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

CLONING AND CHARACTERIZATION OF BILE SALT HYDROLASE GENES (*bsh*) FROM *LACTOBACILLUS PLANTARUM* GD2 STRAIN

MASTER OF SCIENCE

YASİN AYDIN

BOLU, JUNE 2015

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APPROVAL OF THE THESIS

MOLECULAR CLONING AND CHARACTERIZATION OF BILE SALT HYDROLASE GENES (*BSH*) FROM *LACTOBACILLUS PLANTARUM* GD2 STRAIN submitted by **Yasin AYDIN** 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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To my family,

DECLARATION

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

Yasin AYDIN

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ABSTRACT

MOLECULAR CLONING AND CHARACTERIZATION OF BILE SALT HYDROLASE GENES (BSH) FROM *LACTOBACILLUS PLANTARUM* GD2
MSc THESIS
YASİN AYDIN
ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
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(SUPERVISOR: ASSOC. PROF. DR. MEHMET ÖZTÜRK)
BOLU, JUNE 2015

Bile Salts Hydrolase (BSH) enzymes catalyse the hydrolysis of taurine and glycine-linked bile salts into amino acid residues. The precise function of BSHs is still unknown, but some hypothesis have been proposed about playing role in nutrition, cholesterol lowering and colorectal cancer etc. BSHs are frequently synthesized by intestinal commensal lactobacilli. BSHs from different lactobacilli strains exhibit higher variation in sequence, pH optimum, kinetic properties and substrate specificity. In this study, *bsh*1 gene from *L. plantarum* GD2 was cloned into pET22b expression vector and overexpressed. At first; the gene was amplified by PCR using by species specific oligonucleotide primers and then cloned into pJET1.0 or pBluescript II SK+ cloning vector and pET22b expression vector. After clones were transformed to XL1-Blue and BLR(DE3) *Escherichia coli* host strains respectively, the *bsh*1 gene was expressed in *E.coli* BLR(DE3) host by induction of 0.1 mmol 1^{-1} of isopropylthio galactopyranoside. The overexpressed recombinant protein was purified using B-PER 6xHis Fusion Protein Purification Kit and deconjugation ability of the enzyme was tested with six conjugated bile salts by ninhydrin protein assay. Four genes, *bsh*1, *bsh*2, *bsh*3 and *bsh*4 were identified in the genome sequence of *L. plantarum* GD2 and sequenced. Comparison of the deduced amino acid sequences of Bsh1 with previously known sequences revealed high homology with BSH enzymes of several microorganisms. Biochemical characterization of the purified Bsh1 revealed some distinct characteristics not observed in other species of *Lactobacillus*. The purified Bsh1 enzyme showed preferential activity against glyco-conjugated bile acids and show highest activity against glycodeoxycholic acids. In this study, the primary structure of Bsh1 enzyme was characterized and analysed to further studies. It is thought that site-directed mutagenesis on conserved amino acids of BSH enzyme and structural analysis will be required for the future studies to investigate key residues of the active site, substrate binding pocket and substrate selectivity of BSH enzyme.

KEYWORDS: *Lactobacillus plantarum*, Bile Salt Hydrolase Enzymes, Conjugated Bile Salts, Deconjugation Activity, Substrate Specificity.

ÖZET

LACTOBACILLUS PLANTARUM GD2'DEN SAFRA TUZU HİDROLAZ GENLERİNİN (_BSH_) MOLEKÜLER KLONLANMASI VE KARAKTERİZASYONU
YÜKSEK LİSANS TEZİ
YASİN AYDIN
ABANT İZZET BAYSAL ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
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(TEZ DANIŞMANI: DOÇ. DR. MEHMET ÖZTÜRK)
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Safra Tuzu Hidrolaz (BSH) Enzimleri taurin ve glisin bağlı safra tuzlarını aminoasitlere parçalayan enzimlerdir. BSH'ların kesin fonksiyonu halen bilinmezliğini korumakla birlikte; beslenme, kolesterol düşürücü etki ve bağırsak kanserindeki rolü ile ilgili bazı hipotezler geliştirilmiştir. BSH'lar bağırsak florasında bulunan kommensal laktobasiller tarafından da sıklıkla sentezlenirler. Farklı laktobasil suşlarından elde edilen BSH'ların genomik varyasyonları, optimum pH, kinetik özellikler ve substrat seçiciliği gibi farklılıklar gösterdikleri görülmektedir. Bu çalışmada, *Lactobacillus plantarum* GD2 suşundan elde edilmiş *bsh*1 geni pET22b ekspresyon vektörüne klonlanmış ve yoğun şekilde ekspres edilmiştir. Gen öncelikle PZR vasıtasıyla türe özgü primerler kullanılarak çoğaltılmış ve sırasıyla pJET1.0 veya pBluescript II SK+ klonlama ve pET22b ekspresyon vektörlerine aktarılmıştır. Klonlar sırasıyla XL1-Blue ve BLR (DE3) *Escherichia coli* suşlarına aktarılmış, daha sonra 0.1 mM isopropyl-β-D-1-thiogalactopyranoside ile indüklenerek ekspres edilmiştir. Yoğun olarak ekspres edilen rekombinant protein, B-PER 6xHis Füzyon Protein Pürifikasyon Kiti kullanılarak saflaştırılmış ve ninhidrin protein analizi yöntemi kullanılarak altı adet farklı konjuge safra tuzu ile dekonjugasyon testi yapılmıştır. *bsh*1, *bsh*2, *bsh*3 ve *bsh*4 olmak üzere dört gen *L. plantarum* GD2 suşunun genom dizisinde tanımlanmış ve sekanslanmıştır. *bsh*1 geninin dizileme sonuçları, daha önceden bilinen diziler ile karşılaştırıldığında; birçok mikroorganizmanın BSH enzimleri ile yüksek oranda homoloji gösterdiği görülmüştür. Saflaştırılmış Bsh1 enziminin biyokimyasal karakterizyonunda enzimde diğer *Lactobacillus* türlerinde görülmeyen bir takım özellikler ortaya çıkmıştır. Enzim gliko-konjuge safra asitlerini daha yüksek oranda parçalamaktadır ve en yüksek oranda glycodeoxycholic ile aktivite göstermiştir. Bu çalışmada, ileri düzey çalışmalar için Bsh1 enziminin primer yapısının karakterizasyonu ve analizleri yapılmıştır. Gelecekte aktif bölge, substrat bağlanma bölgesi, substrat seçiciliği gibi özelliklerin anlaşılması için, BSH enziminin korunmuş amino asitleri üzerinde yönlendirilmiş mutagenez ve yapısal analizler yapılması gerektiği düşünülmektedir.

ANAHTAR KELİMELER: Dekonjugasyon Aktivitesi, Konjuge Safra Tuzları, *Lactobacillus plantarum,* Safra Tuzu Hidrolaz Enzimleri, Substrat Seçiciliği.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Pages

LIST OF TABLES

Pages

LIST OF ABBREVIATIONS AND SYMBOLS

Amp^R : Ampicillin resistance

BSH : Bile Salt Hydrolase

bp : Base pair

CA : Cholic acid

EDTA : Ethylenediaminetetraacetic acid

DCA : Deoxycholic acid

G+ : Gram positive

GCA : Glycocholic acid

GCDCA : Glycochenodeoxycholic acid

GDCA : Glycodeoxycholic acid

GIT : Gastrointestinal Tract

IPTG : Isopropyl-β-D-thiogalactopyranoside

LAB : Lactic Acid Bacteria

LB : Luria-Bertani

LCA : Lithocholic acid

kbp : Kilo base pair

kDa : Kilo Dalton

mg : Miligram

ml : Mililiter

ng : Nanogram

SCFA **:** Short Chain Fatty Acids

SDS-PAGE : Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TCA : Taurocholic acid

TCDCA : Taurochenodeoxycholic acid

TDCA : Taurodeoxycholic acid

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

µg : Microgram

µl : Microliter

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1. INTRODUCTION

1.1 Human Gut Microbiota

Gastrointestinal tract (GIT) is harbour of microorganisms mostly bacteria in various amounts called as "gut microflora". As an ecosystem which represents higher complexity, the microflora of humans is the most intimate portion of their biological environment (Holzapfel et al. 1998). Colonisation of the GIT of newborn infants starts with contamination from birth canal and environment. After two year, intestinal microflora formed completely as to that of adults (Bullen et al. 1977). It is estimated that 10^{14} of viable bacteria live in GIT of adults (Luckey and Floch 1972). This number is ten times greater than the total number of eukaryotic cells in the body (Guarner and Malagelada, 2003). When compared to 2 m^2 skin surface of body (Van Dijk, 1997), GIT provide much larger place for microorganisms to colonize on mucosal surfaces with its 150 – 200 m^2 area (Waldeck, 1990). Mucosal surface area of small intestine increase by circular folding, epithelial folding (intestinal villi) and folding of microvilli of enterocytes in the resorptive luminal membrane (Holzapfel et al. 1998; Waldeck, 1990). It is estimated that 300-500 different species live in GIT (Guarner and Malagelada, 2003; O'Hara and Shanahan 2006). Probably 99% of bacteria come from 30-40 species of bacteria (Beaugerie and Petit, 2004).

The gut microflora has a number of physiological functions associated to overall health. Therefore, may be detrimental if disturbance occurs in ecological balance of GIT (Holzapfel et al. 1998). There is a mutualistic relationship between gut flora and human. Bacteria of normal flora of human may possess a wide range of benefits. Guarner and Malagelada (2003) summarizes the main function of gut microflora in three main topic; metabolic, trophic and protective function. (i) Fermentation of non-digestable dietary residue and non-digestable mucus produced by epithelia are performed by the flora (Guarner and Malagelada, 2003). Considerable amount of energy is recovered by passively absorbing of short-chain fatty acids (SCFA) (Holzapfel et al. 1998). SCFA's are produced by colonic microflora and passively absored by the enterocytes (Hoverstad, 1989). The microflora also plays roles in absorbing of ions (Guarner and Malagelada, 2003), producing of vitamin K and B, and

metabolising of xenobiotics, sterols and bile acids (Cummings et al. 1997). (ii) The gut flora is an effective barrier against pathogenic and opportunistic bacteria (Holzapfel et al. 1998). (iii) In addition, it has a function in controlling of epithelial cell proliferation, differentiation, and, development and homeostasis of the immune system (Guarner and Malagelada, 2003). The functions of these microorganism resemble to functions of an organ, leading some to considering gut bacteria as a forgotten organ (O'Hara and Shanahan, 2006). The genera of predominant bacteria isolated from regions of small and large intestine (Table 1.1) summarized by Ridlon et al. (2006).

Table 1.1. Microbiological and biochemical characteristics of small and large intestine.

Section	Isolated Bacteria
Small Intestine	
Duodenum (25cm) pH 5.7 – 6.4 ~10^3 bacteria/ml	*Lactobacillus, Streptococcus*
Jejenum (1.0 m) pH 5.9– 6.8 ~10^4 bacteria/ml	*Lactobacillus, Streptococcus,Veillonella, Staphylacoccus*
Ileum (2.0 m) pH 7.3 – 7.7 ~10^8 bacteria/ml	*Enterobacteria, Enterococcus, Bacteroides, Clostridium, Lactobacillus, Veillonella*
Large Intestine	
Cecum/Colon (150 cm) pH 5.7 – 6.8 ~10^{11} bacteria/g	*Bacteroides, Eubacterium, Bifidobacterium, Ruminococcus, Peptostreptococcus, Propionibacterium, Clostridium, Lactobacillus, Escherichia, Streptococcus, Methanobrevibacter*

1.2 What are Probiotics?

The concept of probiotics evolved from Greek language "pro bios" mean "for life" and are defined as live microorganisms that confer health benefits to the host when consumed in adequate amounts. The history of probiotics began with history of mankind. Bible and sacred books of Hinduism mention about the cultured dairy products (Soccol et al. 2010); before bacteria recognized traditional milk products like yogurt was known by Turks.

Metchkinoff (1910) was the first who mention about probiotic organisms, he stated that long life of Bulgarian is related their consumption of fermented milk products containing lactobacilli. However, the term was first used by Lilly and Stillwell (1965) and defined as "substances secreted by one microorganism that stimulate the growth of another". In 1974, Parker described the term as "substances and organisms which contribute to intestinal microbial balance". In 1989 the definition was improved by Fuller, who proposed that probiotics are living microbes affect host health positively by improving intestinal microbial balance. Havenaar et al. (1992) stated a broadened definition "mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora". Salminen et al. (1998) defined them as "food which contains live bacteria beneficial to health". Besides these definitions, a definition derived by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) and approved by International Scientific Association for Probiotics and Prebiotics, today used frequently by majority of authors "live microorganisms which, when administered adequate amounts, confer a health benefit on the host".

1.2.1 Selection Criteria for Probiotics

Probiotics has a mechanism which exert some biological effects still poorly understood, however colonization resistance or competitive exclusion are often used to explain their mode of action (Elo et al. 1991). Oelschleager (2010) reported that effect of probiotics can be classified in three modes of action. (i) Probiotics might be able to get through the host's defense mechanism both of innate and acquired immunity. (ii) Probiotics may have direct effect on other microorganisms like commensal and pathogenic ones. (iii) Probiotics may have some effects on microbial products like toxins and host products e.g. bile salts and food ingredients.

More than the definitions, potential probiotic strains may show some properties for can be considered as probiotic organism. Havenaar et al. (1992) proposed some criteria to select probiotics are; total safety for host, surviving in gastric acidity and pancreatic secretions, adhesion to epithelial cells, antimicrobial activity, inhibition of adhesion of pathogenic bacteria, evaluation of resistance to antibiotics, tolerance to

food additives and stability in the food matrix. The potential probiotic strains would be able to perform effectively in GIT, and, are generally selected based on in vitro tolerance of related physiological stresses: eg, low pH and bile (Klaenhammer and Kullen, 1999).

It is a fact that probiotic properties of microorganisms are strain specific. Modern probiotic research aims to characterization of the normal, healthy gut microbiota in each individual and investigation of composition and concentration of strains in different portion of intestine (Soccol et al. 2010). The target is to define and characterize the gut microflora aims both nutritional management of specific gut-related diseases and finding new microbe sources for future biotechnological applications (Soccol et al. 2010). There are several genera of microorganisms which are used in probiotic researches; therefore lactobacilli and bifidobacteria are most popular genera which have attracted much attention with noticeable probiotic features (Collins and Gibson, 1999).

1.3 The Genus *Lactobacillus*

The genus *Lactobacillus* is member of lactic acid bacteria (LAB). LAB usually found in decomposing of dead plants and animals, they produce lactic acid as end product of carbohydrate metabolism by fermentation. Lactic acid-producing fermentation is an old invention. Scientific world have been aware of health benefit of LAB since Metchkinoff's reports. These bacteria are associated with fermentation of dairy products e.g. cheese, butter and yogurt. LAB strains exemplify main characteristics of probiotics, and they are frequently used in food and pharmaceutical industry. Some LAB strains inhabit in oral cavity, GIT and vagina of human and have healthful effects on host (Holzapfel and Schillinger, 2002). In probiotic applications, LAB strains have been used with increased interest.

The genus *Lactobacillus* is Gram-positive (G+), facultative aerobe, non-spore forming and non-flagellated rod shaped bacteria. Like LABs, lactobacilli inhabit in vagina and GIT, and are distributed in different niches both of there. They are colonized in different concentration in different parts of intestine. According to general assumptions a large number of *Lactobacillus* species form stable and grand number of lactobacilli populations are found in human intestinal tract, mostly in small intestine

(Velez et al. 2007). In human, colonization of *Lactobacillus* flora starts with at the end of ileum and well developed in intestine (Maerteau et al. 1995). There are 17 species that are associated with the human GIT (Table 1.2), most of the species are common inhabitants of oral cavity as well as GIT (Walter, 2008). Colonizing in human gut - specifically in intestine- increases the popularity of lactobacilli as a probiotic.

Table 1.2. Human associated *Lactobacillus* species which can isolate from saliva and feces.

Species	Feces	Oral cavity
L. acidophilus	+	+
L. crispatus	+	+
L. gasseri	+	+
L. johnsonii	+	
L. salivarus	+	+
L. ruminis	+	
L. casei	+	+
L. paracasei	+	+
L. rhamnosus	+	+
L. plantarum	+	+
L. reuteri	+	
L. fermentum	+	+
L. brevis	+	+
L. delbrueckii	+	
L. sakei	+	
L. vaginalis	+	+
L. curvatus	+	

1.4 Bile and Bile Salts

Bile is a yellow-green aqueous solution which play an important role in digestion and absorption of fats and lipid soluble vitamins in vertebrates. The main components of the bile are bile acids, cholesterol and phospholipids (Hoffman, 1994). Bile is synthesized in liver hepatocytes and secreted into gallbladder, stored and concentrated in there. After ingestion, gallbladder is stimulated to secrete bile into duodenum via bile duct.

The synthesis of bile acids starts with synthesizing of the primary bile acids. Cholic acid and chenodeoxycholic acid are primary bile acids and are synthesized from cholesterol (Chiang, 2009). After these two bile acids are secreted into the lumen of the intestine, intestinal bacteria dehydroxylate them through 7α-dehydroxylation to

form the secondary bile acids (Figure 1.1), deoxycholic acid and lithocholic acid respectively (Chiang, 2009). The liver cells may also conjugate primary and secondary bile acids with one of two amino acids, glycine or taurine, these conjugated bile acids are generally called as “bile salts” (Chiang, 2009). The solubility of the hydrophobic steroid nucleus of bile acid molecule is increased by conjugation of N-acyl amidate with either of amino acids (Begley et al. 2006). The resulting molecules act in emulsfying lipids in lumen by their amphipathic molecule structure, they can solubilize lipids to form mixed micelles.

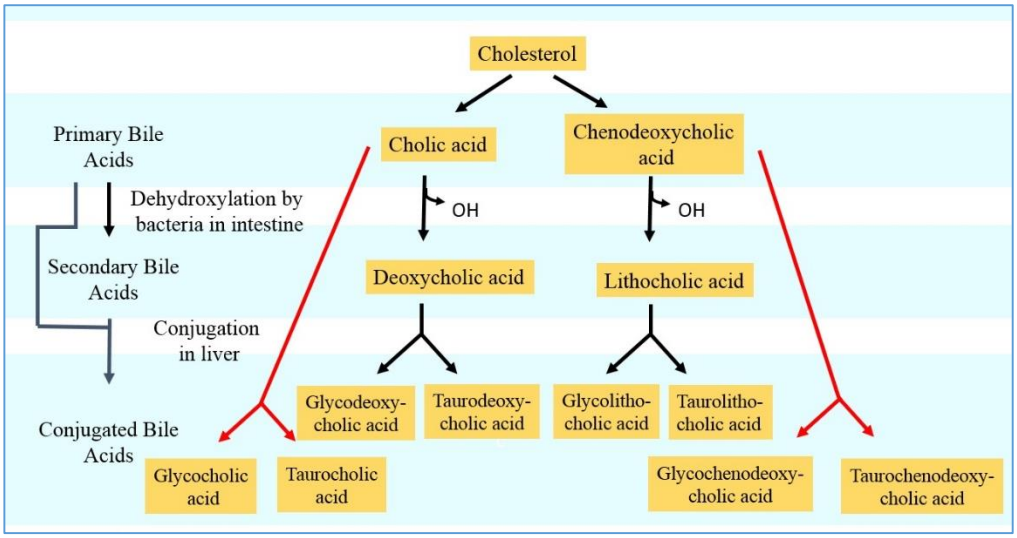

Figure 1.1. Primary and secondary bile salts (acids) and their transformation.

1.5 Enterohepatic Cycling of Bile Salts and Relation with some Diseases

Elevated cholesterol level in blood (hypercholesterolemia) is the major risk factor for developing of cardiovascular diseases and heart attacks. Therefore, decreasing serum cholesterol level is so important to prevent various diseases. It has been proposed that probiotics in human gastro-intestinal tract have the ability to decrease serum cholesterol level by bile salt hydrolase (BSH) activity. The reduction in serum cholesterol level 1% might reduce the risk of coronary disease by 2-3% (Liong and Shah, 2005). Oral administration of probiotics decrease the cholesterol levels in the range of 22 to 33% (Perreira and Gibson, 2002). Conjugated bile salts are secreted into the small intestine to assist in the absorption of dietary fat and cholesterol

(Hoffman, 1964), and then normally they are reabsorbed by intestine and returned to the liver by hepatic portal circulation in higher percentage, unabsorbed portion is lost in feces (Gorbach et al. 1969). BSH transportation in ileum is highly efficient (~95%), therefore 400 – 800 mg of bile salts lost to enterohepatic circulation daily (Vlahcevic et al. 1996). Deconjugated bile salts (free bile acids) are less soluble and less efficiently reabsorbed by intestine, therefore deconjugation of bile salts may lead to reduction in serum cholesterol level by expending of cholesterol for *de novo* synthesis (Reynier et al. 1981).

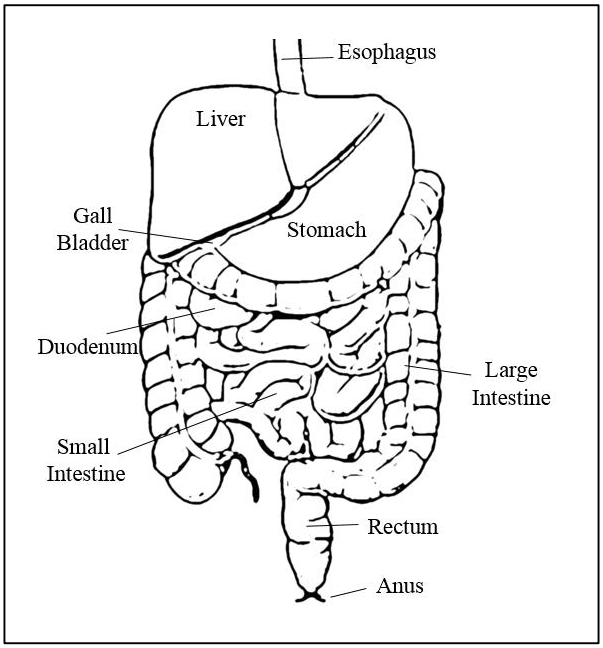

Figure 1.2. Anatomy of gastro-intestinal tract.

Primary bile salts (CA and CDCA) are synthesized from cholesterol by hepatocytes and conjugated with taurine and glycine in liver, then deposited in gall bladder. After stomach digestion, chyme enter the duodenum and bile secreted from gall bladder with its high bile salt contents. The majority of conjugated bile salts (95%) is actively reabsorbed from intestinal wall and transported to liver by portal circulation. A small part of primary, conjugated bile salts is deconjugated into free bile salts by BSH activity of intestinal microbiota and may be dehydroxylated into secondary bile salts (DCA and LCA). Secondary bile salts are passively absorbed from large intestine and 300 – 600 mg of them is excreted with feces daily.

It can be evaluated as therapeutic alternative instead of pharmaceutical treatments which are expensive and have side effects (Schuster, 2004), De Smet et al. (1994) suggest that consumption of BSH-active strains or cultured products might play a critical role in serum cholesterol level with interaction bile salt metabolism of the host. The reduction of cholesterol has been demonstrated with several microorganism species in different organisms which have similar to humans in cholesterol and bile metabolism, plasma protein distribution and regulation of hepatic cholesterol enzymes (Fernandez et al. 2000). Specifically, the cholesterol lowering effect of *Lactobacillus* has been determined in human (Anderson an Gilliand, 1999; Larsen et al. 2000; Nguyen et al. 2007), rats (Kawase et al. 2000; Park et al. 1996, 2007; Tannock 1995), hamsters (Chiu et al. 2006) and pigs (De Smet et al. 1998; Haberer et al. 2003).

However, the typical western type of diet is a high fat intake that leads to increased level of bile acid in large bowel (Reddy et al. 1976). After excretion of bile acids into the digestive tract, bacteria from enteric microflora metabolize the bile acids and secondary bile acids are arised (Hylemon and Glass, 1983). The secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA) -primarily deoxycholic acid in humans- are cytotoxic to colon cells and promote tumours to carcinogenesis although these acids are not carcinogens by themselves (Bernstain et al. 2005). In human, DCA accumulates in bile acid pool at high levels in some individuals and under normal physiological conditions there is no methabolic pathway to removing DCA from bile acid pool in humans (Ridlon et al. 2005). Another bile acid, cholic acid also has been reported as associated with carcinogenesis in rats (Magnuson et al. 1993), but the promoting effect of cholic acid (CA) may because of the formation of DCA from CA by bacterial dehydroxylation in the colon (Weidema et al. 1985). However some researchers claim that some bile acids may cause DNA damage and act as carcinogens in humans (Bernstein et al. 2005). In colon cancer patients DCA level in blood is higher than the control patients (Bayerdörferr et al. 1993). In addition, secondary bile acids have been reported to cause apoptosis in colonic epithelial cells (Bernstein et al. 2005). However, LCA is an activator to vitamin D receptor, activation the receptor in intestinal epithelial cells cause activation of the gene which metabolize the LCA (Adachi et al. 2005). When deconjugation of bile salts has been related to reduction in serum cholesterol level (Klaver and van der Meer, 1993), excessive deconjugation and dehydroxylation of primary bile salts play an important role in gall

stone formation (Dowling & Murphy, 1990) and enhances the risk of colon cancer (Marteau et al. 1995).

1.6 Bile Salt Hydrolases and Their Activity

BSHs are enzymes which catalyze the hydrolysis of taurine- and glycine-conjugated bile salts into free bile acids and amino acid residues. These enzymes have been classified in the family of choloylglycine hydrolase enzymes, which also enclose penicillin amidases (EC-3.5.1.11). Both of families are N-terminal nucleophilic hydrolases with an N-terminal cysteine residue. BSH (choloylglycine hydrolase; EC-3.5.1.24) catalyzes the hydrolysis of amide bond in conjugated bile acids (Patel et al. 2010). Some bacteria have possess two to four BSH-homologue, such as *L. plantarum* (Kleerebezem et al. 2003), *L. acidophilus* (McAuliffe et al. 2005) and *L. johnsonii* (Elkins and Savage, 1998). The precise role of these homologues are unknown but according to predictions these genes are transfered horizontally from other microorganisms which are share same microenvironment during their life cycle (Dussurget et al. 2002). Lambert et al. (2008) show that *L.plantarum* WCFS, carrying four BSH genes (*bsh*1, *bsh*2, *bsh*3, *bsh*4) where only *bsh1* exhibit BSH activity and other genes are involved to encode penicilin V acylase rather than BSH activity.

There is a strong interest between the habitat of a specific bacterial strains and BSH activity. BSH activity is a typical feature for inhabitants of the gastro-intestinal tract. (Tanaka et al. 1999). The capability to deconjugate bile salts suggests additional advantages for survival and persistence under gastro-intestinal conditions. It is claim that BSH activity plays a role in the detoxification (Grill et al. 2000), it can be said that bacteria deconjugate the bile salts in order to survive well. Altough exact mechanism is little known, BSH activity is rare among most Gram-negative bacteria and these bacteria are more resistant to bile salts which are so toxic materials (Lambert et al. 2008). They do not need detoxification against bile salts by their strong cell wall structure.

It has been concluded that deconjugation may provide a nutritional advantage using the released amino acids as carbon, nitrogen and energy source. (van Eldere et al. 1996). BSH activity facilitate the integration of cholesterol into bacterial membranes (Taranto et al. 1999), in this way membrane tension may increase or

membrane charge and fluidity may change (Bernstain et al. 2005). Altering membrane properties leads to increase resistance against bile, intestinal defensins and lysozyme etc. (Begley et al. 2005).

Distinct from conjugation, which occur in liver, deconjugation is performed in intestine and mostly by bacteria. BSH activity has been widely detected in several bacterial genera of the GI microbiota of mice, rats, chickens, swine and humans (Savage, 1977). BSH activities was reported from several genera of microorganisms; such as *Clostridium* (Coleman and Hudson 1995), *Bacteroides* (Kawamato et al. 1989), *Bifidobacterium* (Kim et al. 2004), *Enterococcus* (Wijaya et al. 2004) and *Lactobacillus* (Elkins et al. 2001, Yıldız et al. 2011). LAB strains are critically important for BSH activity and they have been frequently studied so far. Tanaka et al. (2000) reported that screening analysis of more than 300 LAB strains studied in their laboratory showed that BSH activity found primarily in organisms isolated from the GI tract of mammals (*Bifidobacterium spp*., *L. acidophilus*, *L. gasseri* and *L. johnsonii* and *L. plantarum*).

1.7 Genetical Properties of BSH

BSH enzyme at first was partially purified from *Clostridium perfingens* and found to be catalytically active against glyco- and taurocholic acids (Nair et al. 1967), then the enzyme purified and characterized (Gopal-Srivastava & Hylemon, 1988). Dashkevicz and Feihner (1989) developed a selective medium for the screening of BSH activity from the genomic library (direct plate assay). Genes which encoding BSH activity was cloned first by Christiaens et al. (1992), they cloned the BSH gene from genomic library of *Lactobacillus plantarum* 80 and developed a differential medium for BSH-active *E.coli* clones using Luria Bertani (LB) medium with some modifications. Tanaka et al. (1999) used a method to determine the substrate specificity of BSH enzymes. According to the method, amino acids from deconjugation of bile salts are measured using ninhydrin assay after then enzyme reaction released.

Coleman and Hudson (1995) have showed that BSH enzyme from *C. perfingens* was homologous to penicillin V acylase (PVA) from *Bacillus sphaericus*. Nair et al. (1967) was reported that cysteine is associated with catalytic function of *C. perfingens*,

and exchange of Cys-2 with alanine in the *Bifidobacterium longum*, organism could not produce active BSH. Rossocha et al. (2005) reported that sequence alignments of PVA with some other BSHs indicated that five of amino acids are strictly conserved which are catalytically important in BSHs. These five amino acids are Cys-2, Asp-20, Tyr-82, Asn-175 and Arg-228. Kumar et al. (2010) have showed that four highly conserved amino acid motifs (YFGRNXD, NEXGLXXAGLNF, VXVLTNNPXF and SXSRFVRXAF) are located around the active site.

1.8 Substrate Specificity of BSH

BSHs deconjugate both of primary and secondary bile salts and how they recognize their substrates is under discussion. Moser and Savage (2001) reported that *L. buchneri* JCM1069 deconjugates TDCA but not TCA. These bile salts both have taurine as their amino acid moiety and differ in cholate moieties. McAuliffe et al. (2005) have showed that inactivation of *bshA* of *L. acidophilus* NCFM cause reduction in deconjugation activity of the strain against chenodeoxycholic acids (GDCA, TDCA) as steroid moiety. It has been claimed according to kinetic properties of the enzyme, substrate is recognized predominantly at the amino acid moieties (Coleman and Hudson, 1995; Tanaka et al. 2000). In addition, bacteria display different preferencials against tauro- and glyco-conjugated bile salts. BSH from *L. johnsonii* 100-100 (Elkins & Savage, 1998; Lundeen and Savage, 1990) show preferencial hydrolysis of tauroconjugated bile salts; however majority of strains such as: *B. bifidum* ATCC 11863 (Kim et al. 2004); *B. longum* SBT 2928 (Tanaka et al. 2000); *L. plantarum* WCSF1 (Christiaens et al. 1992), BBE7 (Dong et al. 2012); *L. gasseri* Am1 (Jiang et al. 2010); *L. acidophilus* LA4 and LA11 (Jiang et al. 2010) preferences glycoconjugated bile salts. In addition, *L. acidophilus* NCFM can conjugate both of tauro- and glyco-conjugated bile salt (Mc Auliffe et al. 2005), and *L. johnsonii* PF01, has three of BSH enzyme (BSH A, B and C), when BSH A and B has affinity to tauroconjugated bile salts BSH C display preferencial glycoconjugated bile salts (Chae et al. 2012; Oh et al. 2008;).

2. AIM AND SCOPE OF THE STUDY

BSHs are enzymes which are produced by liver hepatocytes and act in dietary lipid absorption in the the small intestine. They have been reported which have critical role in enterohepatic circulation of bile salts and secondary metabolites from deconjugation of BSHs related the proliferation of cancer cells in colon. Some of intestinal bacteria constantly display BSH activity. BSH activity is an important selection criteria for probiotic organisms to reduce serum cholesterol level. There have been increased interest to LAB strains which are popular probiotic strains*;* *Bifidobacterium* and *Lactobacillus* are most popular probiotic organisms. BSHs from different *Lactobacillus* species have been cloned and characterized several times. Sequence aligments of BSHs with the enzymes from choloylglycine hydrolase family show that five amino acid are conserved in open reading frame, and, different conserved amino acid motifs have been determined around them. Like other probiotic properties, BSH activity is also strain specific. The substrate specificities of BSH enzymes among intestinal bacteria varies even in same species. Reported data show that there is a relation between substrate specificity and amino acid sequence of BSHs. Therefore, BSHs have been frequently cloned by researchers to find the organism which carry the ideal *bsh* gene to human health.

L. plantarum GD2 strain was isolated from breast-fed human infant feces. The strain was identified as *L. plantarum* using by API 50CHL kit and 16S rRNA gene sequence analysis method, have higher cholesterol removing activity and bile resistance (Yıldız et al. 2011). In present study, to investigate the relation of substrate specificity and amino sequence of *bsh* genes, the *bsh*1 gene was cloned, overexpressed, purified and characterized with six bile salts. At first, oligonucleotid primers was designed according to sequences of BSH genes from *L. plantarum* strains (Sequence data was downloaded from GenBank). After proliferation using PCR, *bsh*1 gene from *L. plantarum* GD2 was cloned into pET22b expression vector, expressed in *E. coli* BLR (DE3) and purified, followed by ninhydrin protein assay as in Tanaka's protocol (1999). The *bsh*1 gene, encoding bile salt hydrolase enzyme, were identified in the genome sequence of *L. plantarum* GD2. Comparison of the deduced amino acid sequences of the *bsh*1 gene with previously known sequences; revealed high homology

with BSH enzymes of several microorganisms. Biochemical characterization of the purified BSH from *L. plantarum* GD2 revealed some distinct characteristics not observed in other species of *Lactobacillus*.

3. MATERIALS AND METHODS

3.1 Bacterial Strain and Growth Conditions

All *Escherichia coli* strains and plasmids used in this work are listed in Table 2.1. *Escherichia coli* strains and their plasmid-containing derivatives were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotics (ampicillin, 100 µg/ml per ml*)* for plasmid selection at 37°C on plates or liquids (by shaking 170-175 rpm). All ingredients of the culture media and the preparation of the antibiotic solutions are listed in the Appendices.

3.2 Plasmid DNA Isolation

A freshly grown single colony of *E. coli* strains were inoculated to 5-10 ml culture of LB medium with suitable antibiotic provided to each sample and grown at 37°C with shaking at 170-175 rpm for 16-18 hours. 5 ml cultures were prepared for the strains containing high copy (pJet1.2 and pBluescript II SK+ and 10 ml cultures were used for low copy (pET22b) number of plasmids. DNAs were isolated using GeneJET™ Plasmid Miniprep Kit by following the manufacturers' protocol.

3.3 Enzyme Digestion

Insert and plasmid DNAs were digested by using the endonucleases (Fermentas) according to manufacturers' instructions. For cloning, 2 µg plasmid DNAs (pBluescript II SK+ or pET22b) were digested with 2 µl of restriction enzymes (10 U/µl) in 50 µl final volume at 37°C overnight. Dephoshorylation was achieved to prevent self cycling of fragments by incubating digested plasmids at 37°C for 45 minutes. For orientation test to control the clones ~200 ng plasmid DNA was digested with 0.5 µl of restriction enzyme (5 U/µl) in 20 µl final volume at 37°C for 1 hour. The name and catalog number of restriction endonucleases are listed at Appendices.

Table 3.1. Bacterial strains and plasmids used in this study.

Strain	Genotype - Origin	Phenotype	Reference
Lactobacillus plantarum			
GD2	Human faeces		Yıldız et al. 2011
Escherichia coli			
XL1-Blue	rec A end A 1 gyr A986 thi-1hsdr17supE44 rel A1 lac		Stratagene
XL1-Blue/pYA302		Amp^r	This work
XL1-Blue/pYA303		Amp^r	This work
XL1-Blue/pYA304		Amp^r	This work
XL1-Blue/pYA101		Amp^r	This work
BLR (DE3)	F-ompT gal dcm lon hsdSB (rB- mB-) λ(DE3)		Novagene
BLR (DE3) /pYA201			This work
Plasmid	**Genotype**	**Phenotype**	**Reference**
pBluescript	II SK+	Amp^r	Fermentas
pYA302	pBluescript II SK+ with 1.0 kb bsh2 gene insert	Amp^r	This work
pYA303	pBluescript II SK+ with 1.0 kb bsh3 gene insert	Amp^r	This work
pYA304	pBluescript II SK+ with 1.0 kb bsh4 gene insert	Amp^r	This work
pJet1.2/blunt end		Amp^r , Lethal gene	Fermentas
pYA101	with 1.0 bsh1 gene insert	Amp^r , Lethal gene	This work
pET22b	pelB, T7 lac, ApR, pBR322 ori (C-terminal His-tagged protein)	Amp^r	Novagene
pYA201	with 1.0 kb bsh1 gene insert and fragment from MCS site of pJet1.2	Amp^r	This work

3.4 Purification of Digested DNA Samples

Plasmid DNAs were isolated from the transformants and to be used for sequencing which were purified and concentrated by High Pure PCR Product Purification Kit (Roche cat #: 11732668001) following the manufacturers' instructions. Final volume of the sample was adjusted according to the concentration of the DNA.

3.5 Isolation of DNA Fragments from Agarose Gel

For the isolation of target DNA fragments from agarose gel, digested DNA solution was loaded on 1% agarose gel and the gel was run approximately one hour. After the different sized DNA bands were separated to each other, the target bands were excised from agarose gel (minimization of UV exposure was taken care to protect DNA). The approximate volume of gel slice was determined by its weight (1 g equals approximately 1 ml) and placed into 1.5 ml eppendorf tube. Finally to elute DNA in silica membrane of column, 50 µl EB buffer (10 mM Tris-HCl, pH 8.5) or dH_2O was added to center of the membrane and centrifuged for 1 min at maximum speed.

3.6 Ligation Reaction

T4 ligase (Fermentas cat. # EL0331) was used for ligation reaction. The concentrations of vector and insert DNA could be determined by either spectrophometry at OD_{260} or deduction of its value using with 1 kbp marker on agarose gel. The following equation is used for calculating the amounts of insert and vector DNAs before set up ligation reaction.

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

~50 ng vector and ~150 ng insert DNA was used for the ligation mixture and incubated at 22°C for 1 hour. After ligation process, T_4 DNA Ligase was inactivated by incubating at high temperature (65°C for 10 minutes).

Table 3.2. The reactants of a standard ligation reactions.

Reactants	Volumes
Insert DNA (150 ng)	5 µl
Vector DNA (50 ng)	2 µl
T_4 DNA Ligase (2U-4U/µl Fermentas)	1 µl
T_4 DNA Ligase Buffer (10X -Fermentas)	1,5 µl
ddH_2O	5,5 µl
Total reaction volume	15 µl

3.7 Competent Cell Preparation

3.7.1 XL1-Blue and BLR(DE3) Cell Preparation

Both of XL1-Blue and BLR(DE3) *E. coli* competent cells are prepared following same procedure. 500 µl overnight culture of *E. coli* strains is inoculated to 50 ml LB in a 500 ml flask. The inoculated cultures were incubated at 37°C with vigorous shaking until bacteria reaches log phase (OD_{600} 0.4-0.5). Then, cells were taken on ice and transferred to ice cold Falcon tubes for centrifugation. Centrifugation was occured at 5000 rpm for 10 minutes at 4°C. After centrifugation, supernatant was discarded and pellet was resuspended in 20 ml (1/2 of the culture volume) of ice cold $CaCl_2$ (100mM), finally incubated on ice for 30 minutes. After 30 minutes incubation on ice, cells centrifuged at 5000 rpm and 4°C for 10 minutes. Supernatant was discarded carefully and pellet was resuspended in 2 ml (1/20 of the culture volume) of ice cold solution containing $CaCl_2$ (100 mM) and glycerol (10% of final concentration). Consequently, competent cells were aliquoted as 100 µl volumes into sterile Eppendorf tubes and stored at -80°C.

3.7.2 $CaCl_2$ Transformation

~ 10 µl of ligation reaction mixture was added to 100 µl of competent cells and incubated on ice for 45 minutes. Then, heat shock was applied to the cells by incubating them at 37°C for 5 minutes. Samples were re-taken on ice and incubated for 5 minutes. 1 ml of LB was added to the samples and cells were incubated for 1 hour at 37°C with vigorous shaking. After incubation, cells were centrifuged at 13.000 rpm for 1 min at RT and pellet was resuspended in 100 µl LB. Transformant cells were spread on selection plates and incubated at 37°C for 16-18 hours.

3.7.3 Proliferation of BSH Genes (*bsh*1, *bsh*2, *bsh*3, *bsh*4) by Polymerase Chain Reaction (PCR)

Species specific forward and reverse oligonucleotid DNA primers were designed according to *bsh* DNA sequences from NCBI (National Center of Biotechnology) to synthesize four *bsh* genes (RefGen, Ankara). In this method, *Pfu* DNA polymerase which has a proofreading activitiy compared to the other polymerases and avoids second site-mutations (Flaman et al., 1994; Cline et al., 1996) was used as polymerase enzyme and PCR reactions were performed by using thermal temperature cycler (Techne TC-3000). PCR reactions were prepared as defined on Table 3.4 and Table 3.5 for each *bsh* genes. Reactant concentrations and number of recyling were same for all PCR reactions, only annealing temperatures are different because of different melting temperatures of the primers (Table 3.3). Annealing temperatures was 39°C for proliferation of *bsh*1, 36°C for *bsh*2 reaction and 42°C for *bsh*3 and *bsh*4 reactions. PCR reactions (Table 3.5) were recycled 35 times for every *bsh* genes.

Table 3.3. Forward and reverse primers used for the proliferation of *bsh* genes.

Primers	**Sequence of the primers**	**Used for reaction**
Bsh-1F	5'-ATGTGTACTGCCATAAC-3'	*bsh*1
Bsh-2F	5'-ATGTGCACTAGTCTAAC-3'	*bsh*2
Bsh-34F	5'-ATGTGTACTAGTTTAAC-3'	*bsh*3 and *bsh*4
R1	5'-GCTTCTGATCGTAAT-G-3'	*bsh*1
R2	5'-GGACGTATTCTAACGG-3'	*bsh*2
R3	5'-GGGTTGTCTTTAATTC-3'	*bsh*3
R4	5'-AATCGGCAGGAAAG-3'	*bsh*4

Table 3.4. PCR reactions for proliferation of *bsh* genes.

Reactants	**Volumes**
Template DNA (10 ng/µl)	1.5 µl
Buffer (10X *Pfu* Reaction buffer)	5 µl
Forward Primer (125 ng/µl)	2 µl
Reverse Primer (125 ng/µl)	2 µl
dNTP mix (10mM) (Fermentas)	2 µl
$MgCl_2$ (50mM)	4 µl
Pfu polymerase (5u/ µl)	0.5 µl
ddH_2O	32 µl
Total reaction volüme	50 µl

Table 3.5. PCR conditions for proliferation of *bsh* genes.

Cycle	Temperature	Time
First denaturation	95 °C	10 min
Denaturation	95 °C	1 min
Annealing	39 °C	1 min
Extension	72 °C	2 min
Final extension	72 °C	5 min
Final hold	4 °C	-

3.8 Construction of *bsh*1, *bsh*2, *bsh*3, *bsh*4 Carrying Vectors

3.8.1 The Selection of Plasmid Vectors

In this study, pBluescript II SK+ and pJet1.2 plasmid cloning vectors and pET22b plasmid expression vectors were used. These vectors were preferenced because of some advantageous features. pBluescript II SK+and pJet1.2 are small and high copy vectors. Transformants with pBluescript II SK+ can be selected easily by Blue-White Colony Assay in the presence of IPTG and X-Gal. Transformants' colonies without any insert DNA appear in blue color by the function of β-galactosidase enzyme wihich break down X-gal; but transformants' colonies with insert DNA were seems in white color. Insert DNA fragment disrupts LacZ gene and production of β-galactosidase enzyme is paused.

pJet1.2 cloning vector has a multiple cloning site (MCS) in lethal gene. Therefore, after transformation of *E. coli* cells which take a pJet1.2 without any insert can not live and reproduce. When plasmid enter the cell, two ends of linear plasmid ligated by cell enzymes and the lethal gene recombined. On the other hand, inserted plasmids has no complete lethal gene because insert DNA divide the lethal gene to two parts and does not express. Therefore, after transformation, only cells which have insert containing plasmids can live and reproduce.

pET22b plasmid vector was used for expression of *bsh*1 gene. pET22b plasmid vector has 6x Histidine amino acids codons which code the fusion protein. When *bsh*1 gene was expressed in the vector, fusion protein is tagged the protein product. During protein purification with Lithium-chelated columns, target protein – which His-Tag

fusion protein is binded in his C-terminal end – binds the column by the attraction of histidine of fusion protein and Li^+ ions embedded column.

3.8.2 Cloning of *bsh*1 Gene to pJET1.2 Vector

For ligation reaction, the pJET1.2 vector was obtained commercially (ClonJet PCR Cloning Kit) in linear form with two blunt ends. After PCR reaction, *bsh*1 gene fragments - almost 1 kb – was cloned to the vector. According to pJET1.2 vector kit protocole non-purified PCR products were added to reaction mixture. 975 bp *bsh*1 fragment was inserted into pJet1.2 vector by ligation reaction at 22°C for 1 hour. After ligation reaction, 10 µl of ligation mixture were transformed into XL1-Blue competent cells by $CaCl_2$ transformation. Transformants were selected on the basis of lethal gene and their size (4.0 kbp). The new clone was saved and named as XL1-Blue/pYA101 (Figure 3.1).

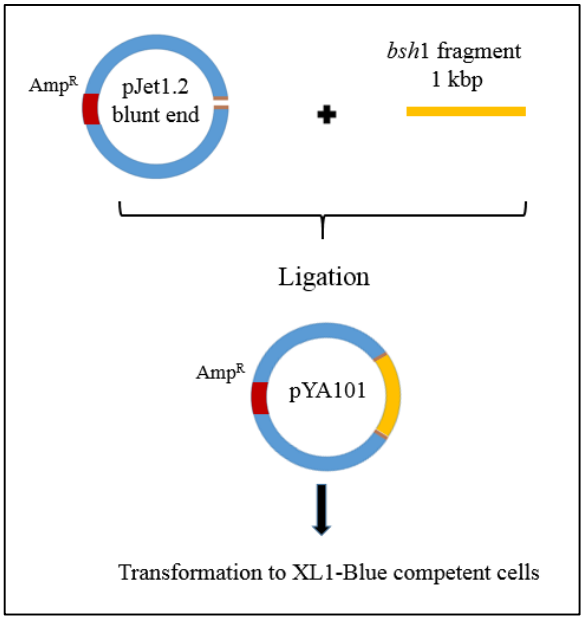

Figure 3.1. Cloning of *bsh*1 fragment into pJet1.2 blunt-end vector.

3.8.3 Orientation Test with *Xba*I Restriction Endonuclease for PYA101

To select the desired XL1-Blue/pYA101 clones, plasmid DNAs were isolated from transformants by Plasmid DNA Isolation Kit (Fermentas). To select the clone that have correct oriented insert, plasmid DNAs were isolated from XL1-

Blue/pYA101 transformants and digested with *Xba*I which cuts once vector and once insert resulting 3.3 kbp and 0.6 kbp fragments if insert is correct oriented (Figure 3.2), 3.7 kbp and 0.3 kbp fragments if insert is wrong oriented (Figure 3.3).

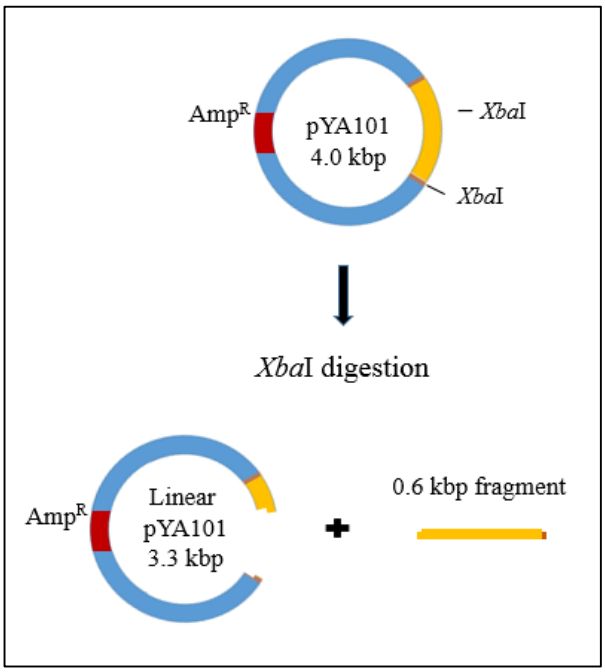

Figure 3.2. Digestion of pYA101 with *Xba*I when insert in correct orientation.

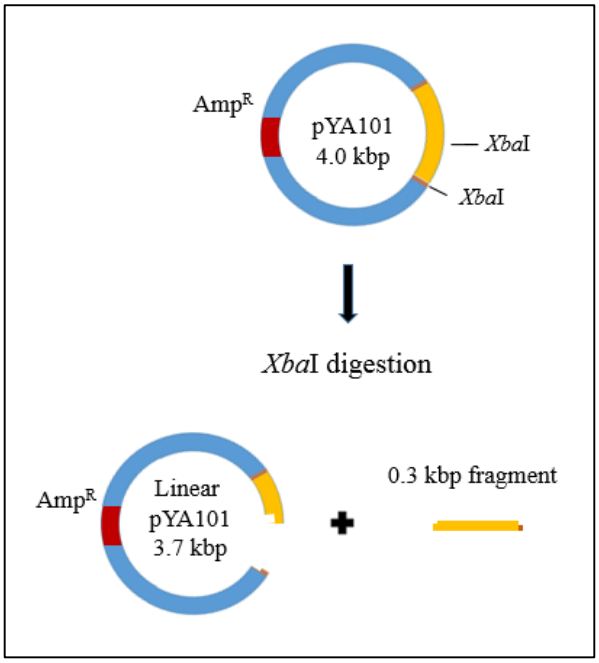

Figure 3.3. Digestion of pYA101 with *Xba*I when insert in incorrect orientation.

3.8.4 Transfering of *bsh*1 Fragment from pYA101 to pET22b Expression Vector

When transfering of *bsh*1 from pYA101 to pET22b expression vector; two of restriction enzymes, *Xho*I and *Nco*I, were selected. These enzymes digest the both of the plasmids in a reverse order. Transfering of *bsh*1 gene from pYA101 plasmid to pET22b plasmid, caused inversion in the direction (Figure 3.4 and 3.5). Therefore, the wrong oriented pYA101 is selected in beginning to provide correct orientation in pET22b.

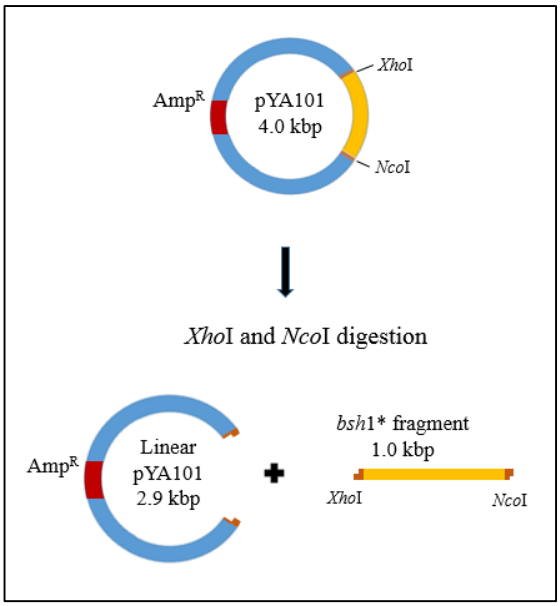

Figure 3.4. Ceasing of *bsh*1 site from pYA101 by digestion of pYA101 with *Xho*I and *Nco*I.

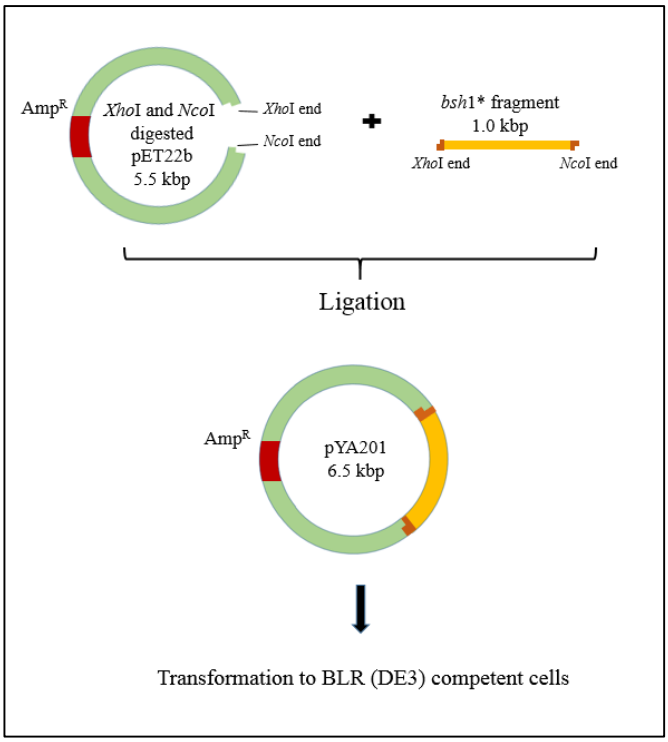

Figure 3.5. Cloning of *bsh*1 gene to pET22b expression vector.

Both of pYA101 and pET22b plasmids were treated with *Xho*I and *Nco*I enzymes, resulting *bsh*1 site was ceased from pYA101and pET22b plasmid was linearized. Desired fragments were separated on the 1% agarose gel and isolated from agarose gel. Then 1031 bp fragment (carry *bsh*1 gene) was inserted into linear pET22b vector by ligation reaction at 22°C for 1 hour. After ligation reaction, 10 µl ligation mixtures were transformed into BLR(DE3) competent cells by $CaCl_2$ transformation. Transformants were selected on the basis of ampicillin resistance and their size (6.5 kbp). The new clone was saved and named as BLR(DE3)/pYA201.

3.8.5 Orientation Test with *Xba*I Restriction Endonuclease for pYA201

To select the clone that have correct oriented insert, plasmid DNAs were isolated from BLR(DE3)/pYA201 transformants using by Plasmid DNA Isolation Kit and digested with *Xba*I which cuts once vector and once insert resulting 5.7 kbp and 0.8 kbp fragments if insert is correct oriented, 6.1 kbp and 0.4 kbp fragments if insert is wrong oriented.

3.8.6 Cloning of *bsh*2, *bsh*3 and *bsh*4 Genes to pBluescript II SK+ Cloning Vector

pBluescript II SK+ plasmid vector was linearized by digestion of *Sma*I enzyme producing two blunt ends. The gel-extracted PCR products, *bsh*2, *bsh*3 and *bsh*4, were inserted into pBluescript II SK+ by ligation process at 22°C for 1 hour. After ligation process, 10 µl ligation mixtures were transformed into XL1-Blue competent cells by $CaCl_2$ transformation. Transformants were selected on the basis of Blue-White Colony Assay and their size (~4.0 kbp). The new clones were saved and named as XL1-Blue/pBGD2bsh2, XL1-Blue/pBGD2bsh3 and XL1-Blue/pBGD2bsh4.

3.8.7 DNA Sequencing

To see complete sequence of BSH genes (*bsh*1, *bsh*2, *bsh*3 and *bsh*4), pYA101, pYA302, pYA303 and pYA304 plasmids were sequenced by RefGen (Ankara, Turkey) using universal primers T3 (5'- GCGCGAAATTAACCCTCACTA-AAG-3'; 24-bp) and T7 (5'- TAATACGACTCACTATAGGG -3'; 20-bp).

3.9 Analysis of Bsh1 Protein Activity

3.9.1 Preparation of Bsh1 Protein

Bsh1, his-tagged protein, was purified using by B-Per^{TM} 6xHis Fusion Protein Purification Kit. A bacterial colony of *E. Coli* BLR(DE3)/pYA201 were inoculated to 200 ml culture of LB broth with suitable ampicillin providing (100 µg ml^{-1}) and incubated at 37°C with shaking at 175 rpm until $O.D._{600}$ (optical density at 600 nm) reached to between 0.5 and 0.6. At this point expression was induced by the addition of IPTG (0.1 mmol 1^{-1}) and incubated again until $O.D._{600}$ reached to 2.0 – 3.0. Next, cells were harvested by centrifugation at 8000 g and +4 °C for 10 minutes. The pellet was washed and suspended in 10 ml of B-PER Reagent by either pipetting and vortexing under until cell suspension homogenous. The homogenous mixture shaked at RT for 10 minutes. Soluble proteins were separated from insoluble proteins by centrifugation at 27000 g and +4 °C for 15 minutes. Pellet was removed and 10 ml of supernatant applied to Nickel Chelated Column. The column washed by 6 ml of Wash Buffer I and 9 ml of Wash Buffer II respectively. Finally, 6xHis-tagged protein was

eluted by using by 6 ml of Elution Buffer. All solutions which flow through the column were allowed to analysis directly by SDS-PAGE.

3.9.2 Determination of Amount of Purified Protein by Lowry Assay

Protein concentration was determined by Lowry method with BSA as the standard protein (Lowry et al. 1951). At first, standard BSA (Bovin Serum Albumin) protein solution is prepared (1.0 g/ml). Then the stock BSA solution is added to six Eppendorf tubes (0, 4, 8, 12, 16 and 20 µl of BSA stock) respectivelly and final volume completed to 200 µl by adding required amount of 1% SDS/0.1 N NaOH. For sample, 50 µl of Bsh1 protein sample diluted with 150 µl of 1 % SDS/0.1 N NaOH solution and then reagent E was added to BSA standard proteins' tubes and Bsh1 protein sample tube and they were incubated at RT for 5 minutes. Then 100 µl 1 N Folin and Ciocalteu's Phenol reagent was added to all protein samples and they were incubated at RT for 30 minutes. The curve of standard BSA samples concentration was made by spectrophotometric analysis which was conducted with optical density at 660 nm ($O.D._{660}$) (Figure 3.6). The optical density of the Bsh1 protein sample were also determined at $O.D._{660}$ nm and concentration of the Bsh1 protein sample were determined by the following formula: y =0.049x + 0.013, (y is absorbance and x is amont of the protein as a µg/ml).

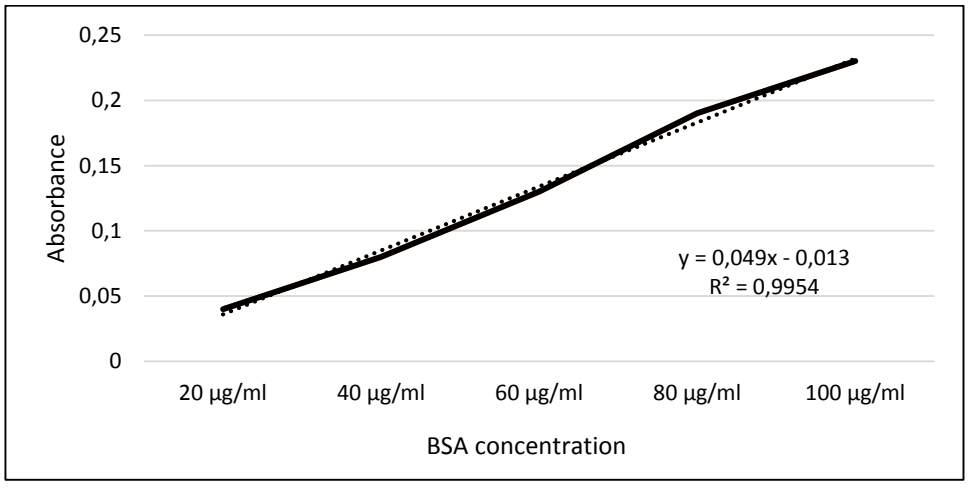

Figure 3.6. Standard curve of Bovin Serum Albumin (BSA) concentrations.

3.9.3 SDS-PAGE and Coomassie Stain

Sodium dodecyl sulfate SDS-PAGE were performed using 15% (w/v) polyacrylamide solutions according to Laemmli protocole (Laemmli, 1970). The gel consist of two parts; separating and stacking gels. First separating (running gel) was prepared and poured in gel apparatus. After 45 minutes staking gel was prepared and poured in. Polymerization of the gel takes at least two hours, samples were not loaded before polymerization was completed.

After protein purification, protein samples were solubilized in 5% β-mercaptoethanol by incubating at 37°C for 10 minutes before loading on gels. After that, samples were solubilized in equal volume of Loading Dye then they were loaded with on 15 % Laemmli type SDS-PAGE and runned at 60 V for 1 hour, then runned at 120-150 V to all dyes passed out from the gel.

Table 3.6. The preparation of 15% Laemmli type SDS-PAGE.

Gel Contents	**Separating Gel (ml)**	**Stacking Gel (ml)**
ddH_2O water	7.05	6.10
Gel Buffer	7.50	2.50
10% SDS	0.30	-
Acrylamide mix (30%)	15.00	1.30
10% APS	0.15	0.10
TEMED	0.015	0.005
Total	30 ml	10 ml

After removing the polyacrylamide SDS-PAGE gel from glass, it was rinsed in ddH_2O in a suitable container for staining protein lines. Coomassie Staining solution (Appendix B) was added until jel was covered with the staining buffer (~ 1.5 cm). It was heated by microwave on high power for 1 minute until the Coomassie Stain boiled. The gel was incubated in the Coomassie Staining solution for 5 to 10 minutes on rocking table. Then Coomassie Stain was poured off and rinced twice in ddH_2O and fresh Destain solution (Appendix B) to cover the gel (~ 1.5 cm). It was heated by microwave on high power for 1 minute until the destain solution boiled and the gel was incubated on a rocking table and destain solution was refreshed until protein bands seem apparent (Roland, 2007).

3.9.4 Substrate Specificity Test for Bsh1 Protein by Ninhydrin Protein Assay

The method, modified from Tanaka et al. (1999), was used to determine the substrate specificity of Bsh1 enzyme against six bile salts; taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GA), glycodeoxycholic acid (GDCA) and glycochenodeoxycholic acid (GCDCA). At first, glycine stock solution was prepared (40 mmol 1^{-1}), and standard series were prepared from the stock. The six of standard series each of them also contains 10 mmol 1^{-1} of dithiotreitol (DTT) and 100 mmol 1^{-1} sodium phosphate (pH 6.0) comprised of in an order of 0, 4, 8, 12, 16 and 20 mmol 1^{-1} of glycine concentration. For each of reaction tubes, 10 µl of Bsh1 enzymes was added to 190 µl of reaction mixtures consist of 100 mmol 1^{-1} sodium phosphate (pH 6.0), 10 m mol 1^{-1} conjugated bile acids (glyco- or tauro-conjugated bile salts) and 10 mmol 1^{-1} of dithiotreitol (DTT). Reaction tubes were incubated at 37°C for 10 minute. Each reaction was stopped using 15% trichloroacetic acid. The mixture was centrifuged at 20 000 **g** for 5 minute to obtain and recover the reaction samples from precipitates.

The ninhydrin protein assay was applied to measure the amount of released amino acids using ninhydrin reagent. Standard series and reaction samples was boiled for 15 minutes and then cooled to room temperature. Spectrophotometric analysis was performed at $O.D._{570}$ and according to these results relative enzyme activities was calculated with the highest value designated as 100%.

4. RESULTS AND DISCUSSIONS

4.1 Results

4.1.1 Proliferation of *bsh* Genes

The genomic DNA isolated from *L. plantarum* was used as template DNA to amplify *bsh* genes (Figure 4.1). Using species specific oligonucleotid primers blunt ended *bsh*1, *bsh*2, *bsh*3 and *bsh*4 genes were proliferated by PCR reactions. PCR reactions were set up by different annealing temperatures because of different melting temperatures of forward and reverse oligonucleotid primers. Adjusting the appropriate temperatures *bsh* genes were produced. After PCR reactions 10 µl of PCR products of each reaction were visualized on gel.

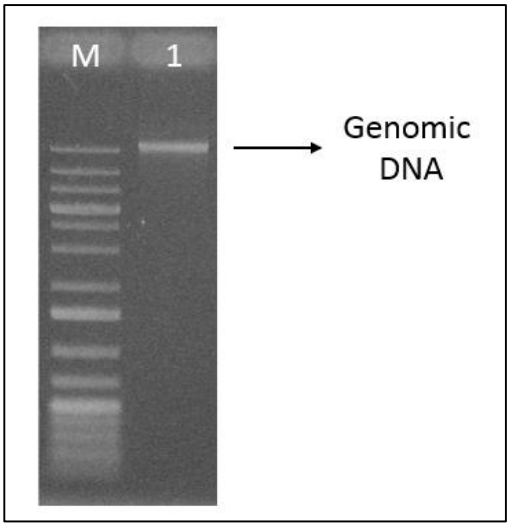

Figure 4.1. Determination of the amount of genomic DNA. The line indicates the genomic DNA of *L. plantarum* GD2 which was used as template DNA in the four of PCR reactions; marker is designated as M.

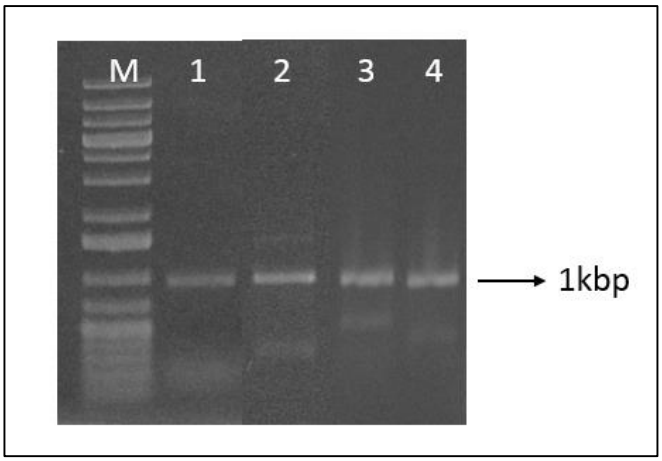

Figure 4.2. Proliferations of blunt-ended *bsh* genes by PCR reactions. The four of lines contain the four of *bsh* genes (*bsh*1, *bsh*2, *bsh*3 and *bsh*4) respectively; marker is designated as M.

4.1.2 Cloning of *bsh*1 Gene to pJET1.2 Vector

After PCR reaction, PCR product fragments (*bsh*1) were cloned to pJET1.2 cloning vector using by CloneJET™ PCR Cloning Kit. According to kit protocole, non-purified PCR products were added to reaction mixture directly without purification. After incubation at 22°C for 1 hour for ligation reaction, 10 µl ligation mixture was transformated to *E. coli* XL1-Blue competent cells by $CaCl_2$ transformation. The transformation mixture were poured off to LB agar plate with ampicillin providing and incubated at 37°C overnight. After incubation, three of different transformants colonies inoculated to three different 5ml of LB broth and incubated at 37°C and 175 rpm overnight. Plasmid DNAs were isolated from the three transformants and analysed using gel electrophoresis (Figure 4.3). All of the transformants' plasmids were same in size (4.0 kbp) and carry 975 bp DNA fragments. The new clones designated as XL1-Blue/YA101 before check of the clones.

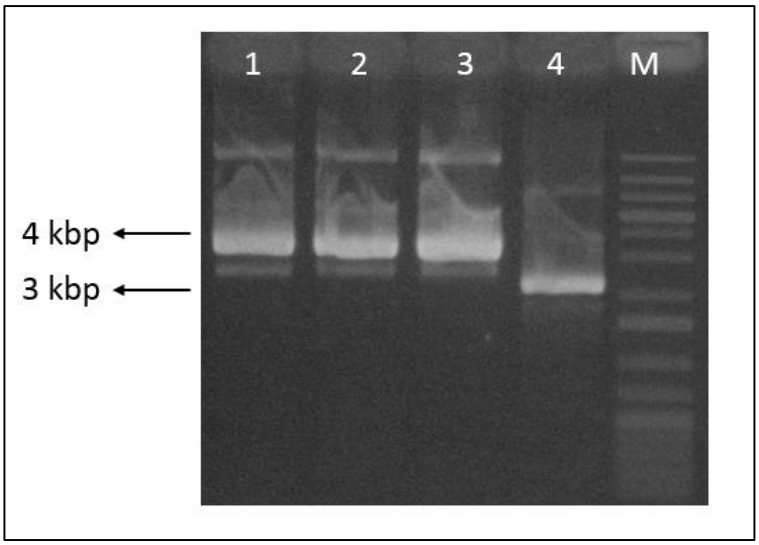

Figure 4.3. Plasmid DNA isolation from three of different pYA101 clones. Line 1, 2 and 3 indicate the pYA101 plasmids which were same in size (4.0 kbp), and Line 4 indicates circular pJet1.2 plasmid DNA (3.0 kbp) as a control; marker is designated as M.

4.1.2.1 Orientation Test with *Xba*I Enzyme for pYA101

To select the clone that have correct oriented insert, orientation test was carried out with *Xba*I restriction enzyme which cuts once insert and once pJET1.2 vector, results two DNA fragments. After *Xba*I digestion, two of the transformants were correct oriented with 3.3 and 0.6 kbp (Figure 4.4, Line 1 and 2) fragments and one of the transformants were wrong oriented with 3.7 and 0.3 kbp fragments (Figure 4.3, Line 3).

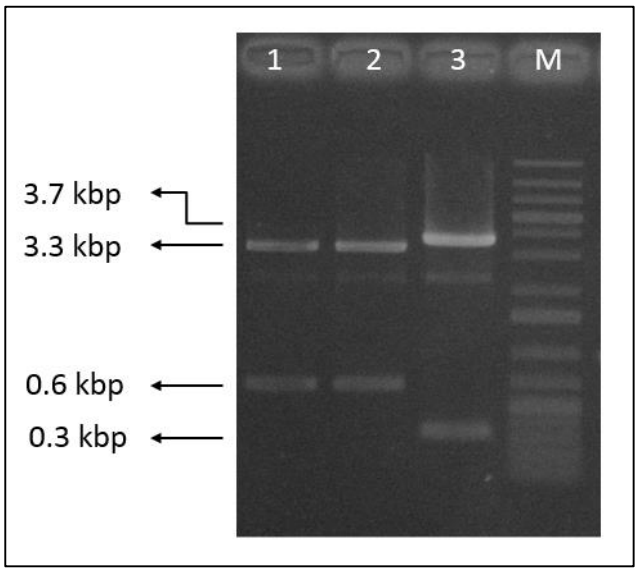

Figure 4.4. Orientation test by *Xba*I digestion for 1 kbp *bsh*1 fragment in pYA101. Line 1 and 2 contain 3.3 and 0.6 kbp of linear fragments, Line 3 contains 3.7 and 0.3 kbp linear DNA fragments; marker is designated as M.

4.1.3 Transfering of *bsh*1 Fragment from pYA101 to pET22b Expression Vector

For the transfer of the 1.0 kbp *bsh*1 fragment from pYA101 to pET22b expression vector, both of plasmids were digested with *Nco*I and *Xho*I enzymes. Wrong oriented pYA101 plasmid was selected for digestion and *bsh*1 fragment was ceased from pYA101. 5.5 kbp fragment was produced by the enzyme digestion of pET22b. After digestion, DNA fragments were separated using 1% agarose gel electrophoresis and desired fragments (1 kbp from pYA101 and 5.5 kbp from pET22b) purified from agarose gel (Figure 4.4). The amounts of isolated insert and vector were determined by using gel electrophoresis (Figure 4.5). Finally, 1.0 kbp fragment from pYA101 inserted to *Xho*I and *Nco*I digested pET22b expression vector by ligation reaction and transformed to *E. coli* BLR (DE3) competent cells. Four different transformants were selected on the basis of their size (6.5 kbp) and their ampicillin resistance (Figure 4.6). The new clones designated as BLR(DE3)/pYA201.

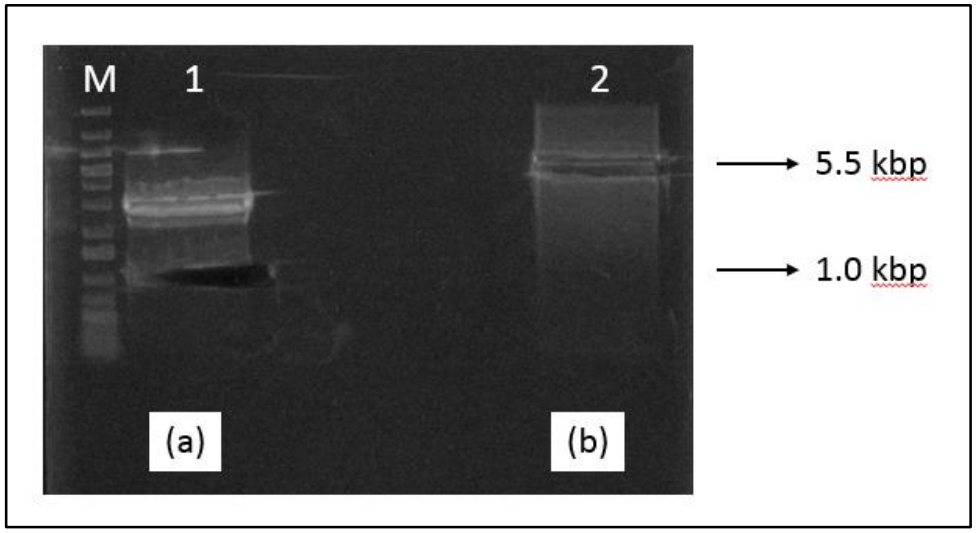

Figure 4.5. Isolation and separation of *Xho*I and *Nco*I digested pYA101 and pET22b. **(a)** Separation of 2.9 kbp and 1.0 kbp DNA fragments from *Xho*I and *Nco*I digested pYA101 and isolation of 1.0 kbp fragment from 1% agarose gel. **(b)** Separation of 5.4 kbp and 64 bp DNA fragments from XhoI and *Nco*I digested pET22b and isolation of 5.5 kbp DNAfragment from 1% agarose gel.

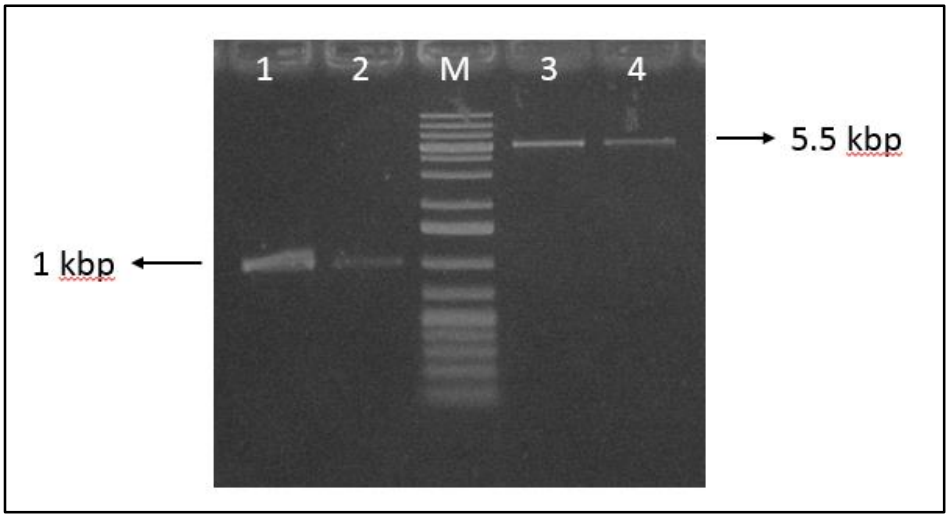

Figure 4.6. Determination of the amount of DNA fragments after DNA extraction from 1% agarose gel. Line 1 and 2 indicates first and second elution 1 kbp fragment from pYA101; Line 3 and 4 indicates first and second elution of 5.5 kbp fragment from pET22b; and marker is indicated as "M".

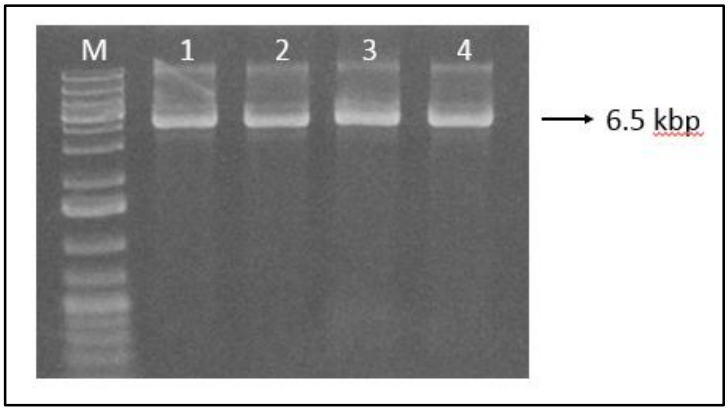

Figure 4.7. Determination of the amount of plasmid DNAs after plasmid DNA isolation from BLR(DE3)/pYA201 transformants. Lines indicate the four of different transformants; and marker is indicated as "M".

4.1.3.1 Orientation Test with *Xba*I Enzyme for pYA201

After plasmid DNA isolation from four BLR(DE3)/pYA201 transformants, all of four plasmid DNA digested with *Xba*I enzyme to verify the orientation. Normally 1.0 kbp and 5.5 kbp fragment must be ligated in correct orientation because of sticky ends both of them have same ends (*Xho*I and *Nco*I ends). *Xba*I enzyme cuts the once vector and once insert and resulting 5.7 kbp and 0.8 kbp fragments if insert is correct oriented, 6.1 kbp and 0.4 kbp fragments if insert is wrong oriented. After *Xba*I

digestion, all of the transformants was in correct orientation with 5.7 kbp and 0.8 kbp fragments (Figure 4.8).

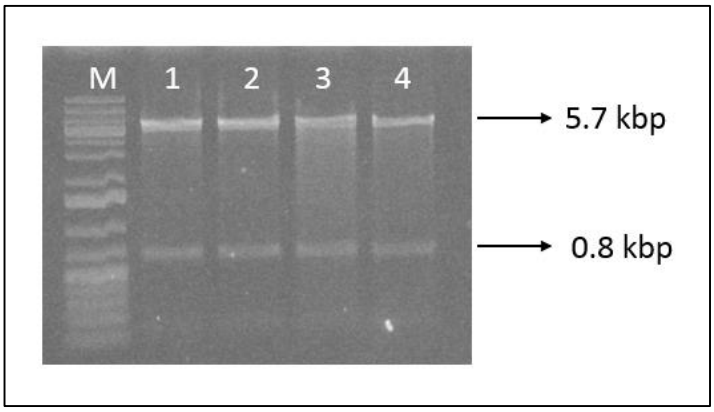

Figure 4.8. Orientation test by *Xba*I digestion for 1 kbp *bsh*1 fragment in pYA201. Line 1, 2, 3 and 4 contain 5.7 and 0.8 kbp linear DNA fragments of *Xba*I digested pYA201* from four different clones; marker is designated as M.

4.1.4 Cloning of *bsh*2, *bsh*3 and *bsh*4 Genes to pBluescript SKII Vector

The fragments of *bsh*2, *bsh*3 and *bsh*4; which are PCR products; were cloned to pBluescript SKII plasmid vector. At first, the vector linearized by *Sma*I digestion. Then, the three gel-extracted PCR products were inserted into pBluescript II SK+ by ligation process at 22°C for 1 hour. After ligation process, 10 µl ligation mixtures were transformed into XL1-Blue competent cells by $CaCl_2$ transformation. The three transformation mixtures was plated on LB plates containing IPTG, X-Gal and ampicillin. Transformants were selected on the basis of Blue-White colony assay and their sizes (Figure 4.9).

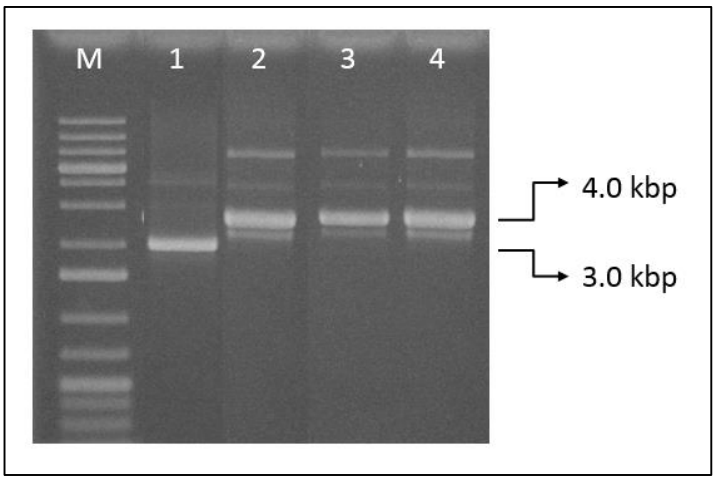

Figure 4.9. Determination of the amount of plasmid DNA from XL1-Blue/pYA302, XL1-Blue/pYA303 and XL1-Blue/pYA304. Line 1, indicates circular pBluescript II SK+ DNA (3.0 kbp) as control; Line 2, indicates pYA302 (4.0 kbp); Line 3, indicates pYA303 DNA (4.0 kbp) and Line 4 indicates pYA304 (4.0 kbp).

4.1.5 DNA Sequencing

The complete DNA sequence of *bsh*1, *bsh*2, *bsh*3 and *bsh*4 genes in plasmid DNAs of pYA101, pYA302, pYA303 and pYA304 were sequenced by RefGen (Ankara) by using universal T3 and T7 oligonucleotid primers. Open reading frame (ORF) of 975, 1017, 987 and 954 nucleotides were translated to amino acid sequences. ORFs begin with a methionine starting codon (ATG) and translational terminal codons (-TAA for *bsh*1, *bsh*2 and *bsh*3; -TGA for *bsh*4) encoding 324, 338, 328 and 318 amino acids.

```
>GD2_bsh1

1     ATG TGT ACT GCC ATA ACT TAT CAA TCT TAT AAT AAT TAC TTC GGT   45
1     Met Cys Thr Ala Ile Thr Tyr Gln Ser Tyr Asn Asn Tyr Phe Gly   15

46    AGA AAT TTC GAT TAT GAA ATT TCA TAC AAT GAA ATG GTT ACG ATT   90
16    Arg Asn Phe Asp Tyr Glu Ile Ser Tyr Asn Glu Met Val Thr Ile   30

91    ACG CCT AGA AAA TAT CCA CTA GTA TTT CGT AAG GTG GAG AAC TTA   135
31    Thr Pro Arg Lys Tyr Pro Leu Val Phe Arg Lys Val Glu Asn Leu   45

136   GAT CAC CAT TAT GCA ATA ATT GGA ATT ACT GCT GAT GTA GAA AGC   180
46    Asp His His Tyr Ala Ile Ile Gly Ile Thr Ala Asp Val Glu Ser   60

181   TAT CCA CTT TAC TAC GAT GCG ATG AAT GAA AAA GGC TTG TGT ATT   225
61    Tyr Pro Leu Tyr Tyr Asp Ala Met Asn Glu Lys Gly Leu Cys Ile   75

226   GCG GGA TTA AAT TTT GCA GGT TAT GCT GAT TAT AAA AAA TAT GAT   270
76    Ala Gly Leu Asn Phe Ala Gly Tyr Ala Asp Tyr Lys Lys Tyr Asp   90

271   GCT GAT AAA GTT AAT ATC ACA CCA TTT GAA TTA ATT CCT TGG TTA   315
91    Ala Asp Lys Val Asn Ile Thr Pro Phe Glu Leu Ile Pro Trp Leu   105

316   TTG GGA CAA TTT TCA AGT GTT AGA GAA GTG AAA AAG AAC ATA CAA   360
106   Leu Gly Gln Phe Ser Ser Val Arg Glu Val Lys Lys Asn Ile Gln   120

361   AAA CTA AAC TTG GTT AAT ATT AAT TTT AGT GAA CAA TTA CCA TTA   405
121   Lys Leu Asn Leu Val Asn Ile Asn Phe Ser Glu Gln Leu Pro Leu   135

406   TCA CCG CTA CAT TGG TTG GTT GCT GAT AAA CAG GAA TCG ATA GTT   450
136   Ser Pro Leu His Trp Leu Val Ala Asp Lys Gln Glu Ser Ile Val   150

451   ATT GAA AGT GTT AAA GAA GGA CTA AAA ATT TAC GAC AAT CCA GTA   495
151   Ile Glu Ser Val Lys Glu Gly Leu Lys Ile Tyr Asp Asn Pro Val   165

496   GGT GTG TTA ACA AAC AAT CCT AAT TTT GAC TAC CAA TTA TTT AAT   540
166   Gly Val Leu Thr Asn Asn Pro Asn Phe Asp Tyr Gln Leu Phe Asn   180

541   TTG AAC AAC TAT CGT GCC TTA TCA AAT AGC ACA CCC CAA AAT AGT   585
181   Leu Asn Asn Tyr Arg Ala Leu Ser Asn Ser Thr Pro Gln Asn Ser   195

586   TTT TCG GAA AAA GTG GAT TTA GAT AGT TAT AGT AGA GGA ATG GGC   630
196   Phe Ser Glu Lys Val Asp Leu Asp Ser Tyr Ser Arg Gly Met Gly   210

631   GGA CTA GGA TTA CCT GGA GAC TTG TCC TCA ATG TCT AGA TTT GTC   675
211   Gly Leu Gly Leu Pro Gly Asp Leu Ser Ser Met Ser Arg Phe Val   225

676   AGA GCC GCT TTT ACT AAA TTA AAC TCG TTG CCG ATG CAG ACA GAG   720
226   Arg Ala Ala Phe Thr Lys Leu Asn Ser Leu Pro Met Gln Thr Glu   240

721   AGT GGC AGT GTT AGT CAG TTT TTC CAT ATA CTA GGG TCT GTA GAA   765
241   Ser Gly Ser Val Ser Gln Phe Phe His Ile Leu Gly Ser Val Glu   255

766   CAA CAA AAA GGG CTA TGT GAA GTT ACT GAC GGA AAG TAC GAA TAT   810
256   Gln Gln Lys Gly Leu Cys Glu Val Thr Asp Gly Lys Tyr Glu Tyr   270

811   ACA ATC TAT TCT TCT TGT TGT GAT ATG AAC AAG GGA GTT TAT TAC   855
271   Thr Ile Tyr Ser Ser Cys Cys Asp Met Asn Lys Gly Val Tyr Tyr   285

856   TAT AGA ACT TAT GAC AAT AGT CAA ATT AAC AGT GTC AAT TTA AAC   900
286   Tyr Arg Thr Tyr Asp Asn Ser Gln Ile Asn Ser Val Asn Leu Asn   300

901   CAT GAG CAC TTG GAT ACG ACT GAA TTA ATT TCT TAT CCA TTA CGA   945
301   His Glu His Leu Asp Thr Thr Glu Leu Ile Ser Tyr Pro Leu Arg   315

946   TCA GAA GCA CAA TAC TAT GCA GTT AAC TAA   975
316   Ser Glu Ala Gln Tyr Tyr Ala Val Asn End
```

Figure 4.10. Nucleotide and amino acid sequence of *bsh*1 gene from *L. plantarum* GD2 Strain.

```
>GD2_bsh2

1     ATG TGC ACT AGT CTA ACT TAT ACA AAC AGT CAT GGA GGC CAC TTC   45
1     Met Cys Thr Ser Leu Thr Tyr Thr Asn Ser His Gly Gly His Phe   15

46    TTA GCT CGT ACA ATG GAT TTT AAC GTT GAC TTT GAG ACC CGT ATT   90
16    Leu Ala Arg Thr Met Asp Phe Asn Val Asp Phe Glu Thr Arg Ile   30

91    ATG TTC ATG CCT CGA CAT TAC CGC GTG ACG GGT GAC CTT GGT GAT   135
31    Met Phe Met Pro Arg His Tyr Arg Val Thr Gly Asp Leu Gly Asp   45

136   TTC ACC ACG ACT TAT GGC TTT ATT GGC GCG GGT CGC CAA CTG AAC   180
46    Phe Thr Thr Thr Tyr Gly Phe Ile Gly Ala Gly Arg Gln Leu Asn   60

181   CAT GAA ATT TTC ACG GAC GGC GTT AAC GAA TGT GGT GTC AGC ATC   225
61    His Glu Ile Phe Thr Asp Gly Val Asn Glu Cys Gly Val Ser Ile   75

226   GCA GCA CTC TAC TTT CCG AAT CAT GCA ATT TAC CAG CCT CAC AGT   270
76    Ala Ala Leu Tyr Phe Pro Asn His Ala Ile Tyr Gln Pro His Ser   90

271   AAC CAA GAC AAA ATC GAT CTC GCA CCC CAC GAT TTC GTC GCC TGG   315
91    Asn Gln Asp Lys Ile Asp Leu Ala Pro His Asp Phe Val Ala Trp   105

316   GTA CTC GGA AAA ATC ACT AGT GTT GCT GAC TTA CGT GAG CGC GTC   360
106   Val Leu Gly Lys Ile Thr Ser Val Ala Asp Leu Arg Glu Arg Val   120

361   AAA GAC GTT CAA TTG ATT AGT AGC ACG GCA GAA TTA ATT AAC GAA   405
121   Lys Asp Val Gln Leu Ile Ser Ser Thr Ala Glu Leu Ile Asn Glu   135

406   ATT CCA CCA CTT CAC TTT ATC ATT AGT GAC CAA ACC GGT GAA ACC   450
136   Ile Pro Pro Leu His Phe Ile Ile Ser Asp Gln Thr Gly Glu Thr   150

451   GCC GTC TTG GAA CCA ACT AGT GGC GAG CTT CGC CTG ATT AAT AAC   495
151   Ala Val Leu Glu Pro Thr Ser Gly Glu Leu Arg Leu Ile Asn Asn   165

496   CCA GTC GGC GTC CTG ACC AAT TCA CCG AAC CTC AAA TGG CAG CTA   540
166   Pro Val Gly Val Leu Thr Asn Ser Pro Asn Leu Lys Trp Gln Leu   180

541   CAA AAC TTA AGT AAG TAT GGC ACC CTG ACT AAC ACC GAG CGG CCA   585
181   Gln Asn Leu Ser Lys Tyr Gly Thr Leu Thr Asn Thr Glu Arg Pro   195

586   CTA AAT AAA TTC ATT AAC TAC CAA CCT GGT TCA CAG GGA CCT GGT   630
196   Leu Asn Lys Phe Ile Asn Tyr Gln Pro Gly Ser Gln Gly Pro Gly   210

631   ACG GGC GCA TTA GGT CTA CCT GGT GAC TAT ACT TCG ATG TCT CGC   675
211   Thr Gly Ala Leu Gly Leu Pro Gly Asp Tyr Thr Ser Met Ser Arg   225

676   TTT GCA CGG ACC GTC TTC TTG AAA CAC TAT GCG CAA GTG CCA GCG   720
226   Phe Ala Arg Thr Val Phe Leu Lys His Tyr Ala Gln Val Pro Ala   240

721   ACA ACA ACA GAT ACA GTC AAC TTA CTT CAA CAC ATC TTG AAT GCT   765
241   Thr Thr Thr Asp Thr Val Asn Leu Leu Gln His Ile Leu Asn Ala   255

766   GTG ACT ATT CCC AAG GGC GCC AAA GTA GCC GCC AAC GGC CAA GCA   810
256   Val Thr Ile Pro Lys Gly Ala Lys Val Ala Ala Asn Gly Gln Ala   270

811   ACC TAT ACT GAG TAC CGT AGC TAC ATG GAC TTG AAT CAT CAA ACG   855
271   Thr Tyr Thr Glu Tyr Arg Ser Tyr Met Asp Leu Asn His Gln Thr   285

856   TAC GCA CTA GAA CTG TAC GAA AAT CCG GGA GTG ATT CAG CAA GTT   900
286   Tyr Ala Leu Glu Leu Tyr Glu Asn Pro Gly Val Ile Gln Gln Val   300

901   AAC TTA ACT GAT CAT TTA TTA GAA AAA CAG ACC GTT CCG TTA GAA   945
301   Asn Leu Thr Asp His Leu Leu Glu Lys Gln Thr Val Pro Leu Glu   315

946   TAC GCC CTT AGT CGC ACC CCG CAC GTC CAA TTA CTA CCT CCC GAT   990
316   Tyr Ala Leu Ser Arg Thr Pro His Val Gln Leu Leu Pro Pro Asp   330

991   ATT GCC ACC TTG CCA GCG GCC CAT TAA   1017
331   Ile Ala Thr Leu Pro Ala Ala His End
```

Figure 4.11. Nucleotide and amino acid sequence of *bsh*2 gene from *L. plantarum* GD2 Strain.

```
>GD2_bsh3

1     ATG TGT ACT AGT TTA ACG ATT CAA ACC ACG GCG GGT GAT CAG TTT   45
1     Met Cys Thr Ser Leu Thr Ile Gln Thr Thr Ala Gly Asp Gln Phe   15

46    TTA GCA CGC ACC ATG GAC TTT GCT TTT GAA CTT GGT GGT CGA CCA   90
16    Leu Ala Arg Thr Met Asp Phe Ala Phe Glu Leu Gly Gly Arg Pro   30

91    GTG GCA ATC CCA CGG AAT CAC CAC TTT GAC AGT GTT ACC AAT GCG   135
31    Val Ala Ile Pro Arg Asn His His Phe Asp Ser Val Thr Asn Ala   45

136   GAC GGT TTT GAT AGC CCG TAT AGC TTT GTT GGA ACG GGC CGT GAC   180
46    Asp Gly Phe Asp Ser Pro Tyr Ser Phe Val Gly Thr Gly Arg Asp   60

181   TTA AAT GGC TAT ATC TTT GTC GAT GGT GTC AAT GAG CAC GGG GTC   225
61    Leu Asn Gly Tyr Ile Phe Val Asp Gly Val Asn Glu His Gly Val   75

226   AGT GCT GCT GCA CTC TAT TTC TCG GGA CAA GCT CAC TTT ACT CAG   270
76    Ser Ala Ala Ala Leu Tyr Phe Ser Gly Gln Ala His Phe Thr Gln   90

271   CAG ACT AAG GCT GGC AAG GTT AAC TTG GCA CCC CAC GAA GTT TTA   315
91    Gln Thr Lys Ala Gly Lys Val Asn Leu Ala Pro His Glu Val Leu   105

316   ATG TGG ATT TTA GGA AAC GTG AAG AGC ACC GCT GAA TTA GGC GAA   360
106   Met Trp Ile Leu Gly Asn Val Lys Ser Thr Ala Glu Leu Gly Glu   120

361   CGG ATT GCT GAC TTG AAC GTG ATG GAA GCC GCC GCA CCA CTA TTG   405
121   Arg Ile Ala Asp Leu Asn Val Met Glu Ala Ala Ala Pro Leu Leu   135

406   AAT ATT GTG GTA CCA CTA CAC TGG ATC ATT AGT GAC AAG AGT GGT   450
136   Asn Ile Val Val Pro Leu His Trp Ile Ile Ser Asp Lys Ser Gly   150

451   TCT ACT TAC GTC TTA GAA TTG GAA AAT GAC GGT GTT CAC TAC ATG   495
151   Ser Thr Tyr Val Leu Glu Leu Glu Asn Asp Gly Val His Tyr Met   165

496   AAG AAT CCG GTG GGC GTC ATG ACG AAC ACA CCA GAT TTT GAA TGG   540
166   Lys Asn Pro Val Gly Val Met Thr Asn Thr Pro Asp Phe Glu Trp   180

541   CAT CTC AAG AAT TTG AGT AAT TAC GTC AAC TTA CAA CCC GGC CCT   585
181   His Leu Lys Asn Leu Ser Asn Tyr Val Asn Leu Gln Pro Gly Pro   195

586   CAT CCT AGC CGT CAA TAC GGT GAC ATG ACG GTG AAT CCT TTC GGT   630
196   His Pro Ser Arg Gln Tyr Gly Asp Met Thr Val Asn Pro Phe Gly   210

631   CCT GGA ACT GGG GCG TTG GGA ATG CCT GGT GAC TAT ACG TCA GTT   675
211   Pro Gly Thr Gly Ala Leu Gly Met Pro Gly Asp Tyr Thr Ser Val   225

676   GCA CGC TTC GTT CGG ACG GTC TTC ATG CGT GAA CAT ACG GAT GCA   720
226   Ala Arg Phe Val Arg Thr Val Phe Met Arg Glu His Thr Asp Ala   240

721   GTA ACG ACT GAT GCA GAA GCT GTC AAC GCA TTA TCA CAC ATG CTG   765
241   Val Thr Thr Asp Ala Glu Ala Val Asn Ala Leu Ser His Met Leu   255

766   AAC TCA GTG GAG ATT CCT AAG GGC GTT AAG ATG CAA GAT AAC GGG   810
256   Asn Ser Val Glu Ile Pro Lys Gly Val Lys Met Gln Asp Asn Gly   270

811   ACG CCA GAT TAT ACC CAG TAC CGC GCC TAT ATG AGC ATG AAT GAA   855
271   Thr Pro Asp Tyr Thr Gln Tyr Arg Ala Tyr Met Ser Met Asn Glu   285

856   CCA GCA TTT TAC ATG CAA CCA TAC GCG GAT CAG ACG ATT ACG CGG   900
286   Pro Ala Phe Tyr Met Gln Pro Tyr Ala Asp Gln Thr Ile Thr Arg   300

901   GTC GAA TTG ACA CCA GCT TTA ATG ACG GCC GCG CAA CCG ACT GAA   945
301   Val Glu Leu Thr Pro Ala Leu Met Thr Ala Ala Gln Pro Thr Glu   315

946   TTT GAA TTA AAG ACA ACC CAA CAG TTC CGG TTA GCA AAC TAA   987
316   Phe Glu Leu Lys Thr Thr Gln Gln Phe Arg Leu Ala Asn End
```

Figure 4.12. Nucleotide and amino acid sequence of *bsh*3 gene from *L. plantarum* GD2 Strain.

```
>GD2_bsh4

1     ATG TGT ACT AGT TTA ACT TAT CTT GAT ACT GAC AAT CAC CGC TAC   45
1     Met Cys Thr Ser Leu Thr Tyr Leu Asp Thr Asp Asn His Arg Tyr   15

46    TTC GCC CGC ACC ATG GAC TTT CCA ACA ACG ACA CCT TGG CGG CCA   90
16    Phe Ala Arg Thr Met Asp Phe Pro Thr Thr Thr Pro Trp Arg Pro   30

91    ATT TTT TTG CCG CGC CGT TAT CCG TGG CCA ACT GGG TTA GCG ACG   135
31    Ile Phe Leu Pro Arg Arg Tyr Pro Trp Pro Thr Gly Leu Ala Thr   45

136   ACG CGT ATG ACG CAG TAT GCC ATT CTC GGT GGT GGT CGG CTA CCT   180
46    Thr Arg Met Thr Gln Tyr Ala Ile Leu Gly Gly Gly Arg Leu Pro   60

181   GAC CAC TTT AAG GCT TGT TTG ATG GCT GAC GGC ATT AAC GAA GCT   225
61    Asp His Phe Lys Ala Cys Leu Met Ala Asp Gly Ile Asn Glu Ala   75

226   GGT TTG GTG TGT GCT GAA CTG TAC TTG CCC CAC GCC GTT GAA TAC   270
76    Gly Leu Val Cys Ala Glu Leu Tyr Leu Pro His Ala Val Glu Tyr   90

271   GCC ACT CAA CCA CAA GTC AAC CAA ATT AAT TTA ACA CCC CAA GCC   315
91    Ala Thr Gln Pro Gln Val Asn Gln Ile Asn Leu Thr Pro Gln Ala   105

316   TTC ATC AAC TGG GCT TTA GGT GAA CAC CAA TCA GTC GCA GCC GTG   360
106   Phe Ile Asn Trp Ala Leu Gly Glu His Gln Ser Val Ala Ala Val   120

361   ATC GCC GAT CTG CCA AGT GTT AAC CTG GTC GGT GCG TCC TGG GGT   405
121   Ile Ala Asp Leu Pro Ser Val Asn Leu Val Gly Ala Ser Trp Gly   135

406   GAT GAC ACT GGT GAA GTC TAT CCC TTT CAC TGG TAT CTC AGT GAT   450
136   Asp Asp Thr Gly Glu Val Tyr Pro Phe His Trp Tyr Leu Ser Asp   150

451   GCA CAC ACC AGT GCC GTC ATC GAA CCC ACT GGT GGC CCA CTG ACG   495
151   Ala His Thr Ser Ala Val Ile Glu Pro Thr Gly Gly Pro Leu Thr   165

496   GCG CAA CCG AAT CCA GCC GGC GTC CTG ACC AAT ACA CCA GTC CTA   540
166   Ala Gln Pro Asn Pro Ala Gly Val Leu Thr Asn Thr Pro Val Leu   180

541   AGC GAC CAT CAG CGC CGA CTA AAT CGT TAT TTA GCA GTA TCT GGC   585
181   Ser Asp His Gln Arg Arg Leu Asn Arg Tyr Leu Ala Val Ser Gly   195

586   AAC CAG ATT ACA ACT GCC ACT CGT CAG GCT GCT CAG CAC GTG ATT   630
196   Asn Gln Ile Thr Thr Ala Thr Arg Gln Ala Ala Gln His Val Ile   210

631   CAG ACT AAG CAA CCA TTA CCG AGC GGG CCG ATT CCC ACT GAT CGT   675
211   Gln Thr Lys Gln Pro Leu Pro Ser Gly Pro Ile Pro Thr Asp Arg   225

676   TTC ATT CAC ATG GCA CTT CGA CGA CTG GGA ACA CCG CAG CTA GCA   720
226   Phe Ile His Met Ala Leu Arg Arg Leu Gly Thr Pro Gln Leu Ala   240

721   CCG CAA CAA GTG CCG ACC ACT TTA TTC CGC TGG TTA CAA GAA GTA   765
241   Pro Gln Gln Val Pro Thr Thr Leu Phe Arg Trp Leu Gln Glu Val   255

766   AGC TTG CCA TAC CAC GCC GAC CGT CGC CAT CTC ATC AGC CAC AAC   810
256   Ser Leu Pro Tyr His Ala Asp Arg Arg His Leu Ile Ser His Asn   270

811   TAC ACG CAC TAT CGT TGT TTG ATC ACG TTA GCG ACT CGT ACT TAC   855
271   Tyr Thr His Tyr Arg Cys Leu Ile Thr Leu Ala Thr Arg Thr Tyr   285

856   CGC TTT ATT CCA CGC ACG ACT GGT CAC GAA CAA CGA CTG ACA CTA   900
286   Arg Phe Ile Pro Arg Thr Thr Gly His Glu Gln Arg Leu Thr Leu   300

901   ACA CCT GAA ATG GCA ACA ACC TGG CGA ACA CCG TAC CTC TTT CCT   945
301   Thr Pro Glu Met Ala Thr Thr Trp Arg Thr Pro Tyr Leu Phe Pro   315

946   GCC GAT TGA   954
316   Ala Asp End
```

Figure 4.13. Nucleotide and amino acid sequence of *bsh*4 gene from *L. plantarum* GD2 Strain.

4.1.5.1 Alignment of *bsh*1 gene from *L. plantarum* GD2 with *bsh* Genes from different Strains

The deduced amino acid sequence of Bsh1 from *L. plantarum* GD2 was aligned with reported sequences of BSH from some other bacteria; it shared 39.5, 48.5, 51.5, 52.1, 52.1, 99.1 and 99.3% identities with BSHs from *C. perfingens* 13, *L. Johnsonii* PF01 (BSH-A), *L. johnsonii* PF01 (BSH-C), *L. johnsonii* 100-100, *L. plantarum* WCSF1 and *L. plantarum* BBE7 respectively (Table 3.5). According the crystal structure of *C. perfingens* there are five putative active sites (Cys-2, Asp-21, Asn-79, Asn-170 and Arg-223) has been identified on the genomic sequence of *bsh1*, which are catalytically important (Rossocha et al. 2005). In comparison with BSH from other bacteria all of these active sites are conserved. In addition four amino acid motifs (FGRNFD, GLGCAGLN, LTNNPNF and LPGDLSSMSRF) are located around the active sites were conserved and semiconservative in some sites. Bsh1 enzyme have a Cys-2 active site (Figure 3.8) which serve a nucleophile and proton donor and is important for catalysis (Rossocha et al. 2005, Chae et al. 2012) and is homologous to well studied *C. perfingens* (Coleman and Hudson 1995).

Table 4.1. BSH proteins used for amino acid sequence alignment.

Code in alignment	Strain	BSH	Relative Activity	Reference
*C.perfingens*_BSH	*C. perfingens* 13	CBAH-1	TC	Coleman and Hudson (1995)
La_NCFM_BSHA	*L. acidophilus* NCFM	BSH A	TC/GC	McAuliffe et al. (2005)
Lj_PF01_BSH-A	*L. johnsonii* PF01	BSH A	TC	Chae et al. (2012)
Lj_PF01_BSH-C	*L. johnsonii* PF01	BSH C	GC	Chae et al. (2012)
Lj_100-100_CBSHα	*L. johnsonii* 100-100	CBSH-α	TC/GC	Lundeen and Savage (1990)
Lp_BBE7_BSH	*L. plantarum* BBE7	BSH	TC/GC	Dong et al. (2012)
Lp_GD2_bsh1	*L. plantarum* GD2	BSH1	TC/GC	This study
Lp_WCSF1_BSH1	*L. plantarum* WCSF1	BSH1	GC	Lambert et al. (2008)

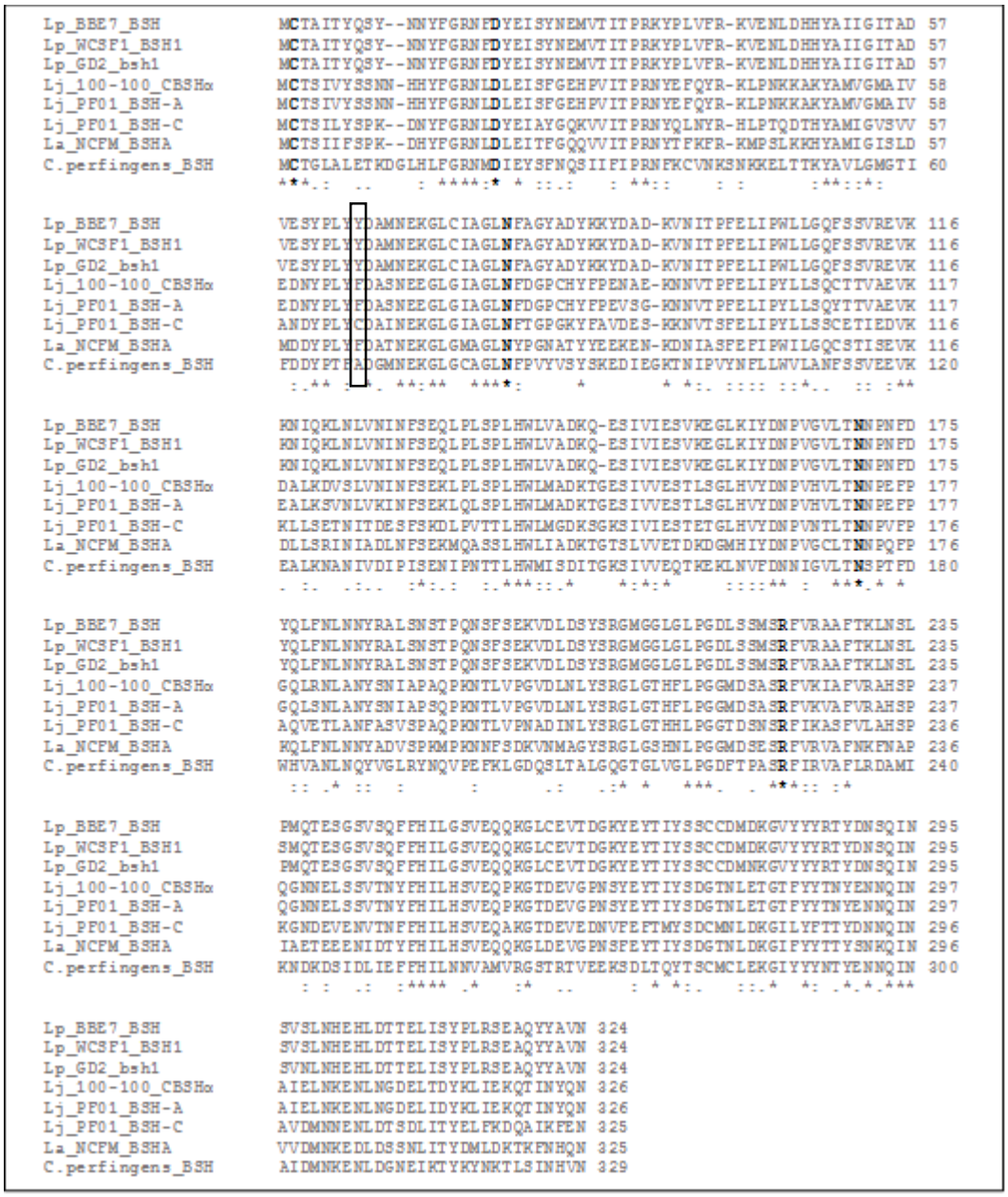

Figure 4.14. Multiple sequence alignment of Bsh1 from *L. plantarum* GD2 with BSH proteins from *Lactobacillus* species and *C. perfingens*: (Name codes are on the Table 3.5). Bold highlights represent active sites. Boxed residue indicates Ala-68, a notable substrate binding site according to *C. perfingens* crystal structure. (*) conserved residue, (:) conservative residue, (.) less conservative residue.

4.1.6 Analysis of Bsh1 Protein Activity

4.1.6.1 SDS-PAGE and Coomassie Stain

Before the coomassie staining, the solutions of purification steps and purified Bsh1 elution loaded the gel (Figure 4.15). According to gel, Bsh1 protein was concentrated in total cell lysates of BLR(DE3)/pYA201* (Line 1 and 2). Bsh1 protein was almost not found in flow-through fraction (Line 3) and wash fraction (Line 4) by the binding of the agarose column, and Bsh1 was highly concentrated in eluted fraction with higher purification rate. Bsh1 is a monomeric enzyme (has no subunits) with a molecular mass of approximately 43 kDa was observed as a prominent band . This BSH enzyme of 324 amino acids was estimated to be about 37,008 Da. This is slightly higher than the predicted molecular weight of Bsh1 of *L. plantarum* GD2, probably by unnecessarry vector derived portion and fusion protein part (6xHis).

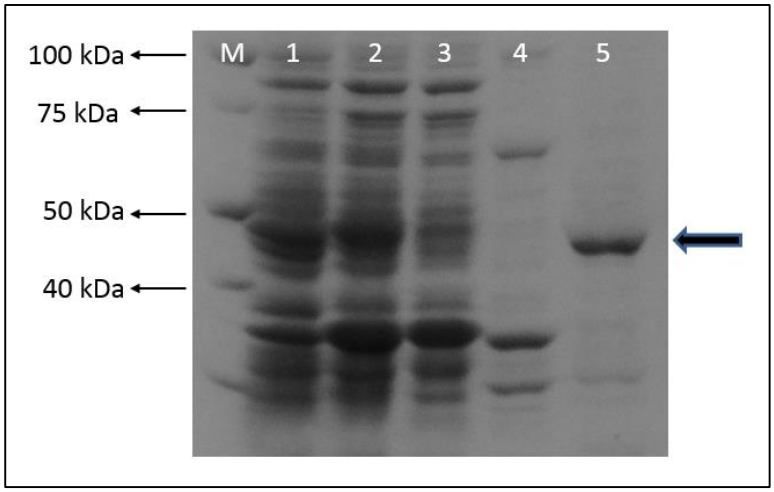

Figure 4.15. SDS-PAGE analysis for Bsh1 enzyme and steps of purificaitons. Line 1 and 2, contain total cell lysate of BLR(DE3)/pYA201*; Line 3, contains flow-through fraction; Line 4, contains wash fraction and Line 5 contains eluted fraction. The arrow shows the purified Bsh1 protein and broad range protein marker indicated as M.

4.1.6.2 Substrate Specificity Test for Bsh1 by Ninhydrin Protein Assay

The Ninhydrin Protein Assay was used to test the substrate specificity of the Bsh1using by six of human bile salts including primary and secondary bile salts and both of tauro and glyco-conjugated. According to spectrophotometric analysis, the purified Bsh1enzyme exhibited activity against six bile salts, it could deconjugate both

of taurine and glycine conjugated bile salts in different level. The enzyme shows prefential activity against glyco-conjugated bile salts and showed highest activity against glycodeoxycholic acids. The enzyme show highest acticity against to deoxycholic acids; moderate activity against to chenodeoxycholic acids, and minimum activity against cholic acids. After the spectrophotometric analysis, "relative activity" of the enzyme was calculated with the highest value designated as 100% (Table 4.2).

Table 4.2. Spectrophotometric analysis of Bsh1 deconjugating activity against six conjugated bile salts. All reactions was repeated three times, standard deviations designated as "± value".

	Bsh1 Activity
Bases	**Spectrophotometric analysis**
GCA	0,520 ± 0,045
GDCA	0,641 ± 0,060
GCDCA	0,545 ± 0,021
TCA	0,161 ± 0,016
TDCA	0,267 ± 0,005
TCDCA	0,168 ± 0,008

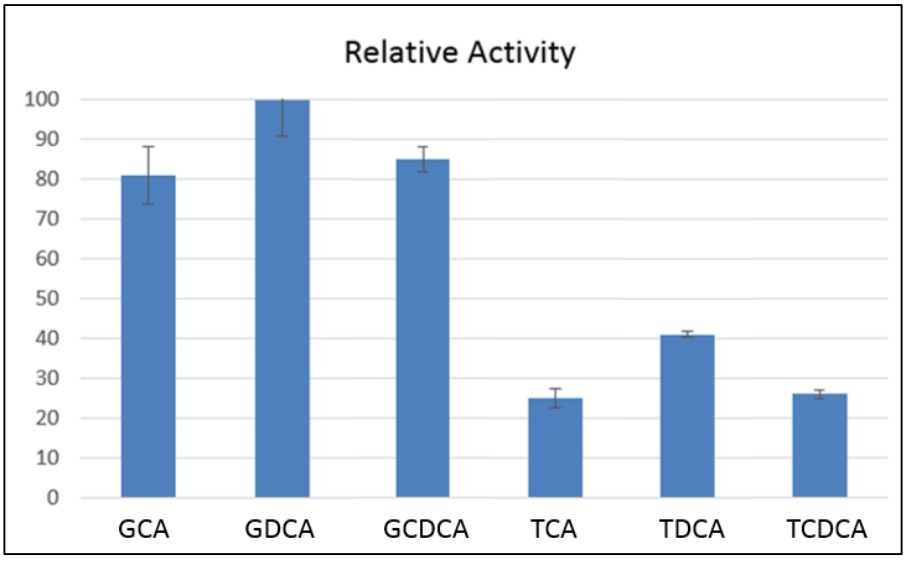

Figure 4.16. Substrate specificity of recombinant Bsh1 enzyme against to six major bile salts; taurocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid, glycocholic acid, glycodeoxycholic acid and glycochenodeoxycholic acid. Relative activity was calculated for each substrate compared to highest bile salt hydrolase activity, defined as 100%. Error bars represent standard deviatons.

4.2 Discussions

BSH enzymes have been identified from several numbers of taxa in the last decades, but the substrate hydrolysing capabilties have been not yet fully understood. The diversity of the BSH enzymes and their amino acid sequences are highly complicated among genera, species and even strains. In this study, the four BSH genes were identified on the genomic DNA of *L. plantarum* GD2. Recombinant Bsh1 enzyme was overexpressed, purified and characterized using by six bile salts in the basis of specific amino acid moieties. The substrate specificity of Bsh1is different from the majority of BSHs. It showed hydrolysing activity to both tauro- and glyco-conjugated bile salts and show preferential activity against glyco-conjugated bile salts.

BSHs from intestinal lactic acid bacteria generally show higher affinity for glycoconjugated bile salts (Coleman and Hudson 1995, Kim et al. 2004; Oh et al. 2008; Tanaka et al. 2000). It is emphasized on the reported data that substrate specificity may occur at amino acid (Coleman and Hudson, 1995; Tanaka et al. 2000) or steroid moieties (McAuliffe et al. 2005), kinetic data suggest that these enzyme reconjugate their substrates predominantly at amino acid moieties (Rossocha et al. 2005, Chae et al. 2012). Rossocha and his colleagues (2005) pointed out that BSH enzyme substrate specificity could not be deduced for cholyl moieties. BSHs had variable relative activity for primary (CA, CDCA) and secondary types (DCA) of bile acids; they also showed low deconjugation against secondary bile acids. In this study *L. plantarum* GD2 Bsh1 enzyme showed highest activity against GDCA (secondary type), and show preferential activity against glyco-conjugated bile acids.

The boxed residue in Figure 3.14 highlights Ala-68 is one of the substrate binding pocket residue of CBAH enzyme from *C. perfingens* (Rossocha et al. 2005). According to crystal structure; Ala-68 lies within the site where hydrogen bonds are formed and indirectly interacted with catalysis and substrate binding of Ntn-hydrolyses (Oinonen and Rouvinen, 2000). In this site all amino acids are neutral but have different polarity. BSH enzymes which hydrolyse glyco-conjugated bile salts had polar amino acids Cys (C) or Tyr (Y); while others which hydrolases tauro-conjugated bile salts had a nonpolar Phe (F) or Ala (A) residue (Chae et al. 2012). However,

L. plantarum GD2 Bsh1 enzyme has Tyr (Y) residue in this site and can deconjugate both of tauro- and glyco-conjugated bile salts.

Lambert (2008) show that *L. plantarum* WCFS, carrying four BSH genes (*bsh*1, *bsh*2, *bsh*3, *bsh*4) where only *bsh1* exhibit BSH activity and other genes are involved to encode penicilin V-acylase rather than BSH activity. In this study, four BSH genes also have been proliferated from *L. plantarum* GD2 and cloned; but only Bsh1 could deconjugate conjugated bile salts (data not shown). Gu et al. (2014) reported that *bsh*2, *bsh*3 and *bsh*4 from *L. plantarum* CGMCC-8198 had hydrolysing activity against both of tauro and glyco-conjugated bile salts. According to dual sequence comparison of Bsh2, Bsh3 and Bsh4 genomes from *L. plantarum* GD2 and *L. plantarum* CGMCC-8198 are highly different from each others (data not shown).

L. plantarum GD2 strain was isolated from breast-fed human infant feces. The strain was identified and characterized with higher cholesterol removing activity and bile resistance (Yıldız et al. 2011). BSH activity of enteric bacteria is an important role for persistent colonization in the intestinal tract (Elkins et al. 2001); and bile salt resistance is important for probiotic organisms to survive and colonize in the GIT and show their health promoting effect on the host.

5. CONCLUSION AND RECOMMENDATIONS

In this study, we report the cloning and sequence analysis bile salt hydrolase genes (BSH) from *Lactobacillus plantarum* GD2. Four genes (*bsh*1, *bsh*2 *bsh*3 and *bsh*4) were proliferated with PCR and cloned for sequence analysis. *bsh*1 gene was also cloned to expression vector, pET22b, to overexpression of Bsh1 enzyme. Bsh1 enzyme was characterized by the testing of affinity of the enzyme against six bile salts. The Bsh1 enzyme shows different affinities to bile salts from the some of reported studies. *L. plantarum* GD2 have preferable probiotic properties and it is an ideal model with its powerful deconjugate ability for the researches about the relation of substrate specificity and amino acid sequence of BSHs. For the future studies, site-directed mutagenesis on conserved amino acids of BSH enzyme and structural analysis will be implemented to investigate key residues of the active site, substrate binding pocket and substrate selectivity of BSH enzyme.

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APPENDICES

7. APPENDICES

Appendix A Bacterial Growth Media

A1 Liquid Luria-Bertani (LB) Medium

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

Table A. 1. Contents of liquid Luria Bertani growth medium

Contents	**For 1 L**
Bacto tryptone	10 g
Yeast extract	5 g
NaCl	10 g

A2 LB Plate

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

Appendix B Solutions and Antibiotics

B1 Solutions

- **BSA (1mg/ml)**

0.010 g BSA was dissolved in 10 ml of dH_2O and stored at -20°C.

- **EDTA (Ethylenediamine-tetraacetic acid) (0.5 M)**

For 250 ml EDTA solution, 46.5 g EDTA is dissolved in 150 ml dH_2O, and pH is adjusted with NaOH to 8,00. The volume is completed to 250 ml with H_2O and autoclaved.

- **Solutions Used for Lowry Method**

Solution A: 2 g Na_2CO3 is dissolved in 0.1 N NaOH than volume completed to 100 ml with ddH_2O.

Solution B: 1 g $CuSO_4$. H_2O is dissolved in 1000 ml ddH_2O.

Solution C: 2 g Sodium potassium tartarate are dissolved in ddH_2O

Solution D: Solution B and C are mixed (1/1 ratio) (It is daily prepared).

Solution E: 1 ml solution D was added in 50 ml solution A (It is prepared just before used).

- **Solutions Used for SDS-PAGE**

  - **Acrylamide (30%)**

Acrylamide and bis-acryamide were dissolved in 70 ml ddH_2O, then volume was made up to 100 ml and stored at +4°C in dark.

Table B. 1. Contents of Acrylamide used for SDS-PAGE

Contents	For 100 ml
Acrylamide	29.2 g
Bis-acrylamide	0.8 g

- **10% SDS Solution**

1 g Sodium Dodecyl Sulphate (SDS) was dissolved in 1ml of ddH2O.

- **Ninhydrin (1%)**

1 g Ninhydrin was dissolved in 30 ml water and stored in dark at RT.

- **Trichloroacetic acid (TCA) (15%)**

1.5 g Tricholoroacetic acid was dissolved in 10 ml of water stored in dark at RT.

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

0,1 g Ammonium persulfate (APS) was dissolved in 1ml of ddH_2O, prepared as fresh.

- **0.25 M Na-Acetate Solution (pH: 5)**

17.01 g Na-Acetate was dissolved in 400 ml ddH_2O. pH was adjusted to 5 with glacial acetic acid. The volume was completed to 500 ml.

- **Stain Solution for Coomassie**

Mixture was prepated and stored at RT.

Table B. 2. Contents of Coomassie Stain solution.

Contents	For 1 L
Coomassie	1.0 g
Glacial acetic acid	100 ml
Methanol	400 ml
ddH_20	500 ml

- **De-Stain for Coomassie**

Mixture was prepared and stored at RT.

Table B. 3. Contents of De-Stain for Coomassie

Contents	For 1 L
Methanol	100 ml
Glacial acetic acid	100 ml
ddH_20	700 ml

B2 Antibiotics

- **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 0.22µm filter and stored at -20 °C.

Appendix C Buffers

- **TAE Buffer (50X)**

The volume is completed to 1 L with H_2O, and sterilized by autoclaving.

Table C. 1. Contents of TAE buffer (50X)

Contents	For 1 L
Trizma Base	242 g
Glacial Acetic Acid	57.1 ml
EDTA (0.5 M pH:8)	100 ml

- **TE Buffer**

The volume was completed to 100 ml with ddH_2O, and sterilized by autoclaving.

Table C. 2. Contents of TE buffer.

Contents	For 100 ml
Tris Base (1M, pH: 7.50)	1 ml
$EDTA.2H_2O$ (0.5 M, pH: 8)	200 µl

- **Stacking Gel Buffer (4x)**

6.1 g Tris-base dissolved in 80 ml water. pH was adjusted to 6.8 adding with HCl. Volume was made up to 100 ml and stored at +4 °C.

- **Separating Gel Buffer (4x)**

18.2 g Tris-base dissolved in 80 ml water. pH was adjusted to 8.8 adding with HCl. Volume was made up to 100 ml and stored at +4°C.

- **Electrode Buffer (10x)**

30 g Tris-base and 14.98 g Glycine were dissolved in 800 ml water. pH was adjusted to 8.3 adding with HCl. Volume was made up to 1000 ml and stored at +4 °C. When diluting to 1X SDS stock was added to a final concentration of 0.1%.

- **DTT (40 mM)**

0.0124 g Sodium Dithiothreitol (DTT) was dissolved in 1 ml dH_2O and stored at 20°C.

- **Sodium Phosphate Buffer (0.1 M, pH: 7.0)**

1.42 g of sodium phosphate dibasic (Na_2PO_4) was dissolved in 80 ml in dH_2O, pH adjusted to 7.0. Volume was made up to 100 ml and stored at RT.

- **Sodium Phosphate Buffer (0.2 M, pH: 6.0)**

2.84 g of sodium phosphate dibasic (Na_2PO_4) was dissolved in 80 ml in dH_2O, pH adjusted to 6.0. Volume was made up to 100 ml and stored at RT.

- **Sodium Citrate Buffer (0.5 M, pH: 5.5)**

14.7 g of sodium citrate dihydrate ($C_6H_5Na_3O_7.2\ H_2O$) was dissolved in 80 ml dH_2O, pH adjusted to 5.5. Volume was made up to 100 ml and stored at RT

- **Loading Dye for SDS-PAGE**

Table C. 3. Contents of loading dye for SDS-PAGE.

Contents	**For 1 ml**
ddH_2O	340 µl
10 % SDS	400 µl (4%)
100 % Glycerol	120 µl (12%)
0.5 M Tris/HCl (pH; 6.8)	100 µl (50mM)
0.5 % Bromophenol Blue	40 µl (0.01%)

Appendix D Chemicals

30% Acrylamide-Bis Acrylamide (Biorad, 161-0159)

40 % Acrylamide-Bis Acrylamide (Biorad, 161-0148)

Acetic acid (Merck, 100056)

Agar (Merck, 101614)

Agarose (Sigma, 9012-36-6)

Ammonium persulfate [APS] (Sigma, A-9164)

Ammonium sulfate [$(NH4)_2SO_4$] (Sigma, 7783-20-2)

Ampicillin (Sigma, A-0166)

Bacto peptone (Merck, 884113)

Bacto tryptone (Merck, 667114)

Bromophenol Blue (Sigma, 115-39-9)

Calcium Chloride [$CaCl_2$] (Merck, 208290)

Calcium chloride dihydrate [$CaCl_2.2H_2O$] (Sigma, 10035-04-8)

Copper (II) sulfate pentahydrate [$CuSO_4.5H_2O$] (Sigma, 7758-99-8)

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

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

Glucose (Merck, 346351)

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

Glycerol, Electrophoresis grade (Sigma G-8773)

Glycine (Merc, 56-40-6)

Glycochenodeoxycholicacid (Calbiochem, 640-79-9)

Glycocholic Acid, Sodium Salt (Calbiochem, 863-57-0)

Glycodeoxycholic Acid, Sodium Salt (Calbiochem, 16409-34-0)

Hydrogen peroxide [(H_2O_2), 30%] (Aldrich, Chemical Co., 21, 676.3)

Methanol (Carlo Erba reagent, 309203)

Potassium chloride [KCl] (Sigma, 7447-40-7)

Sodium acetate (Merck, 6268)

Sodium Citrate Dihydrate [$C_6H_5Na_3O_7.2H_2O$] (Merc, 6132-04-3)

Sodium chloride [NaCl] (Merck, 106404)

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

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

Sodium hyroxide [NaOH] (Merck, 106462)

Sodium Phosphate dibasic [Na_2HPO_4] (Sigma 7558-79-4)

Ninhydrin (Sigma, 485-47-2)

N, N'-Metylene-bis-Acrylamide (Sigma, M-7256)

N,N,N′,N′-Tetramethyl-p-phenylenediamine [TMPD] (Sigma, 100-22-1)

Potassium chloride [KCl] (Sigma, 7447-40-7)

Potassium sodium tartrate tetrahydrate [KOCOCH(OH)CH(OH)COONa .$4H_2O$] (Sigma, 6381-59-5)

Taurodeoxycholic Acid, Sodium Salt (Calbiochem, 1180-95-6)

Taurochenodeoxycholic acid (Calbiochem, 516-35-8)

Taurocholic Acid, Sodium Salt (Calbiochem, 145-42-6)

Trichloroacetic Acid (Merc, K43153010 237

Trizma® base (Sigma, 77-86-1)

Trizma® hydrochloride (Sigma, 1185-53-1)

Yeast Extract (Merck, 103750)

Appendix E Enzymes

- **Restriction Endonucleases**

*Nco*I (Thermo) : cat. # ER0572

*Sma*I (Thermo) : cat. # ER0661

XhoI (Thermo) : cat. # ER0691

- **Polymerases**

Pfu DNA Polymerase (MBI) : cat. # EP0501

- **Lıgases**

T4 DNA Ligase (MBI) : cat. # EL0335

- **Phosphatases**

Shrimp Alkaline Phosphatase (SAP) (Strategene) : cat. # ER EF0341

- **dNTP Set**

dNTP (MBI) : cat. # R0181

Appendix F Equipments Used in This Study

PCR (Techne, TC 3000)

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

34ºC and 37ºC shaker-incubator (Gerhardt)

Shaker- Heidolph Unimax 2010

Modular Double Vertical Electrophoresis System (Beijing Liuyi Instrument Factory)

DNA Electrophoresis System (Thermo Scientific)

Power supply (Thermo EC250-90)

Dry block (VWR Digital Dry-Block)

Shaker-heater (IKA RCT basic)

Autoclave (Hirayana)

pH meter (HANNA HI 221)

Micropipettes (Finnpipette) 0,5-10, 1-10, 10-100, 100-1000 µl and 1- 10 ml pipettes

Desktop centrifuges (Hettich Micro 120)

Centrifuge (Hettich Rotina 38R)

+4ºC refrigerators (Arçelik)

-20ºC deepfreeze (Arçelik)

-80ºC deepfreeze (Thermo Scientific)

Spectrophotometer (HITACHI U-1900)

UV Transilluminator (UVP)

Imaging system (UVP Photo Doc-It™)

Vortex (Yellowline TTS2)

Water Purification System (Human Corporation)

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List of Publications

Oral Presentations:

International:

1. **Aydın, Y.** and Öztürk, M. (2014), Cloning and Characterization of Bile Salt Hydrolase (BSH) from *Lactobacillus plantarum* GD2, 3rd International Molecular Biology and Biotechnology Congress, June 02 – 06, Sarajevo, Congress Book Pg. 35.

National:

2. Öztürk, M., **Aydın, Y.** and Çakır, İ. (2010), Yakın *Lactobacillus* türlerinin 16S rRNA PCR-RFLP metodu ile ayrıştırılması, XXII. Ulusal Biyokimya Kongresi 27-30 September, Eskişehir, Congress Book Pg. 89.

Poster Presentations:

National:

1. **Aydın, Y**., Kaya, Y., and Öztürk, M. (2013), *Lactobacillus plantarum* GD2 suşundan izole edilmiş Safra Tuzu Hidrolaz Enziminin (BSH) Direk Besi Yeri Uygulaması, XIII. Ulusal Tıbbi Biyoloji ve Genetik Kongresi, Aydın-Kuşadası 27- 30 September, Congress Book Pg. 234.

Completed / Ongoing Projects

National

1. Öztürk, M., Aydın, Y. and Kaya, Y., Cloning and Characterization of Bile Salt Hydrolase Genes (BSH) from Human Originated Lactobacillus Species, AIBU-Scientific Research Project, Code # 2011.03.01.406, 2012 – 2013 (Completed).

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