membrane may change hence host immune defence molecules can be affected (Wilson et al., 1999).

3) Bile detoxification

BSH may have detoxification effect through its ability of deconjugation. However, scientist refuted this hypothesis. Moser and Savage (2001) did not find connection between bile salt hydrolase activity and resistance to toxicity in Lactobacilli. But Grill and coworkers' (2000) result showed that mutant *Lactobacillus amylovorus* displayed decreased growth rates in the present of bile salts.

1.5 Positive Effects of BSH on Host

1.5.1 Alternative to Antibiotic Growth Promoters

Antibiotic growth promoters (AGPs) are low- dose antibiotics which used as feed additives on animals. Wegener (2003) found that there is a connection between usage of APGs and emergence of antibiotic resistant bacteria. Antibiotic resistant bacteria can spread from food animals to human therefore treating of human with antibiotics may become ineffective. This is dangerous for public health (Wegener, 2003). Consequently, AGPs usage has been narrowed down all around the world (Turnidge, 2004; Dibner and Richards, 2005).

Usage of AGPs decreased in number of Lactobacillus species in chickens (Guban et al., 2006) in which lactobacillus are major BSH producing species (Begley et al., 2006). BSH enzyme regulates lipid metabolism on host and plays important role in weight gain. Some antibiotics, such as tetracycline and roxarsone, have direct inhibitory effect on BSH activity and these antibiotics have been used as AGPs. However some AGPs have low inhibitory effect on BSH for example bacitracin

(Smith et al., 2014). Therefore BSH is a good microbiome target for generating alternatives to AGPs for improving productivity of food animals (Lin, 2014).

1.5.2 Cholesterol Lowering

Cholesterol is a very important molecule which has several life-sustaining roles such as precursor for synthesis of bile acids and steroid hormones and also maintaining membrane structure (Ma, 2004). However, high blood cholesterol has been investigated as major risk factor for cardiovascular disease like coronary heart diseases (Aloğlu and Öner, 2006). Cholesterol levels were reduced by 22% to 33% through taking probiotics orally (De Smet et al., 1998; Pereira and Gibson, 2002). These reduction effects may be attributed to BSH activity (Liong and Shah, 2005). Many studies show that taking lactobacilli with BSH activity may reduce cholesterol level in humans (Jones et al., 2013), mice (Park et al., 2014; Miyoshi et al., 2014), rats (Kumar et al., 2011) and pigs (De Smet et al., 1998). Dong and his coworkers (2012) compared to sixteen lactobacillus strains and found that *Lactobacillus plantarum* BBE7 had highest BSH activity and also this strain had higher cholesterol-removing activity then other tested strains on MRS agar.

BSH deconjugated primary bile acids and produced secondary (deconjugated) bile acids which are less soluble and more difficult to reabsorb from intestine of hosts. Because of this property, large amount of deconjugated bile acids are lost by defecation. The new bile acids must be synthesized from cholesterol to homeostasis of bile pool. Therefore deconjugation of bile acids could help to reduction of cholesterol in serum (Begley et al., 2006).

1.6 Negative Effect of BSH on Host

1.6.1 BSH Relates with Colorectal Cancer

In the colon, uncontrolled cell growth cause colorectal cancer (CRC) (Sabel et al., 2013) which is the third most prevalent cancer type (Siegel et al., 2012). According to Platz et al. (2000), 50% to 90% of colon cancers are related with diet. Several studies show that omnivores have higher mortality rate of colorectal cancer than vegetarians (Phillips et al., 1980; Kinlen et al., 1983) and vegans (Reddy and Wynder, 1973). Other studies show that, the native African people whose diet is low in animal protein and fat has low colorectal cancer risk (< 1:100.000), on the contrary South African whites and African American have high colorectal cancer risk (17:100.000 and 65:100.00) (O'Keefe et al., 1999; Sharman and O'Keefe, 2007).

Diets high in fat like Western diet increase the production of bile acids in GIT as a result more secondary bile acids are synthesized by BSH producing bacteria. These secondary bile acids have carcinogenic properties (Bernstein et al., 2011). Jounhai and coworkers' experiment result (2013) showed that Americans had high secondary bile acids in their GIT than Africans. Therefore, Americans having a high fat in their diet have high CRC risk. Another study showed that vegan people had a lower enterococci and lactobacilli than western diet consumed people (Van Faassen et al., 1987). Lactobacilli are a major BSH producing bacterial stain. As a result, there should be connection between BSH enzyme and developing CRC.

1.6.2 BSH Relates with Cholesterol Gallstone

Patients with cholesterol gallstones (CGs) show much more secretion of cholesterol which is related with bile acids in bile (Nilsell et al., 1985). Therefore bile is supersaturated with cholesterol. This lets formation and growth of CGs (Paumgartner and Sauerbruch, 1991).

Intestinal bacteria with BSH activity can be formed deoxycholic acid (DCA) from cholic acid (CA) after 7α-dehydroxylation pathway (Ridhon et al, 2016). Excessive levels of DCA in bile have correlation with increased secretion of cholesterol in some CGs patients (Shoda et al., 1995). According to Berr et al.

(1992), excessive DCA in bile acid pool occurs in 20%-30% of patients with CGs. CA is quickly 7α-dehydroxylated to DCA in these patients' intestine. Then, more than 70% of these DCA absorbed and flows into the bile pool. But in healthy humans only about 30% of DCA is absorbed (Berr et al., 1992). Herewith excessive production of DCA by intestinal microflora can be a factor for cholesterol gallstones formation or growth (Berr et al., 1996).

1.6.3 BSH Relates with Bowel Diseases

Connections between BSH and bowel diseases are not clear but there are many studies that have been trying to understand these connections. For example Fereira-Pereira and coworkers (2014) published well-rounded study in which bile acids from colic content, portal serum and gallbladder of control animals and animals with short bowel syndrome (SBS) were compared and found that SBS animals have generally lower secondary bile acids and higher primary bile acids than control animals. Some other studies with bowel diseases came up with similar results (Shin et al., 2013; Duboc et al., 2013). Fecal primary bile salts were higher than control group in patients with diarrhea-predominant irritable bowel syndrome (IBS-D). What is more, fecal secondary bile acids were lower than control group in patients with constipation-predominant irritable bowel syndrome (IBS-C). Only one of secondary bile acid (Lithocholic acid) was higher in IBS-C patients (Shin et al., 2013). Duboc and coworkers (2013) also got similar results by working with inflammatory bowel diseases. Fecal primary bile acids were higher in patients with inflammatory bowel diseases, whereas, secondary bile acids were lower. Moreover their results showed that amount of total gut bacteria were decreased in patients. The main decreased species was members of the Firmicutes which is one of the deconjugating bacteria. All these results show that decreasing the amount of BSH producing bacteria might cause increasing primary bile acids excretion in feces.

2. AIM AND SCOPE OF THE STUDY

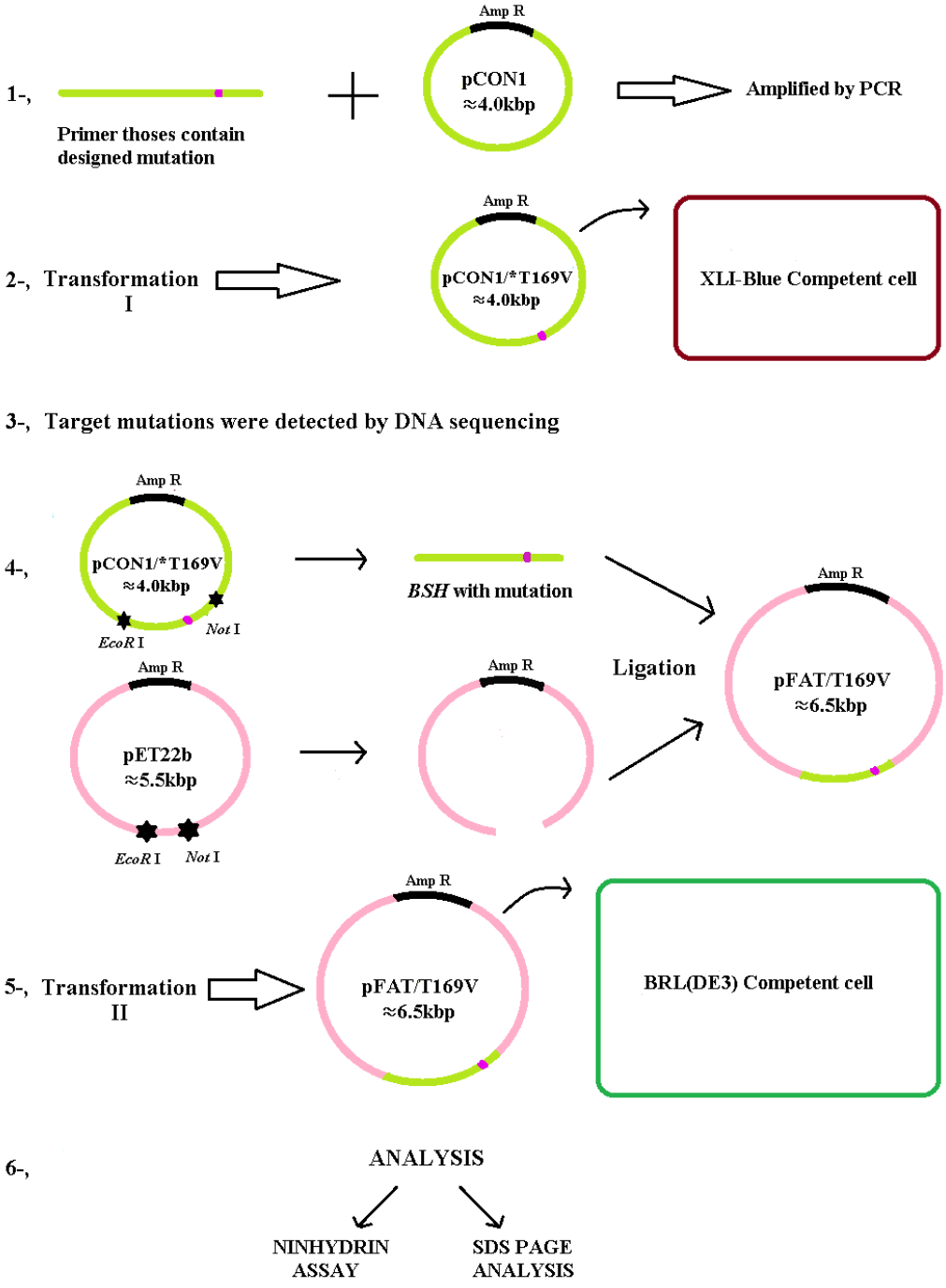
Taurine or glycine conjugated form of bile salts (BSs) are required for digestion of lipids in mammals (Begley et al. 2004). The primary bile acids (BAs) in human, mostly cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from hepatic cholesterol followed by conjugation with either taurine or glycine (Begley et al. 2004) and stored in the gall bladder. The BSs, secreted into the gastro intestinal track (GIT) of human, can be metabolized into free bile acids and glycine or taurine amino acids by bile salt hydrolases (BSHs) mainly derived from species of *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus* and *Listeria* in the GIT. Although almost 95% of the secreted primary BSs is recycled via the enterohepatic circulation (McAuliffe et al 2005), 5% of BSs escape to the colon because of their deconjugation by microbial BSHs. Thus, the gastrointestinal bacteria carrying active BSH could reduce serum cholesterol levels by accelerating cholesterol metabolism (Huang et al., 2013; Chand et al., 2017). On the other hand, the functional bile salt hydrolase enzyme allows the bacteria to colonize in human intestine in which at least six different bile salts, toxic for most of the microorganisms, were secreted. Therefore, the expression of functional bile salt hydrolase ability is one of the criteria for the selection of the candidate probiotic strains.

To date, the crystal structure of BSHs from four species, *Clostridium perfringens* (Rossocha et al., 2005), *Bifidobacterium longum* (Kumar et al., 2006), *Lactobacillus salivarus* (Xu et al., 2016) and *Enterococcus faecalis* (Chand et al., 2018) have been reported. Structural analyses of BSHs members from these species led to the discovery of the, critical residues in catalysis. Amino acid sequences of BSHs resemble those of penicillin V acylase (PVA). BSH and PVA family members are belong to the N-terminal nucleophile (Ntn) hydrolase superfamily that contain N-terminal nucleophile amino acid acting as the catalytic residue (Oinonen and Rouvinen, 2000). Although the chemical structure of substrates of BSH and PVAs is quite different, homology analysis of both groups indicated that the catalytic residues of them appear to be highly conserved. Five out of the six catalytic residues, C2, R16, D19, N79, N170 and R223 (*Lb. plantarum* numbering), are identical in

members of the Ntn superfamily. However, the catalytic residues at the position 169 and 172 of BSH and of PVA are mostly T and P respectively. Previous in silico structural studies and comparative proteomics revealed that there might be a relationship between these two conserved amino acids, T169 and P172, and catalytic activity of BSH, there is no experimental investigation supporting the relationship between these residues and catalytic activity of BSH. Therefore, the function of the partially conserved T169 and P172 amino acids of BSH enzyme is not fully known.

Structural analyses of BSHs and PVAs led to the discovery of the critical residues in catalysis. Site-directed mutagenesis of targeted amino acids can also provide the key information on the catalytic activity of BSHs. Studies of the site directed mutagenesis on N169 and P172 of BSH and analysis of catalytic activity of mutant BSHs from *Lb. plantarum* B14 may facilitate the identification of desired probiotics. Because of the strong implications between deconjugated bile salts and positive or negative health consequences, characterization of the BSH enzymes is required. Since there is little to no information on the mechanism of the catalytic activity of the BSH, N169 and P172 amino acids are good candidate for achievements and functional studies of the BSH. It is the aim of this study to substitute the conserved polar N169 and P172 of the BSH from *Lactobacillus plantarum* B14 strain for the hydrophobic V169 and H172 by PCR-based site directed mutagenesis and to analyze mutant enzymes in respect to their catalytic activity and assembly.

Summary of the study



PS: This figure represents only one mutation.

3. MATERIALS AND METHODS

3.1 In Silico Analysis of BSH

To find out conserved amino acids of *bsh*, many *bsh* genes from different strains were aligned with T-Coffe Expresso program (http://tcoffee.crg.cat/apps/tcoffee/do:expresso) (Figure 4.1). Some penicillin v acylase (*pva*) genes were also added to check, if conserved amino acids are same with *bsh*. These same organisms were used in alignment and phylogenic tree making. The organisms and their accession numbers were given on following table (Table 3.1).

3-D construction of *bsh* from *Lactobacillus plantarum* B14 was viewed and desired amino acids were labelled on image by pMoL 0.99rc6 program (Figure 4.4). On the same image, desired mutations were made and showed hypothetically (Figure 4.5).

Table 3.1. Organisms which used in alignment

Organisms with *bsh*	**Accession numbers**
Lactobacillus acidophilus	ACL98176.1
Lactobacillus crispatus	EST03469.1
Lactobacillus johnsonii	EGP12391.1
Lactobacillus reuteri	ACH81023.1
Lactobacillus plantarum	KY080706.1
Lactobacillus salivarus	ACL98203.1
Bifidobacterium bifidum	AAT11513.1
Bifidobacterium longum	AAF67801.1
Clostridium perfingens	EDS79409.1
Enterococcus feacalis	PDB: 4WL3_B
Lactobacillus gasseri	ABJ60345.1
Lysinibacillus sphaericus	P12256.1
Bacillus subtilis	Q2HPP6

3.2 Construction of pCON1/*T169V Mutant

3.2.1 Site Directed Mutagenesis of Threonine 169 by PCR

First, forward and reverse primers shown in Table 3.2 were designed to make T169V mutation. The codon 169 of *bsh* gene, threonine, was substitiuted for the valine aminoacid by PCR based site direct mutagenesis kit (stratagene) and Techne TC-3000 PCR. pCON1 (*bsh*/pBluescript) was used as a template DNA during the PCR reaction. The pCON1 DNAs from the clones were isolated by GeneJET^{TM} Plasmid Miniprep Kit (#K0503 Thermo Scientific, Europe) and the clone containing right oriented insert DNA was detected by digestion of the isolated DNA with *Xba*I restriction enzyme (RE).

The PCR reaction was proceeded in the 50 µl total volume which contains 25 µM forward and reverse primers, 25 mM $MgCI_2$, 5 mM dNTP and 2.5 u/µl Pfu DNA polymerase enzyme (Fermentas, Europe) (Table 3.3). The *Pfu* DNA polymerase was preferred to reduce unwanted mutations because of its proofreading activity. After PCR cycling (Table 3.4), 40 µl PCR sample was digested with *Dpn*I RE (Fermantas) at 37 °C for 2 hours. The PCR product was loaded on 0.8% agarose gel and separated by electrophoresis at 75 volt for 1 hour.

Table 3.2. Designed primers used for valine mutation

Primer Name	**Sequence**	**Tm**
T169V-F	5'-CAGTAGGTGTGTTA**GTG**AACAATCCTAATTTTG-3'	60 ºC
T169V-R	5'-CAAAATTAGGATTGTT**CAC**TAACACACCTACTG-3'	60 ºC

Table 3.3. PCR conditions

Reactants	Quantities
Template DNA (135 ng/µl)	0.75 µl
$MgCI_2$ (25 mM)	4 µl
dNTP mix (5 mM)	2 µl
Primer Forward (25 µM)	2 µl
Primer Reverse (25 µM)	2 µl
Buffer (10X *Pfu* polymerase buffer)	5 µl
Pfu polymerase (2.5 u/µl)	1 µl
ddH_2O	33.25 µl
Total reaction volume	50 µl

Table 3.4. PCR cycle conditions

Cycle	Temperature	Time
Initial denaturation	95 ºC	30 sec
Denaturation	95 ºC	1 min
Annealing	55 ºC	1 min
Extension	72 ºC	9 min
Final extension	72 ºC	5 min
Number of cycles	30 ºC	

3.2.2 Purification of PCR Products

The PCR product of pCON1/*T169V was loaded and separated on 1% agarose gel (Sigma, USA) by electrophoreses at 75 volt for 1 hour and then it was purified by Gel DNA Recovery Kit (#GF-GP-050 Vivantis, Europe) according to its protocol.

3.2.3 Preparation of BLR(DE3) and XLI-Blue Competent Cell

BLR(DE3) and XLI-Blue cells were incubated at 175 rpm for 14-16 hours in 3 ml Luria-Broth (LB) medium and then 1% of this culture was inoculated into 50 ml

LB medium and incubated at 37 ºC until the optical density at 600 nm (O.D600) reached 0.04-0.06. This culture was separated into 20 ml each in the sterile falcon tubes. Samples were centrifuged at 4 ºC and 4000 rpm for 5 minutes. The pellets were resuspended in 10 ml $CaCI_2$ (100 mM, pH: 7.0) and centrifuged again at 4 ºC and 4000 rpm for 10 minutes. The obtained pellets were resuspended in 1 ml $CaCI_2$ containing 10% glycerol and then allocated in 100 µl cultures in the Eppendorf tubes and stored in -80 ºC.

3.2.4 Transformation of pCON1/*T169V DNAs into *E. coli* XLI-Blue Strain

Gel purified PCR amplicons were loaded on 0.8% agarose gel and run at 75 V for 1 hour. By the help of DNA kb ladder view, concentration of PCR amplicons were detected and relevant PCR amplicons was calculated and mixed with 100 µl XLI-Blue competent cell for ligation reaction. The mixtures were incubated on ice for 45 minutes. Right after mixtures were incubated at 37 ºC for 5 minutes, they were incubated on ice for 5 minutes again. Ten µl of this culture was transformed into 1 ml of LB (Luria-Broth) and grown in shaker at 37 ºC for 1 hour. Then samples were harvested by centrifugation at 6000 rpm for 3 minutes. Pellets were kept and dissolved with 100 µl LB (Luria-Broth). These samples were spread on LB Agar plate containing ampicillin (25 mg/ml) and incubated at 37 ºC for overnight. Four different colonies from the pellets were picked randomly and transferred into 5 ml LB containing ampicillin (25 mg/ml) and grown at 175 rpm and 37 ºC in shaker for overnight. The Plasmid DNAs obtained from colonies were isolated by Plazmid Miniprep Kit (Thermo Scientific) for the sequencing of *bsh* gene to detect the requested T169V mutation.

3.2.5 Sequencing of T169V *bsh* Gene of *Lactobacillus plantarum*

The DNA picked from obtained clones was sequenced by Biomers (Germany) to detect the requested mutation (XLI-Blue/ pCON1/*T169V). During sequencing, Universal primers, M13/pUC forward and M13/pUC reverse, were used. Nucleotide sequence and amino acid sequence of pCON1/*T169V were aligned with wild-type *bsh* nucleotides and amino acid residues by SIB (Swiss Institute of

Bioinformatics) ExPASy sequence translate tool to detect the requested T169V mutation.

3.3 Construction of pFAT/T169V Mutant

3.3.1 Digestion of pCON1/*T169V Mutant and pET22b Vector via the Restriction Endonucleases Enzyme

pCON1/*T169V clones which containing requested mutation on pET22b vector were cut with *Eco*RI (Thermo Scientific) and *Not*I (Thermo Scientific) at 37 ºC for at least 30 min (Figure 3.1 and 3.2). The condition of this digestion was given in Tables 3.5 and 3.6. These samples were loaded on 1% of agarose gel and separated by electrophoresis at 75 volt for 1 hour. The mutant *bsh* part of the pCON1/*T169V DNA and needed part of pET22b fragment were sliced from agarose gel and purified by Gel DNA Recovery Kit (#GF-GP-050 Vivantis, Europe) according to its protocol.

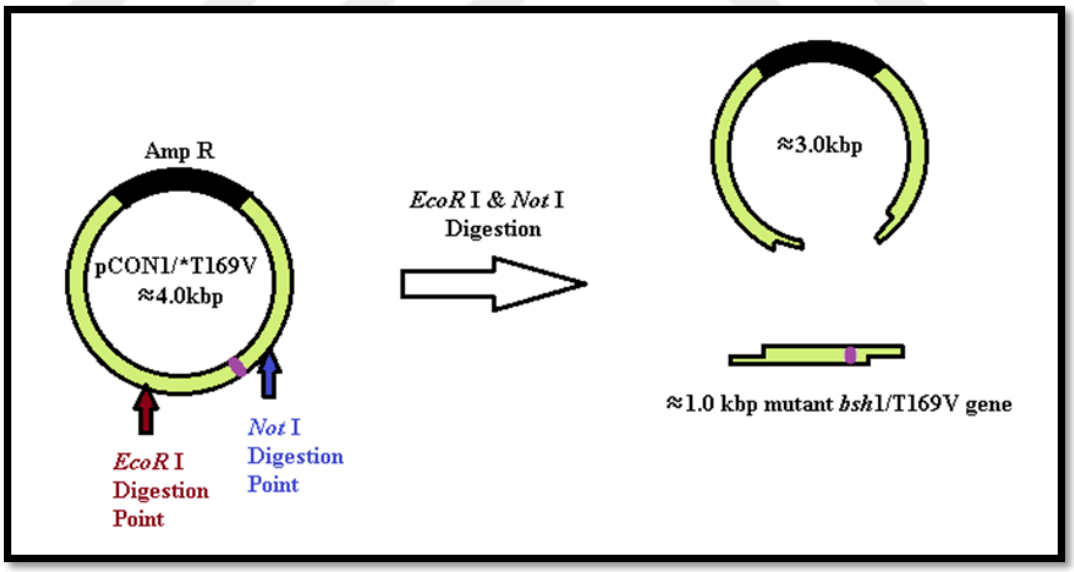


Figure 3.1. Digestion of pCON1/*T169V with *Eco*RI and *Not*I Res

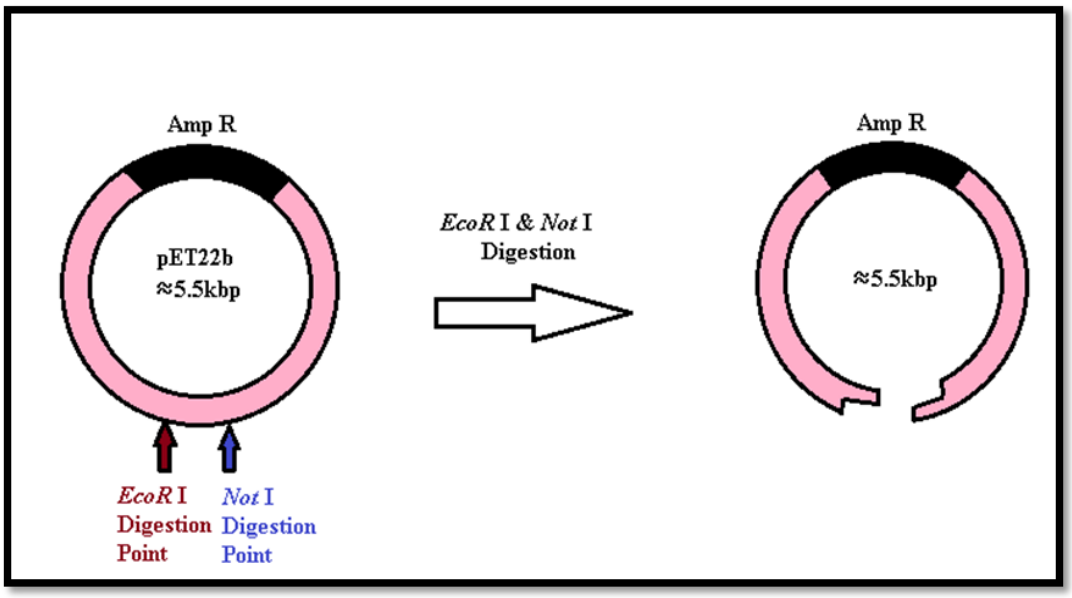


Figure 3.2. Digestion of pETT22b with *Eco*RI and *Not*I REs

Table 3.5. Digestion conditions of pCON1/*T169V

Substances	Reaction volumes
pCON1/*T169V(mutant)	40 µl
Fast Digest Buffer	7 µl
*Not*I Enzyme	2,5 µl
*Eco*RI Enzyme	2,5 µl
ddH_2O	18 µl
Total Volume	70 µl

Table 3.6. Digestion conditions of pET22b

Substances	Reaction volumes
pET22b (vector)	30 µl
Fast Digest Buffer	6 µl
*Not*I Enzyme	2 µl
*Eco*RI Enzyme	2 µl
ddH_2O	20 µl
Total Volume	60 µl

3.3.2 Ligation of Mutant *bsh*/T169V DNA with pET22b Vector

Purified mutant *bsh*/T169V DNA fragments were ligated into *Eco*RI/*Not*I site of pET22b by T4 DNA ligase enzyme (Thermo Scientific) at 22 ºC for 1 hour (Figure 3.3). The conditions of this digestion were given in Table 3.7. The vector-insert ratio was taken as 4/1 in ligation reaction. The amount of DNAs was calculated by using following equation.

Insert mass ng= ratio X (insert length in kb) / (vector length in kb) X vector mass in ng

Table 3.7. Ligation reaction conditions of *bsh*/T169V and pET22b

Substances	Reaction volumes
DNA (insert)	7.30 µl
pET22b (*Eco*RI & *Not*I)	6.25 µl
Buffer (T4 ligase 10X)	2.00 µl
T4 DNA ligase	1.00 µl
ddH_2O	3.45 µl
Total volume	20.00 µl

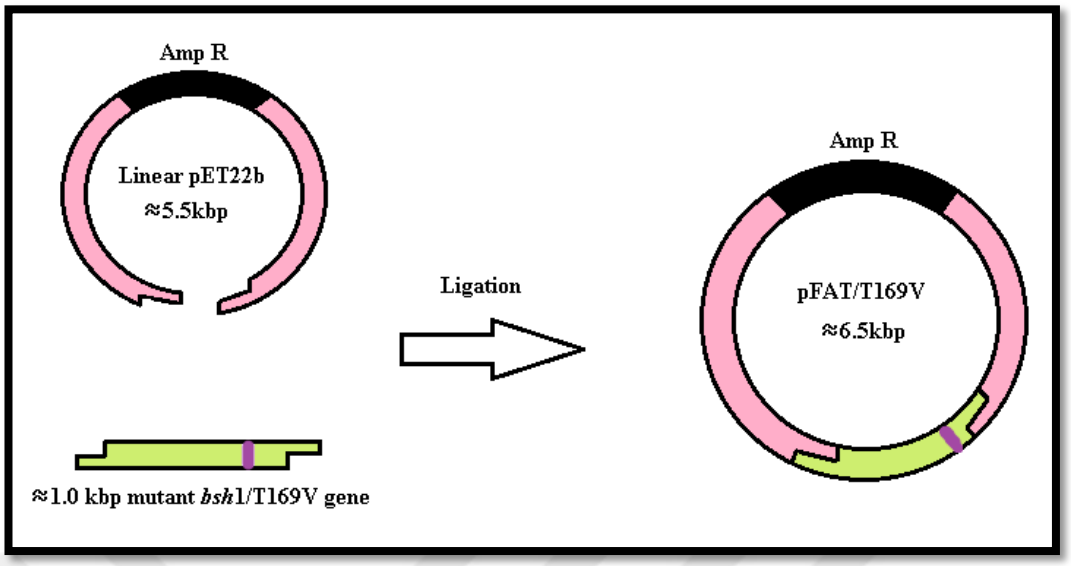


Figure 3.3. Ligation of pET22b vector and mutant *bsh*1/T169V gene

3.3.3 Transformation of pCON1/*T169V DNA into *E.coli* BLR(DE3)

10 µl ligation sample was mixed with 100 µl BLR(DE3) competent cell. This mixture was incubated on ice for 45 minutes and then mixture was incubated at 37 ºC for 5 minutes. Right after mixture was incubated on ice for 5 minutes, 1 ml of LB was added into this mixture and grown at 37 ºC and 175 rpm in shaker. Grown samples were harvested by centrifugation at 6000 rpm for 3 minutes. Obtained pellets were dissolved in 100 µl LB. These samples were spread on LB Agar plate which contains ampicillin (25ng/ml) and then incubated at 37 ºC for overnight. Four different colonies were picked randomly from these plates and colonies were transferred into 10 ml LB medium containing ampicillin. Samples were grown at 175 rpm and 37 ºC in shaker and then isolated by Plazmid Miniprerp Kit (Thermo Scientific) according to its protocols. The new construct containing the requested mutation was called pFAT/T169V.

3.4 Construction of pCON1/*P172H Mutant

The 516th codon of *bsh* gene that is coding proline amino acid was substituted for the codon coding histidine amino acid by site direct mutagenesis (stratagene) protocol and Techne (TC-3000) device at the conditions shown in Table 3.9 and

Table 3.10 respectively. After PCR reaction, 40 µl of sample was digested with *Dpn*I (Fermantas) at 37 ºC for 2 hours. Rest of the sample was kept for analysis on agarose gel.

Table 3.8. Designed primers used for proline mutation

Primer Name	**Sequence**	**Tm**
P172H-F	5'-GTTAACAAACAAT**CAT**AATTTTGACTACC-3'	53 ºC
P172H-R	5'-GGTAGTCAAAATT**ATG**ATTGTTTGTTAAC-3'	53 ºC

Table 3.9. PCR conditions

Reactants	**Reaction volumes**
Template DNA (135 ng/µl)	0.75 µl
$MgCI_2$ (25 mM)	4 µl
dNTP mix (5 mM)	2 µl
Primer Forward (25 µM)	2 µl
Primer Reverse (25 µM)	2 µl
Buffer (10X *Pfu* polymerase buffer)	5 µl
Pfu polymerase (2.5 u/µl)	1 µl
ddH_2O	33.25 µl
Total reaction volume	50 µl

Table 3.10. PCR cycle conditions

Cycle	**Temperature**	**Time**
Initial denaturation	95 ºC	30 sec
Denaturation	95 ºC	1 min
Annealing	48 ºC	1 min
Extension	72 ºC	9 min
Final extension	72 ºC	5 min
Number of cycles	30	

PCR product of pCON1/*P172H was loaded and separated on 1% agarose gel (Sigma, USA) by electrophoreses at 75 volt for 1 hour. Right after sample was purified by using GF-1 Gel DNA Recovery Kit (#GF-GP-050 Vivantis, Europe) according to company's instructions.

Gel purified PCR amplicons were mixed with 100 µl XLI-Blue competent cell. These mixtures were incubated on ice for 45 minutes and then 5 minutes at 37 ºC. After that mixtures were incubated on ice for 5 minutes. 1 ml of LB (Luria-Broth) was added to mixtures and grown at 37 ºC in shaker for 1 hour. Then samples were centrifuged at 6000 rpm for 3 minutes. Pellets were dissolved with 100 µl LB (Luria-Broth). These samples were separated on LB agar plate containing ampicillin (25mg/ml) and then incubated at 37 ºC for 14-16 hours. Four different colonies were chosen randomly on LB agar plate and transferred into 5 ml LB containing ampicillin. After this sample was grown at 175 rpm and 37 ºC in shaker for 14-16 hours, DNAs were isolated from grown colonies by Plazmid Miniprep Kit (Thermo Scientific).

Mutant *bsh* gene part of the pCON1/H172 DNA was sequenced using universal primers, M13/pUC forward and M13/pUC reverse, by Biomers Company (Germany). Obtained nucleotide sequence of *bsh*/H172 was aligned with that of wild-type *bsh* gene by SIB (Swiss Institute of Bioinformatics) ExPASy sequence translate tool.

3.5 Construction of pFAT/P172H Mutant

pCON1/*P172H clones which have requested mutation and related vector (pET22b) were digested with *Eco*RI and *Not*I restriction enzymes (Figure 3.4 and 3.5) at the same conditions used for first mutation pCON1/*T169V (quod vide page 22).

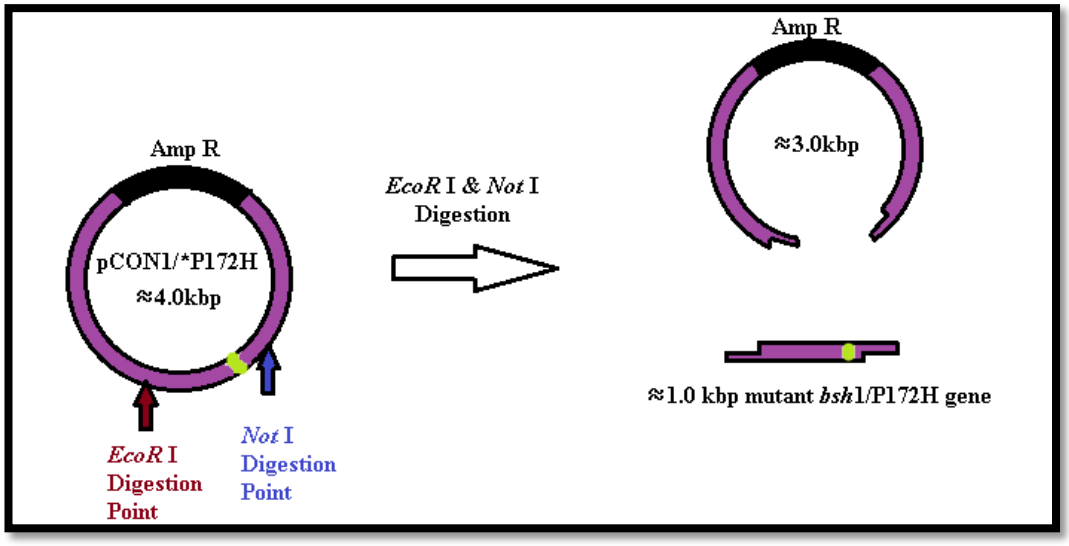


Figure 3.4. Digestion of pCON1/*P172H with *Eco*RI/*Not*I Res

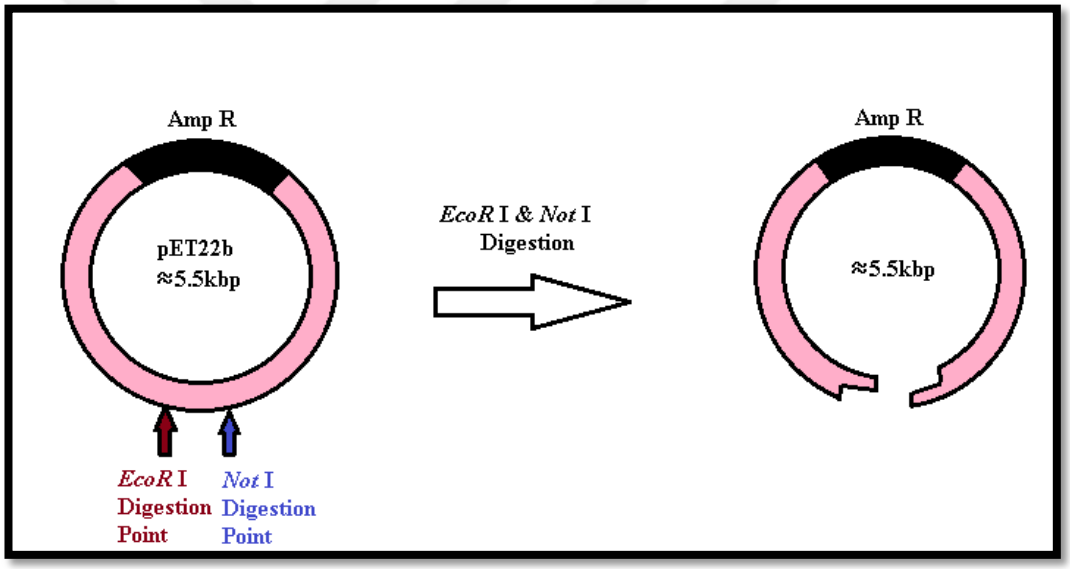


Figure 3.5. Digestion of pETT22b with *Eco*RI/*Not*I Res

After separation of *Eco*RI/*Not*I digested sample by electrophorese, mutant *bsh*/V172 DNA fragment and linearized vector pET22b were purified from agarose gel and then ligated at 22 ºC for 1 hour (Figure 3.6). Ligation conditions were shown in following Table (Table 3.11)

Table 3.11. Ligation reaction conditions of *bsh*/P172H and pET22b

Substances	Reaction volumes
DNA (insert)	3.70 µl
pET22b (*Eco*RI & *Not*I)	6.25 µl
Buffer (T4 ligase 10X)	2.00 µl
T4 DNA ligase	1.00 µl
ddH_2O	7.05 µl
Total volume	20.00 µl

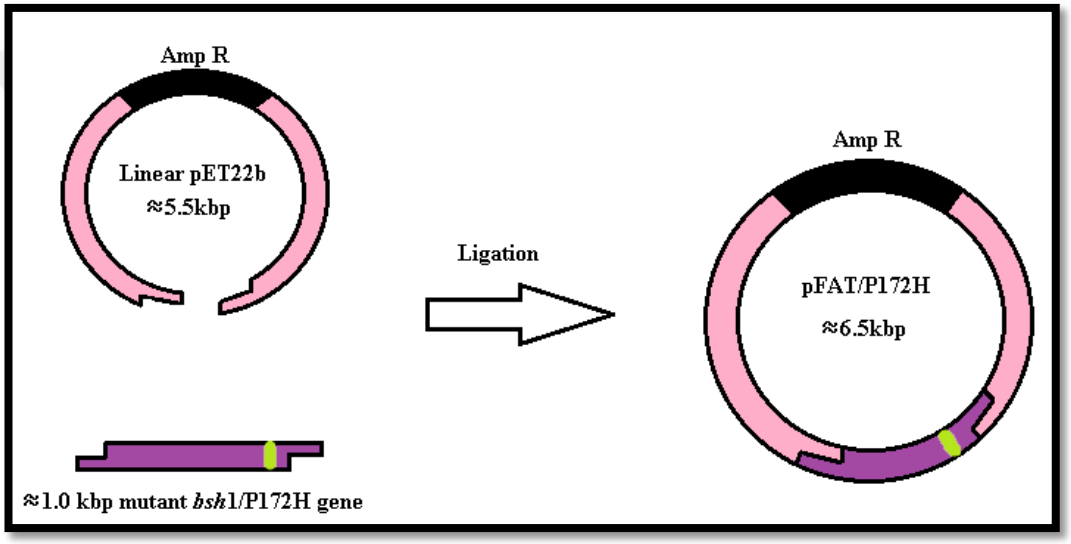


Figure 3.6. Ligation of pET22b vector and mutant *bsh*1/P172H gene

Ten µl of ligation product was transformed into *Escherichia coli* BLR(DE3) competent cells. This mixture was separated on LB agar plate containing ampicillin and incubated at 37 ºC for overnight. Four colonies were picked and isolated from this plate. The clone containing requested mutation was called pFAT/P172H.

3.6 Determination of Mutant BSH Enzyme Activities by Ninhydrin Assay

3.6.1 Preparation of Cell Extract

10 ml LB containing mutant *bsh* gene in BRL(DE3) strain of *Escherichia coli* liquid cultures were started with transformants which had desired wild-type and mutants *bsh* genes and then they were incubated overnight at 37 ºC and 175 rpm. Five ml of overnight cultures were added in 250 ml LB medium containing amphicillin and 4% glycerol and then they were grown at 37 ºC and 175 rmp until the OD_{600} values of cultures reached 0.45-0.6. In this stage, *bsh* gene expression was induced with 0.3 mM of Isoproplyβ-D-1-thiogalactopyranoside (IPTG) and re-incubated at 37 ºC and 175 rpm until the OD_{600} values of cultures reached 1.5-3.0. Cultures were alaquated into five 50 ml falcon tubes. To collect cell pellects, these tubes were centrifuged at 4 ºC and 8000g for 15 minutes. After supernatants were removed, 4 of the cell pellets were kept at -80 ºC freezer, only one tube was resuspended with 2 ml of binding buffer at pH:7.8. Then, lysozyme (1 mg/ml) was added to resuspenden solution and the mixture was incubated on a rocky platform for 10 minutes at 4 ºC. After the incubation, Tween20 (1%), DNase (5 µl/ml) and RNase (5 µl/ml) were added into the tube and re-incubated on the rocking platform for 10 minutes at 4 ºC. The insoluble debris was removed by centrifugation at 13520g and 4 ºC for 20 minutes. The supernatant was collected with a 0.45 µl filter into a new tube. The protein concentration of the cell extract was determined by the Lowry method. Bovine Serum Albumin (BSA) was used as a standard protein.

3.6.2 Ninhydrin Assay

BSH enzyme activity was determined by Ninhydrin assay. In this assay, 10 µl of the cell extract, 100 µl of 1.0 M sodium phoshate buffer, 50 µl of 40 mM Dithiotheitol (DTT) and 50 µl of 40 mM of one of the six different bile salts (glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA)) were mixed for each reaction and incubated

at 37 ºC for 30 minutes. Then 210 µl of Trichloroacetic acid (TCA) was added (for a ratio 1:1) and the tube was centrifuged at 14000 rpm for 15 minutes. Twenty µl of supernatant was mixed with 80 µl of ultra pure water and 1.9 ml of ninhydrin reagent in the new tube and boiled for 15 minutes. Finally, absorbance of samples were measured at OD_{570} with glycine as a standard. The BSH activity was expressed as 1 µmol of aminoacids liberated from the substrate per minute per mg of BSH.

4. RESULTS AND DISCUSSIONS

4.1 RESULTS

4.1.1 In Silico Analysis

Eleven different amino acid residues of BSH enzyme and three different amino acid residues of PVA enzyme were obtained from NCBI GenBank and aligned with that of *Lactobacillus plantarum* B14 stain by T-Coffe Expresso program. The selected organisms containing BSH enzyme are; *Lactobacillus acidophilus* (AN: ACL98176.1), *Lactobacillus crispatus* (AN: EST03469.1), *Lactobacillus johnsonii* (AN: EGP12391.1), *Lactobacillus reuteri* (AN: ACH81023.1), *Lactobacillus plantarum* (AN: KY080706.1), *Lactobacillus salivarus* (AN: ACL98203.1), *Bifidobacterium bifidum* (AN: AAT11513.1), *Bifidobacterium longum*, (AN: AAF67801.1), *Clostridium perfingens* (AN: EDS79409.1), *Enterococcus feacalis* (AN: 4WL3_B). Organisms which contain PVA enzyme; *Lactobacillus gasseri*, GenBank: ABJ60345.1; *Lysinibacillus sphaericus*, GenBank: P12256.1; *Bacillus subtilis*, GenBank: Q2HPP6)(Figure 4.1). On this alignment figure, totally conserved amino acids, Cys-2, Asp-19, Asn-79, Arg-223 and our target amino acids, Thr-169 and Pro-172 were labeled with black arrow and green boxes respectively. Phylogenic tree was also generated using same data by the help of MAB (Methodes et algorithmes pour la bio informatique available at phylogeny.fr) (Figure 4.2). To understand our conserved amino acids location, *bsh* DNA sequence chain view of *Enterococcus feacalis* was obtained from RCSP Protein Data Bank (https://www.rcsb.org/) (Figure 4.3). Because of Cys-2 is totally conserved and well-studied, Cys-2, Thr-169 and Pro-172 are labelled on 3-D construction of wide BSH enzyme (Figure 4.4). Then Thr169 and Pro172 of amino acids were substituted to Val169 and His172. These new amino acids also labelled on the 3-D image by pMoL 0.99rc6 program (Figure 4.6). 3-D images showed that Thr-169/Val-169 and Pro-173/His-172 amino acids were very close to Cys-2.

Figure 4.1. Multiple sequence alignment of BSH enzyme by T-Coffe Expresso program (http://tcoffee.crg.cat/apps/tcoffee/do:expresso). One of the fully conserved amino acids Cys-2, Asp-19, Asn-79, Asn170 and Arg-223 are labelled with black arrow and conserved amino acids Thr169 and His172 are showing in purple and green boxes respectively.

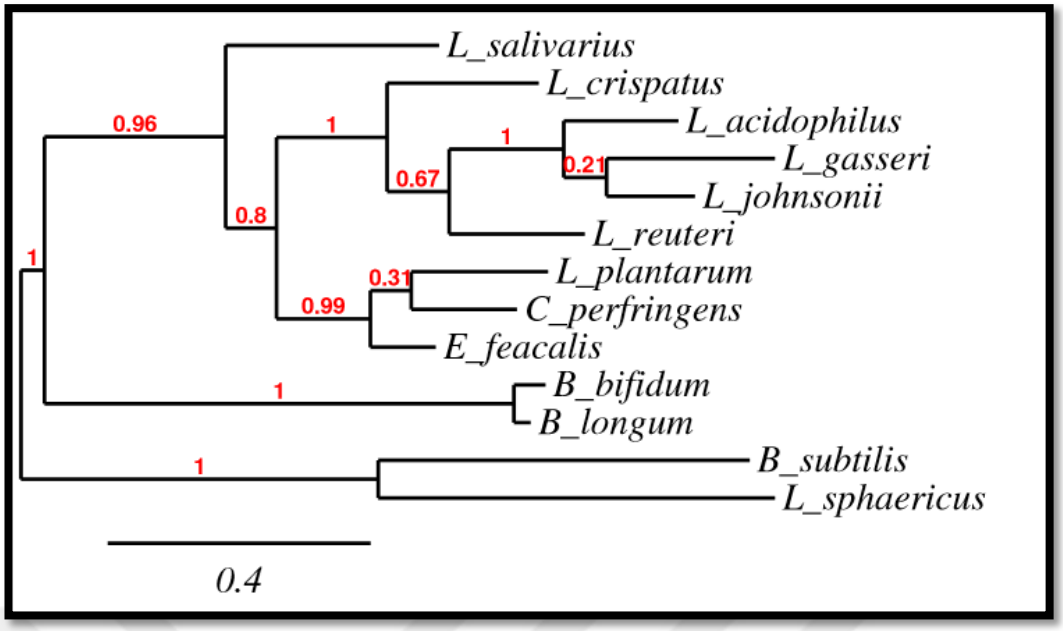


Figure 4.2. Phylogenetic tree of BSH enzyme from different species generated by MAB: Methodes et algorithmes pour la bio informatique with "One Click" Mode available at phylogeny.fr.

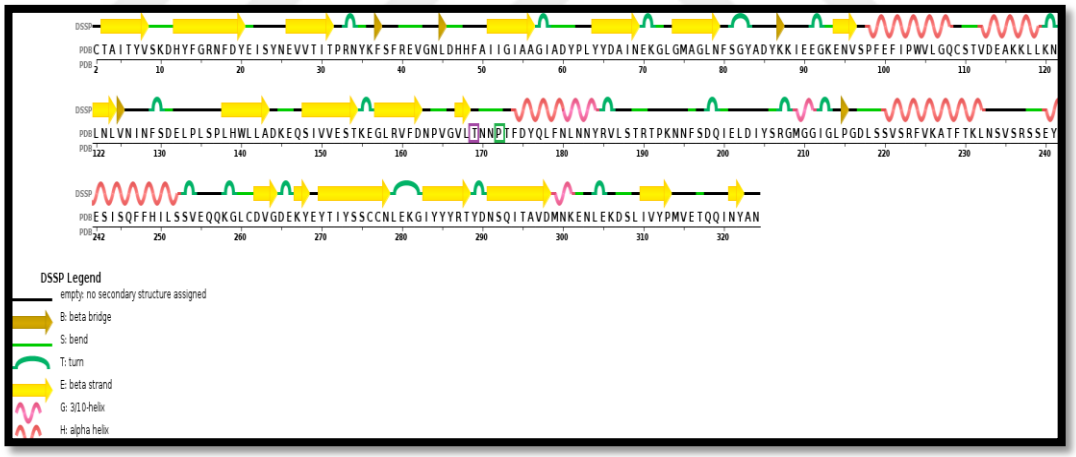


Figure 4.3. *Bsh* sequence chain view of *Enterococcus faecalis* obtained from RCSP Protein Data Bank (https://www.rcsb.org/).

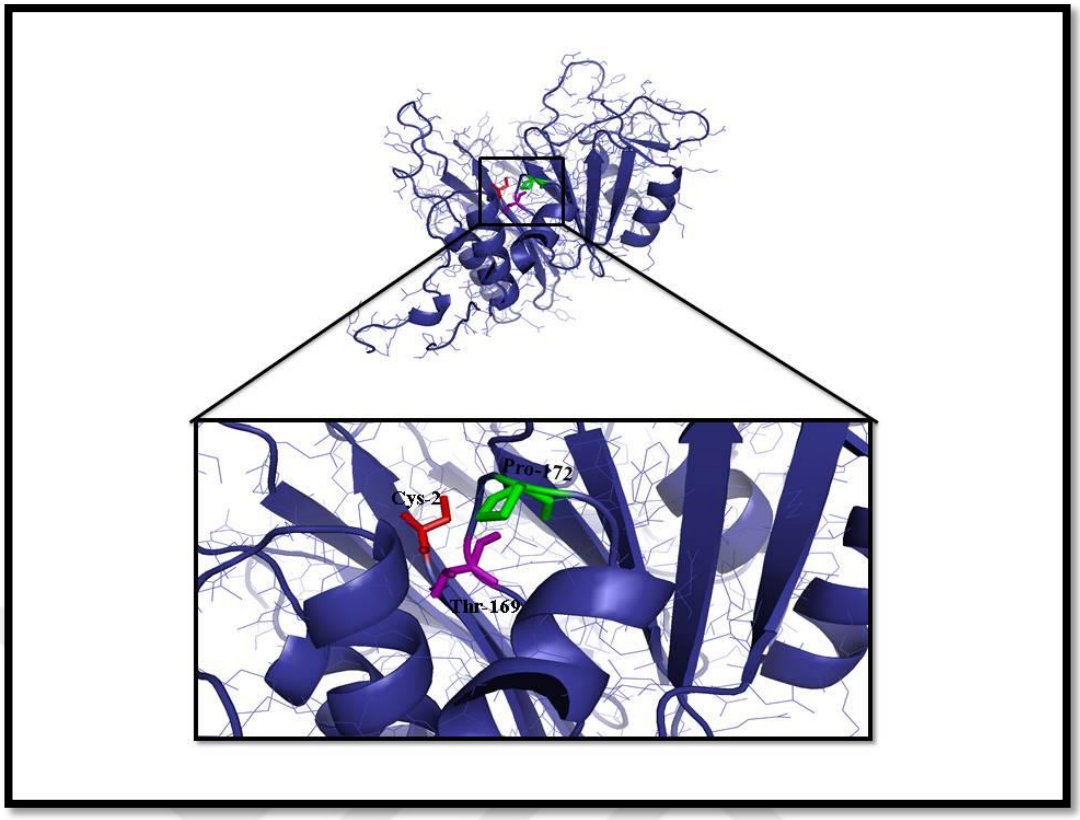


Figure 4.4. 3-D construction of part of BSH enzyme from wide *Lactobacillus plantarum* by pMoL 0.99rc6 program. Fully conserved amino acid Cys-2 is showing with orange and conserved amino acids Thr-169 and Pro-172 are showing with purple and green respectively.

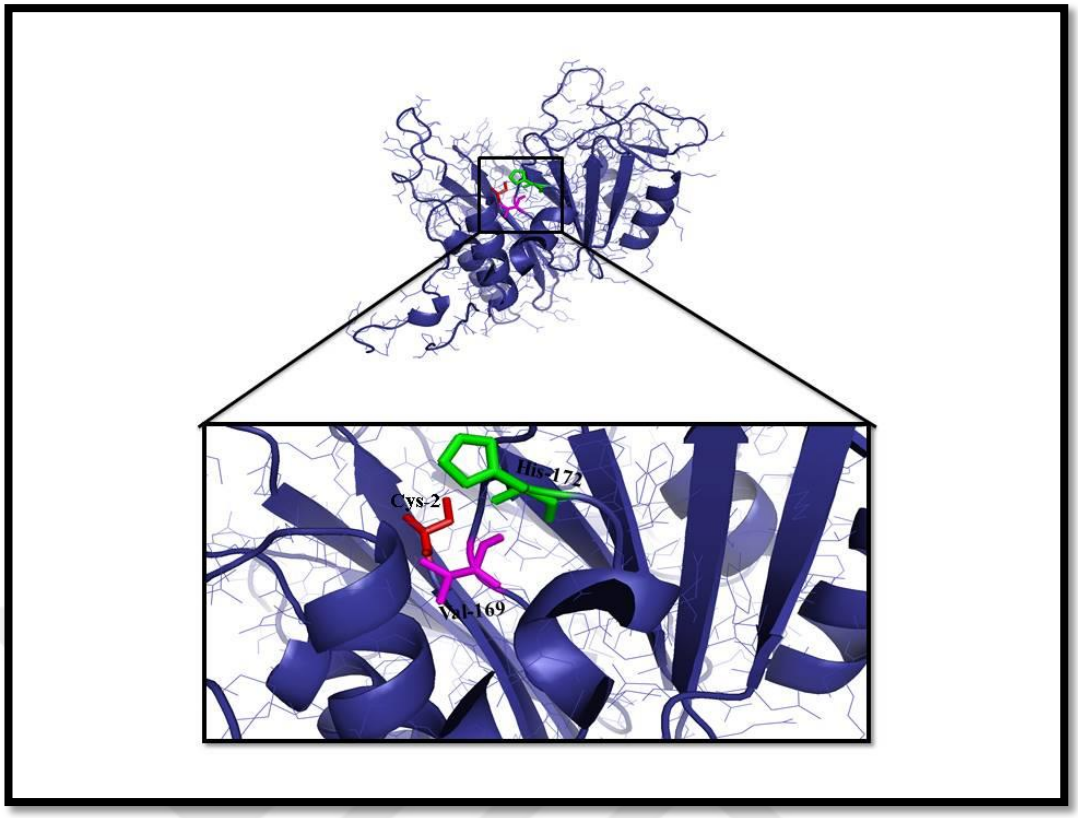


Figure 4.5. 3-D construction of part of BSH enzyme from mutated *Lactobacillus plantarum* by pMoL 0.99rc6 program. Substituted Val-169 and His-172 amino acids are showing in purple and green respectively.

4.1.2 Construction of pCON1/*T169V and pCON1/*P172H Mutants

4.1.2.1 Site Directed Mutagenesis of Threonine-169 and Proline-172 by PCR

The 507th and 516th codons of *bsh* gene coding threonine and proline were substituted for the codons coding valine and histidine, respectively. One hundred sixty ninth threonine amino acid of BSH enzyme on pCON1 (*bsh*1/pBluescript) was substituted for valine and one hundred seventy second proline amino acid was substituted for histidine amino acid by using designed primers. pCON1 DNAs were used as a template to make mutation by PCR-based site direct mutagenesis.

The PCR products of mutated bile salt hydrolase gene were loaded on a 0.8% agarose gel and separated by electrophorese at 75 volt for one hour. After staining of agarose gel with Ethidium bromide, PCR amplicon (975 bp) was visualized on UV-transilluminator (Figure 4.6).

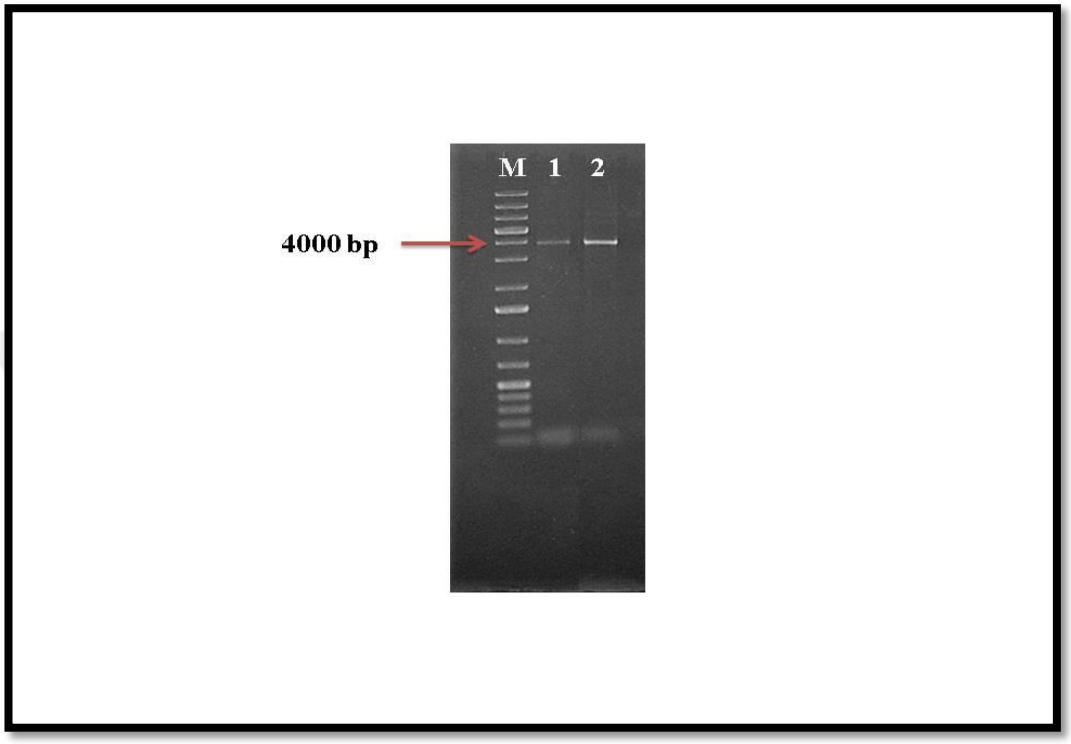


Figure 4. 6. Site Directed Mutagenesis of threonine-169 and proline-172 by PCR. M represent the Marker; line 1 is pCON1/*T169V PCR product and line 2 is pCON1/*P172H PCR product.

The PCR amplicons were treated with *Dpn*I restriction enzyme (RE) which cleaves the methylated DNA. The pCON1 template DNA was firstly cleaved with *Xho*I RE to evaluate PCR products and then treated with *Dpn*I RE. All *Dpn*I treated samples were loaded on 0.8% agarose gel to check digestion efficiency of the PCR product (Figure 4.7). Finally, *Dpn*I treated mutant gene samples were purified with GF-1 Gel DNA Recovery Kit (#GF-GP-050 Vivantis, Europe) (Figure 4.8).

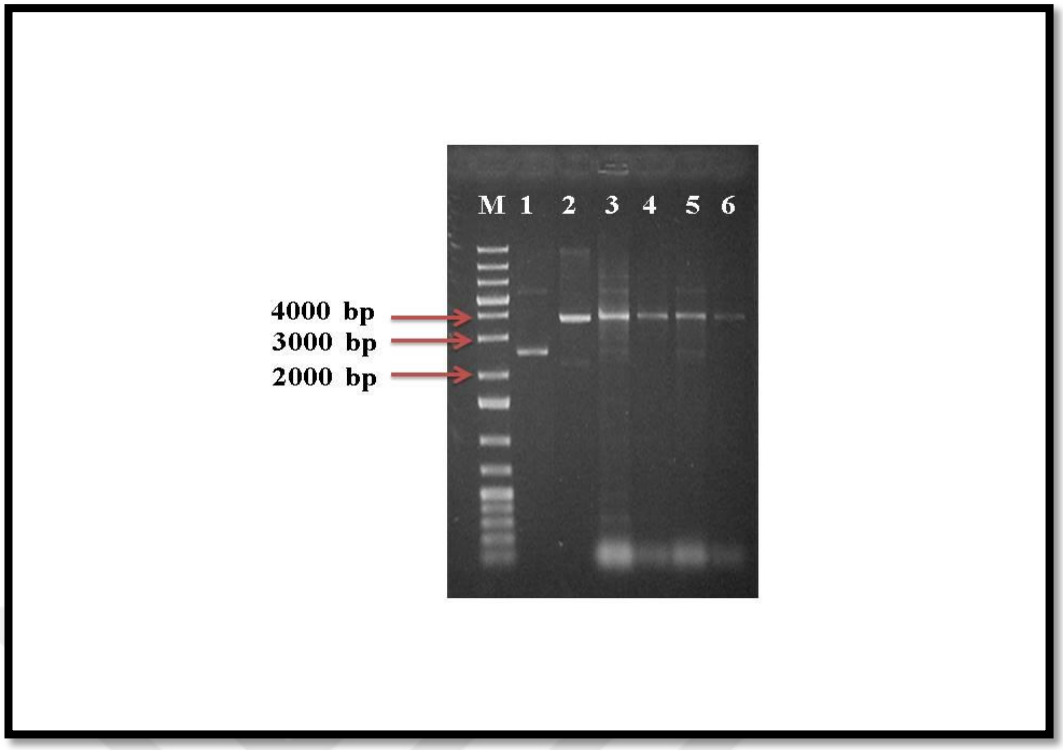


Figure 4.7. Digestion of pCON1 template DNA, pCON1/*T169V and pCON1/*P172H DNAs with *Xho*I and *Dpn*I. M represent the Marker; line 1: pCON1 template DNA; line 2: pCON1 template DNA digested by *Xho*I restriction enzyme; line 3: pCON1/*T169V PCR product; line 4: pCON1/*T169V PCR product digested by *Dpn*I restriction enzyme; line 5: pCON1/*P172H PCR product; line 6: pCON1/*P172H PCR product digested by *Dpn*1 restriction enzyme.

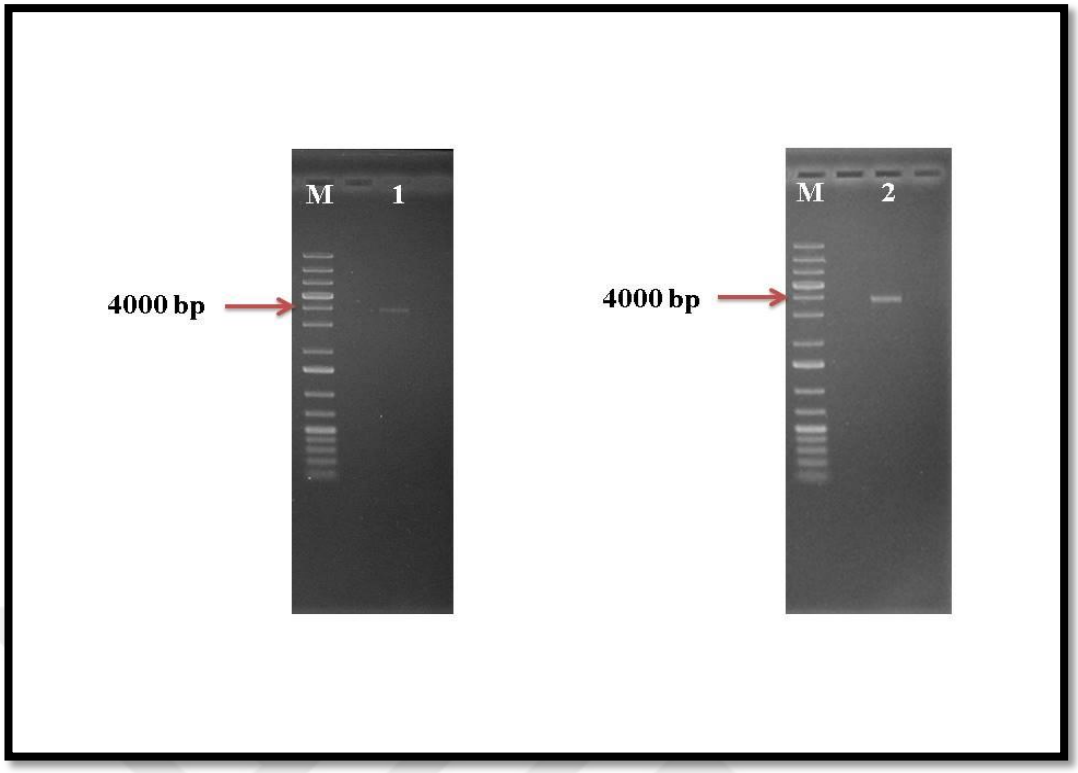


Figure 4.8. Purification of *Dpn*I digested products. M represents the Marker; line 1: Purification of pCON1/*T169V DNA; line 2: Purification of pCON1/*P172H DNA.

4.1.2.2 Transformation of pCON1/*T169V and pCON1/*P172H DNAs into *E. coli* XLI-Blue Strains

Agarose gel purified PCR amplicons, pCON1/*T169V and pCON1/*P172H, were transferred into XLI-Blue competent cells treated with $CaCI_2$. Four random colonies were selected and isolated from LB plate supplemented with ampicillin (25 mg/ml). These isolated samples were loaded on 0.8% agarose gel to see whether clones had a desired mutations or not (Figure 4.9).

Figure 4. 9. View of pCON1/*T169V and pCON1/*P172H DNAs isolated from the picked clones. M represent the Marker; line 1:pCON1 DNA; line 2: transformed product of pCON1/*T169V DNA; line 3: Transformed product of pCON1/*P172H DNA.

4.1.2.3 Sequencing of pCON1/*T169V and pCON1/*P172H DNAs

The *bsh* genes on pCON1/*T169H and pCON1/*P172H constructs were sequenced by using the universal primers, M13/pUC Forward and M13/pUC Reverse (Figure 4.10 and 4.11). Sequence results were aligned with that of pCON1 to check desired mutations and provide that there is no mutation rather than desired ones. After confirmation of the desired mutations, construct was named as a pCON1/*T169H and pCON1/*P172H.

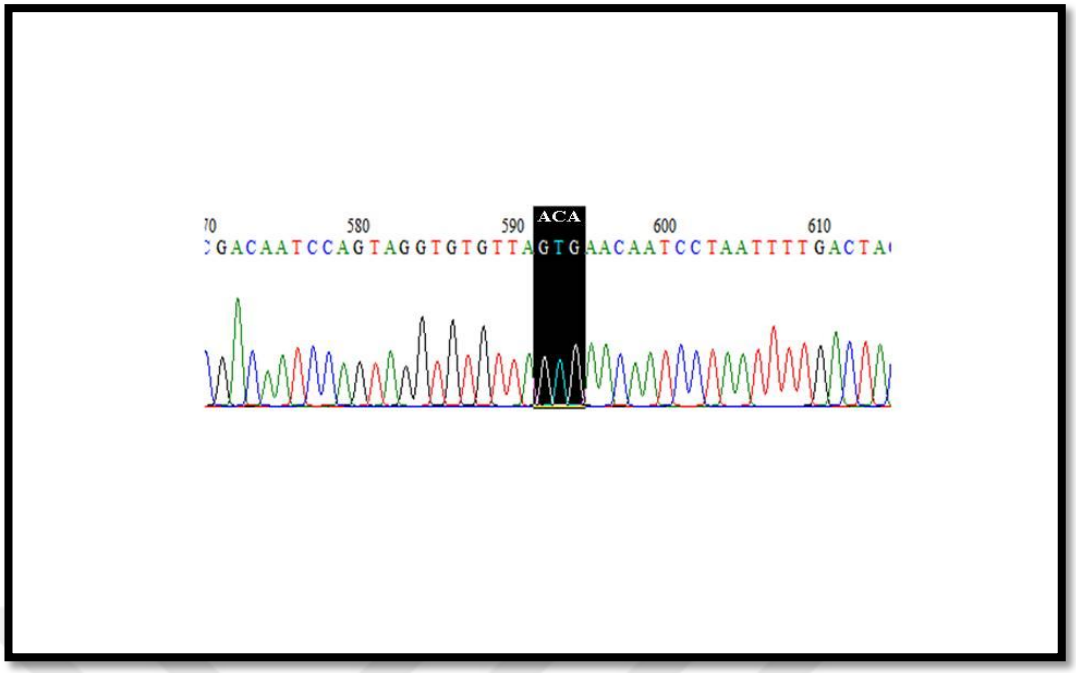


Figure 4. 10. Partial sequence of pCON1/*T169V DNA from transformant. The ACA codon coding threonine was substituted for the GTG codon coding valine amino acid.

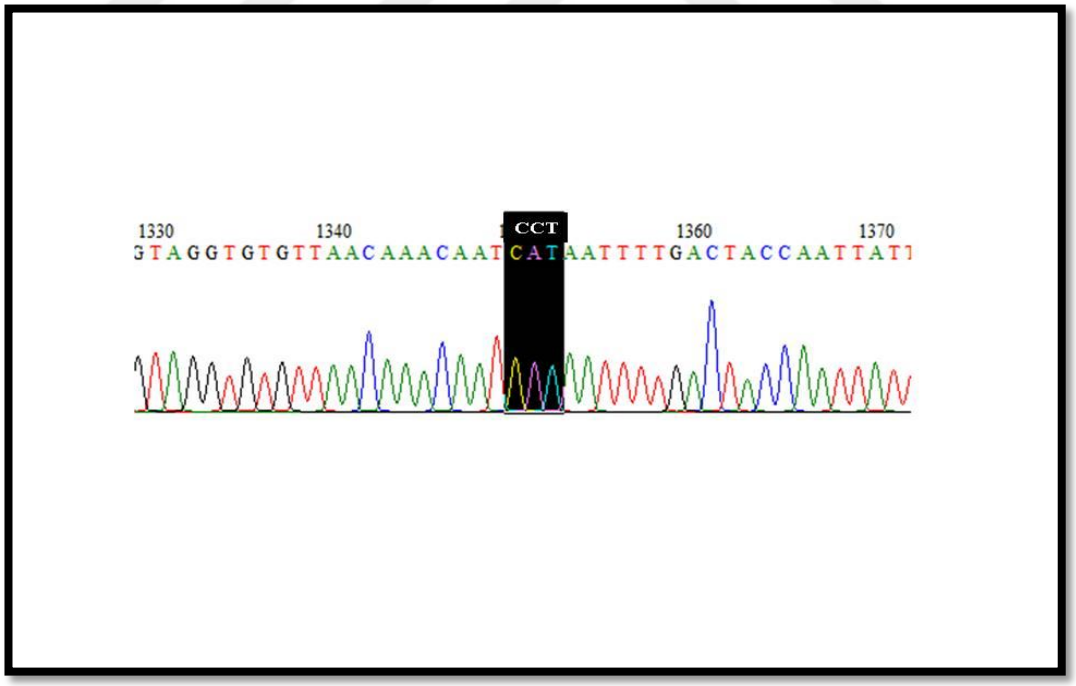


Figure 4. 11. Partial sequence of pCON1/*P172H DNA from transformant. The CCT codon coding proline was substituted for the CAT codon coding Histidine amino acid.

4.1.3 Construction of FAT/T169V and FAT/P172H Plasmids

4.1.3.1 Digestion of pCON1/*T169V, pCON1/*P172H and pETT22b DNAs by *Eco*RI and *Not*I Restriction Enzymes

The constructs containing desired mutations pCON1/*T169V, pCON1/*P172H, and pET22b DNAs were digested with *Eco*RI and *Not*I RE. The digestion products were separated by agarose gel electrophoresis (Figure 4.12). Then our desired *bsh* gene and the pET22b vector were purified from the 1% agarose gel (Figure 4.13).

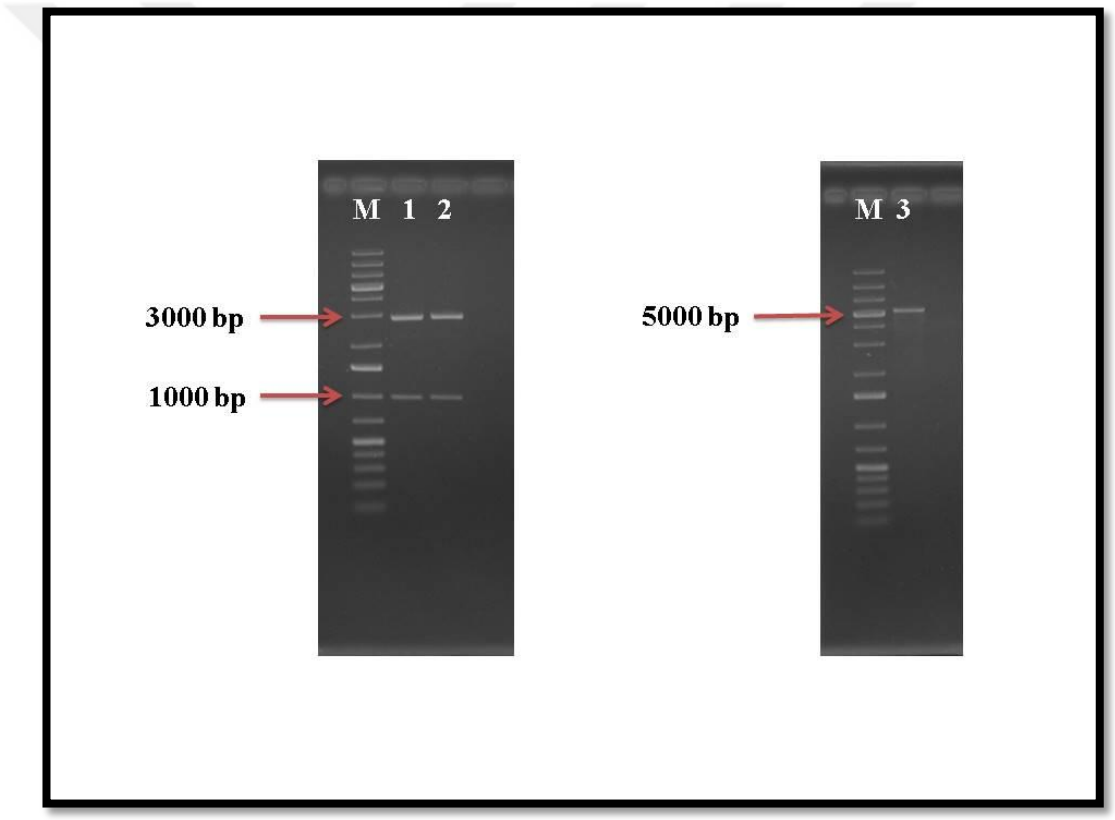


Figure 4. 12. Digestion of pCON1/*T169V, pCON1/*P172H and pETT22b DNAs by *Eco*RI and *Not*I Restriction Enzymes. M represent the Marker; line 1: Digestion of pCON1/*T169V with *Eco*RI and *Not*I; line 2: Digestion of pCON1/*P172H with *Eco*RI and *Not*I; line 3: Digestion of pET22b with *Eco*RI and *Not*I.

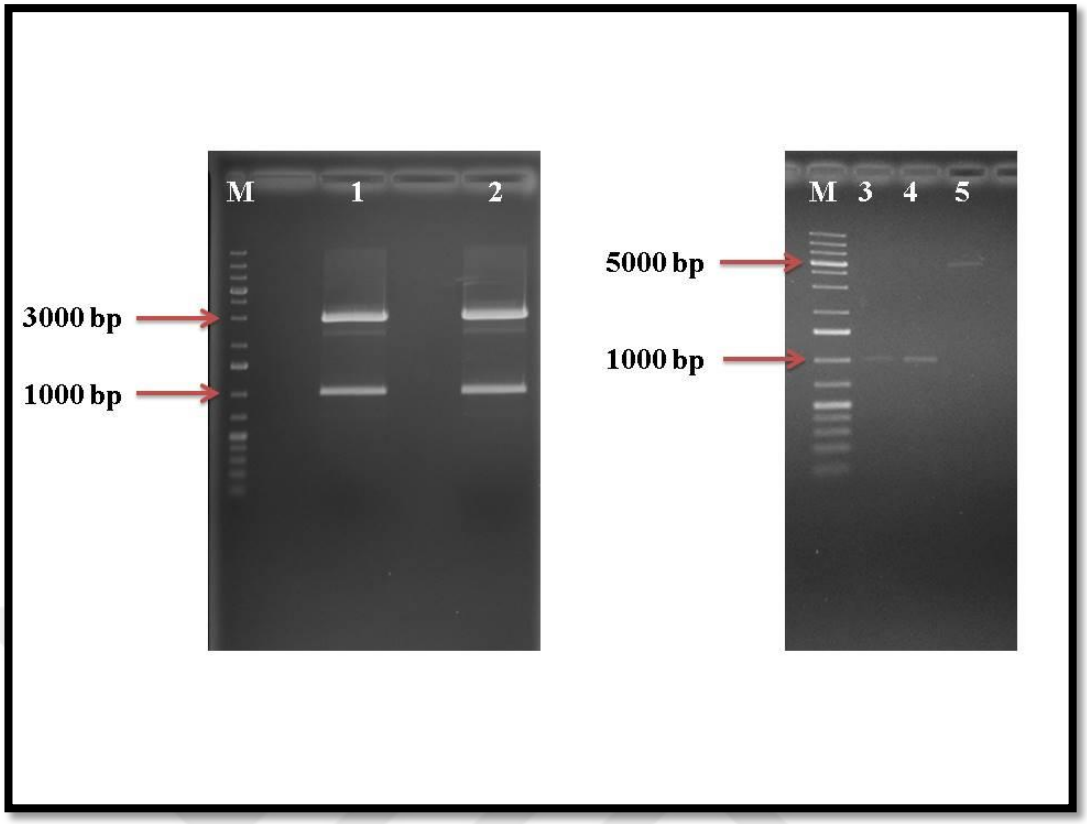


Figure 4. 13. Purification of *Eco*RI/*Not*I digested pCON1/*T169V, pCON1/*P172H and pET22b DNAs. M represent the Marker; line 1: *Eco*RI/*Not*I digested pCON1/*T169V; line 2: *Eco*RI/*Not*I digested pCON1/*P172H; line 3: Agarose gel purified mutant T169V *bsh* gene; line 4: Agarose gel purified mutant P172H *bsh* gene; line 5: Agarose gel purified pET22b DNA.

4.1.3.2 Transformation of pFAT/T169V and pFAT/P172H DNAs into *E. coli* BRL(DE3) Strains

*Eco*RI/*Not*I digested mutant *bsh* genes from pCON1/*T169V and pCON1/*P172H constructs were inserted into *Eco*RI/*Not*I site of expression vector pET22b DNA. These new plasmids were named as pFAT/T169V and pFAT/P172H. Ligation products were transferred into BRL(DE3) competent cells for the expression of mutants *bsh* genes. DNAs for each mutation were isolated from obtained clones and loaded on agarose gel for electrophoresis (Figure 4.14).

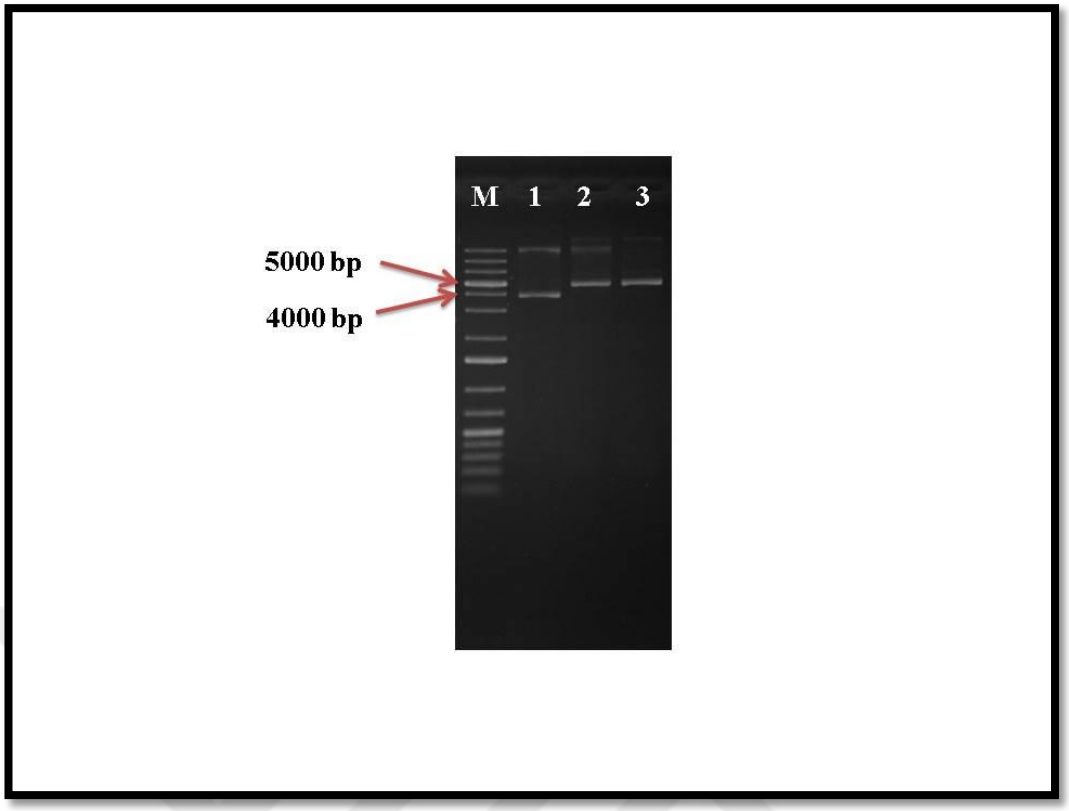


Figure 4. 14. Transformation of pFAT/T169V and pFAT/P172H into *E. coli* BRL(DE3) strains. M represent the Marker; line 1: pFAT/T169V transformant DNA; line 2: pFAT/P172H transformant DNA; line 3: Uncut pET22b DNA.

4.1.3.3 Detection of the Inserted pFAT/T169V and pFAT/P172H *bsh* Genes

The inserted mutant *bsh* genes were detected by digestion of the DNA of the clones isolated from transformants with *Eco*RI and *Not*I restriction enzymes. The digested plasmid DNA products containing the vector and insert *bsh* genes were separated by agarose gel electrophoresis (Figure 4.15).

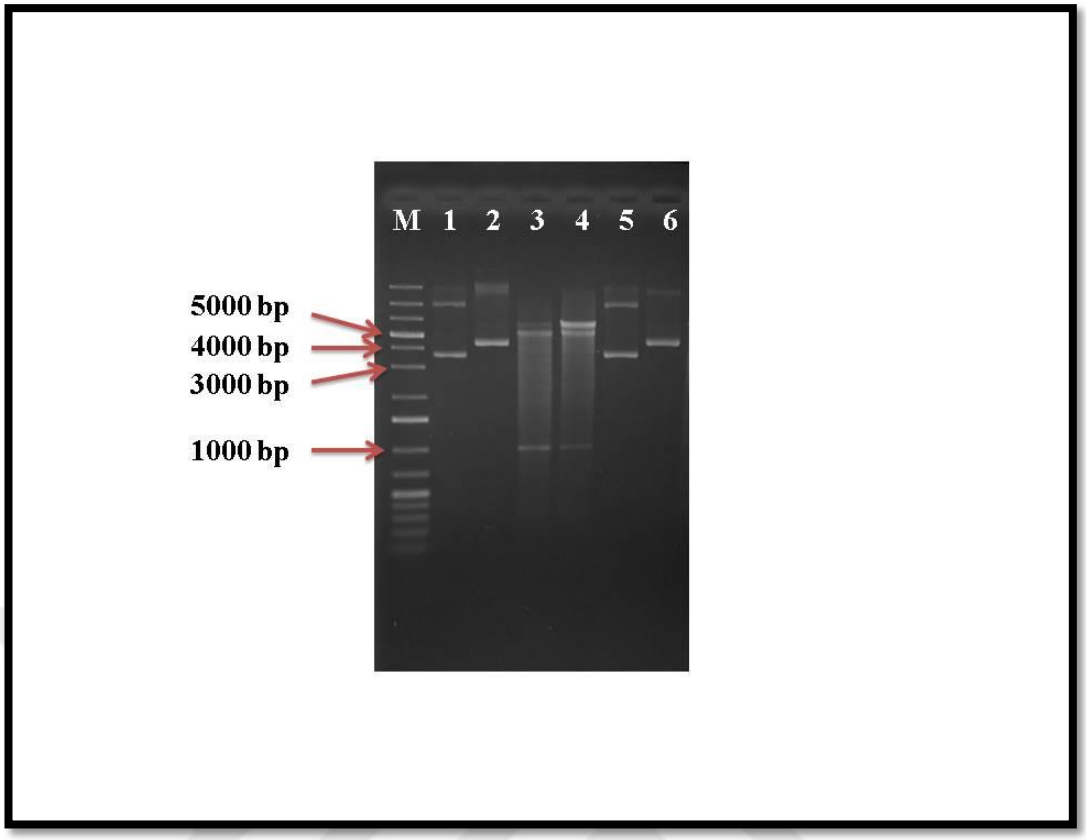


Figure 4. 15. Detection of the inserted *bsh* genes from pFAT/T169V and pFAT/P172H by digesting with *Eco*RI and *Not*I restriction enzymes. M represents the Marker; line 1: Uncut pET22b DNA; line 2: Uncut pFAT/T169V DNA; line 3: *Eco*RI/*Not*I digested DNA of pFAT/T169TV; line 4: *Eco*RI/*Not*I digested DNA of pFAT/P172H; line 5: Uncut pET22b DNA; line 6: Uncut pFAT/P127H DNA.

4.1.4 Determination of BSH Enzyme Activities from pFAT/T169V and pFAT/P172H by Ninhydrin Assay

Ninhydrin assay test was used for determination of BSH enzyme activity by a reaction between ninhydrin molecules and hydrolyzed bile salts. In this test, six different human bile salts, glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA) were used as a substrate. The BSH enzyme activities of pFAT/T169V and pFAT/P172H decreased in each of the six bile salts. More particularly P172H mutation almost completely inactivated the BSH enzyme (Figure 4.16).

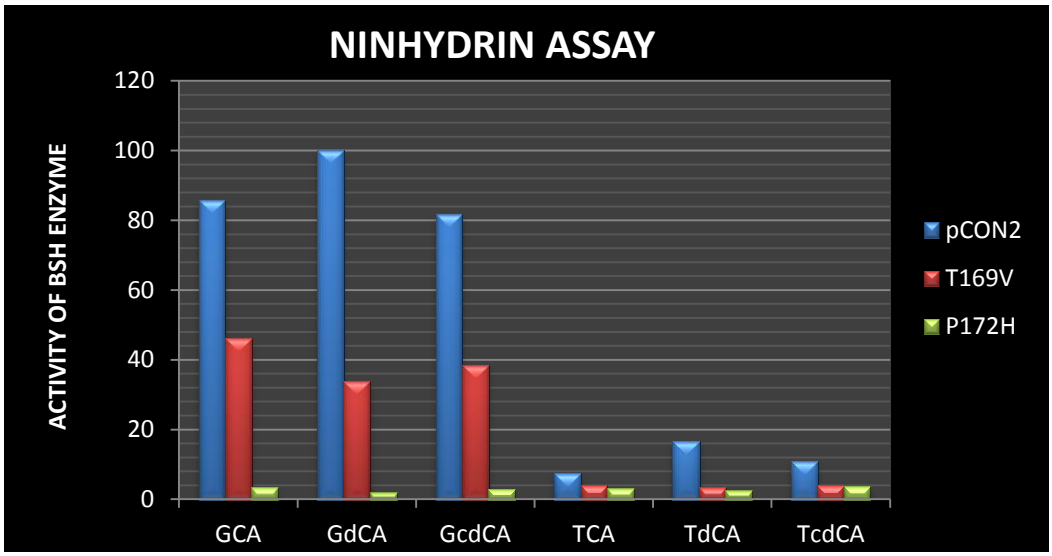


Figure 4. 16. Determination of BSH enzyme activities from pFATT169V and pFATP172H. Blue columns represent wild-type BSH activity as a positive control, pCON2; red columns represent BSH activity of pFAT/T169V; green columns represent BSH activity of pFAT/P172H.

4.1.5 Analysis of pFAT/T169V and pFAT/P172H BSH Enzymes by SDS-PAGE

The pET22b, pCON2, pFAT/T169V and pFAT/P172 constructs were expressed in 200 ml liquid LB medium supplemented with ampicillin (25 mg/ml) and induced with IPTG (0.3 mM). Partially purified cell extract were loaded on 12% polyacrylamide gel (Figure: 4.17). The pET22b was used as negative control because there is no *bsh* gene on it. The pCON2 has wild type *bsh* gene so it was used as positive control. The BSH protein bands were observed for both mutations on gel. This means they are not critical for assembly or folding of the BSH enzyme but for activity.

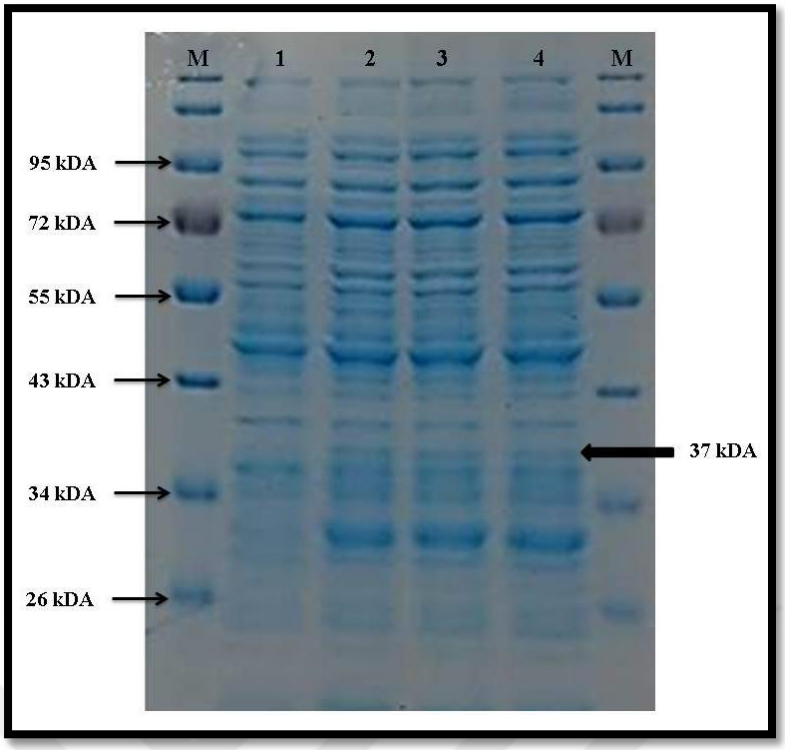


Figure 4. 17. SDS-PAGE analysis gel. M represent the protein molecular weight marker; line 1: pET22b the empty vector; line 2: wild BSH enzyme from pCON1; line 3: BSH enzyme from pFAT/T169V; line 4: BSH enzyme from pFAT/P172H.

4.2 DISCUSSION

BSH enzyme has very important role in host metabolism. It can deconjugate either taurine or glycine amino acids of bile salts. This property is directly connected with lipid metabolism of host. Because lipids are essential for mammalian, failure of lipid metabolism can cause many diseases. Herewith BSH can be a factor for Bowel diseases (Fereira-Peraira et al., 2014; Duboc et al., 2013; Shin et al., 2013), colorectal cancers (Bernstein et al., 2011) and gallstones production (Berr et al., 1992; Berr at el., 1996). This enzyme may also be a potential alternative for AGPs in food of animal poultry on account of relation with lipid metabolism (Wegener et al., 2003). To understand the role and function of BSH, more information should be required.

Bsh gene is found various bacterial genus such as *Lactobacillus* (De Smet et al., 1995), *Bifidobacterium* (Jarocki and Targonski, 2013), *Enterecoccus* (Wijaya et

al., 2004) and *Clostridium* (Coleman and Hudson, 1995). The species of these genus can carry one or more different type of *bsh* genes. According to Begley et al. (2006), *bsh* genes from different strains have conserved amino acids, even some of them are totally conserved in all BSH. These strictly conserved amino acids are Cys-2, Asp-19, Asn-79, Asn-170 and Arg-223 in BSH of *Lb. plantarum* B14 stain. The results of in silica analysis showed that these strictly conserved residues had played a role in catalytic activity of BSH. The Cys-2 residue is highly conserved in BSH and Penicillin V Acylase (PVA). Both enzymes are belongs to N-terminal nucleophile (Ntn) hydrolase group. Tanaka and his colleagues (2000) replaced Cys-1 with alanine in BSH of *Bifidobacterium longum* and they got an inactive protein. Using PCR-based site-directed mutagenesis, likewise Kim and his coworkers (2004) were exchanged Cys-1 with serine or threonine in BSH of *Bifidobacterium bifidum*. Their mutations also resulted in inactive BSH proteins but mutant BSH protein bands were observed on SDS-Page gel.

The other totally conserved amino acid residues, Arg-16 and Asp-19, in loop I of the BSH from *Lactobacillus plantarum* B14 strain were studied by Hacıbeyoğlu (2016). These amino acids were substituted for phenylalnine and leucine amino acids respectively (Hacıbeyoğlu, 2016). Although she observed stable BSH proteins for Asp19Leu mutant on SDS-PAGE, she obtained almost totally inactive enzyme. However, Arg16Phe mutation resulted in disassembled enzyme. On the other hand, Asn-170 and Arg-223 located in loop 3 were replaced with valine and phenylalanine respectively by Kılıçsaymaz (2017). She obtained disassembled enzymes. These results revealed that while Arg-16 amino acid is responsible for the catalytic activity of BSH enzyme, Asp-19, Asn-170 and Arg-223 amino acid are required for the folding or stability of the BSH enzyme.

Before starting to experimental analysis, some in silico analysis done. Many BSH and PVA genes were aligned and it was seen that two amino acids, Thr-169 and Pro-172, were totally conserved for both enzymes (Figure 4.1). These two amino acids are located in bend part of enzymes (Figure 4.3). After checking BSH 3-D construction, it was seen that Thr-169 and Pro-172 were very close to Cys-2 which might responsible for catalytic activity according to Begley (2006) (Figure 4.4).

In our experimental study, two totally conserved amino acids of BSH, Thr-169 and Pro-172, from *Lb. plantarum* B14 were chosen. These residues were exchanged with valine and histidine respectively by PCR-based site-directed mutagenesis. Similar to Arg16Phe mutant BSH enzyme we obtained assembled BSH proteins for both mutants (Figure 4.17). The Thr172Val mutation showed good protein expression but, had almost completely eliminated BSH activity; however, T169V mutant retained the 52% of wild type BSH activity (Figure 4.16).

Briefly, based on our in silico and experimental results, we suggested that amino acids position of 169 and 172 of BSH from *Lb. plantarum* B14 might have a critical role for the configuration of the catalytic domain of the BSH, but not for folding or stability of it.

5. CONCLUSIONS AND RECOMMENDATIONS

Although many bacterial BSHs from different genus, species and even strains were isolated and characterized the roles and functions of them still were not well understood. In previous studies, BSH was found as a factor for both diseases causing and diseases remedial on mammalian. Besides, BSH may be alternative to antibiotics which are used in animal industry due to their hypocholesterolemia properties. Therefore, knowing the mechanism of BSH enzyme's catalytic activity and substrate preferences is critical.

In this study, two conserved amino acid residues BSH of from *Lactobacillus plantarum* B14 were substituted with different suitable amino acids by PCR-based site-directed mutagenesis. Effects of these mutations were checked with ninhydrin assay and SDS-Page gel electrophoresis. Six different human bile salts were used to show catalytic activity of mutant enzyme for ninhydrin assay. Our ninhydrin assay results showed that Thr-169 and Pro-172 amino acid residues had a significant role on catalytic activity of BSH although they are conserved. On the other hand, our SDS-PAGE analysis revealed that these two amino acid residues might not be responsible for stability of the enzyme. However, to better understand the exact functions of the Thr-169 and Pro-172 amino acids and other strictly or partially conserved amino acids, more biochemical investigations and further multiple site-directed mutagenesis studies are needed.

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APPENDICES

7. APPENDICES

Appendix A Bacterial Growth Media

Liquid LB (Luria Bertani) Media

Contents	for 1 Liter
Tryptone	10 g
Yeast extracts	5 g
NaCI	10 g

These contents are dissolved in 900 ml upH_2O and pH is adjusted to 7.5 with NaOH. Than final volume is completed to 1 L with up H_2O and sterilized at 121 ºC for 15-20 minutes.

LB Plate

15 g of agar-agar is added in 1 L liquid LB medium and sterilized at 121 ºC for 15-20 minutes.

Ampicillin

0.25 g Ampicillin Sodium Salt and 10 ml up H_2O are mixed then final solution is sterilized by using a 0.22 µm filter and stored at -20 ºC.

LB Plate with Ampicillin

4 ml of ampicillin is added in 1 L of sterilized LB medium.

Appendix B Buffers and Solutions

➢ For Primer Resuspension

TE Buffer

- ✓ 3.634 g of Trisma base
- ✓ 30 ml ddH_2O

The pH is adjusted to 8.0.

➢ For Electrophoresis

TEA Buffer

Contents	for 1 Liter
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 final buffer volume of 1 L.

➢ For Competent Cell Preparation

0.1 M $CaCI_2$

- ✓ 1.48 g of $CaCI_2$
- ✓ 100 ml of upH_2O

The pH is adjusted to 7.0.

➢ **For Cell Extract Preparation**

- **1 M Monobasic Sodium Phosphate (NaH_2PO_4) Buffer**

12 g of NaH_2PO_4 are dissolved in 100 ml upH_2O. The pH measured was 4.12. Then this solution was sterilized at 121 ºC for 15-20 minutes.

- **1 M Dibasic Sodium Phosphate (Na_2HPO_4) Buffer**.

14.2 g of Na_2HPO_4 are dissolved in 100 ml upH_2O and pH recorded was 8.80. Then solution was sterilized at 121 ºC for 15-20 minutes.

- **0.1M Sodium Phosphate Buffer**

NaH_2PO_4 and Na_2HPO_4 are mixed with changeable amounts to obtain pH: 6.0. Then enough ddH_2O is added to make final volume buffer of 50 ml.

- **Binding Buffer**

Contents	**for 1 Liter**
20 mM NaPi Buffer	20 ml of 1 M NaPi
500 mM NaCI	29.22 g

➢ **For Ninhydrine Assay**

0.1 M NaOH

- ✓ 0.4 g NaOH
- ✓ 100 ml ddH_2O

1% SDS in 0.1 M NaOH

- ✓ 0.5 g Sodium dodecyl sulfate
- ✓ 5 ml 0.1 M of NaOH

Reagent A

- ✓ 1 g Sodium carbohydrate ($NaCO_3$)
- ✓ 50 ml 0.1 M of NaOH

Reagent B

- ✓ 5 mg Cupper II sulphate pentahydrate ($CuSO_45H_2O$)
- ✓ 0.5 ml of ddH_2O

Reagent C

- ✓ 10 g Potassium sodium tartarate ($C_4H_4KNaO_64H_2O$)
- ✓ 0.5 ml of ddH_2O

Reagent D

- ✓ 500 µl of reagent B
- ✓ 500 µl of reagent C

Reagent E

- ✓ 50 ml of reagent A
- ✓ 1 ml of reagent D

1 mg/ml Bovine Serum Albumin (BSA)

- ✓ 0.001 g BSA
- ✓ 1 ml of ddH_2O

0.2 M Glycine Solution

- ✓ 0.7507 g glycine
- ✓ 50 ml of ddH_2O

0.5 M Sodium Citrate Buffer

- ✓ 7.353 g Sodium citrate
- ✓ 50 ml of ddH_2O

The pH is adjusted to 5.5 and then buffer is sterilized at 121 ºC for 15-20 minutes.

15% Trichloroacetic Acid (TCA)

- ✓ 1.5 g TCA
- ✓ 10 ml of ddH_2O

1% Ninhydrine Solution

- ✓ 0.2 g Ninhydrine
- ✓ 20 ml Sodium citrate buffer

Ninhydrine Reagent

- ✓ 0.5 ml 1% of Ninhydrine solution
- ✓ 1.2 ml Glycerol
- ✓ 0.2 ml 0.5 M of Sodium citrate buffer

1 M DTT

- ✓ 0.154 g Sodium dithiothreitol ($C_4H_{10}O_2S_2$)
- ✓ 1 ml of ddH_2O

➢ For SDS-PAGE Analysis

4X Separating Gel Buffer

- ✓ 9.1 g Trizma base (MW: 121.14g/mol)
- ✓ 50 ml of ddH_2O

  The pH is adjusted to 8.8.

4X Stacking Gel Buffer

- ✓ 3.05 g Trizma base (MW: 121.14g/mol)
- ✓ 50 ml of ddH_2O

  The pH is adjusted to 6.8.

10X Electrode Buffer

- ✓ 6.05 g Trizma base (MW: 121.14g/mol)
- ✓ 28.83 g Glycine (MW: 75.07g/mol)
- ✓ 200 ml of ddH_2O

  The pH is adjusted to 8.3.

- **1X Electrode Buffer in 0.1% SDS**

✓ 100 ml of 10X Electrode buffer
✓ 1 g 0.1% of SDS
✓ 900 ml of ddH_2

- **10% Sodium Dodecyl Sulfate (SDS)**

✓ 1 g SDS
✓ 10 ml of ddH_2O

- **30% Acrylamide**

✓ 14.4 g Acrylamide
✓ 0.4 g Bisacrylamide
✓ 50 ml of ddH_2O

- **10% Ammonium Persulfate (APS)**

✓ 0.05 g APS
✓ 500 µl ddH_2O

Appendix C Chemicals

Acrylamide (Sigma)

Agar (Merck)

Agarose (Sigma)

Ammonium persulfate [APS] (Sigma)

Ampicillin (Sigma)

Bovine Serum Albumin (Sigma)

Buthanol

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

Calcium Chloride Dehydrate [CaCl2] (Merk)

Comassie Brilliant Blue G-250 (Fluka)

Cupper II SulphatePentahydrate (Sigma)

DTT (BioChemica)

EDTA [Ethylenediaminetetraacetic acid] (Sigma)

EtBr [Ethidium Bromide] (Sigma)

Folin ciocalteu Phenol Reagent (Sigma)

Glacial Acetic acid (Carlo Erba)

Glycerol, cell culture tested (Sigma)

Glycine (Merck)

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

Methanol (Tekkim)

MgCI2 (Thermo Scientific)

N,N'-Methyenebisacrylamide (Sigma)

Ninhydrin (Sigma)

Potassium Sodium Tartarate (Sigma)

Sodium Carbohydrate (Sigma)

Sodium Chloride [NaCl] (Merck)

Sodium Citrate Dehydrate (Merck)

Sodium Dithiothreitol [DTT] (Aplichem)

Sodium Dodecyl Sulfate [SDS] (Sigma)

Sodium Hydroxide [NaOH] (Merck)

Sodium Phosphate Dibasic (Sigma)

Sodium Phosphate Monobasic (BDLab)

TEMED (Bio-Rad)

Tetrachloro Acetic Acid (Sigma)

Trichloroacetic Acid (Merck)

Trizma Base (Sigma)

Trizma Hydrochloride (Sigma)

Yeast Extract Granulated (Merck)

Tryptone (Lab-M)

Tween® 20 (Sigma)

X-Gal (5bromo4chloro3indolylbetaDgalactopyranoside) (Fermentas)

Appendix D Enzymes and Other Chemicals

Not I (Fermentas)

*Eco*R I (Fermentas)

Fast Digest Pack (Fermentas)

Dnase (Sigma)

Dpn I (Thermo Scientific)

Rnase (Sigma)

Lysozyme (Fluka)

T4 DNA Ligase (Fermentas)

Page Ruller Prestain Protein Ladder (Thermo Scientific)

Pfu DNA Polymerase (Fermentas)

dNTP Mix (Fermentas)

1 kb Plus DNA Ladder (Fermentas)

6X Loading Dye Solution (Fermentas)

Glycocholic Acid, Sodium Salt (Calbiochem)

Glycochenodeoxycholic Acid, Sodium Salt (Sigma)

Glycodeoxycholic Acid, Sodium Salt (Calbiochem)

Taurocholic Acid, Sodium Salt (Calbiochem)

Taurochenodeoxycholic Acid, Sodium Salt (Sigma)

Taurodeoxycholic Acid, Sodium Salt (Calbiochem)

Appendix E Equipment Used in This Study

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

-20°C deepfreeze (Arçelik)

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

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

+4°C refrigerators (Arçelik)

Autoclave (Hirayama)

Centrifuge (Hettich Rotina 38R)

Desktop centrifuge (Hettich Micro 120)

Electrophoresis system (Thermo Scientific)

Imaging system (UVP Photo Doc-It TM)

Micropipettes (Finn pipette)

PCR (Techne, TC 3000)

pH meter (HANNA HI 221)

Power supply (Thermo EC 250-90)

Shaker-heater (IKA RCT basic)

Spectrophotometer (HITACHI U-1900)

UV Transilluminator (UVP)

Vortex (Yellowline TTS2)

Water Purification System (Human Corporation)

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