ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

CONSTRUCTION AND CHARACTERIZATION OF ARG16PHE AND ASP19LEU MUTATIONS ON BILE SALT HYDROLASE ENZYME ISOLATED FROM *LACTOBACILLUS PLANTARUM* IN *E.COLI*

MASTER OF SCIENCE

KÜBRA HACIBEYOĞLU

BOLU, JANUARY 2016

APPROVAL OF THE THESIS

CONSTRUCTION AND CHARACTERIZATION OF ARG16PHE AND ASP19LEU MUTATIONS ON BILE SALT HYDROLASE ENZYME ISOLATED FROM *LACTOBACILLUS PLANTARUM* IN *E.COLI* submitted by **Kübra HACIBEYOĞLU** in partial fulfillment of the requirements for the degree of Master of Science in **Department of Biology, Abant Izzet Baysal University** by,

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January 22, 2016

Prof. Dr. Duran KARAKAS .....

Director, **Graduate School of Natural and Applied Sciences**

To my grandfather, Hüseyin HACIBEYOĞLU

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.

KÜBRA HACIBEYOĞLU

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ABSTRACT

CONSTRUCTION AND CHARACTERIZATION OF ARG16PHE AND ASP19LEU MUTATIONS ON BILE SALT HYDROLASE ENZYME ISOLATED FROM *LACTOBACILLUS PLANTARUM* IN *E.COLI*

MSC THESIS
KÜBRA HACIBEYOĞLU
ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: ASSOC. PROF. DR. MEHMET ÖZTÜRK)

BOLU, JANUARY 2015

Probiotics are thought to have an important role in the lowering of the high cholesterol level that is shown as the main reason for cardiovascular diseases. Drugs used for treatment are expensive and have a lot of side effects so promoting the importance of the probiotics. There are a lot of studies proving that probiotics especially, intestinal bacteria such as *Lactobacillus* and *Bifidobacterium,* are lowering the amount of cholesterol with their Bile salt hydrolase (BSH) enzymes, but we do not know much about this enzyme. To characterize BSH enzyme one of the methods is; site direct mutagenesis and the other method is crystallography. However, no mutagenesis study on BSH enzyme was found in literature except Cys2 amino acid. In this study, fully conserved amino acids, Arg-16 and Asp-19 of *L. plantarum* species, were substituted for phenylalanine and leucine amino acids respectively in pCON1 construct using appropriate primers. Afterwards, mutant *bsh* genes were transferred to pET22b expression vector. The recombinant mutant proteins were purified using a Ni^{2+} chelated column and then were analyzed in respect to their activity and stability by direct plate assay, ninhidrin assay and polyacrylamide gel electrophoresis, respectively.While Direct plate assay and Ninhidrin assay results indicated that all of the mutations resulted in inactive BSH enzymes, polyacrylamide gel electrophorese results showed that Arg-16 mutant BSH protein was folded, but Asp-19 mutant protein was not. Our results revealed that while Arg-16 amino acid is responsible for the catalytic activity of BSH enzyme, Asp-19 amino acid is required for the folding of the BSH enzyme and its stability. Therefore, this work emphasized that further site directed mutagenesis studies, especially on the amino acids supposed to be responsible for catalytic activity and bile salt substrate specificity of the BSH enzyme, are required.

KEYWORDS: Bile salt hydrolase, Probiotics, *Lb.plantarum*, Site directed mutagenesis

ÖZET

E.COLİ DE *LACTOBACILLUS PLANTARUM* DAN İZOLE EDİLEN SAFRA TUZU HİDROLAZ ENZİMİNDE ARG16PHE AND ASP19LEU MUTASYONLARININ OLUŞTURULMASI VE KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

KÜBRA HACIBEYOĞLU

ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ

BIYOLOJİ ANABİLİM DALI

(TEZ DANIŞMANI: DOÇ. DR. MEHMET ÖZTÜRK)

BOLU, OCAK - 2016

Kardiyovasküler hastalıkların temel sebebi olarak gösterilen yüksek kolesterol seviyesinin düşürülmesinde probiyotiklerin önemli role sahip oldukları düşünülmektedir. Tedavi için kullanılan ilaçların pahalı ve çok fazla yan etkilerinin olması probiyotiklerin önemini artırmaktadır. Probiyotiklerin özellikle bağırsak bakterilerindeki *Lactobacillus* ve *Bifidobacterium*'un Safra Tuzu Hidrolaz (STH) enzimi ile kolesterol miktarını düşürdüğünü gösteren birçok çalışma bulunmaktadır ancak STH enzimi hakkında yeterince bilgimiz yoktur. STH enzimini karakterize etmek için kullanılan metotlardan biri yönlendirilmiş mutagenez yöntemi, diğeri de kristalografidir. Bununla birlikte, literatürde Cys2 aminoasiti dışında STH enzimi ile ilgili mutagenez çalışması bulunmamaktadır. Bu çalışmada, *Lactobacillus plantarum* türündeki tamamen korunmuş olan Arg-16 ve Asp-19 aminoasitleri uygun primerler kullanılarak pCON1 konstraktı içinde sırasıyla fenilalanin ve lösin aminoasitlerine dönüştürülmüştür. Daha sonra da mutant *bsh* genleri, pET22b ekspresyon vektörüne aktarılmıştır. Rekombinant mutant proteinler Ni^{2+} temelli kolon kullanılarak saflaştırılmış ve sonra da aktiteleri ve yapısı sırasıyla direk besi yeri ve ninhidrin testleri ile ve poliakrilamid jel elektroforezi ile analiz edilmiştir. Direk besiyeri ve Ninhidrin test sonuçları, tüm mutasyonların enzim inaktivasyonu ile sonuçlandığını gösterirken, poliakrilamid jel elektroforez sonuçları, Arg-16 mutant BSH proteinin katlandığını, ancak Asp-19 mutant proteinin katlanmadığını göstermiştir. Elde edilen sonuçlar; Arg-16 aminoasidinin BSH enziminin katalitik aktivitesinden sorumlu olduğunu, ortaya koyarken, Asp-19 aminoasitinin ise BSH enziminin katlanması ve stabilitesi için gerekli olduğunu göstermektedir. Bu çalışma; BSH enziminin, özellikle katalitik aktiviteden ve safra tuzlarına olan özgüllüğünden sorumlu olduğu varsayılan aminoasitler üzerinde daha fazla yönlendirilmiş mutagenez çalışmalarının gerekli olduğunu ortaya koymaktadır.

ANAHTAR KELİMELER: STH, Probiyotik, *Lb.plantarum,* Yönlendirilmiş mutagenez

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LIST OF ABBREVIATIONS AND SYMBOLS

GIT	**:** Gastrointestinal Tract
IBD	**:** Inflammatory Bowel Disease
LAB	**:** Lactic Acid Bacteria
GRAS	**:** Generally recognized as safe microorganisms
CA	**:** Cholic acid
CDCA	**:** Chenodeoxycholic acid
BSH	**:** Bile Salt Hydrolases
Ntn	**:** N-terminal nucleophilic
IPTG	**:** Isopropyl-β-D-1 thiogalactopyranoside
X-gal	**:** 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Amp^R	**:** Ampicilline resistance
PCR	**:** Polymerase Chain Reaction
SDS-PAGE	**:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis
LB	**:** Luria-Bertani
OD	**:** Optic Density
RPM	**:** Revolutions per minute
kDa	**:** Kilo Dalton
Ni^{+2}	**:** Nickel (II)
kbp	**:** Kilo base pair
bp	**:** Base pair
µl	**:** Mikroliter
µM	**:** Micromolar
mM	**:** Millimolar
U	**:** Enyme unit
M	**:** Molar

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1. INTRODUCTION

1.1 Probiotics

A complex community of microorganisms live in the gastrointestinal tract (GIT) (Figure 1.1). The colon is the main site of microbial colonization and contains more than 500 different species (Blaut et al., 2002). These different species, which are called microbiota are localized soon after birth and remain for life (Backhed et al., 2005). Microbial groups that are localized in the human gut are important for maintaining health. When these microbial groups are changed, host microbe relationship leads to disease. Change in gut microflora causes specific diseases, such as inflammatory bowel diseases, irritable bowel disease and is also antibiotic associated diarrhea (Gibson et al., 1995; Juntunen et al., 2001) and also associated with allergy (Kalliomäki et al., 2001), obesity and diabetes (Ley et al., 2005; Turnbaugh et al., 2006;). Therefore, the microbiota equilibrium of gut flora is essential to preserve and inhibit diseases (Figure 1.2).

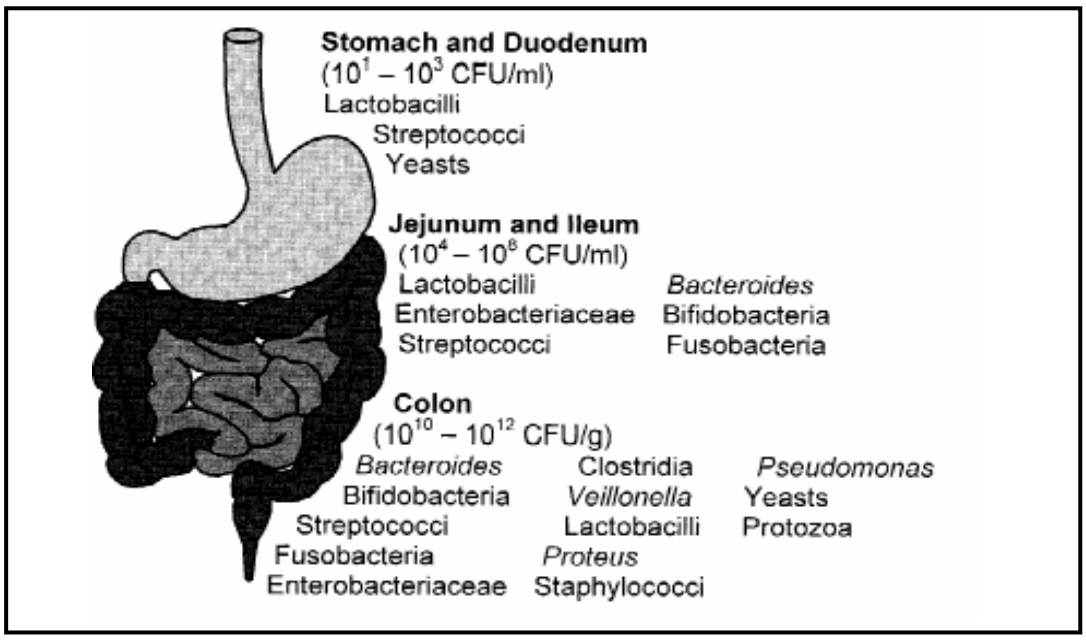


Figure 1.1. Microorganisms of gastrointestinal tract (modified after Simon and Gorbach, 1982)

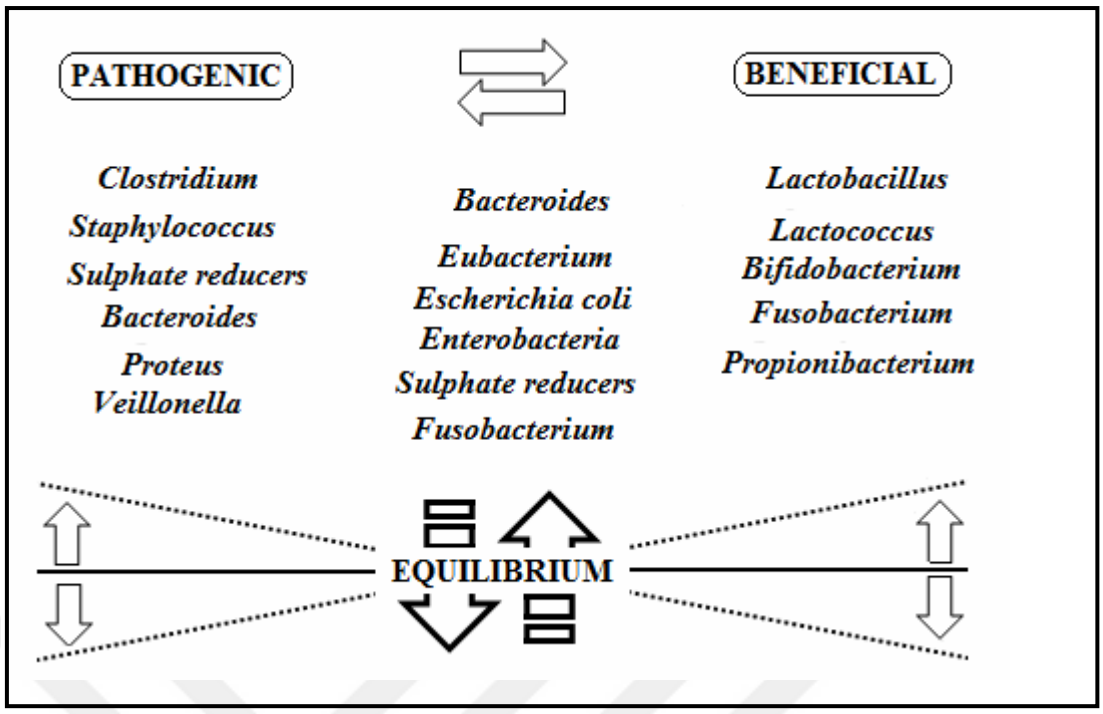


Figure 1.2. Bacterial equilibrium (modified from Gibson et al., 1995).

Microflora have a beneficial effect in the GI tract are termed probiotics (Marteau et al., 1995). Probiotic is derived from the Greek word probiosis which means "for life." A probiotic is a live microorganism and indicates beneficial effects on the host's health beyond inherent fundamental nutrition (Sultan et al., 2006). *Lactobacillus* and *Bifidobacterium* are used as the most common probiotics (Meurman and Stamatova, 2007) (Table 1.1).

Table 1.1. Most of the common probiotic microorganisms modified from (Holzapfel et al., 2001).

***Lactobacillus* species**	***Bifidobacterium* species**	**Other LAB**	**Non-lactics**
Lb.acidophilus	*B.adolescentis*	*Ent.faecalis*	*Bacillus cereus var.toyoi*
Lb.amylovorus	*B.animalis*	*Ent.faecium*	*Escherichia colistrain nissle*
Lb.casei	*B.bifidum*	*Lactoc.lactis*	*Propionibacterium freudenreichii*
Lb.crispatus	*B.breve*	*Leuc.mesenteroides*	*Saccharomyces cerevisiae*
Lb.delbrueckii subsp.bulgaricus	*B.infantis*	*Ped.acidilactici*	*Saccharomyces boulardii*
Lb.gallinarum	*B.lactis*	*Sporolactobacillus inulinus*	
Lb.gasseri	*B.longum*	*Strep.thermophilus*	
Lb.johnsonii			
Lb.paracasei			
Lb.plantarum			
Lb.reuteri			
Lb.rhamnosus			

1.2 Mode of Action of Probiotics

The beneficial effects of probiotics may be mediated by a direct antagonistic effect against specific groups of organisms resulting in a decrease in numbers or by an effect on their metabolism or by stimulation of immunity (Hentges, 1983). These effects of probiotics are shown in (Table 1.2).

Table 1.2. Mode of action of probiotics (Fuller, 1989).

1. Suppression of viable count by:
(a) Production of antibacterial compound
(b) Competition for nutrients
(c) Competition for adhesion sites
2. Alteration of microbial metabolism
(a) Increased enzyme activity
(b) Decreased enzyme activity
3. Stimulation of immunity
(a) Increased antibody levels
(b) Decreased antibody levels

1.3 Selection Criteria of Probiotics

Probiotics are members of many different genera but the most commonly used strains are members of heterogenous group of lactic acid bacteria such as lactobacilli, enterococci and bifidobacteria. There is no one agreed set of selection criteria for classifying bacterial strain as probiotics but the most common criteria are shown in (Table 1.3) (Ouwehand et al., 2002). In addition probiotic bacteria do not need to fulfil all of these selection criteria (Ouwehand et al., 1999).

Table 1.3. Main criteria for probiotics bacteria (Ouwehand et al., 2002).

Property	Benefit
Resistance to pancreatic enzymes, acid and bile	Survival of passage through the intestinal tract
Adhesion to the intestinal mucosa	Immune modulation
	Pathogen exclusion
	Enhanced healing of damaged mucosa
	Prolonged transient colonisation (?)
Human origin	Species specific interactions with the host
Documented health effects	Proposed health effects are 'true'
Safe	No health risk to consumer
Good technological properties	Strain stability
	Production at large scale
	Oxygen tolerance

Probiotic bacteria must be resistance to low pH, bile and pancreatic enzymes in order to survive passage through the gastrointestinal tract. Acid and bile tolerance can be easily observed and they are also the main properties of lactic acid bacteria (Ouwehand et al., 2002).

1.4 Beneficial Effect of Probiotics

1.4.1 Antimicrobial Activity

Antimicrobial activity is a very important role for probiotic bacteria due to the inhibition activities of harmful and pathogenic intestinal microbes. Antimicrobial substances which include organic acids (lactic and acetic acid), hydrogen peroxide (in the presence of oxygen), diacetyl, β-hydroxypropionaldehyde (*Lactobacillus reuteri*) or bactericidal or bacteriostatic peptides and proteins are produced by probiotic bacteria (DeVuyst and Vandamme, 1994). For instance, bacteriocins are proteins or protein complexes produced by probiotic bacteria which also demonstrate their bactericidal activity. There are a lot of studies related to the characterization of bacteriocins or bacteriocin-like compounds from lactic acid bacteria especially *Lactobacillus, Pediococcus* and *Enterococcus* as natural preservatives in foods (Chen and Hoover, 2003).

1.4.2 Antibiotic-Associated Diarrhoea

A major problem of antibiotic treatment is diarrhea, often caused by *Clostridium difficile*. This bacterium is found as a small count in the healthy intestinal flora and it is increased when the gastrointestinal tract is affected by any factor such as antibiotic. Treatments are fixed GIT flora but diseases are relapsed again. Hence, probiotic bacteria are useful for treatment of this disease. For example, *Lactobacillus* GG inhibits diarrhea and yeast preparation containing *Saccharomyces cerevisiae* ('boulardii') also prevents diarrhea (Shah, 2004, 2006b).

1.4.3 Lactose Intolerance

Lactose intolerance or lactose maldigestion is occasioned by reduction of *β*-galactosidase synthesis (Launiala, 1968). It is thought that approximately two-thirds of the world adult population has aggrieved lactose maldigestion, with the prevalence particularly high in Africa and Asia. Lactose maldigestion is variable in Europe such as about 2% in Scandinavia and about 70 % in Sicily (Vesa et al., 2000). When lactose is consumed, the osmotic load in the small intestine is increased with subsequent secretion of fluids which causes loose stools (Launiala, 1968). As well as loose stools, lactose maldigestion also caused abdominal bloating, pain, flatulence, nausea and borborygmi (Marteau et al., 2002). The basis of abdominal pain associated with lactose consumption by lactose maldigesting subjects is not well understood and it does not appear to relate to the production of gasses from the fermentation of lactose by the intestinal microflora (Lasser et al., 1975). Many lactose intolerant individuals are better able to consume fermented dairy products such as yogurt. Although yogurt contains the same amount of lactose as milk, yogurt shows fewer symptoms than the same amount of unfermented milk. When lactic acid bacteria are used to make yogurt, lactase is produced and digests the lactose before it reaches the colon (Ljungh and Wadstrom, 2006).

1.4.4 *Helicobacter pylori* Infection

Helicobacter pylori can resist acidic environments and also colonize the epithelial cell lining (Sheu et al., 2006). Though its role in the etiology of these diseases is still under investigation, *Helicobacter pylori* is related to gastric ulcers and gastric cancer (Dilnawaz et al., 2011). Antibiotic treatments can destroy *H.pylori* but these treatments have a lot of side effect such as antibiotic resistance. Probiotic organisms do not destroy the *H.pylori* but they are able to reduce *H. pylori* infection. To illustrate, *L. johnsonii* La1 and *L. gasseri* OLL2716 have been found to reduce *H. pylori* colonization and also inflammation (Felley et al., 2001). Besides this, *L. casei* Shirota and *L. acidophilus* also inhibit growth of *H. pylori* (Cats et al., 2003).

1.4.5 Inflammatory Bowel Disease

Inflammatory bowel diseases (ulcerative colitis and Crohn's disease) are associated with intestinal microflora (Shah, 2007) and its multi-factorial chronic aggressive disorders (Schultz et al., 2003). Overgrowth of entero-adhesive and entero-emorrhagic *Escherichia coli* and low levels of *Bifidobacterium* causes these diseases (Saarela et al., 2002; Rath, 2003). Donabedian (2006) reported that there exists strong evidence that early enteral feeding to patients may assist in prevention of infections and surgical illnesses. Due to probiotic administration, regulation of the imflammatory response, enhancement of barrier function or modulation of composition or activity of gut microbiota might bring about relief in IBD symptoms or maintain remission from symptoms (Tuohy et al., 2003; Santosa et al., 2006).

1.4.6 Anticarcinogenic Properties

Bacteria and metabolic products like genotoxic compounds (nitrosamine, heterocyclic amines, phenolic compounds and ammonia) cause colorectal cancer. The consumption of cooked red meat and low consumption of dietary fibre are a major role in causing colorectal cancer (Yoon et al., 2000) and also cause increasing levels of *Bacteroides* and *Clostridium* and decreased levels of *Bifidobacterium* in the intestinal microflora (Benno et al., 1991). The colonic flora is also reported to cause

carcinogenesis mediated by microbial enzymes like β-glucuronidase, azoreductase and nitroreductase. This enzymes cause precarcinogenic compounds to turn into the carcinogens.

Some strains such as *L. acidophilus* and *Bifidobacterium* spp. are reported to decrease the levels of enzymes like β-glucuronidase, azoreductase and nitroreductase responsible for activation of precarcinogens and also decrease the risk of tumour development (Yoon et al., 2000). In addition, *L. acidophilus* and *Bifidobacterium*, *L. plantarum* and *L. rhamnosus* produce short chain fatty acids and are reported to inhibit the generation of carcinogenic products by reducing enzymes activities (Cenci et al., 2002).

As a result of the removal of sources of procarcinogens by probiotic bacteria (or the enzymes that lead to their formation) improvement in the balance of intestinal microflora, normalized intestinal permeability (leading to prevention or delaying of toxin absorption), strengthening of intestinal barrier mechanisms, and activation of non-specific cellular factors (like macrophages and natural killer cells) via regulation of γ-interferon production. Orally administered Bifidobacterium play a role in increasing production of IgA antibodies and functions of Peyer's patch cells (Singh et al., 1997).

1.4.7 Reduction of Serum Cholesterol

Cholesterol is essential for a lot of functions in the human body. It is a precursor of certain hormones and vitamins and a component of cell membranes and nerve cells. However, high levels of total blood cholesterol or other blood lipids lead to risk factors for developing coronary heart disease (Parvez et al., 2006).

Many commercial food and dairy producers claim that their products can benefit health especially in relation to cholesterol reduction. Most of probiotic products have been used in clinical trials of serum lipid modulation. To illustrate, in the early 1970's, Mann and Spoerry administered fermented milk to the Maasai population and demonstrated a reduction in serum cholesterol (Mann et al., 1974).

There are many studies showing that probiotic bacteria especially *Lactobacillus* and *Bifidobacterium* reduce serum cholesterol levels (Pereira and Gibson, 2002). Hence, interest in probiotic bacteria, as a diet and pharmacologic agent, as well increase day by day for reduction of serum cholesterol. Many studies have been made to understand the reduction of serum cholesterol levels by lactic acid bacteria. Two mechanisms have been forward (Tok and Aslım, 2007);

1) *Lactobacillus* reduce the assimilation of cholesterol absorbed from the intestine

2) *Lactobacillus* plays a major role at deconjugation of bile salts

1.5 General Characteristic of Lactic Acid Bacteria

The term Lactic Acid Bacteria (LAB) was accepted in the beginning of 20th century (Carol et al., 2010). Other terms like" milk souring" and "lactic acid producing" bacteria had been previously used for same bacteria and these caused slight confusion.

Orla-Jensen (1919) classified LAB according to their morphological properties (cocci or rods, tetrad formation), mode of glucose fermentation (homo- or heterofermantative), growth of certain temperature (e.g., 10°C and 45°C) and form of lactic acid produced (D, L or both) (Kenji et al., 2009). The core of LAB consists by the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Strecptococcus* but these four genera revision and identified again as *Aerococcus*, *Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weisella* (Axelsson, 1998).

Lactic Acid Bacteria (LAB) are low G+C content, gram-positive, catalase-negative, non-sporulating, acid tolerant and facultative anaerobic organisms (Mayo et al., 2010). Most of LAB members are non-pathogenic and generally recognized as safe microorganisms (GRAS). It is generally associated with habitats rich in nutrients, such as various food products (milk, meat, vegetables), but some are also

members of the flora of the mouth, intestine and vagina of mammals (Whittenbury, 1964).

They involve the most important part of normal intestinal microbiota, which contribute to a variety of functions including intestinal integrity, immunomodulation and pathogen resistant (Ouwehand et al., 2002; Gibson and Roberfroid, 1995; Reid, 1999). Consequently, the most commonly studied intestinal bacteria for potential probiotic use are members of the genera *Lactobacillus* and *Bifidobacterium* spp (Nagpal et al., 2007).

1.6 The Genus *Lactobacillus*

The genus *Lactobacillus* members of LAB are also characterizied by carbohydrate metabolism. They are gram-positive, strictly fermantative, microaerophilic and chemo-organotrophic, non-spore forming, rod and coccobacilli with G+C content between 33 mol% and 55 mol% (Stiles and Holzapfel, 1997; Felis and Dellaglio, 2007). *Lactobacillus* are generally catalase negative microorganisms but some strains show pseudocatalase activity and found in environment where carbonhydrates are present, like foods (dairy products, fermented meat, sour doughs, vegetables, fruits and beverages), gastrointestinal, respiratory and genital tracts of human and animals, in sewage and plant material (Felis and Dellaglio, 2007).

Traditionally *Lactobacillus* divided into three groups according to type of sugar fermentation as homofermentative (producing more than 85% lactic acid by fermantation of hexose sugars via glycolysis), heterofermantative (producing lactic acid, carbon dioxide, ethanol and/or acetic acid via the 6-phosphogluconate/ *phosphoketolase* pathway) and finally facultative heterofermantative (ferment hexose via glycolysis and pentoses via the 6-phosphogluconate/ phosphoketolase pathway) (Walter, 2005).

The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Lactobacillaceae* according to *Taxonomic Outline of the Procaryotes* (Garrity et al., 2004). Besides, the genus *Lactobacillus* and the

genus *Pediococcus* constitute the family Lactobacillaceae and presently involve 80 recognized species and 15 subspecies (Hammes and Hertel, 2006).

According to Hammes and Hertel (2003), *Lactobacillus* group could be obtained as seven phylogenetic group, i.e. the ***L. buchneri* group**, *L. buchneri*, *L. diolivorans*, *L. ferintoshensis*, *L. fructivorans*, *L. hilgardii*, *L. homohiochii*, *L. kefiri*, *L. kunkeei*, *L. lindneri*, *L. parabuchneri*, *L. parakefiri* and *L. sanfranciscensis*; ***L. delbrueckii* group**, *L. acetotolerans*, *L. acidophilus*, *L. amylolyticus*, *L. amylophilus*, *L. amylovorus*, *L. crispatus*, *L. delbrueckii*, *L. fornicalis*, *L. gallinarum*, *L. gasseri*, *L. hamsteri*, *L. helveticus*, *L. iners*, *L. intestinalis*, *L. jensenii*, *L. johnsonii*, *L. kefiranofaciens*, *L. kefirgranum* and *L. psittaci*; ***L. casei* group**, *L. casei*, *L. manihotivorans*, *L. pantheris*, *L. paracasei*, *L. rhamnosus*, *L. sharpeae* and *L. zeae*; ***L. plantarum* group**, *L.alimentarius*, *L. arizonensis*, *L. collinoides*, *L. farciminis*, *L. kimchii*, *L. malefermentans*, *L. mindensis*, *L. paralimentarius*, *L. paraplantarum*, *L. pentosus*, *L. plantarum* and *L. versmoldensis*; ***L. reuteri* group**, *L. coleohominis*, *L. durianis*, *L. fermentum*, *L. frumenti*, *L. ingluviei*, *L. mucosae*, *L. oris*, *L. panis*, *L. pontis*, *L. reuteri*, *L. suebicus*, *L. thermotolerans*, *L. vaccinostercus* and *L. vaginalis*; ***L. sakei* group**, *L. curvatus*, *L. fuchuensis*, *L. graminis* and *L. sakei*; ***L. salivarus* group**, *L. acidipiscis*, *L. agilis*, *L. algidus*, *L. animalis*, *L. aviarius*, *L. cypricasei*, *L. equi*, *L. mali*, *L. murinus*, *L. nagelii*, *L. ruminis*, *L. salivarus*.

1.7 *Lactobacillus plantarum*

Lactobacillus plantarum is one of the most common species of *Lactobacillus* genus and it is used in food related technologies (Brinques et al., 2010; Sauvageau et al., 2012). This microorganism is facultative heterofermentative (Bove et al., 2012; Siezen RJ and van Hylckama Vlieg JET, 2011) and also it is tolerating of bile salt and considered as a safe microorganism (Brinques et al., 2010).

Lactobacillus plantarum is found microbiota of starchy foods, cereals, meats, dairy products, vegetables, fruits and drinks (Ricciardi et al., 2012). Besides, *Lactobacillus plantarum* is also commonly found in human gastrointestinal tract.

There are a lot of benefits of *Lactobacillus plantarum* namely probiotics in literature (Ningegowda and Gurudutt, 2012; Zago et al., 2011). According to Axling (2012) one of the benefits of *L. plantarum* is that it can affect gut microbiota, lipid metabolism and inflammation as demonstrated in high-fat fed mice. The other example of health benefits of *L. plantarum* PH04 strain is cholesterol lowering effect (Nguyen et al., 2007).

1.8 Physiology of Bile Production

1.8.1 Bile

Bile is a yellow/green aqueous solution of organic and inorganic compounds which includes bile acids, cholesterol, phospholipids (mainly phosphatidylcholine) and the pigment biliverdin (bili: bile, Verdi: green) (Carey and Duane, 1994; Erlinger, 1994; Hofmann, 1999; Johnson, 1998; McPhee and Greenberger, 1987).

Bile is synthesized in the pericentral hepatocytes of the liver, stored and concentrated in the gallbladder and after food intake; the gallbladder is stimulated by the hormone cholecystokinin (Rehfeld, 2004) releasing bile into the duodenum (Figure 1.3). But the gallbladder is not essential for a functioning bile production because some mammals like horse, most deers and birds do not have a gallbladder. These animals secrete bile directly into the small intestine (Kararli, 1995).

Bile is a biological detergent and it is emulsifies and solubilizes lipids so it is important for fat digestion. This detergent property of bile also involves antimicrobial activity through the dissolution of bacterial membranes (Begley et al., 2005a; Begley, et al., 2005b).

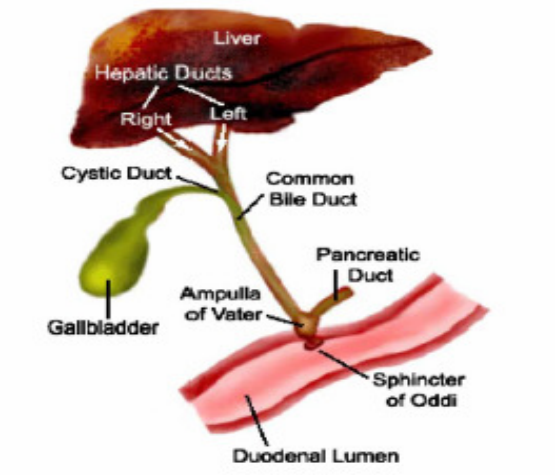


Figure 1.3. This is the biliary system (This figure is reproduced courtesy of the Faculty of Medicine Molson Informatics Project, Mc Gill University, Montreal, Canada).

1.8.2 Cholesterol

Cholesterol is a specialized type of lipid and plays a major role for life (Tok and Aslım, 2007). Cholesterol is the most common steroid in humans and it is responsible for a group of functions such as precursors of bile salts, hormones and vitamin D. Cholesterol is absorbed, or synthesized by the body (Champe and Harvey, 1997).

Cholesterol is a hydrophobic compound and consists of four fused hydrocarbon rings (A, B, C and D). It has an eight-carbon which is branched at C-17 in D ring (Figure 1.4).

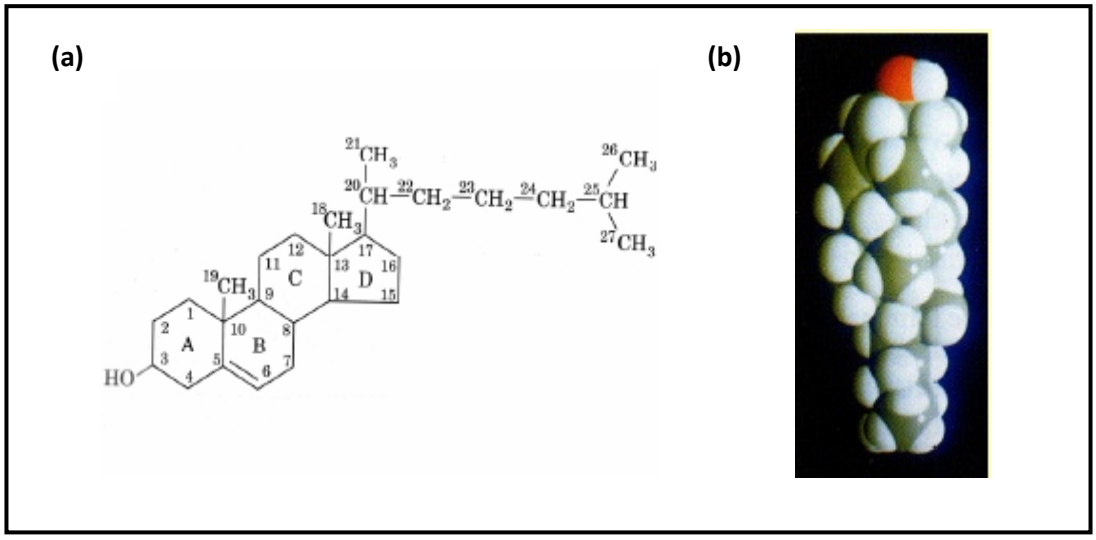


Figure 1.4. (a) Chemical structure of cholesterol **(b)** three dimensional structure of cholesterol (Voet D and Voet JG, 1995).

1.8.3 Bile Acids

1.8.3.1 Biosynthesis, Chemical Structures and Functions of Bile Acids

Bile acids, which are approximately 50% of the organic compenents of bile, are synthesized from cholesterol in the liver via the multienzyme process. Cholesterol is constituted of basically cholic acid and chenocholic acid, called primary bile acids (Figure 1.5a). Generally bile acid substituents are α and β side chains. Alfa side chain makes it hydrophilic beta side makes it hydrophobic. Hence, bile aicid is amphipathic because they have a hydrophilic and hydrophobic side chains. Therefore bile aids can self-associate in water to form micelles and digest fats. (Erlinger, 1994; Hofmann, 1994; Cohen and Carey 1990) (Figure 1.5b).

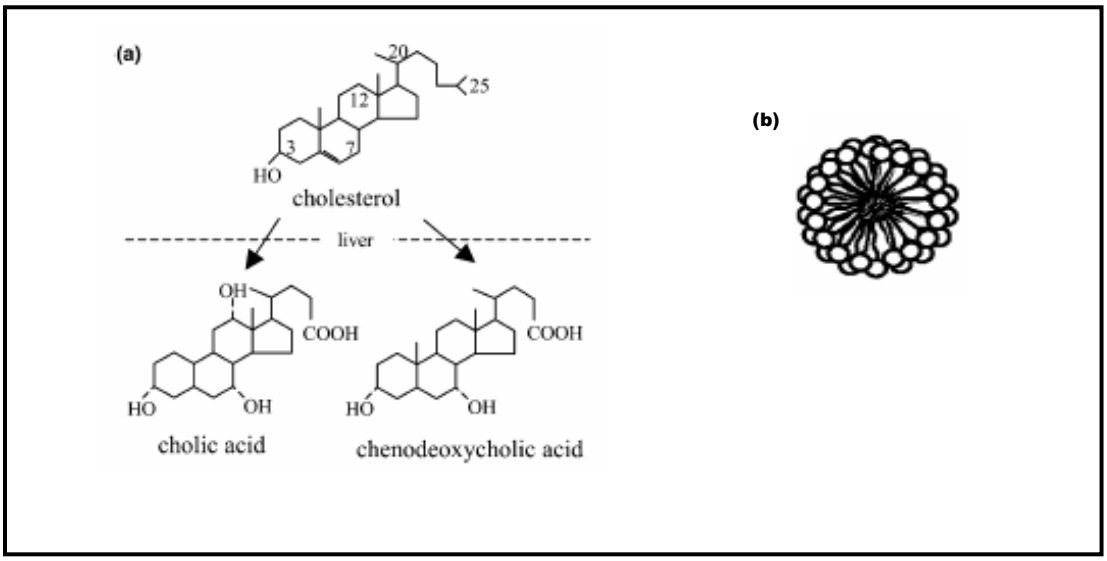


Figure 1.5. **(a)** Chemical structure of bile acids. Cholic acid and chenodeoxycholic acid which are called primary bile acids are synthesized from cholesterol. **(b)** Bile acids are amphipatic so it self-associates and forms micelles. These micelles can solubilize other lipids (Redrawn Carey and Duane, 1994).

Bile acids break down fat globules into minute, microscopic droplets (Hofmann, 1999; Johnson, 1998; Hofmann, 1994). In addition, they act like a detergent and interact with bacterial membrane lipids showing antimicrobial properties on bile.

Correct levels of bile acids are very important. Normally bile acid levels are genetically controlled but several conditions such as acquired defects in bile acid synthesis or transport of abnormal concentrations of bile acids can cause necrosis, apoptosis and also death (Pazzi et al., 1997; Powell et al., 2001). Conversely, decreased bile acid concentration in intestine because of malnourishment or obstructions in enterohepatic circulation or intestinal absorption can cause lipid malabsorption (Kheroua and Belleville 1981; Opleta et al., 1988).

1.8.3.2 Bacterial Alterations of Bile Acids

Three main alterations that affect bile acids are deconjugation, 7α-dehydroxylation and 7α-dehydrogenation. Deconjugation can result from cleavage of the amino acid side chain liberating free bile acids (Christiaens et al., 1992; De Smet et al., 1995; Grill et al., 1995; Lundeen and Savage 1990). Dehydroxylation (removal

of OH group) occurs at the 7 position of nucleus so it is called 7α-dehydroxylation. In this reaction, cholic acid and chenodeoxycholic acid are turned into deoxycholic acid and lithocholic acid respectively (Doerner et al., 1997; Mallonee and Hylemon 1996; Wells and Hylemon 2000).

Bile acid synthesis constitutes of two mechanisms. In the first mechanism, cholic acid and chenodeoxycholic acid, known as primary bile acids, are produced from cholesterol in the liver by de novo synthesis; the second is the production of secondary bile acids. Secondary bile acids occur as a result of modification of primary bile acids by bacterial enzymes in the intestine (Figure 1.6).

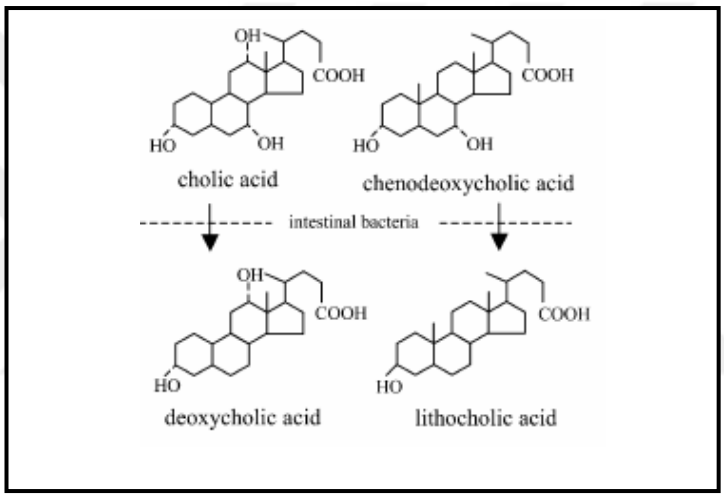


Figure 1.6. This figure represented production of secondary bile acid from primary bile acid (Redrawn Carey and Duane, 1994).

1.8.4 Bile Salts

Bile salts are synthesized from cholesterol and assist in the digestion of fats and also elimination of cholesterol. While they are synthesized from 24 carbon atoms in most vertebrate, in other animals, such as reptiles, elephant and sea cows, bile salts are synthesized more than 24 carbon atoms (Une and Hoshita, 1994).

Bile salts are synthesized by classical and alternate biosynthetic pathways (Agellon, 2002; Russell and Setchell, 1992). The classical pathway is operated entirely in the liver but in the alternate pathway, the first step which is conversion of

cholesterol is carried out in other tissue by oxysterols as intermediate molecules. The last step of bile salt synthesis is always carried out in the liver. The alternate pathway may play an important role in the elimination of excess cholesterol from tissues such as the brain (Bjorkhem and Meaney, 2004). The classical pathway is most important and contains the epimerization of the 3β-hydroxyl group of cholesterol, elimination of the side chain and then saturation and hydroxylation of the steroid nucleus (Agellon, 2002).

Commonly, one or two hydroxyl groups are combined with the bile salts by hydroxylation, the site varying between species. Hydroxylation occurs in one side of steroid nucleus and then occurs in the amphipathic structure which has detergent properties. In humans, bile salts occur from cholic acid (CA) and chenodeoxycholic acid (CDCA) after hydroxylation. Finally, bile salts are formed with the addition of glycine or taurine amino acid (Figure 1.7).

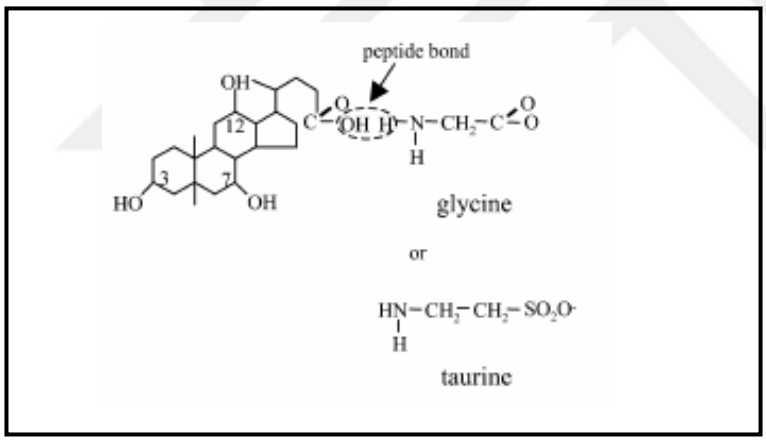


Figure 1.7. Bile acids are combined with glycine or taurine

The type of amino acids in bile salts varies by animal species and diet, in terms of glycine or taurine content. Taurine amino acid is available in meat but not in plants; hence carnivores which eat meat usually produce taurine-conjugated bile salts, whereas herbivores which eat plants produce glycine-conjugated bile salts. Omnivores which eat both meat and plant synthesize both glycine- and taurine- bile salts (Agellon, 2002).

1.9 Enterohepatic Circulation of Bile Acids

Bile acids, which constitute approximately 50% of bile, are synthesized from cholesterol in the liver. Primary bile acids are then conjugated with glycine or amino acid analogue taurine (Hofmann, 1977) and this causes a decrease of the Pk_a to ≈ 5. Hence, conjugated bile acids are fully ionized and may be turned into bile salts (Vlahcevic et al., 1996). Bile salts are secreted actively over the canalicular membrane and then are carried to the gallbladder in bile where they are concentrated in the gallbladder. 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). Bile salts are highly effective detergents and incite solubilization, digestion and absorption of dietary lipids and also lipid soluble vitamins. When bile salts entered the bloodstream, they are complexed to plasma proteins and returned to the liver. Upon reaching the liver, bile salts are cleared efficiently from the circulation by active transporters and then rapidly secreted into bile. This process is called enterohepathic circulation (Figure 1.8) (Vlahcevic et al., 1996).

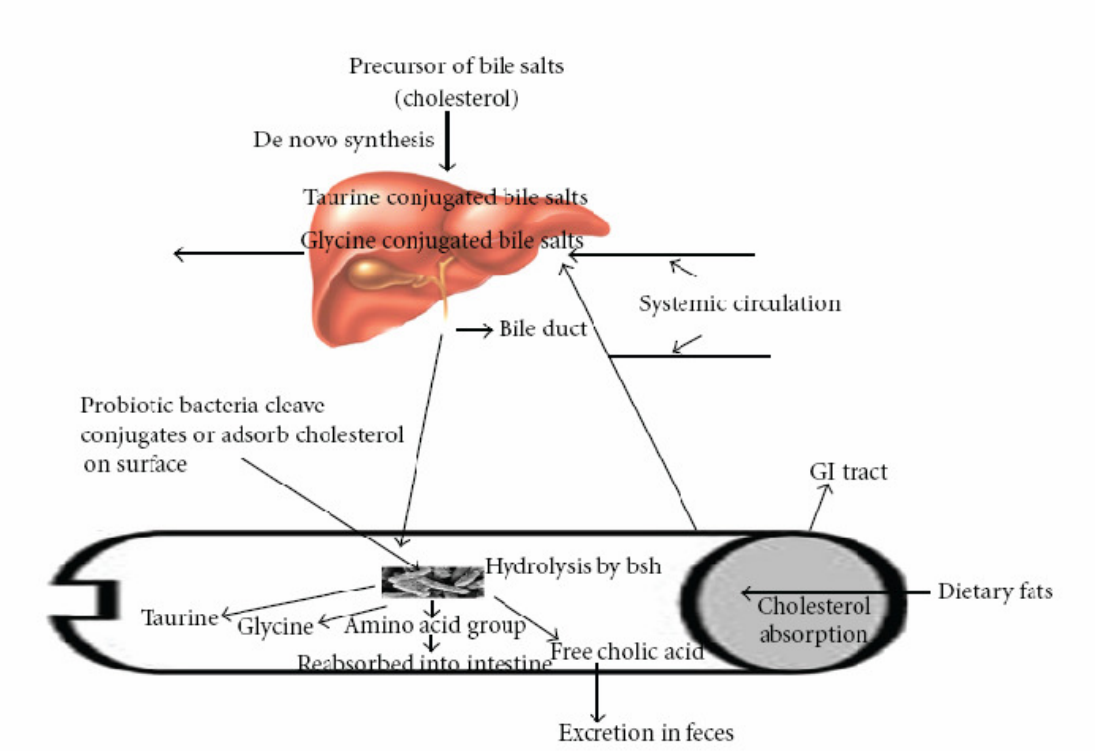


Figure 1.8. Enterohepatic circulation of bile acids (Kumar et al., 2012)

1.10 Bile Salt Deconjugation

1.10.1 Characterization of Bile Salt Hydrolase(s)

Deconjugation means that the conjugate bile salt which is bound with glycine or taurine amino acid, is hydrolyzed at the amide bond between the C-24 positions of the bile acid by bile salt hydrolysis enzymes (BSHs).

Active Site: Bile salt hydrolyses (BSHs) are in the choloylglycine hydrolase family (EC 3.5.1.24) and also contains penicillin amidases. Besides this BSHs have recently been classified as N-terminal nucleophilic (Ntn) hydrolases. Several amino acids such as Cys-1, Asp-20, Tyr-82, Asn-175 and Arg-228 are thought to conserve and also responsible for the catalytic activity (Brannigan et al., 2000; Suresh et al., 1999). For example the thiol (SH) group of Cys-1 is essential for catalysis of BSH. In the *B. longum* Cys-1 amino acid is turned into alanine and as a result BSH is inactivated (Tanaka et al., 2000). In addition oxidize thiol group's agent such as *p*-mercuribenzoate, iodoacetamide, Hg^{2+}, Cu^{2+}, and Cd^{2+} show strong interraction with BSH and inhibition of BSH activity in *Clostridium perfringens* (Gopal-Srivastava and Hylemon P, 1988), *Lactobacillus reuteri* (Taranto and de Valdez, 1999) and *B. longum* (Grill et al., 1995; Tanaka et al., 2000).

Substrate Specificity: BSH can recognize bile acids on both cholate steroid nucleus and also glycine or taurine amino acids. There are several studies concerning substrate specificity. Moser and Savage (2001) revealed that *L. buchneri* JCM1069 showed activity against taurodeoxycholic acid however did not show activity againts to taurocholic acid. These two acids have taurine amino acid but their steroid moieties are different at the 7α position. In addition, the other study related to *L. acidophilus*, inactivation of *bshA* of *L. acidophilus* NCFM reduces the strain's ability to hydrolyze bile salts containing chenodeoxycholic as the steroid moiety, e.g., TCDCA and GCDCA (Mc Auliffe et al., 2005).

1.10.2 Functions of BSH

Although the action of BSH enzymes is not understood exactly, three major hypotheses have been advanced. Firstly, deconjugation may submit a nutritional advantage on hydrolytic strains as carbon, nitrogen and energy sources. While glycine may be metabolized to ammonia and carbon dioxide, taurine metabolized to ammonia, carbon dioxide and also sulphate. Van Eldere (1996) and Huijghebaert (1982) observed that *Clostridia* used released taurine as an electron acceptor. However, according to other studies lactobacilli do not utilize the steroid moiety of bile salts so this function is not universal for BSHs (Gilliland and Speck, 1977; Tannock et al., 1989). Secondly, BSHs facilitate incorporation of cholesterol or bile into bacterial membranes (Dambekodi and Gilliland, 1998; Taranto et al., 2003; Taranto et al., 1997). Finally, one hypothesis claimed that deconjugation of bile salts may be a detoxification mechanism and BSH enzymes may be important in bile tolerance and also in gastrointestinal tract. However, most studies refuted this hypothesis. For example, Taranto et al., (1997) did not find any interaction between bile and bile salts.

1.10.3 Impact of Microbial BSH Activity on the Host

Deconjugation is very important for the physicochemical properties of bile acids. Unconjugated bile acids are less effective in the emulsification of dietary lipids, the formation of micelles, lipid digestion and absorption of fatty acids and monoglycerides than conjugated form of bile acids (De Smet et al., 1994). Effective enterohepatic circulation of bile acids is related to their recognition in the conjugated form in the terminal ileum. Unconjugated bile acids bind with a lower affinity and so may flow into the large intestine. This causes loss of bile salts and so may increase the demand for cholesterol to synthesized bile salts which in turn causes decrease of serum cholesterol levels.

The number of people who were died of various kinds of diseases like cardiovascular illnesses related with hypercholesterolemia is increasing year by year globally. Therefore, nowadays studies which are related of reduction of serum

cholesterol have grown in importance (Bin and Jiang, 2011). Reduction of serum cholesterol has been demonstrated in pigs, guinea pigs and germfree and conventional mice administered with BSH-active bacteria (De Smet et al., 1994; Chikai et al, 1987; De Smet et al., 1998; Du Toit et al., 1998; Pereira et al., 2003; Tannock, 1995). However, some researchers claim that secondary bile acids which are deoxycholic acid and lithocholic acid cause colon cancer in humans (Hill, 1990). In addition deoxycholate is found at higher levels in the serum of patients who have colorectal adenomas than in individuals without adenomas (Bayerdorffer et al., 1994, 1995). BSH activity and secondary metabolites relation have not been understood so far. In order to characterize the functions and structures of BSHs activity, site direct mutageneses as well as crystallography are the reserch techniques that are commonly used.

2. AIMS AND SCOPE OF THE STUDY

Bile salt hydrolase (BSH, Cholyglycine hydrolase, E.C.3.5.1.24) enzyme causes a decrease in serum cholesterol level by deconjugation of bile acid conjugated with glycine or taurine into bile acid and the amino acid residue. Apart from this function of BSH, extreme bile salt deconjugation can have pathological effects as malabsorption of lipids and lipid soluble vitamins in the small bowel (Marteau et al., 1995). In addition to this, deconjugation and 7α-dehydroxylation have a role in the formation of gall stones (Dowling et al., 1990) and also increases the risk of colon cancer in the large intestine (Marteau et al., 1995).

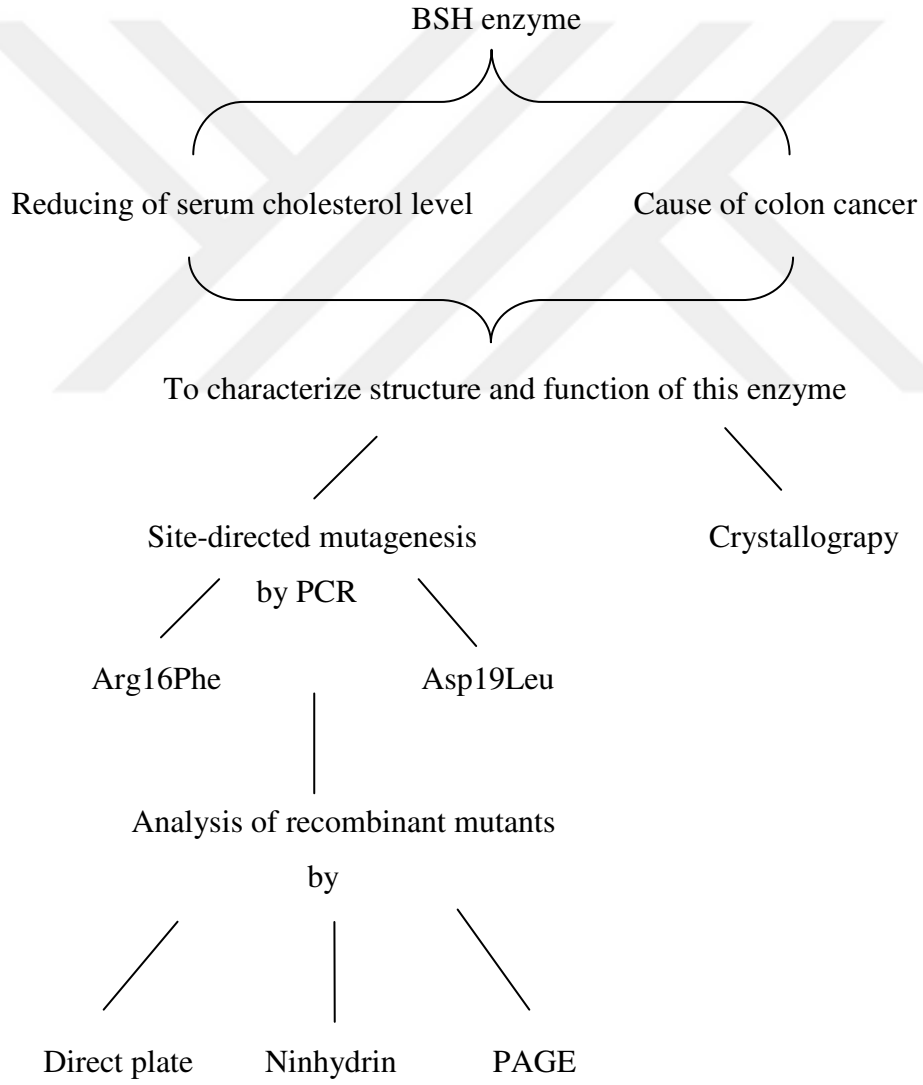
BSH activity is accepted as criteria for probiotic strain selection and provides colonization of bacteria in the intestinal system (Begley et al., 2006). BSH enzyme catalyzes the hydrolysis of glycine and/or taurine-conjugated bile salts into amino acid residues and provides decreasing of the high level cholesterol. Therefore understanding of BSH enzyme's function and structure in the *Lactobacillus* and *Bifidobacterium* are commonly used as probiotics in the dairy industry is essential. In literature, there are a lot of studies about deconjugation of BSH enzymes but there is just one site directed mutagenesis work related with function and structure of the BSH enzyme. This is the first report of BSH enzyme characterization by substitution of two conserved amino acids supposed to be responsible for catalytic activity of BSH enzyme in *L. plantarum*.

Some intestinal bacteria have an excessive deconjugation of tauro-conjugated bile salts and production of secondary bile acid having potential harmful side effects. While BSH enzyme is associated with reduction of serum cholesterol level on the other hand some BSH enzymes are associated with colon cancer. However, the catalytic mechanism and substrate preference of such an important BSH is unclear.

Assosiation of BSH enzyme with reduction of serum cholesterol level and colon cancer has led to increase intrest of this enzyme. To better understand BSH enzyme further functional studies such as site–directed mutagenesis studies are

needed. The first purpose of this study is to substitute strictly conserved Arg-16 and Asp-19 amino acids, thought to be responsible for the catalytic activity or stability of BSH enzyme, for Phe-16 and Leu-19 amino acids by site directed mutagenesis partly to understand function and structure of this enzyme. The second purpose of this stuy is to analyse mutant BSH enzymes in respect to their activities by direct plate assay using LB agar medium containing two common GDCA and TDCA bile salts and ninhydrin assays. The third purpose of this study is to analyse mutant BSH enzymes with respect to their stability by SDS-PAGE.

Shematic summary of this research



3. MATERIALS AND METHODS

3.1 Construction of pCON1R16F Mutant

3.1.1 Site Directed Mutagenesis of Arginine by PCR

For R16F mutation, right orianted pCON1 (*bsh*1/pBluescript and not puplished) were isolated by GeneJET^{TM} Plazmit Miniprep Kit (#K0503 Fermantas, Europe) and pCON1 was used as a template DNA during experiment. The codon of the sixteenth aminoacid of *bsh* gene coding arginine was substitiuted for the codon coding fenilalanine amino acid by PCR based site direct mutagenesis (Stratagene) protocol by PCR (Techne TC-3000). The PCR reaction was proceeded in the 50 µl reaction solution containing 25mM $MgCl_2$, 5mM dNTP, 25µM F and R primers and 2.5u/µl Pfu DNA polymerase enzyme (Fermentas, Europe) (Table 3.2). Pfu DNA polymerase enzyme had proofreading activity thereby it was decreased of risk of second-site mutations (Flaman et al., 1994; Cline et al., 1996). F and R primers were desiged for apropriate R16F mutations (Table 3.1). After PCR cycling (Table 3.3), 10 µl was kept for analysis on agarose gel and the rest of PCR samples was treated with *Dpn*I (Fermentas) at 37°C for 2 hours. Then samples were loaded on %0.7 agarose gel and separated by electrophoresis at 75 volt for 1 hour.

Table 3.1. List of the primers used for Arginine Mutation

Primer name	Sequence (5'–3')	Tm
R16F/ F	5'- TAATTACTTCGGTTTTAATTTCGATTATG-3'	54°C
R16F/ R	5'- CATAATCGAAATTAAAACCGAAGTAATTA-3'	54°C

Table 3.2. PCR reaction conditions for mutation

Reactants	Reactions
Template DNA (135ng/µl)	0.75 µl
$MgCl_2$ (25Mm)	4 µl
dNTP mix (5Mm)	2 µl
Primer Forward (25µM)	2 µl
Primer Reverse (25µM)	2 µl
Buffer (10X *Pfu* polymerase buffer)	5 µl
Pfu polymerase (2.5u/µl)	1 µl
ddH_2O	33.25 µl
Total reaction volume	50 µl

Table 3.3. PCR reaction conditions for R16F mutation

Cycle	Temperature	Time
Initial denaturation	95°C	30 sec
Denaturation	95°C	1 min
Annealing	50°C	1 min
Extension	72°C	9min
Final extension	72°C	5 min
Number of cycles	30	

Table 3.4. Bacterial strains and plasmid used in this study

Plasmid	Genotype	Phenotype	Reference
pBluescript	SKII+	Amp^r	Stratagene
pCON1	pBluescript SKII+ with 1.0 kb *bsh* gene of *Lactobacillus plantarum*	Amp^r	Previous study
pCON1R16F	Substitution of Arginine to Fenilalanine in pCON1		This study
pCON1D19L	Substitution of Aspartic acid to Leucine in pCON1		This study
pET22b+		Amp^r	Novagene
pKUHR16F	Substitution of Arginine to Fenilalanine in pET22b		This study
pKUHD19L	Substitution of Aspartic acid to Leucine in pET22b		This study
Escherichia coli XL1-Blue	recA end A 1 gyr A986 thi-1hsdr 17supE44 rel A1 lac		Stratagene
Escherichia coli BLR(DE3)	E. coli B F– dcm ompT hsdS (rB – mB –) gal λ(DE3)		Stratagene

3.1.2 Purification of PCR Products

PCR products of pCON1R16F were loaded on %1 agarose gel (Sigma, USA) for PCR amplicon purification. PCR samples were separated by electrophorases at 75 volt for 1 hour and then purified by using GeneJET^{TM} Gel Extraction Kit (#K0691 Fermantas, Europe) according to their protocols.

3.1.3 Preparetion of BLR (DE3) and XLI-Blue Competent Cell

3 ml LB (Luria-Broth) medium was inoculated with XLI-Blue and BLR (DE3) cells and grown at 175 rpm for 14-16 hours. After overnight growth, %1 of the grown cells were transfered to 50 ml LB medium and incubated at 37°C for 2-2,5 hours when the culters's value was riched to 0.04-0.05 at $OD_{600.}$ These cultures were taken on the ice and seperated to 20 ml cultures in the sterile falcon tubes. They were centrifugated at 4000 rpm and 4°C for 5 minutes, then pellets were resuspended in 10 ml $CaCl_2$ (100mM, pH: 7.0) and centrifugated at 4000 rpm and 4°C for 10 minutes. Pellets were resuspended with 1ml $CaCl_2$ containing %10 glycerol and then alaqueted in100 µl cultures in the eppendorf tubes and then stored in -80°C.

3.1.4 Transformation of pCON1*R16F DNAs into *E. coli* XLI-Blue strains

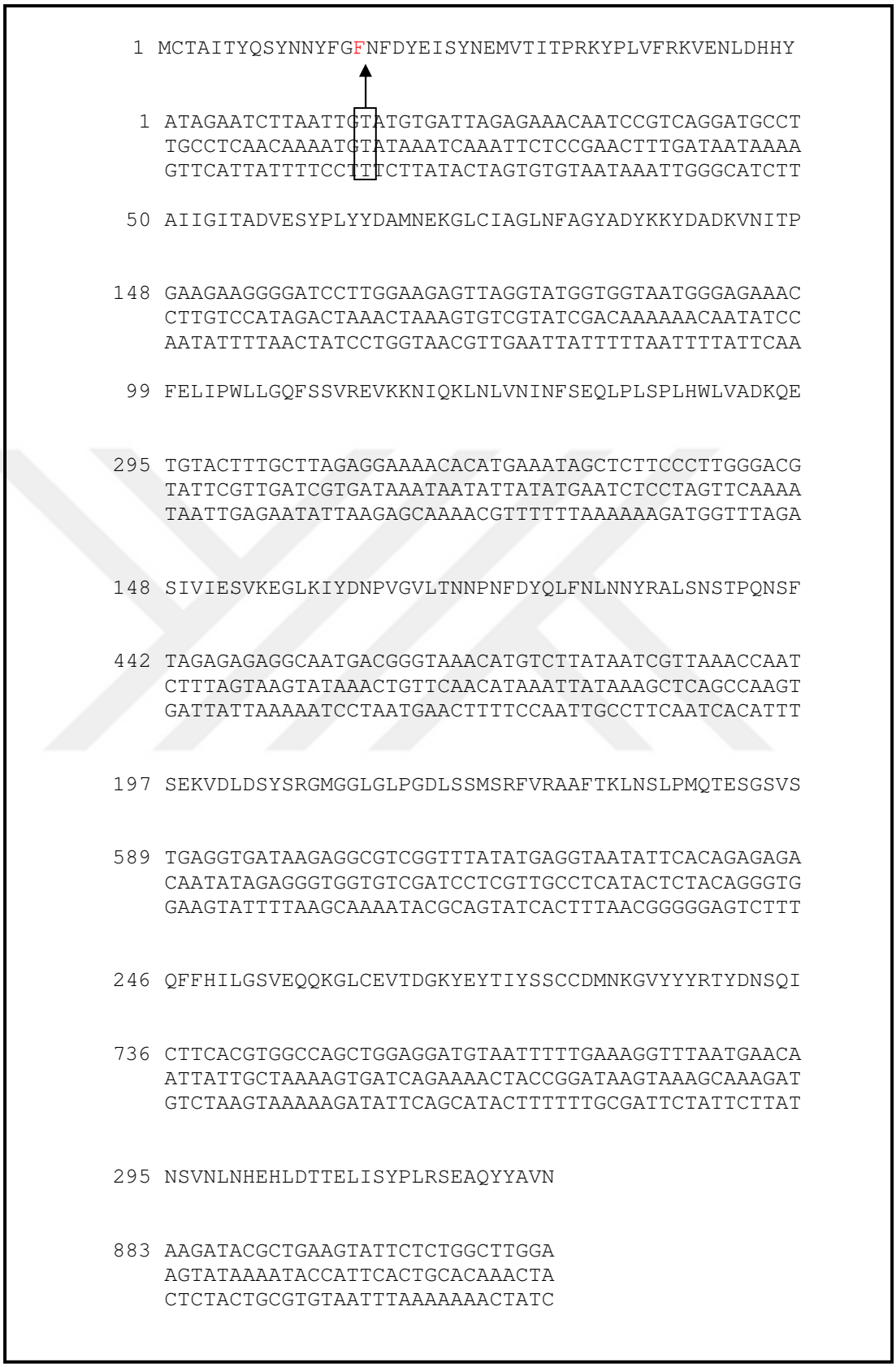
5 µl gel purified PCR amplicons were transfered the 100 µl XLI-Blue competent cells. These samples were incubated on ice for 45 minutes. Then samples were incubated at 37°C for 5 minutes. Finally samples were transferred on ice for 5 minutes again. 1 ml LB (Luria-Broth) was added to each sample and incubated at 37°C in shaker for 1 hour. Then samples were centrifugeted at 6000 rpm for 3 minutes. After centrifugation supernatants were discarded and pellets were dissolved with 100 µl LB (Luria-Broth). Samples were spreaded on LB Agar plate containing Xgal, IPTG and apropriate amphicillin (25mg/ml final concentration) and incubated at 37°C for 16-18 hours (Davis et al., 1988). Four different colonies grown on plates

were selected randomly and plasmid DNAs was isolated by Plazmit Miniprep Kit (Fermantas) for the sequencing of the *bsh* gene to detect the desired R16F mutation. At the same time plasmid DNAs were also digested with *Xho*I to linear and test whether isolated clone is right clone or not by agarose gel electrophorases.

3.1.5 Sequencing of R16F *bsh* gene of *Lactobacillus plantarum* and Sequence Analysis

To detect the desired mutation (XLI-Blue/pCON1*R16F) first DNAs were isolated from first selected clone and then they were sequenced by Macrogen (Korea) using universal primers, M13/pUC Forward (5'-GTAAAACGACGGCCAGT'; 17-bp) and M13/pUC Reverse (5'- CAGGAAACAGCTATGAC-3'; 17-bp). Amino acid sequences of pCON1/R16F were obtained by Bioinformatics Tools for Sequence Translation<EMBL-EBI program and nucleotide sequence and amino acid sequence of pCON1/R16F were aligment by Pairwise Sequence Aligment Tools<EMBL-EBI (Table 3.5). The clone containing desired mutation (R16F) was designated as pCON1R16F.

Table 3.5. Nucleotide and amino acid sequence *of bsh1*R16F mutant from *Lb. plantarum*



3.2 Construction of pKUHR16F

3.2.1 Plasmid DNA Isolation

The day before cloning of mutant *bsh*R16F genes into expression vector (pET22b), pET22b was inoculated in 10 ml LB (Luria-Broth) suplemented with amphicillin (25 mg/ml final concentration) because pET22b was low copy plasmid vector and incubated at 37°C and 175 rpm during 14-16 hours. After 14-16 hours, samples were taken from the shaker and centrifugated at 4000 rpm for 4 minutes. Then the supernatant was poured of and pellet was dissolved in 1 ml (1000 µl) LB (Luria-Broth) and then was centrifugeted again at 5000-6000 rpm for 3 minutes. After this step, supernatant was poured and pellet was dissolved with 250 µl Resuspention solution (at 4°C), then 250 µl Lysis solution was added and inverted 5-6 times. 350 µl Neutralization solutions were added and inverted 5-6 times and then centrifugated at 11000 rpm for 5 minutes. Supernatant was taken and poured in colon and then centrifugated at 11000 rpm for 1 minute. Colon was washed twice with 500 µl wash solution and centrifugated at 11000 rpm for 1 minute. Then centrifugation was made again at 11000 rpm for 1 minute to remove the alcohol from the sample. Then plazmid DNA trapped at colon was eluted via the elution buffer.

3.2.2 Digestion of pCON1R16F Mutant and pET22b Vector via the Restriction Endonucleases Enzymes

The pET22b expression and pCON1/pBluescriptR16F clones containing desired mutation were cleaved with the same enzymes, *Eco*RI (Thermo Scientific) and *Not* I (Thermo Scientific) RE, at 37°C for 1-to 3 hours (Figure 3.1 and Figure 3.2). The fragmnets of digested sampes were separated by agarose gel electrophoreses and then *bsh* mutant genes (***bsh*R16F)** and pET22b vector were purified from the gel by Vivantis Ambiclean Kit according according to its protocol.

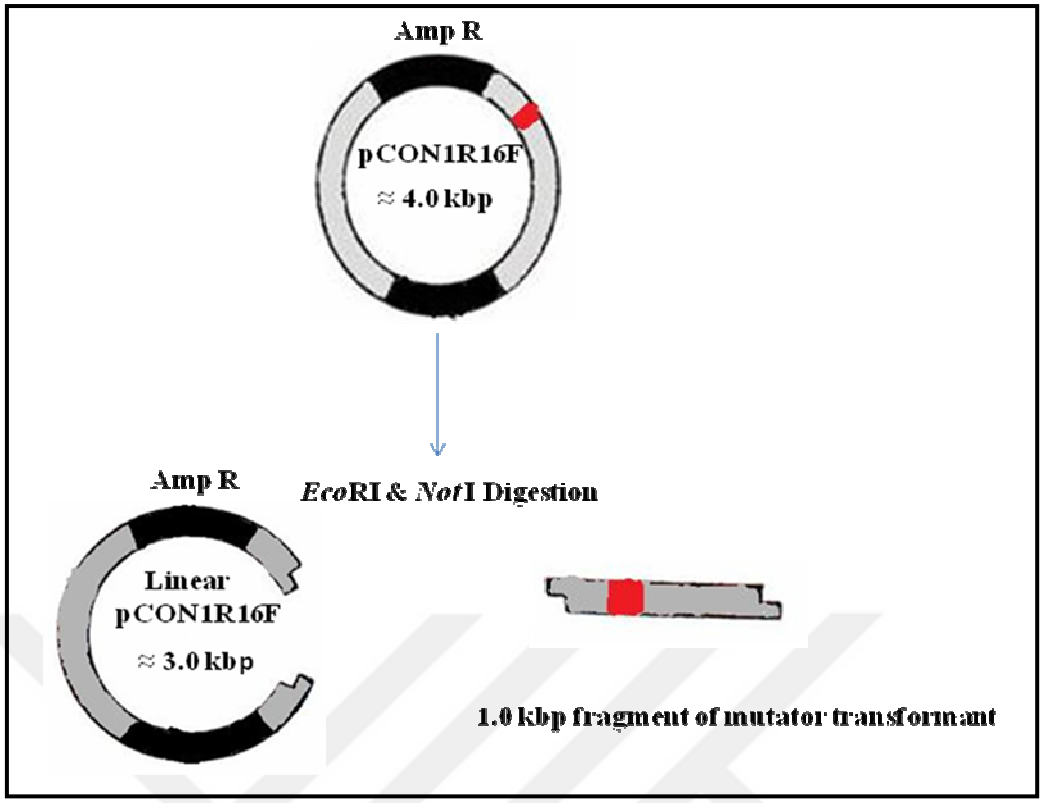


Figure 3.1. Digestion of pCON1R16F with *Eco*RI and *Not*I REs

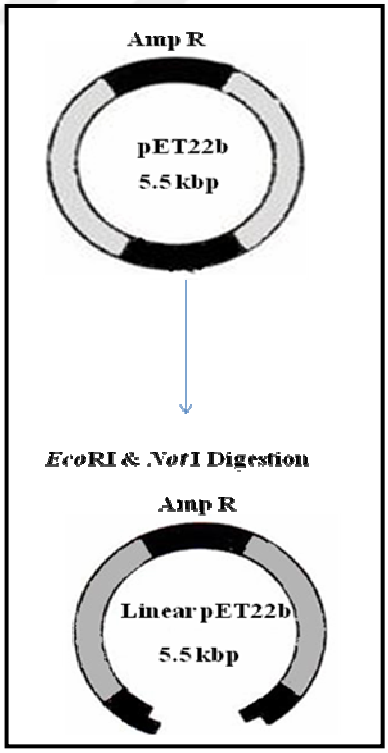


Figure 3.2. Digestion of cloning/expression vector with *Eco*RI and *Not*I REs

3.2.3 Ligation of *bsh*R16F Mutant DNA and pET22b Vector

Almost 1.0 kbp insert mutant *bsh* DNA and vector (pET22b) were ligated at 22°C for 1 hour. During the ligation reaction, T4 DNA ligase enzyme and buffer were used for attachment of the vector and insert DNA ends. The vector-insert ratio to be used in ligation reaction is either 3/1 or 4/1. The amount of the insert and vector DNAs were determined by using following aquation.

$$\text{Insert mass ng} = \text{ratio X } \frac{\text{insert length in kb}}{\text{vector length in kb}} \text{ X vector mass in ng}$$

Table 3.6. Ligation reaction of *bsh*1R16F and pET 22b DNAs

Substances	Reaction
DNA (insert)	3 µl
pET22b (*Eco*R I & *Not* I)	4 µl
Buffer (T4 ligase 10X)	2 µl
T4 DNA ligase	1 µl
ddH_2O	10 µl
Total volume	20 µl

3.2.4 Transformation of pCON1R16F DNAs into *E. coli* BLR(DE3) Strains

10 µl of ligation reaction was transfered to 100 µl BLR (DE3) competent cell. These samples were incubated on ice during 45 minutes and then incubated again on ice for 5 minutes after at 37°C during 5 minutes. Samples were transfered to 1ml LB (Luria-Broth) mediun and inoculated at shaker for 1 hour. After 1 hour, samples were taken and centrifugated at 6000 rpm for 3 minutes. Pellets were resuspended with 100 µl LB culture. Finally, samples were spreaded at LB Agar which was included 25 mg/ml amphicillin and incubated at 37°C overnight. After incubation, four colonies were selected and isolated by Thermo Scientific GeneJET Plasmid Miniprep Kit #K0503 then samples were separated on the %0.7 (Prona) at 75 volt for 1 hour. The clone containing desired mutation was called pKUHR16F (Figure 3.3).

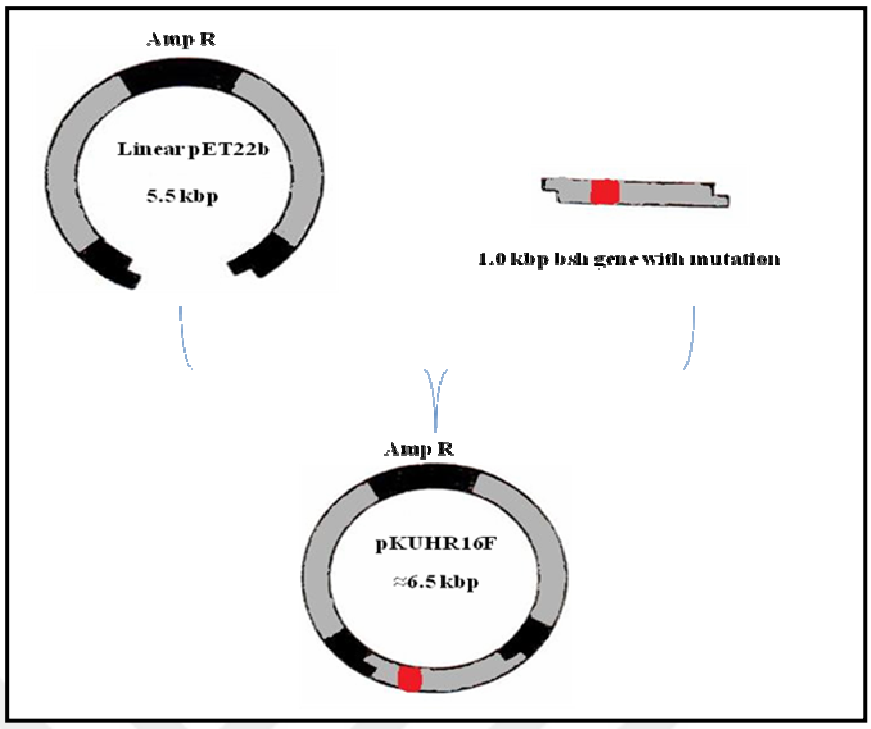


Figure 3.3. Ligation of pET22b cloning/expression vector and mutant *bsh*1R16F gene, and transformation of it into BLR (DE3) competent cell.

3.3 Construction of pCON1D19L Mutant

3.3.1 Site Directed Mutagenesis of Aspartate by PCR

For D19L which was the other mutation, pCON1 DNAs were used as a template DNA. Aspartate which was 19 th substituent of bsh gene was replaced as leucine amino acid using site directed mutagenesis (Stratagene) method by PCR (Techne TC-3000). At this method, 25mM $MgCl_2$, 5mM dNTP, 25µM F and R aproppriate primers for D19L mutation (Table 3.7) and 2.5u/µl Pfu DNA polymerase enzyme (Fermentas, Europe) were used. PCR reactions for D19L mutations were prepared as defined Table 3.2 and their conditions were shown in Table 3.8.

After PCR reactions, 10µl samples were seperated to analysis PCR reactions on agarose gel the rest of samples (approximately 40µl) were digested with *Dpn* I RE enzymes and were loaded on % 0.7 agarose gel to visualized.

Table 3.7. List of the primers used for Aspartate Mutation

Primer name	Sequence (5'–3')	Tm
D19L/F	5'- CGGTAGAAATTTCCTATATGAAATTTCATAC-3'	56°C
D19L/R	5'- GTATGAAATTTCATATAGGAAATTTCTACCG-3'	56°C

Table 3.8. PCR reaction conditions for D19L mutation

Cycle	Temperature	Time
Initial denaturation	95℃	30 sec
Denaturation	95°C	1 min
Annealing	51-53°C	1 min
Extension	72°C	9min
Final extension	72°C	5 min
Number of cycles	30	

3.3.2 Purification of PCR Products

To purification of pCON1/D19L PCR products, Vivantis Ambiclean Kit and GeneJET^{TM} Gel Extraction Kit (#K0691 Fermantas, Europe) were used according to their protocols.

3.3.3 Transformation of pCON1*D19L DNAs into *E. coli* XLI-Blue strains

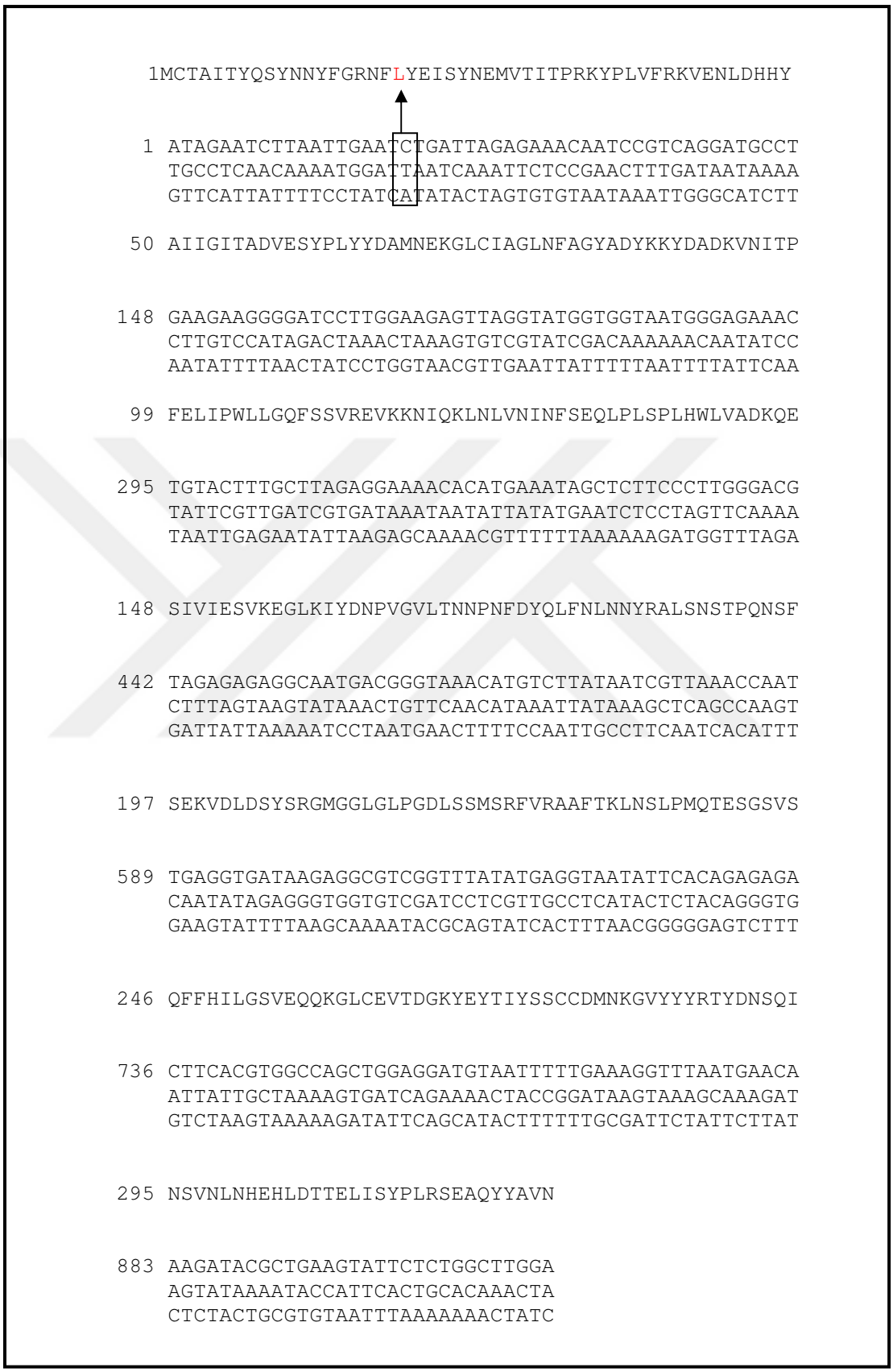
For transformation, 15 µl of purified PCR product and 100 µl XLI- Blue competent cell were incubated on ice for 45 minutes. Then heat shock was applied at 37°C for 5 minutes and then samples were incubated again on ice during 5 minutes. After this incubations, samples which were purified PCR product and competent cell mixture were transfered in 1000 µl LB broth and waited at 175 rpm for 1 minute shaking. After shaking, samples were transferred to eppendorf and centrifugated at 6000 rpm for 3 minutes. Finally, supernatant was discarded and pellet was dissolved in 100 µl LB broth. Dissolved samples were throwed on LB Agar plate containing

Xgal, IPTG and apropriate amphicillin (25mg/ml final concentration) and waited by colonies were grown (approximately 16-18 hours). After growing, four different colonies were selected to detect desired D19L mutation and were isolated and digested as described in section 3.1.4.

3.3.4 Sequencing of D19L *bsh* gene of *Lb. plantarum* and Sequence Analysis

After isolation and digestion, to analyze whether mutation was happen or not samples were sequenced by Macrogen (Korea) using universal primers as followed in section 3.1.5. After sequencing, if sample was desired mutation, it was called pCON1D19L. Nucleotide and amino acid sequencing of pCON1D19L were shown in Table 3.9.

Table 3.9. Nucleotide and amino acid sequence *of bsh1*D19L mutant from *Lb. plantarum*



3.4 Construction of pKUHD19L

After sequencing, desired mutations were called pCON1D19L. Desired mutations and related vector (pET 22b) were isolated by GeneJET^{TM} Plazmit Miniprep Kit (#K0503 Fermantas, Europe) and digested with *Eco*RI (Thermo Scientific) and *Not* I (Thermo Scientific) RE at 37°C for 1 hour (Figure 3.4 and Figure 3.5).

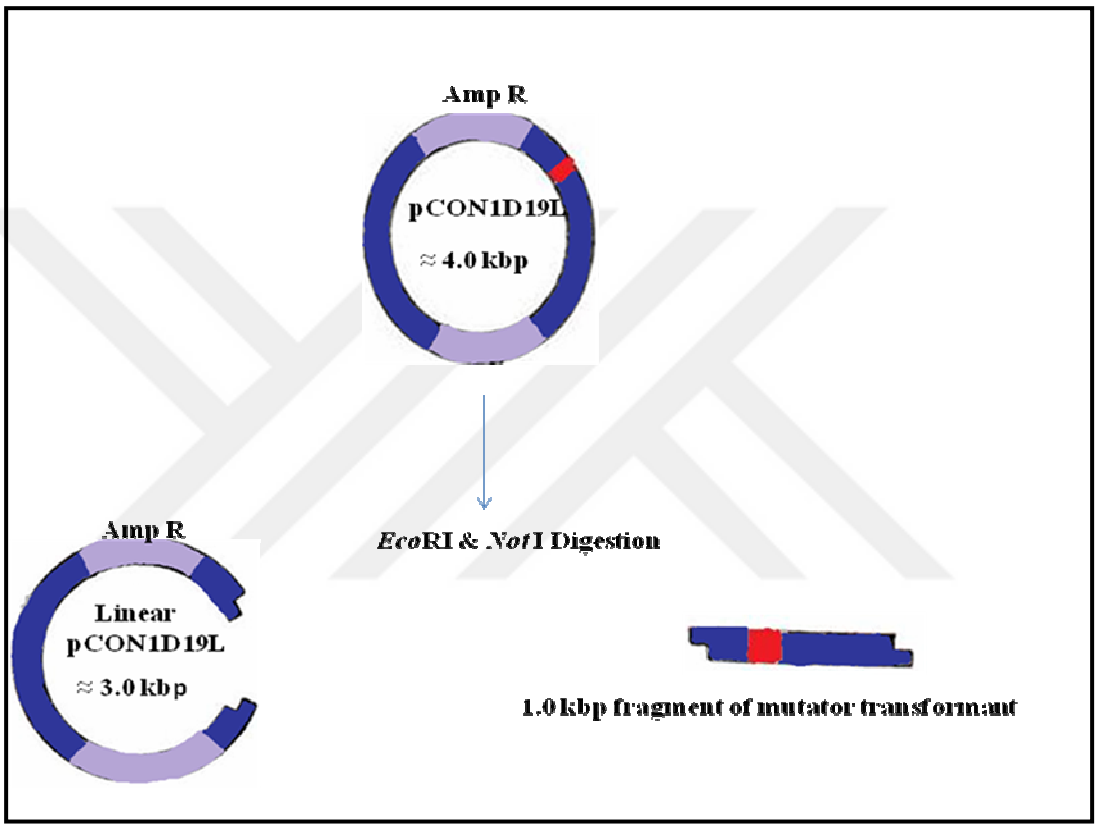


Figure 3.4. Digestion of pCON1D19L with *Eco*RI and *Not*I REs

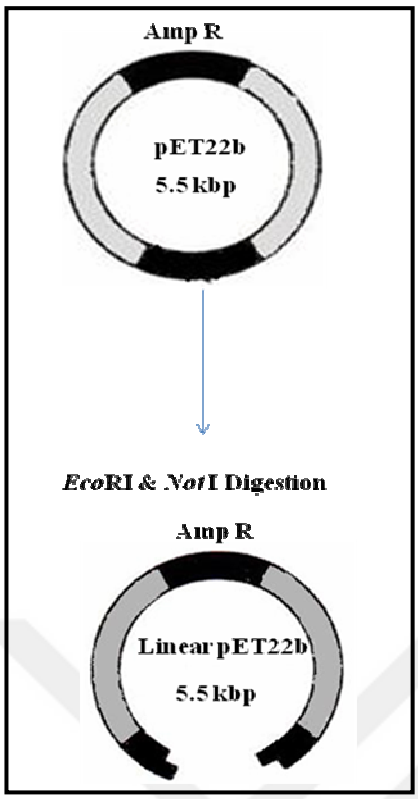


Figure 3.5. Digestion of cloning/expression vector with *Eco*RI and *Not* I REs

Resulting digestion, pCON1D19L and pET 22b linear fragments were purified from agarose gel by using Vivantis Ambiclean Kit. The 1.0 kbp mutated fragment and purified linear vector were ligated at 22°C during 1 hour. Their ligation conditions were shown in Table 3.10. 10 µl of ligation reaction was transformed into BLR(DE3) competent cell and plates on LB agar supplemented with amphicillin and four different colonies were selected randomly. After these four colonies were isolated and desired mutation were called pKUHD19L (Figure 3.8).

Table 3.10. Ligation reactions of *bsh*1D19L and pET 22b DNAs

Substances	Reaction
*bsh*1D19L (*Eco*R I & *Not* I) (insert)	1 µl
pET 22b (*Eco*R I & *Not* I)	4 µl
Buffer (T4 ligase 10X)	2 µl
T4 DNA ligase	1 µl
ddH_2O	12 µl
Total volume	20 µl

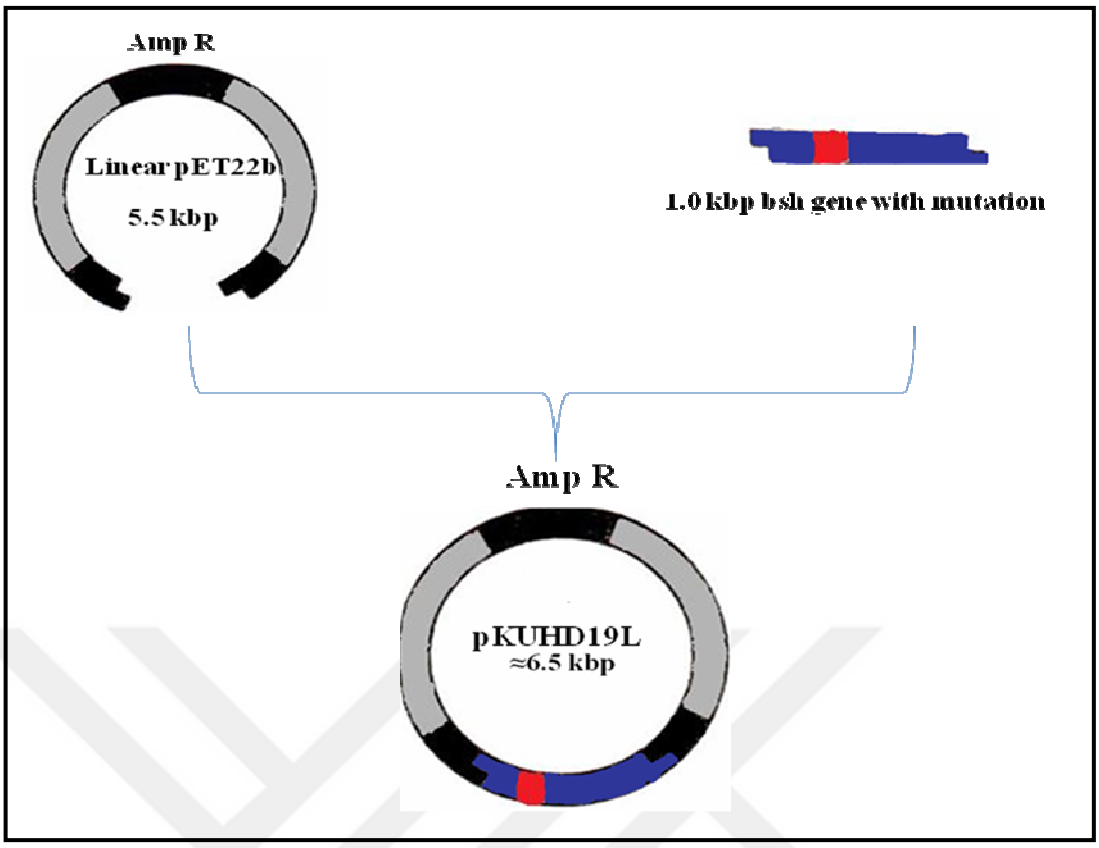


Figure 3.6. Ligation of pET22b cloning/expression vector and mutant *bsh*1D19L gene, and transformation of it into BLR (DE3) competent cell.

3.5 Detection of BSH Activities of pKUHR16F and pKUHD19L in *E. coli* by Direct Plate Assay

To determine pKUHR16F/BlR(DE3) and pKUHD19L/BlR(DE3) activities by using direct plate assay, these two mutant clones were grown in Luria-Bertani (LB) agar which was suplemented with 5 g/lt of taurodeoxycholic acid, 2 mM glycodeoxycholic acid and 0.035% (wt/vol) $CaCl_2.2H_2O$ and 1 mM IPTG (Christiaens et al., 1992) (pH: 6.5) at 37°C for 72 h.

3.6 Determination of BSH Activies of pKUHR16F and pKUHD19L by Ninhydrin Assay

3.6.1 Preparation of *bsh* Cell Extract

Two clones containing our desired mutation on bsh genes, pKUH/pET22bR16F and pKUH/pET22bD19L, were inoculated to 10 ml LB broth supplemented with amphicillin and grown at 37°C with vigorous shaking (175 rpm) for 16-18 hours. 5 ml culture from first inoculation was transfered to 250 ml LB broth including amphicillin and %0.4 glycerol then samples were grown at 175 rpm and 37°C until sample's OD_{600} would reach to 0.5 value. Upon samples were reached to 0.5 value 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induced and grown until sample's OD_{600} would reach to 1.5-3.0 value.

50 ml of these cultures were transformed to falcon tube and centrifugeted at 8000g and 4°C for 15 minutes. After centrifugation every pellet were dissolved in 2 ml of resuspention buffer at pH: 7.49 and then concentrated in the same tubes. After that, samples were centrifugated at 13520g and 4°C for 20 minutes and resuspended in 12 ml resuspention buffer pH: 7.49. Samples were sonicated for 30 minutes (%50 cycle, 60 power) with constant cooling in ice and then centrifugated at 13520g and 4°C for 20 minutes. Supernatants were kept at -20°C.

Protein concentration of cell-free exract was determined by Lowry method using Bovine Serum Albumin (BSA) as a standard protein.

3.6.2 Ninhydrin Assay

To determine of *bsh* activity, ninhidrin assay is one of these methods. In this methods, 10 µl appropriate diluted of cell-free extract, 50 µl (40mM) Dithiothreitol (DTT), 100 µl sodium phosphate buffer (0.1 M, pH: 6.0) and 40 mM one of the six different bile salts (glycocholic acid (GCA), glycodeoxycholic acid (GDCA)

glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA)) for each reaction were used. These mixtures were incubated at 37°C for 30 minutes. Incubation reactions were terminated by adding 210 µl of trichloroacetic acid (TCA) (%15 wt vol^{-1}) and centrifugated at 14000 rpm for 15 minutes.

After centrifugation, 20 µl of supernatant was diluted with 80 µl of dH_2O. Then 1.9 ml of ninhidrin reagent (0.5 ml of %1 ninhidrin in 0.5 M sodium citrate buffer, pH: 5.5), 1.2 ml glycerol and 0.2 ml of 0.5 M sodium citrate buffer (pH: 5.5) was added in the mixture and then samples were vortexed and boiled for 14 minutes. Finally samples were measured the absorbance at 570 nm after cooling by using glycine as a standards. One unit of *bsh* activity was described as amount of protein which was released 1 µmol of amino acid from substrate every minutes.

3.7 Purification of mutant *bsh* Enzymes

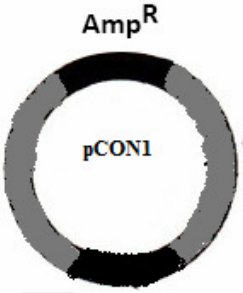
To purify mutant *bsh* enzymes, pKUHpET22bR16F and pKUHpET22bD19L, they were grown at the almost same conditions at which the cell extracts were prepared but some modifications. Samples were transferred to in 100 ml medium including amphicillin and %0.4 glycerol for second inoculation and samples were grown until they would reach to OD_{600} 0.5. When OD_{600} of the samples reached to 0.5, 0.3 mM IPTG was added to induce mutant *bsh* gene expression and grown until their OD_{600} would reach to 1.5-3.0.

The samples grown to OD_{600} 1.5-3.0 were centrifugated at 4°C and 8000g for 15 minutes and then pellet was dissolved in 4 ml of binding buffer at pH: 7.8 (20 mM sodium phosphate, 500 mM NaCl). Then lysozyme was added (final concentration of sample is 1mg/ml) and incubated on ice for 30 minutes. After incubation, these mixtures were incubated again on a rocking platform for 10 minutes at 4°C. During this step, tween-20, DNase and RNase were added in the mixture respectively (final concentrations of %1, 5 µg/ml and 5 µg/ml). Incubation was continued on a rocking for 10 minutes at 4°C. Finally, insoluble debris was removed by centrifugation at 13520g and at 4°C for 20 minutes. Supernatants of

transformants were purified by AKTA Prime Plus Chromotograpy System with Ni^{+2} chelated columns using Affinity Purification any Hi Trap Chelating Program.

Purified samples were mixed with loading dye and 1.5 µl β-mercaptoethanol and incubated at 37°C for 15 minutes. Then samples were loaded on %12 polyacrylamide gel (PAGE) (Laemmli, 1970) and electrophoresed at 120 volt for three hours. To visualize mutant *bsh* enzymes, gels were stained with Coomassie Brilliant Blue G 250 Stain for 15-20 minutes with vigorous shaking then dye was removed form gels for overnight.

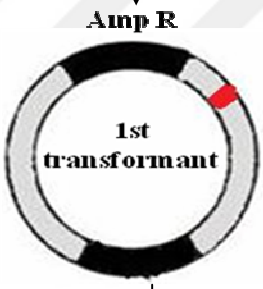
Starting with pCON1 construct



R16F mutation of *bsh* gene

Site directed mutagenesis

D19L mutation of *bsh* gene

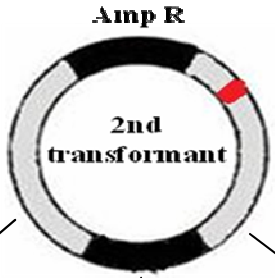


Detection of the target mutants by DNA sequencing

Digestion of vector DNAs with *Eco*RI and *Not*I REs and

insertion of mutants into pET22b expression vector

Transformation recombinant DNAs in to *E. Coli* BLR(DE3) strain



Direct plate assay Ninhydrin assay SDS-PAGE

4. RESULT

4.1 Construction of pCON1R16F Mutant

4.1.1 Site Directed Mutagenesis of Arginine by PCR

To make a mutation on arginine by PCR based site direct mutagenesis, pCON1 DNAs were used as a template. Sixteenth Arginine amino acid of BSH enzyme on pCON1 (*bsh*1/pbluescript) was substituted for Phenylalanine amino acid by using appropriate primers. After site direct mutagenesis, 10 µl samples were kept for the analysis and the rest of the sample was treated with *Dpn*I RE enzyme which cleaves the methylated DNA. To evaluate PCR products, pCON1 template DNA was also cleaved with *Xho*I RE enzyme. After this treatment 10 µl of sample of each mutation and samples treated with *Dpn*I were loaded on %0.7 agarose gels to evaluate *Dpn*I RE enzyme worked or not (Figure 4.1). After these treatment if all template DNA was cleaved by *Dpn*I RE enzyme, samples of each mutation were purified by GeneJET™ Gel Extraction Kit (#K0691 Fermantas, Europe) (Figure 4.2).

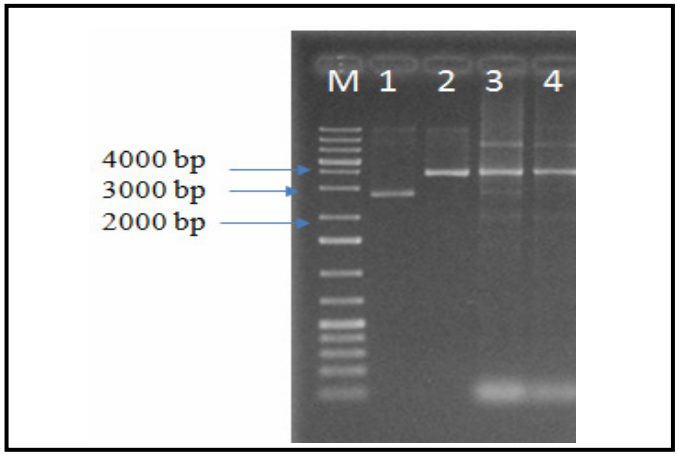


Figure 4.1. Preparation of R16F mutant by PCR based site directed mutagenesis. Line 1 shows pCON1 template DNA; Line 2 shows *Xho*I diested linear pCON1 template DNA; Line 3 shows pCON1*R16F PCR product and Line 4 shows pCON1*R16F incubated with *Dpn*I RE, Marker is represented by M.

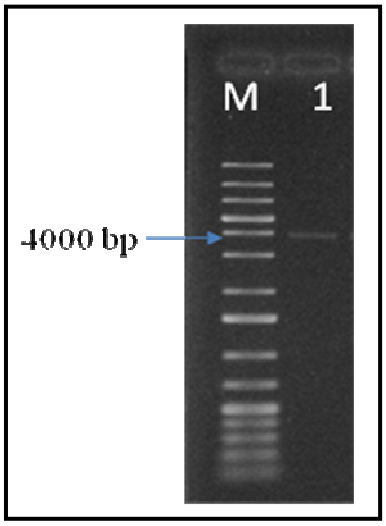


Figure 4.2. Purification of PCR Product. Line 1 represents pCON1*R16F DNA and M represents marker.

4.1.2 Transformation of pCON1*R16F DNAs into XLI Competent Cell

After purification of PCR product, pCON1*R16F (* represents desired mutation) DNAs were transferred into $CaCl_2$ treated XLI-Blue competent cell and four colonies were selected and isolated. After DNA isolation from obtained clones, pCON1 and DNAs isolated from pCON1*R16F were treated with *Xho*I RE enzyme to compare pCON1 linear DNA and pCON1*R16F DNA (Figure 4.3). The clones having 4 kb DNA fragments size of which is equal to pCON1 DNA were kept in –80 °C.

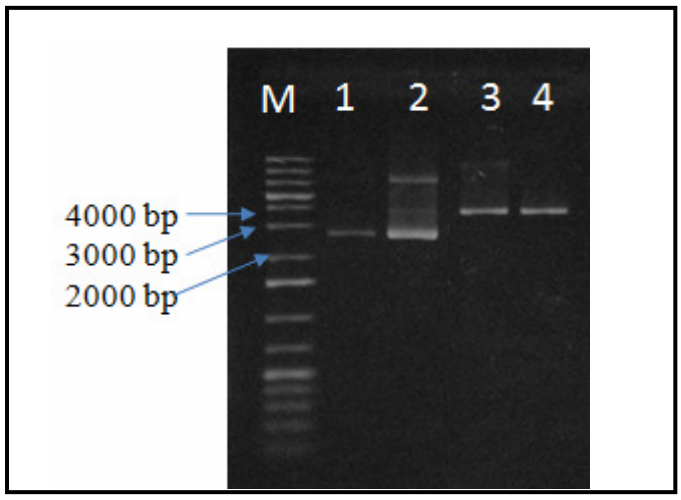


Figure 4.3. Analysis of the pCON1*R16F DNAs isolated from clones. Line 1 contains uncut pCON1 DNA; Line 2 contains uncut pCON1*R16F; Line 3 and Line 4 contain *Xho*I digested pCON1 and pCON1*R16F respectively. Marker is designated as M.

4.1.3 Sequencing of R16F*bsh* gene of *Lactobacillus plantarum* and Sequence Analysis

Plasmid DNA from pCON1*R16F was sequenced to evaluate mutation happened or not by using universal primers. Obtained sequence was compared with template DNA that was pCON1 sequence to see desired mutations. Sequencing indicated that AGA codon coding Arginine 16 amino acid was converted to TTT codon coding phenylalanine 16 amino acid (Figure 4.4). There is no mutation rather than desired one. Desired mutation was labeled and named as pCON1R16F (Figure 4.5).

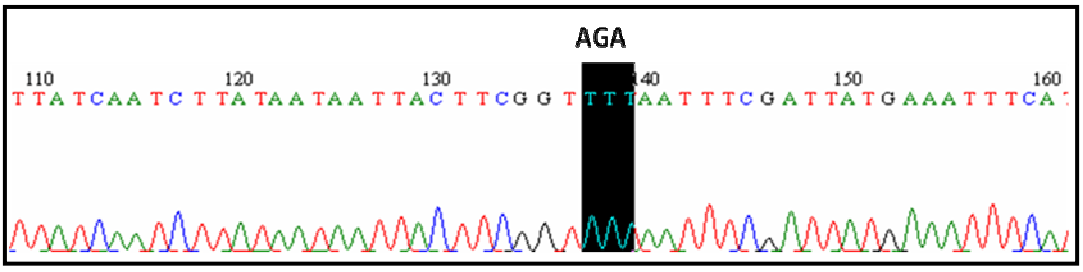


Figure 4.4. Partial sequence of pCON1R16F transformant. AGA codon coding Arginine amino acid was substituent for TTT codon coding phenylalanine amino acid.

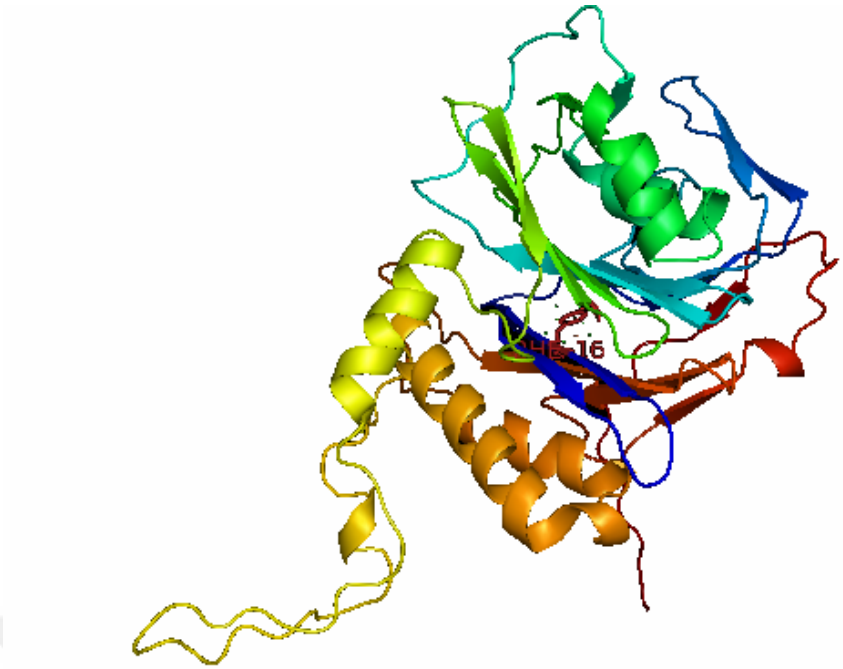


Figure 4.5. Three dimentional model of BSHR16F obtained by pymol program

4.2 Construction and Analysis of pKUHR16F

After creating desired mutation on pCON1 DNA, pCON1R16F DNA and pET22B cloning/expression vector were isolated and digested with *Eco*RI and *Not*I restriction enzymes to construct and characterize mutant *bsh* enzyme. 1 kbp mutated fragment of pCON1R16F was obtained (Figure 4.6).

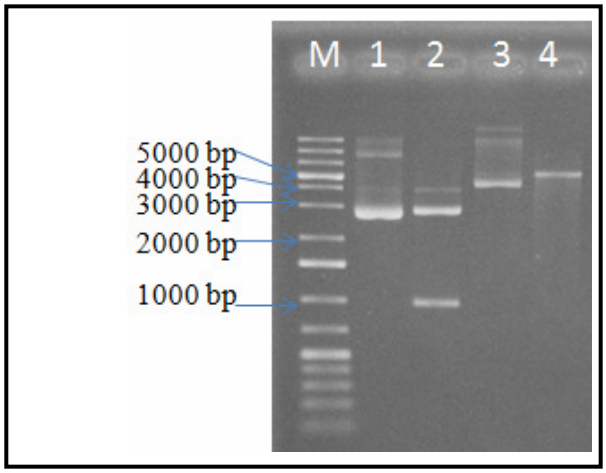


Figure 4.6. Preparation of the mutant *bsh*R16F insert gene and expression vector**.** Line 1 contains circular pCON1R16F; Line 2 contains *Eco*RI- *Not*I treated pCON1R16F DNA; Line 3 contains circular pET22b; Line 4 contains linear pET22b digested *Eco*RI- *Not*I RE. Marker is represented by M.

Digested samples were loaded on %1 agarose gel and 5.5 kbp fragments from pET 22b, 1 kbp fragments from pCON1R16F were seperated and isolated from agarose gel (Figure 4.7 and Figure 4.8). Linear fragments of pCON1R16F (1 kbp) and pET 22b (5.5 kbp) were loaded on %0.7 agarose gel to determine the amount of insert and vector DNAs before ligation (Figure 4.9). 1 kbp fragment of pCON1R16F and 5.5 kbp fragment of pET 22b DNAs were ligated and transformed into $CaCl_2$ treated BLR(DE3) competent cells. Four different colonies were selected randomly and isolated based on clone size. After isolation, DNAs was digested with *Eco*R I and *Not* I restriction enzymes to evaluate whether they receive insert or not and then colonies containing insert were named as pKUHR16F (Figure 4.10).

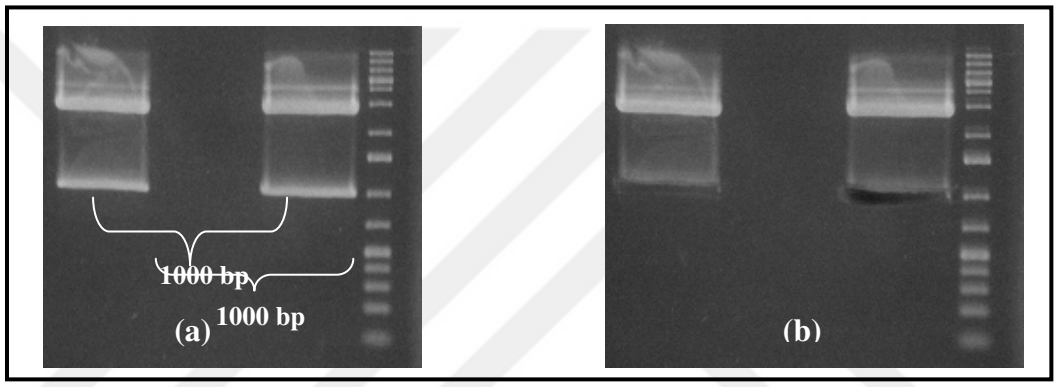


Figure 4.7. Separation and isolation of *Eco*RI and *Not*I RE digested pCON1R16F. (a) Separation of 1 kbp fragment from pCON1R16F. (b) Isolation of 1 kbp fragment from %1 agarose gel.

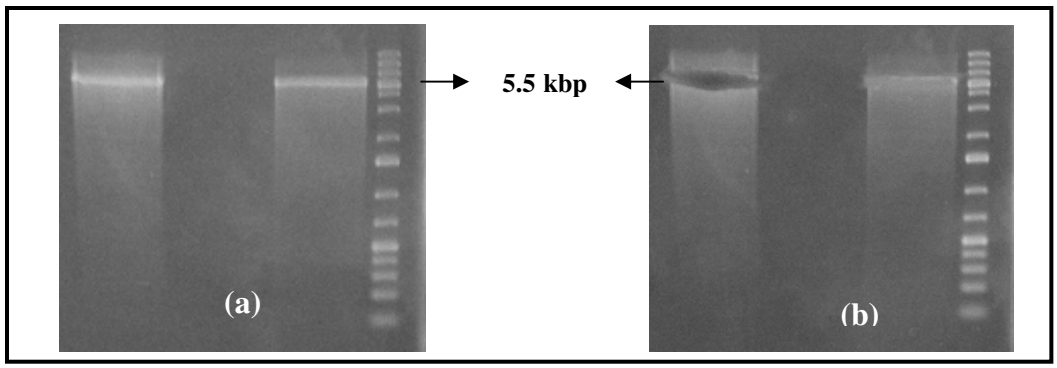


Figure 4.8. Separation and isolation of *Eco*RI and *Not*I RE digested pET22b. (a) Separation of 5.5 kbp fragment from pET22b. (b) Isolation of 5.5 kbp linear pET22b DNA fragments from %1 agarose gel.

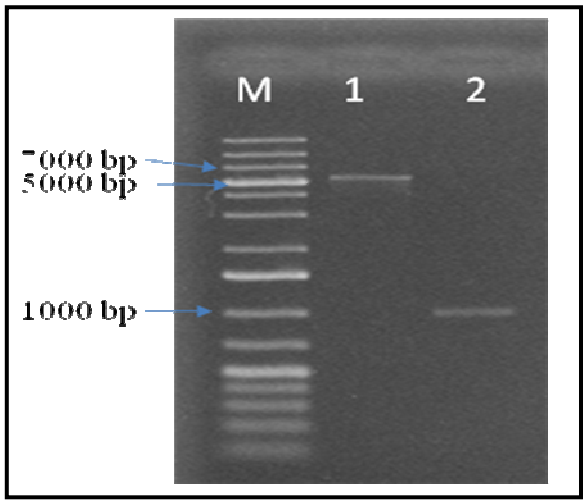


Figure 4.9. Determination of the amount of linear pET22b vector DNA and pCON1R16F insert DNA for ligation. Line 1 shows the 5.5 kbp fragment of linear pET22b vector DNA. Line 2 shows the 1 kbp fragment of linear *bsh*R16F insert DNA. Marker is represented by M.

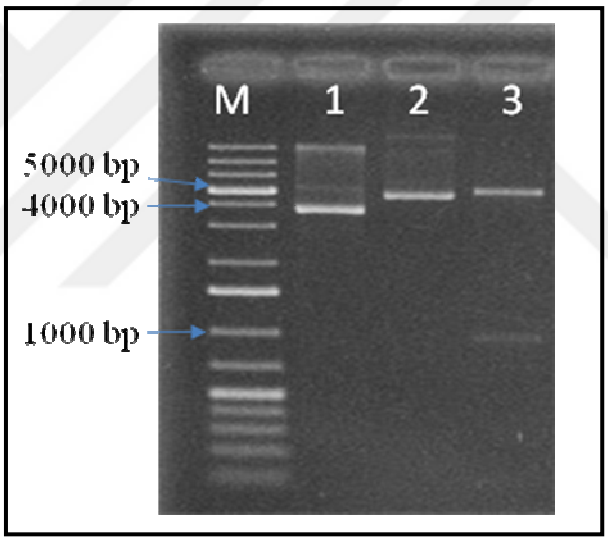


Figure 4.10. Digestion and selection of the pKUHR16F transformants. Line 1 shows circular pET22b vector DNAs; Line 2 shows circular pKUHR16F transformant; Line 3 shows *Eco*RI and *Not*I digested pKUHR16F DNAs. Marker is represented by M.

4.3 Construction of pCON1D19L Mutant

4.3.1 Site Directed Mutagenesis of Aspartate by PCR

The pCON1 DNA was used as a template for the substitution of Aspartat 19 to Leucine 19 in site directed mutagenesis protocol by using designated forward and

reverse primers. After PCR reaction, 10 µl sample was seperated to load on the gel the rest of the sample was treated by *Dpn*I (cleave the methylated DNA) RE enzyme. To evaluate linear PCR reactions, template DNA was also digested with *Xho*I RE cuttting DNA once. After these treatment, samples were loaded on the agarose gel to see if these treatments occured successfully (Figure 4.11). *Dpn*I treated PCR products were purified by Vivantis Ambiclean Kit and GeneJET™ Gel Extraction Kit (#K0691 Fermantas, Europe) (Figure 4.12).

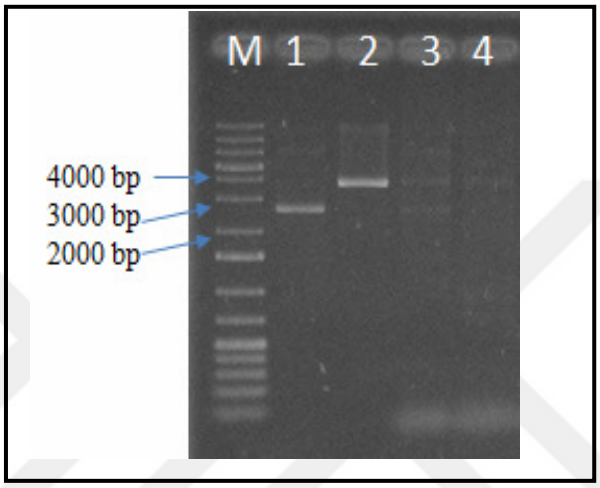


Figure 4.11. Preparation of D19L mutant by PCR based site directed mutagenesis. Line 1 shows pCON1 template DNA; Line 2 shows *Xho*I diested linear pCON1 template DNA; Line 3 shows pCON1*D19L PCR product and Line 4 shows pCON1*D19L incubated with *Dpn*I RE, Marker is represented by M.

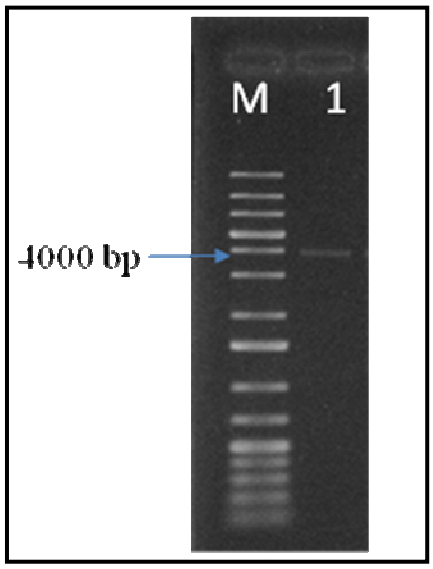


Figure 4.12. Purification of PCR product. Line 1 represents pCON1*D19L DNA and M represents marker.

4.3.2 Transformation pCON1*D19L DNAs into XLI Competent Cell

After purification, sample was transferred into $CaCl_2$ treated XLI competent cells. After incubation four single colonies from pCON1*D19L were selected randomly and isolated. After DNA isolation, template DNA and pCON1*D19L circular DNA were cleaved with *Xho*I RE and compared their linear form with each other on the gel (Figure 4.13). Samples that mutation was happen were kept in -80°C.

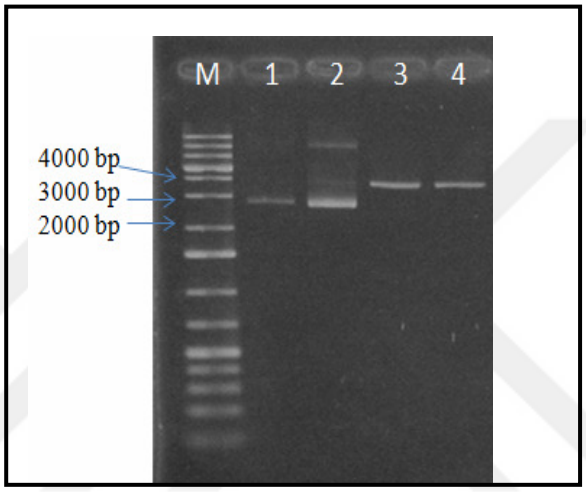


Figure 4.13. Analysis of the pCON1*D19L DNAs isolated from clones. Line 1 contains uncut pCON1 DNA; Line 2 contains uncut pCON1*D19L; Line 3 and Line 4 contain *Xho*I digested pCON1 and pCON1*D19L respectively. Marker is designated as M.

4.3.3 Sequencing of D19L *bsh* gene of *Lactobacillus plantarum* and Sequence Analysis

Plazmid DNA from pCON1D19L mutant was isolated and sequenced by using universal primers as M13/pUC Forward and M13/pUC Reverse primers. Sequencing show that pCON1D19L was desired mutation (CTA) and also there was no other mutation than desired one (Figure 4.14). Desired mutation was labeled and called as pCON1D19L (Figure 4.15).

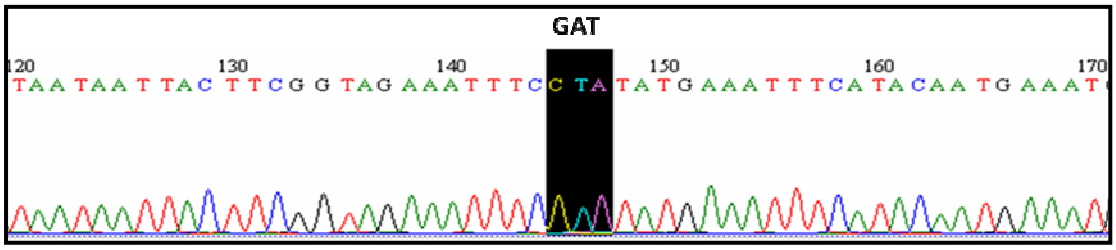


Figure 4.14. Partial sequence of pCON1D19L transformant. GAT codon coding Aspartate amino acid was substituent for CTA codon coding leucine amino acid.

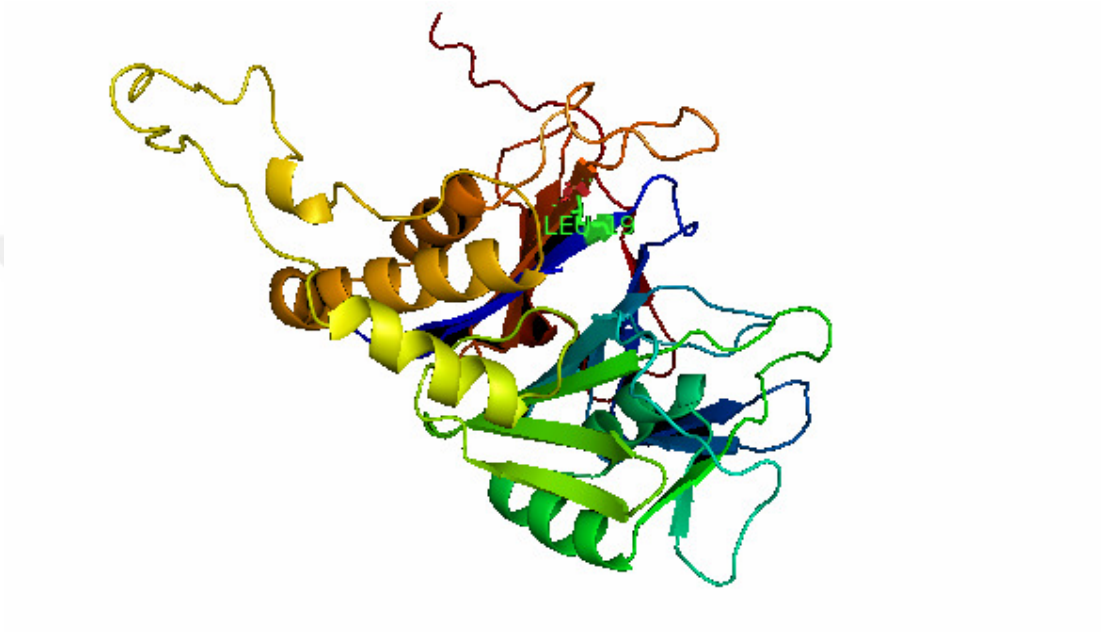


Figure 4.15. Three dimentional model of BSHD19L obtained by pymol program

4.4 Construction and Analysis of pKUHD19L

After desired mutations, pCON1D19L DNA and pET22b vector DNA were isolated according to related Kit's protocol. These plazmid DNAs were digested with *Eco*RI- *Not*I RE enzymes and loaded on the agarose gel. Expected 1 kbp fragment of pCON1D19L was obtained after this treatment (Figure 4.16).

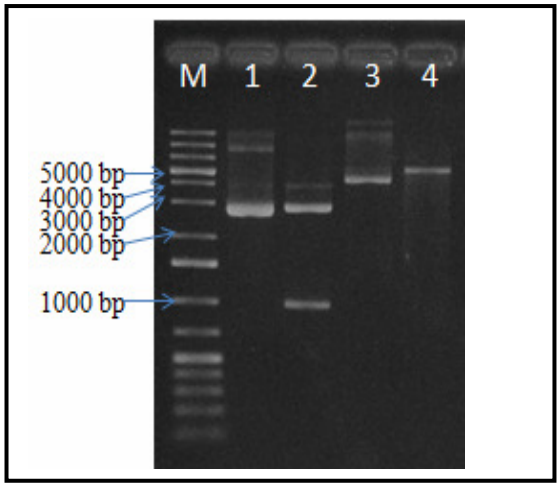


Figure 4.16. Preparation of the mutant *bsh*D19L insert gene and expression vector**.** Line 1 contains circular pCON1D19L; Line 2 contains *Eco*RI- *Not*I treated pCON1D19L DNA; Line 3 contains circular pET22b; Line 4 contains linear pET22b digested *Eco*RI- *Not*I RE. Marker is represented by M.

1 kbp fragment of desired mutation and 5.5 kbp fragment of linear pET22b were loaded on %1 agarose gel to seperation and purification for construction of pKUHD19L (Figure 4.17 and Figure 4.8). *bsh*D19L DNA and linear pET22b DNA was loaded on %0.7 agarose gel to determine amount of these DNAs before ligation (Figure 4.18). 1 kbp fragment of pCON1D19L and 5.5 kbp fragment of pET 22b DNAs were ligated and transformed to BLR(DE3) competent cells. Four single colonies were selected randomly and isolated based on clone size. After isolation, DNAs was digested *Eco*R I and *Not* I restriction enzymes to evaluate whether receive insert and then colonies containing insert was called as pKUHD19L (Figure 4.19).

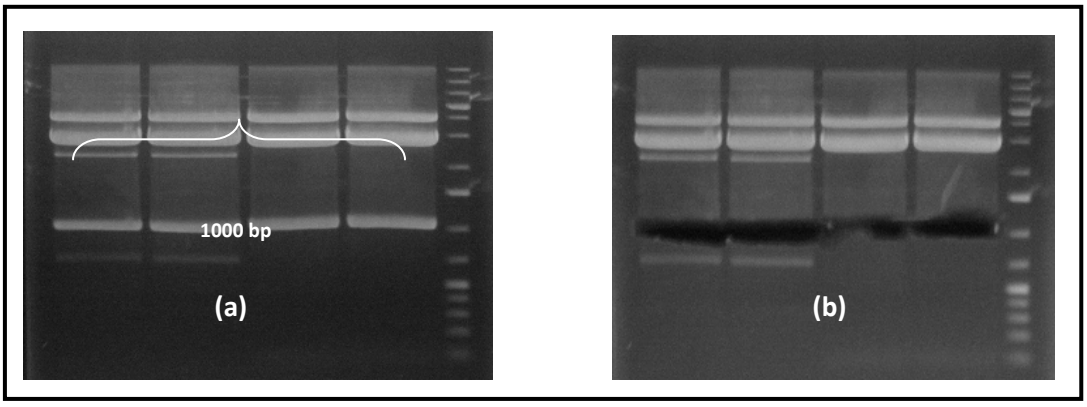


Figure 4.17. Separation and isolation of *Eco*RI and *Not*I digested pCON1D19L. (a) Separation of 1 kbp fragment from pCON1D19L. (b) Isolation of 1 kbp fragment from %1 agarose gel.

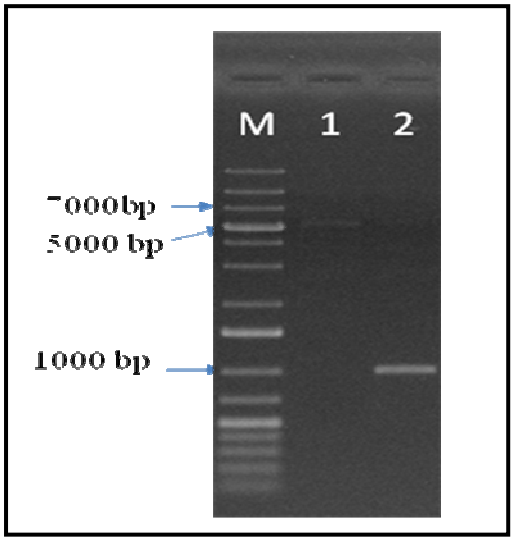


Figure 4.18. Determination of the amount of linear pET22b vector DNA and pCON1D19L insert DNA for ligation. Line 1 shows the 5.5 kbp fragment of linear pET22b vector DNA. Line 2 shows the 1 kbp fragment of linear *bsh*D19L insert DNA. Marker is represented by M.

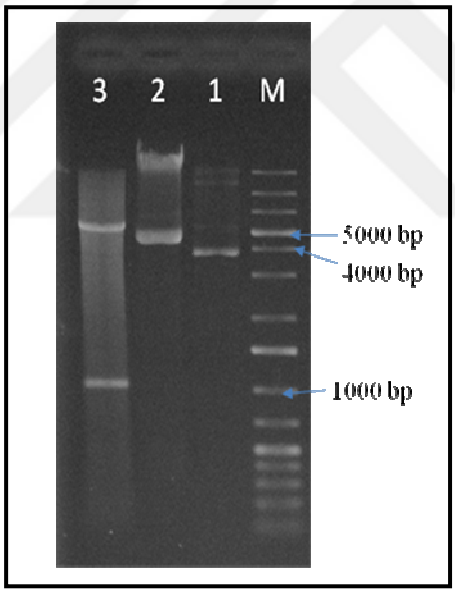


Figure 4.19. Digestion and selection of the pKUHD19L transformants. Line 1 shows circular pET22b vector DNAs; Line 2 shows circular pKUHD19L transformant; Line 3 shows *Eco*RI and *Not*I digested pKUHD19L DNAs. Marker is represented by M.

4.5 Detection of BSH Activities of pKUHR16F and pKUHD19L in *E. coli* by Direct Plate Assay

To analyze quantative activities of BSH enzyme, one of the analysis technique is direct plate assay. In this assay, samples including pKUHR16F, pKUHD19L, pCON2 (*bsh*1*/pET 22b)/BLR(DE3) and pET22b/BLR(DE3) were drawn on LB agar including 5 g/lt of TDCA and 2 mM GDCA with the other supplements. These plates were incubated at 37°C for 72 hours. After incubation, samples were compared with the positive (pCON2 (*bsh*1*/pET 22b)/BLR(DE3)) and negative controles (pET22b/BLR(DE3)) according to their opaque appearance (Figure 4.20 and Figure 4.21). In Figure 4.20 and Figure 4.21, although positive controles had an opaque apperace, negative controles and mutans did not have opaque appearance in LB plate containing GDCA. On the other hand, positive controles did not have aopaque appearance in LB plate containing TDCA.

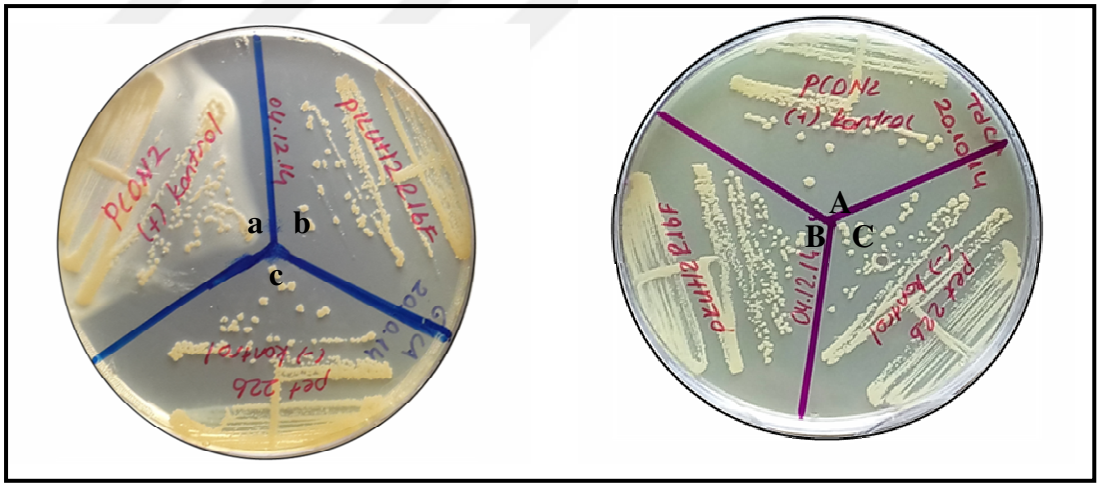


Figure 4.20. Detection of the BSHR16F activities by direct plate asssay on solid medium. (a) represents (*bsh*1/pET22b/BLR(DE3)) positive control inLB plate containing GDCA; (b) represents pKUHR16F/BLR(DE3) mutant; (c) represents pET22b/BLR(DE3) as negative control; (A) represents (*bsh*1/pET22b)/BLR(DE3) as positive control in LB plate containing TDCA; (B) represents pKUHR16F/BLR(DE3) mutant and (C) represents pET22b/BLR(DE3) as negative control.

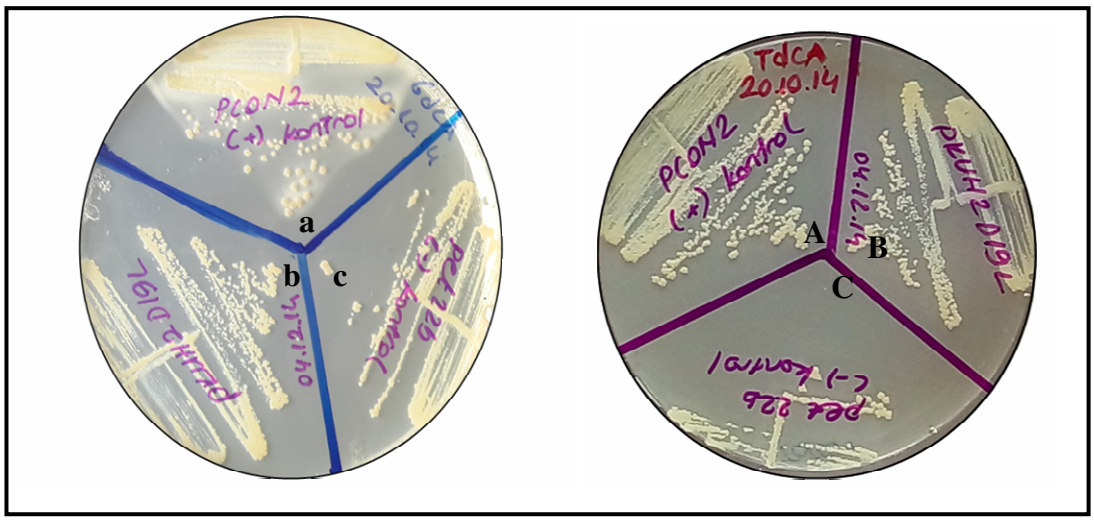


Figure 4.21. Detection of the BSHD19L activities by direct plate asssay on solid medium. (a) represents (*bsh*1/pET22b)/BLR(DE3)) positive control inLB plate containing GDCA; (b) represents pKUHD19L/BLR(DE3) mutant; (c) represents pET22b/BLR(DE3) as negative control; (A) represents (*bsh*1/pET22b)/BLR(DE3) as positive control in LB plate containing TDCA; (B) represents pKUHD19L/BLR(DE3) mutant and (C) represents pET22b/BLR(DE3) as negative control.

4.6 Determination of BSH Activies of pKUHR16F and pKUHD19L by Ninhydrin Assay

To detect BSH activity in cell-free extract, ninhydrin assay was performed by determination of the amount of the liberated amino acids from bile salts based on their color variations. Two common human bile salts, GDCA and TDCA, were used for this protocol whicis based on interaction of the released amino acids with ninhydrin. Normally, while wild type BSH enzyme had a highest activity in a solution containing GDCA, it has low activity in solution containing TDCA. Two of the mutation supposed to be member of the catalytic site, pKUHR16F and pKUHD19L, did not have any activities in the ninhidrin solution containing neither GCDA nor TDCA**.** Boht mutations led to lost of the catalytic activity of the BSH enzyme (Figure 4.22 and Figure 4.23).

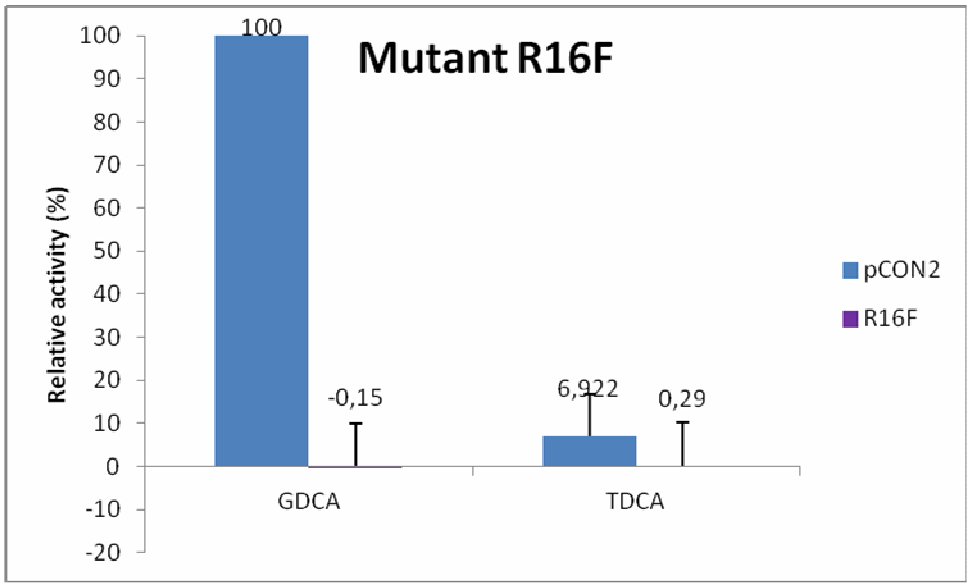


Figure 4.22. Measurement of mutant BSHR16F enzyme activities agains to GDCA and TDCA**.** Graphs show activity of mutant *bsh* enzymes in the presens of 2 major human bile salts, GDCA and TDCA. Relative activity was defined as the measured bile salt hydrolase activity for each substrate compared with the highest activity (taken as %100) in the assay. Error bars represent standard deviation.

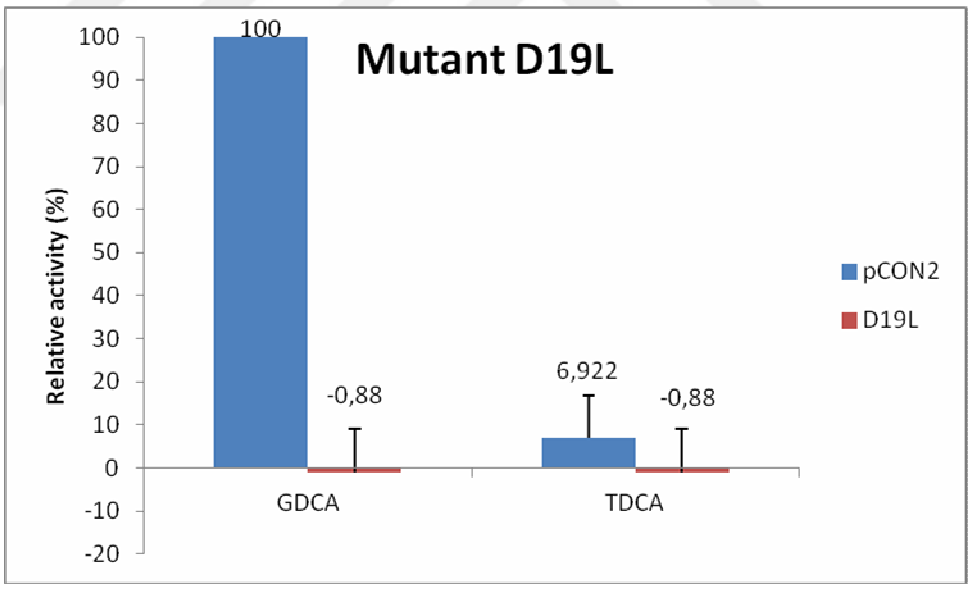


Figure 4.23. Measurement of mutant BSHD19L enzyme activities agains to GDCA and TDCA**.** Graphs show activity of mutant *bsh* enzymes in the presens of 2 major human bile salts, GDCA and TDCA. Relative activity was defined as the measured bile salt hydrolase activity for each substrate compared with the highest activity (taken as %100) in the assay. Error bars represent standard deviation.

4.7 Purification of BSHR16F and BSHD19L Enzymes by SDS-PAGE Analysis

pKUHR16F/BLR(DE3) and pKUHD19L/BLR(DE3) clones were inoculated in 100 ml medium suplamented with amphicillin (100 µg ml-1) and %0.4 glycerol and samples were grown until they would reach to OD_{600} 0.5. After cell culture absorbance reached to 0.5 value at OD600, 0.3 mM IPTG was added to induce mutant *bsh* gene expression and grown until their OD_{600} would reach to 1.5-3.0. The cell extracts reached to 1.5-3.0 absorbance value was purified. After that purified BSH mutant enzymes from BSHR16F/BLRDE3 and BSHD19L/ BLRDE3 cells, negative control pET22b and positive control pCON2 were loaded and visualized on %12 polyacrylamide gel stained with Coomassie Brilliant Blue G 250. Molecular weight of BSH protein is 37 kDa (Figure 4.23). While BSH protein was observed with the positive control in the pCON2, was not observed in the pET22b. After mutation of the R16F, bsh protein was obtained as pCON2 however after mutation of the D19L, bsh protein was obtained as pET22b.

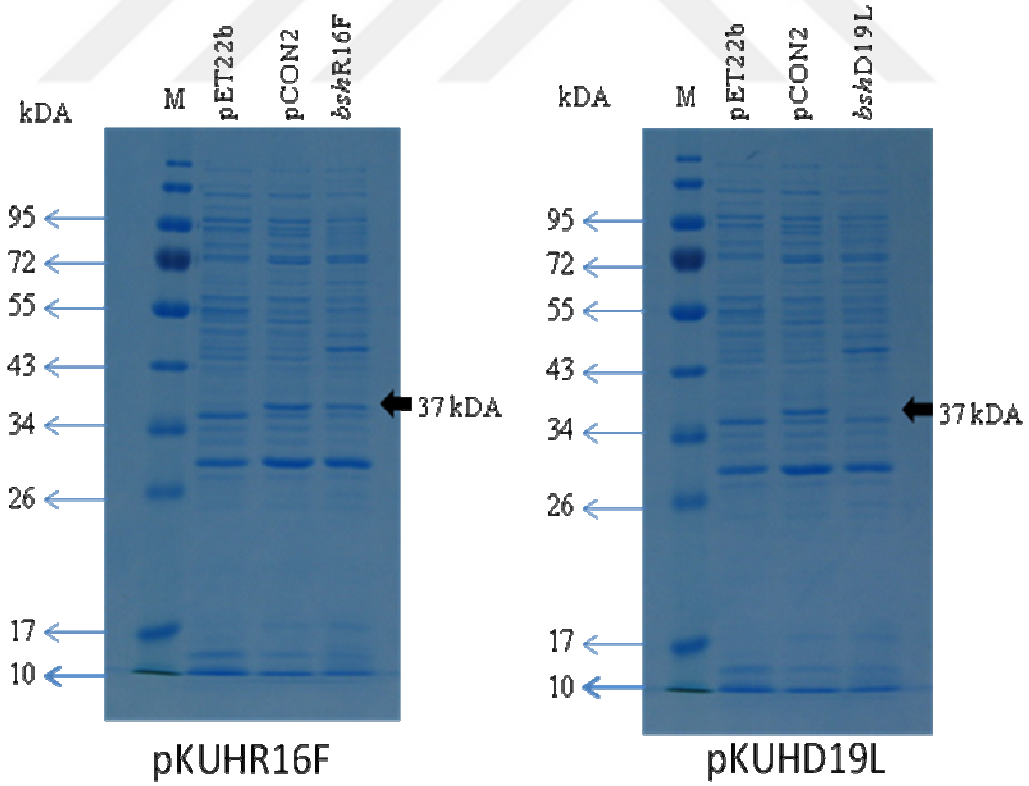


Figure 4.24. Expression and purification of R16F and D19L mutant BSH enzymes from *Lactobacillus plantarum* in *E. coli.*

The *bsh* gene nucleotide sequences of genes belonging to different Lactobacillus species as *Lb.gasseri*, *Lb.reuteri*, *Lb.salivarus*, *Lb.acidophilus*, *Lb.plantarum,* PVA of *Bacillus sphaericus* and conjugated bile acid (CBAH) of *Clostridium perfringens* were obtained from NCBI GenBank and these nucleotide sequences were converted to their amino acid sequence by Bioinformatics Tools for Sequence Translation<EMBL-EBI program and compared with each other by using Jalview 2.8.0b1 (Figure 4.25). Afterthat aligment showed that catalytically active amino acid residues like Cys 2, Arg 16, Asp 19, Asn 69, Asn 170 and Arg 223 amino acids are fully conserved at the nucleotide residues of BSH protein of different Lactobacillus species such as *Lb.gasseri*, *Lb.reuteri*, *Lb.salivarus*, *Lb.acidophilus*, *Lb.plantarum,* PVA of *Bacillus sphaericus* and conjugated bile acid (CBAH) of *Clostridium perfringens*. In addition, alignment of these aminoacid residues indicated that conserved amino acid motifs located around these active sites as YFGRNXD, NEXGLXXAGLNF, VXVLTNNPXF and SXSRFVRXAF in different *Lactobacillus* and *Bifidobacterium* but located around these active sites were partially conserved in *Lactobacillus plantarum* species. (Figure 4.25).

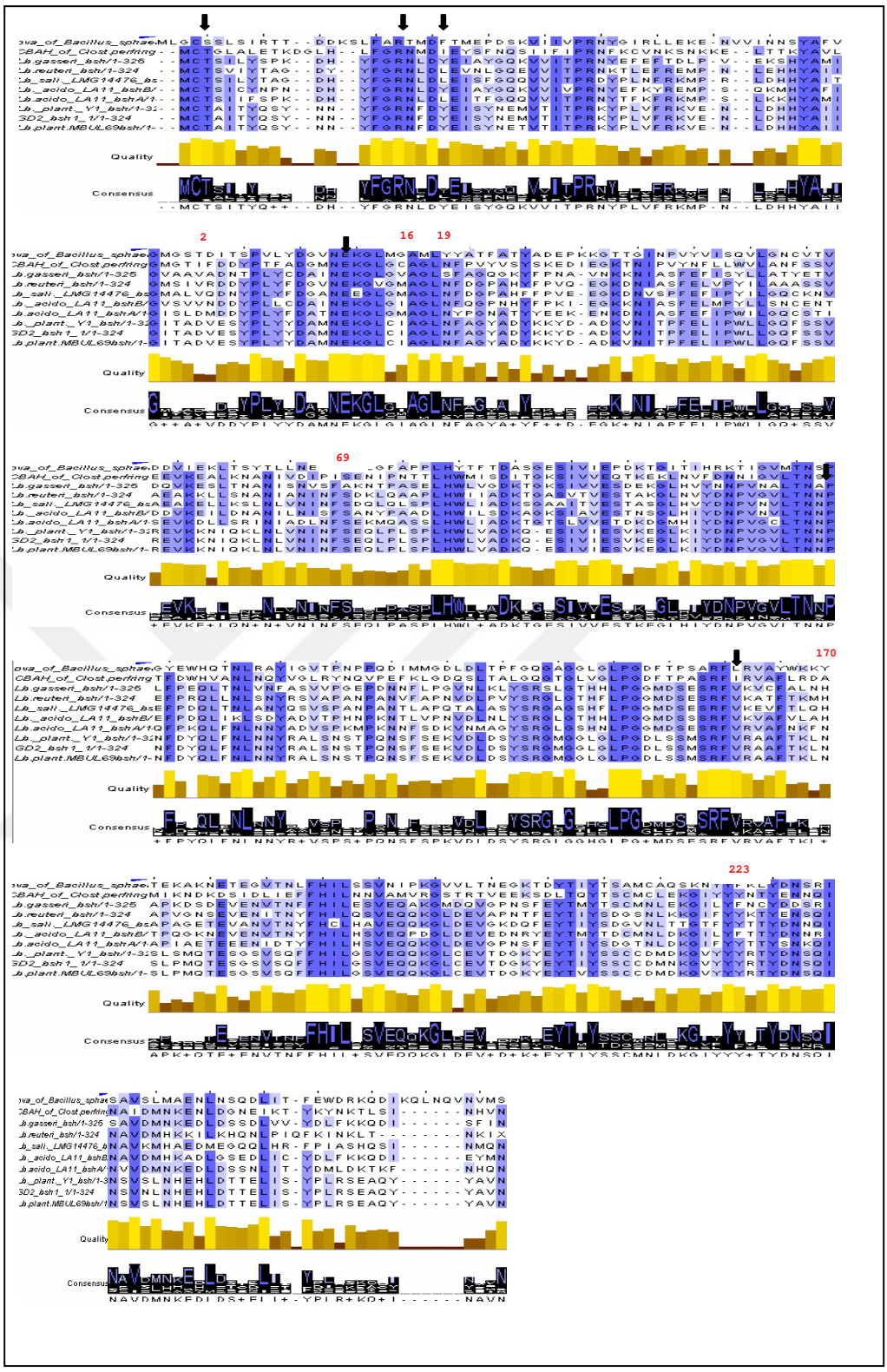


Figure 4.25. Multiple sequence alignment of *bsh* proteins from *Lb.gasseri, Lb.reuteri, Lb.salivarus, Lb.acidophilus* and *Lb.plantarum,* penicillin acylase of *Bacillus sphaericus* and conjugated bile acid (CBAH) of *Clostridium perfringens* species.

5. DISCUSSION

BSH enzymes cause deconjugation of bile salts reducing their reabsorption into the blood stream, resulting in their loss through excretion. This could lead to a reduction in serum cholesterol either by increasing the demand for cholesterol for de novo synthesis of bile acids or by reducing cholesterol solubility and so absorption through the intestinal lumen (du Toit et al., 1998; De Smet et al., 1998; Begley et al., 2006; Park et al., 2007; Sridevi et al., 2009; Kumar et al., 2011; Jones et al., 2011). These mechanisms of some probiotics have been confirmed with a lot of studies in human (Anderson and Gilliland., 1999) as well as in animals (De Rodas et al., 1996; De Smet et al., 1998; du Toit et al., 1998). Contrary to this mechanism, 7α-hydroxylation can convert deconjugated cholates to deoxycholates considered to be cytotoxic and pre-carcinogenic secondary bile acid (Kumar et al., 2012). Hence, BSH enzyme has not yet been sufficiently elucidated.

The *bsh* gene sequences from different *Lactobacillus* species such as *Lb. gasseri, Lb. reuteri, Lb. salivarus, Lb. acidophilus, Lb. plantarum,* penicillin acylase (PVA) of *Bacillus sphaericus* and conjugated bile acid (CBAH) of *Clostridium perfringens* species were aligned using Jalview 2.8.0b1. Amino acid analysis of the BSH protein showed that these enzymes are part of the N-terminal nucleophile (Ntn) hydrolase superfamily and also have an N-terminal catalytic nucleophile which cleaves an amide bond (Oinonen and Rouvinen, 2000). Amino acid aligments of BSH A, B and C from *Lactobacillus johnsonii* PF01 demonstrated that they were homologous to other Ntn-hydrolase enzymes as PVA of *Bacillus sphaericus* and CBAH of *Cl. perfiringens* (Coleman and Hudson, 1995; Tikkanen et al., 1996; Kim et al., 2004; Rossocha et al., 2005). This alignment indicated that Cys 2, Arg16, Asp19, Asn 69, Asn170 and Arg 223 were strictly conserved in all bile salt hydrolase enzymes of *Lactobacillus* species, PVA of *Bacillus sphaericus* and conjugated bile acid (CBAH) of *Clostridium perfringens* species (Figure 4.25) and amino acid motifs were located around YFGRNXD, NEXGLXXAGLNF, VXVLTNNPXF and SXSRFVRXAF sites (Tanaka et al., 2000; Kim et al., 2005; Begley et al., 2006; Oh et al., 2008; Kumar et al., 2010). Cys 2, found in active site as nucleophile and

proton donor further, is important for catalysis (Kabsch et al., 1976; Rossocha et al., 2005). Furthermore, silica analysis indicated that Cys 2, Asp 19, Asn 69, Asn 170 and Arg 223 residues have been identified to be important for the catalytic activity in BSHs. Since carboxyl group of Arg16 binds to tauroCBAH model (Rossocha et al., 2005), Arg16 seems very critical. According to Rossocha (2005), Arg 16 is also strictly conserved and this conservation of Arg16 is due to its involvement in catalysis rather than in conferring substrate specifity.

Although, silica analysis indicated that these five totally conserved amino acids of BSH enzyme might be important for activity of the BSH enzyme, there is no experimental study on those amino acids axcept Cys2 amino acid. Tanaka and his collegues (2000) found that the first N-terminal amino acid sequence of purified mature BSH was cistein amino acid because of the formylmetionin processing. The Important role of Cys-1 was confirmed by changeing of Cys-1 to Ala. Cys-1Ala mutant led to complete inactivation of BSH (Tanaka et al., 2000).

In this study, totaly conserved Arg-16 and Asp-19 amino acids of *bsh*1 genes from *Lactobacillus plantarum* were substituted for phenylalanine (aromatic group) and leucine (nonpolar, aliphatic group) respectively by site directed mutagenesis. This work is the first experimental research on the two of the remaining strictly conserved amino acids in literature. Our findings are identical with findings obtained from BSH of *C. perfirngens,* in which Cys-1 plays a central role at the active site of BSH enzyme (Tanaka et al., 2000).

Generally, BSHs from intestinal lactic acid bacteria have higher affinity to glucoconjugated bile salts (Coleman et al., 1999; Tanaka et al., 2000; Kim et al., 2004). Our ninhidrin and direct plate assay results obtained from wild-type BSH enzyme were also demonstrated that wild type BSH enzyme of *Lb. plantarum* strain had a high activity against GDCA rather than to TDCA substrates. Both of the pKUHR16F/pET22b and pKUHD19L/pET22b mutants lost their activities agains to GDCA and TDCA shown on LB-GDCA and LB-TDCA agar medium (Figure 4.20 and Figure 4.21).

Considering the activity and stability of mutants by ninhidrin assay and PAGE, our results indicated that R16F mutant may be important for catalytic activity of the BSH enzyme whereas D19L mutant may be important just for stability of BSH enzyme. The D19L mutant was not stabiled (Figure 4.22, Figure 4.23 and Figure 4.24).

6. CONCLUSION AND RECOMMENDATIONS

Recent reports show that the BSH enzyme is related with reduction in the level of serum cholesterol. This has caused increased interest in the BSH enzyme for treatment of hypercholesterolemia in humans. It was found that a lot of species have produced bile salt hydrolase or have genes encoding for BSH in their genomes including *Clostridium*, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Listeria monocytogenes*, *Brevibacillus*, *Bacteroides*, *Brucella abartus* and *Streptococcus* genuses. However, there are harmful side effects of secondary bile acid production such as gall stone formation and their carcinogenic activity in the colon. Therefore, BSH enzymes are very important enzyme but structute and mechanism of this enzyme still remains unclear.

In this study, *bsh* gene cloned and expressed in *E. coli* and two conserved amino asids supposed to be responsible for catalytic activity of BSH enzyme were mutated by site directed mutagenesis to understand the effect of these amino acids on the structure and mechanisms of BSH enzyme. Expressed mutant *bsh* proteins were purified with Ni^{2+} chelating column using His tag site. The effect of mutations on catalytic activity of BSH enzyme was analyzed by direct plate assay and ninhydrin assay with two different bile salts.

Briefly, these results showed that the phenylalanine and aspragine amino acid residues at the position of 16 and 19 had a very important role for either catalytic activity or stability of BSH enzymes. Further site-directed mutagenesis studies on critical amino acids are required to better understand the catalytic activity and especially substrate specificity of the BSH enzyme.

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APPENDICES

8. APPENDICES

Appendix A

BACTERIAL GROWTH MEDIA

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

Contents	For 1 Liter
Bacto tryptone	10g
Yeast extracts	5g
NaCl	10g

These contents are dissolved in 900 ml dH_2O and pH was adjusted to 7.5 with NaOH. Than volume is completed to 1L with dH_2O and sterilized at 121°C for 15-20 minutes.

- **LB Plate**

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

- **LB Plate with IPTG, X-gal**

After the 1L plate medium was prepared, 1mM IPTG (Isopropyl-β-D-1 thiogalactopyranoside), 100 μg ml^{-1} ampicillin and 40μg/ml X – gal were added into the medium.

- **LB Plate for Direct Plate Assay**

For 1L plate medium, 0.35g CaCl2.H2O and 10g glucose were added and pH was adjusted to 6.5. All contents were sterilized at 121°C for 15-20 minutes.

For 1L LB medium, 0.25g/ml TDCA (5g/liter), 0.125g/ml GDCA (2mM) were added to different plate with LB medium, containing 1mM IPTG (Isopropyl-β-D-1 thiogalactopyranoside).

Appendix B

SOLUTIONS AND BUFFERS

- **For Competent Cell Preparation**

– 1.11 g CaCl2.H2O was dissolved in 100 ml ddH2O (0.1 M) and pH was adjusted to 7.0.

– 0.55 g CaCl2.H_2O is dissolved in 35 ml ddH_2O and 15 ml glycerol was added.

- **50X TAE**

242 g of Tris base was dissolved in deionized dH_2O. 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) were added to the solution. Final volume of solution was adjusted to 1 L with dH_2O.

- **1X TAE**

20 ml of 50 X TAE buffer was taken and the volume was adjusted to 1 L with dH_2O. The 1X working solution was 40 mM Tris-acetate/1mM EDTA.

- **Preparation for Cell Extract**

1 M NaH_2PO_4

12 g of monobasic sodium phosphate are dissolved in 100 ml ddH_2O and pH was observed as 4.04 and then sterilized at 121°C for 15-20 minutes.

1M Na_2HPO_4

14.2 g of dibasic sodium phosphate are dissolved in 100 ml ddH_2O and pH was aobserved as 8.73 and then sterilized at 121°C for 15-20 minutes.

0,1 M Sodium Phosphate Buffer (pH: 7.2)

3.87 ml of dibasic and 1.13 ml of monobasic were mixed and volume completed to 50 ml.

Resuspention Buffer (20 mM Imidazole, 500 mM NaCl, 20 mM NaPi; pH: 7.49)

0,14 g of Imidazole, 2,922 g NaCl were dissolved in 20 ml of 0,1 M NaPi and volume completed to 100 ml.

- **For Ninhydrine Assay**

15% Trichloroacetic Acid

2.25 g trichloroacetic acid was dissolved in 15 ml dH_2O and sterilized with filtration.

1% Ninhydrine Solution

1 g ninhydrine was dissolved in 100 ml ddH_2O.

0.5 M Sodium Citrate Buffer

5.88 g sodium citrate was dissolved in 20 ml ddH_2O and pH was adjusted to 5.5 and volume was completed to 40 ml. Both of the buffers were sterilized at 121°C for 15-20 minutes.

0.1 M Sodium Phosphate Buffer

0.284 g sodium phosphate was dissolved in 10 ml ddH_2O and pH was adjusted to 6.0 and volume was completed to 20 ml.

100mM Sodium Dithiothreitol (DTT)

0.0174 g sodium dithiothreitol was dissolved in 1 ml 100 mM Tris (pH: 8.0).

- **For Purification of BSH Enzymes**

Binding Buffer pH: 7.8

11,688g of 500 mM NaCl were dissolved in 8 ml of 20 mM NaPi and volume completed to 400 ml.

Elution Buffer pH: 6.0

5 ml of 20 mM NaPi, 7,305 g of 500 mM NaCl and 0,851g of 50 mM imidazole were dissolved in 250 ml.

Acrylamide-Bis Acrylamide Mixture

30% Acrylamide-Bis Acrylamide (Sigma A3553-100G) solution was used.

10% SDS

10g SDS was dissolved in 100ml ddH2O.

Ampicillin

For 10 ml 25 mg/ml stock solution, 0.25 g Ampicillin Sodium Salt (Sigma) was dissolved in 10 ml ddH_2O and sterilized by using 0.22 μm filter and stored at -20°C.

Appendix C

CHEMICALS

Acrlyamide [for electrophoresis=99%] (Sigma, A3553)

Agar (Merck, 101614)

Agarose (Sigma, 9012-36-6)

Ammonium persulfate [APS] (Sigma, A-3678)

Ampicillin (Sigma, A-0166)

Calcium Chloride [minimum 93.0%, granular anhydrous] (Sigma, C1016)

Calcium chloride dihdrate [CaCl2] (Sigma, 10035-04-8)

Comassie Brillant Blue G-250 (Fluka, 27815)

EDTA [Ethylenediaminetetraacetic acid] (Sigma, 60-00-4)

EtBr [Ethidium Bromide] (Sigma, E-8751)

Glycerol, cell culture tested (Sigma, 6-2025)

Glycine (Merck, 396712)

IPTG [isopropyl-beta-D-thiogalactopyranoside] (Thermo Scientific, R0391)

N,N'-Methyenebisacrylamide (Sigma, M7256)

Ninhydrin (Sigma,N4876)

Peptone from casein (Merck, 107213)

Sodium Chloride [NaCl] (Merck, 106404) 88

Sodium Citrate Dihydrate (Merck, 024294)

Sodium Dithiothreitol [DTT] (Sigma, 3483-12-3)

Sodium dodecyl sulfate [SDS] (Sigma, 151-21-3)

Sodium Hydroxide [NaOH] (Merck, 106462)

Sodium Phosphate dibasic (Sigma, S-3264)

Trichloroacetic Acid (Merck, 431530)

Trizma Base(Sigma, T-6066)

Trizma Hydrochloride (Sigma, T5941)

Yeast Extract Granulated (Merck, 103753)

Tween® 20 (Sigma, 9005-64-5)

Appendix D

ENZYMES AND OTHER CHEMICALS

Not I (Fermentas): Cat. # ER1135

EcoR I (Fermentas): Cat. # ER0271

Fast Digest Pack (Fermentas): Cat. # K1991

T4 DNA Ligase (Fermentas): Cat. # EL0335

Page Ruller Prestain Protein Ladder (Thermo Scientific): Cat. # 26617

Pfu DNA Polymerase (Fermentas): Cat. # EP0501

dNTP Mix (Fermentas): Cat. # R1121

X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside)(Fermentas):Cat.# R0404

1 kb Plus DNA Ladder (Fermentas): Cat. # SM1343

6X Loading Dye Solution (Fermentas): Cat. # R0611

Glycodeoxycholic Acid, Sodium Salt (Calbiochem): Cat. # 361311

Taurodeoxycholic Acid, Sodium Salt (Calbiochem): Cat. # 580221

Appendix E

EQUIPMENTS USED IN THIS STUDY

+4°C refrigerators (Arçelik)

-20°C deepfreeze (Arçelik)

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

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

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

AKTAprime^{TM} plus

Autoclave (Hirayana)

Centrifuge (Hettich Rotina 38R)

Desktop centrifuge (Hettich Micro 120)

Dry block (VWR Digital Dry-Block)

Electrophoresis system (Thermo Scientific)

Imaging system (UVP Photo Doc-It TM)

Micropipettes (Finnipipette)

PCR (Techne, TC 3000)

pH meter (HANNA HI 221)

Power supply (Thermo EC 250-90)

Shaker-heater (IKA RCT basic)

Sonopuls Ultraschall-Homogenesisatoren (Bandelin)

Spektrophotometer (HITACHI U-1900)

UV Transalluminator (UVP)

Vortex (Yellowline TTS2)

Water Purification System (Human Corporation)

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Kübra HACIBEYOĞLU, Mehmet ÖZTÜRK, Molecular Identification of Human Originated *Lactobacillus* Species by 16S ARDRA and 16S-23S ISR Techniques, 4th National Molecular Biology and Biotechnology Congress, August 21-24, 2015, Afyonkarahisar, TURKEY.

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