

ABANT İZZET BAYSAL UNIVERSITY

**THE GRADUATE SCHOOL OF NATURAL AND APPLIED
SCIENCES**



**MOLECULAR CLONING, CHARACTERIZATION AND
COMPARISON OF BILE SALT HYDROLASE ENZYME
FROM LACTOBACILLUS GASSERI**

MASTER OF SCIENCE

NDEYE MAREME BA

BOLU, DECEMBER 2016

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APPROVAL OF THE THESIS

**MOLECULAR CLONING, CHARACTERIZATION AND COMPARISON
OF BILE SALT HYDROLASE ENZYME FROM *LACTOBACILLUS
GASSERI*** submitted by **NDEYE MAREME BA** in partial fulfillment of the
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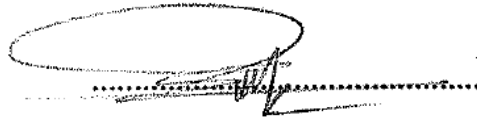
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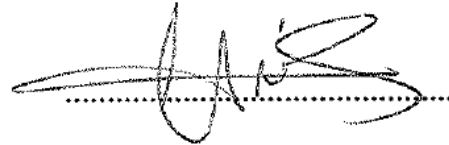
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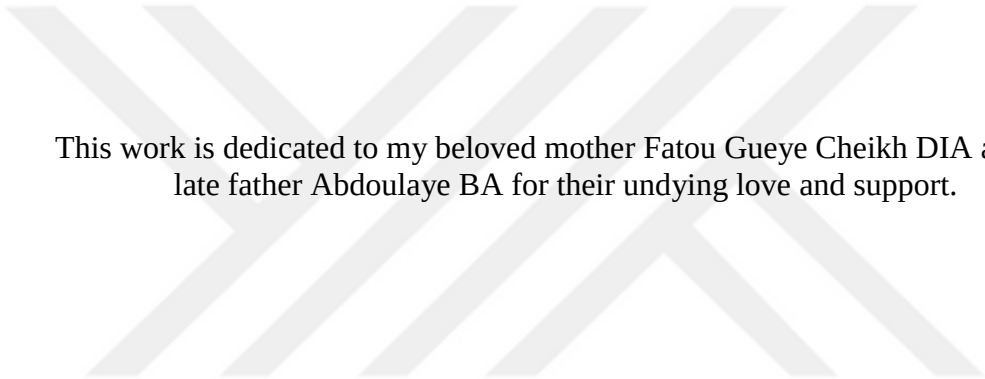
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This work is dedicated to my beloved mother Fatou Gueye Cheikh DIA and my late father Abdoulaye BA for their undying love and support.

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.

NDEYE MAREME BA

ABSTRACT

MOLECULAR CLONING, CHARACTERIZATION AND COMPARISON OF BILE SALT HYDROLASE ENZYME FROM LACTOBACILLUS GASSERI

MSC THESIS

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ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF
NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

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Over the past decade, bile salt hydrolase (BSH) active probiotic strains are being used in the treatment of hypercholesterolemia related diseases. The aptitude of this BSH enzyme to deconjugate bile salts into free bile acids and glycine or taurine moiety has been a major criteria in the selection of probiotics. Not only does this deconjugation provides bile tolerance to the bacteria for its continued existence in the GI tract, it also plays a critical part in the reduction of the blood cholesterol level of the human host. Therefore, knowing and understanding the mechanism of action of the BSH enzymes of the probiotic being used in those treatments is of utmost importance. Even though *Lactobacillus gasseri* is probably one of the most beneficial probiotics on the market, there is little information on the mechanism of action of the catalytic activity and substrate preferences of the BSH enzyme of this microorganism. In this study, the *bsh* gene from *Lb. gasseri* ATCC 33323 strain was cloned, expressed, characterized in *Escherichia coli* BLR(DE3) strain. The hydrolysis activity of the wild-type recombinant BSH (wrBSH) enzyme was then examined in different temperatures ranging from 25°C to 65°C and various pHs from 2.5 to 8.0 along with six different bile acids by ninhydrin assay. The expression of *bsh* gene was detected by SDS-PAGE. The ninhydrin test results indicated that wrBSH enzyme hydrolyzed six major human bile salts with an obvious preference towards glycine-conjugated compared to tauro-conjugated bile salts. Maximun activities of wrBSH enzyme were also recorded at low pH and high temperatures. Our findings support the suggestion that BSH enzymes recognize their substrates predominantly through their amino acid moieties rather than their cholate moieties. However, more extensive studies like site directed mutagenesis are needed to fully understand the catalytic activity as well as the substrate specificities of the BSH enzymes and to set the standards for the selection of probiotic bacteria.

KEYWORDS: Probiotics, *Lactobacillus gasseri*, Bile Salt Hydrolase (BSH), Catalytic activity and Substrate specificity

ÖZET

**LACTOBACILLUS GASSERI SAFRA TUZU HIDROLAZ
EMZİMLERİNİN KLONLANMASI, KARAKTERİZASYON VE
KARSILASTIRMASI
YÜKSEK LİSANS TEZİ
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Geçtiğimiz on yıl içinde, aktif safra tuzu hidrolaz (STH) enzimi taşıyan probiyotikler hiperkolesterolemi temelli hastalıkların tedavisinde kullanılmaktadır. BSH enziminin safra tuzlarını sebest safra asitlerine ve glisin veya taurin amino asitlerine dönüştürme yeteneği, bu STH enziminin probiyotik seçiminde önemli bir kriter oluşturmaktadır. Bu dekonjugasyon sadece bakterilerin gastro intestinal sistemde hayatta kalabilmesi için tolerans sağlamaz, aynı zamanda insan konakçısının kan kolesterol düzeyinin azaltılmasında önemli bir rol oynar. Bu nedenle, tedavilerde kullanılan probiyotiklerin ve safra tuzu hidrolaz enzimlerin etki mekanizmasının bilinmesi ve anlaşılması önemlidir. *Lactobacillus gasseri* muhtemelen piyasadaki en yararlı probiyotiklerden biri olsa da, bu mikroorganizmanın STH enziminin katalitik aktivitesi ve substrat tercihleri etki mekanizması ile ilgili çok az bilgi bulunmaktadır. Bu çalışmada, *Lb.gasseri* ATCC 33323 suşundan elde edilen *sth* geninin *Escherichia coli* BLR(DE3) suşuna klonlanmış, ifade edilmiş ve tanımlanmış. Bu yaban tip rekombinan STH (yrSTH) enzimin hidroliz aktivitesi 25°C ile 65°C arasında değişen farklı sıcaklıklarda ve 4.0 ile 8.0 pH aralıklarında yapılan altı farklı safra tuzunun kullanıldığı ninhidrin testleri araştırılmıştır. Yrsth geninin ifadesi ise SDS-PAGE ile belirlenmiştir. Ninhidrin test sonuçları, yrSTH enziminin altı temel insan safra tuzunu hidroliz etmekle birlikte taurine ekli tuzlardan ziyade glisin ekli tuzları daha fazla tercih ettiklerini göstermektedir. yrBSH enziminin maksimum aktiviteleri düşük pH ve yüksek sıcaklıklarda kaydedildi. Elde edilen bulgular, STH enzimleri substratlarını kolat ekinden ziyade aminoasit eklerine göre tanımaktadır önerisini desteklemektedir. Bununla birlikte, STH enziminin katalitik aktivitesi, substrat özgüllükleri ve probiyotik bakteri seçi kriterlerinin daha iyi anlaşılması için yönlendirilmiş mutagenез gibi daha genişletilmiş çalışmalara ihtiyaç duyulmaktadır.

ANAHTAR KELİMELE: Probiyotik, *Lactobacillus gasseri*, Safra tuzu hidrolaz (STH), Katalitik aktivite ve Sübstrat özgüllüğü

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LIST OF ABBREVIATIONS AND SYMBOLS

bp	: Base pair
BSA	: Bovine Serum Albumin
BSH	: Bile Salt Hydrolase
CHD	: Coronary Heart Disease
DTT	: Dithiothreitol
FAO	: Food and Agriculture Organization
FDA	: Food and Drug Administration
GCA	: Glycocholic Acid
GCDCA	: Glycochenodeoxycholic Acid
GDCA	: Glycodeoxycholic Acid
GIT	: Gastrointestinal Tract
GRAS	: Generally Recognized As Safe
HDL	: High-Density Lipoproteins
IgA	: Immunoglobulin A
IBD	: Inflammatory Bowel Disease
IBS	: Irritable Bowel Syndrome
IPTG	: Isopropyl β -D-1-thiogalactopyranoside
Kbp	: Kilo base pair
LAB	: Lactic Acid Bacteria
LB	: Luria-Bertani -Broth
LCA	: Lithocholic Acid
LDL	: Low-Density Lipoproteins
M	: Molar
MRS	: de MAN, ROGOSA and SHARPE
OD	: Optic Density
PAGE	: Polyacrylamide Gel Electrophoresis
PEG	: Polyethylene Glycol
PCR	: Polymerase Chain Reaction
rpm	: Rotation per minute
SIB	: Swiss Institute of Bioinformatics
TCA	: Taurocholic Acid
TCA	: Trichloroacetic Acid

TCDCA	: Taurochenodeoxycholic Acid
TDCA	: Taurodeoxycholic Acid
Tween20	: Polyethylene Glycol Sorbitan Monolaurate
UTIs	: Urinary Tract Infections
μl	: Mikroliter
μM	: Micromolar
WHO	: World Health Organization
Xgal	: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside



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1. INTRODUCTION

To this day, coronary heart disease (CHD) and cancers are the leading causes of death worldwide with a total of 8.2 million in 2012 (World Cancer Report, 2014). Among the most common causes of cancer deaths are lung, liver and stomach cancers along with colorectal cancer which happens to be the second most diagnosed cancer in developed countries. Unfortunately, it is untreatable when the most advanced stages are reached, making its prevention of utmost importance (Greenlee et al., 2000). There are already many cancer risk factors accentuated by tobacco and alcohol uses, unhealthy diet and obesity as well as the lack of physical activity which when avoided can considerably reduce those deaths. Colorectal cancer along with many other diseases like type I and II diabetes, Inflammatory bowel disease (IBD) and Irritable bowel syndrome (IBS) have been linked with an altered GI (Gastrointestinal) microbiota (Sanders, 2011). Therefore, potential microorganisms can be used to solve or at least alleviate the symptoms by the dietary intake of probiotics. For instance, recent researches suggest that dietary therapy such as the addition of $\omega 3$ fatty acids, soy, red yeast rice and fermented milk in the diet can greatly improve the health of hyperlipidemic and/or hypercholesterolemic patients (Houston et al., 2009; Anderson, 1999). And because of the multiple side effects of the drugs used to treat these diseases, it is understandable why patients would prefer non-drug treatments or natural and alternative therapies.

1.1 History and Definition of Probiotics

The word “probiotic” from the Latin pro meaning “for” and Greek bios meaning “life” was first used in 1954 but the therapeutic effects of probiotics were discovered in the early twentieth century by Metchnikoff (1908). He demonstrated that consuming lactic acid bacteria can result not only in the reduction of toxin-producing bacteria in the gut, but also in the prolongation of the life of the host (Metchnikoff, 1908). And ever since then, many studies have been achieved to try to elucidate the beneficial effects of probiotics as well as their mechanisms of action.

Over the years there has been so many proposed definitions for probiotics that in 2001, the FAO (Food and Agriculture Organization) and the WHO (World Health Organization) came up with a unanimously recognized definition for probiotics: “Live microorganisms which when administered in adequate amounts confer health benefits to the host” (Guarner, et al., 2005; Sanders, 2003, FAO/WHO report, October 2001). These so called microorganisms are most often lactic acid bacteria such as Bifidobacteria, Lactobacilli and Streptococci; but they also include non-lactic acid bacteria like for instance Bacillus and yeasts such as *Saccharomyces boulardii*. A great number of bacteria are normally found within the human body and are involved with many tissues like the skin, the respiratory tract, the genital tract and the GI tract. These microbes are found throughout the entire GI tract with the majority of them residing in the colon (Figure 1).

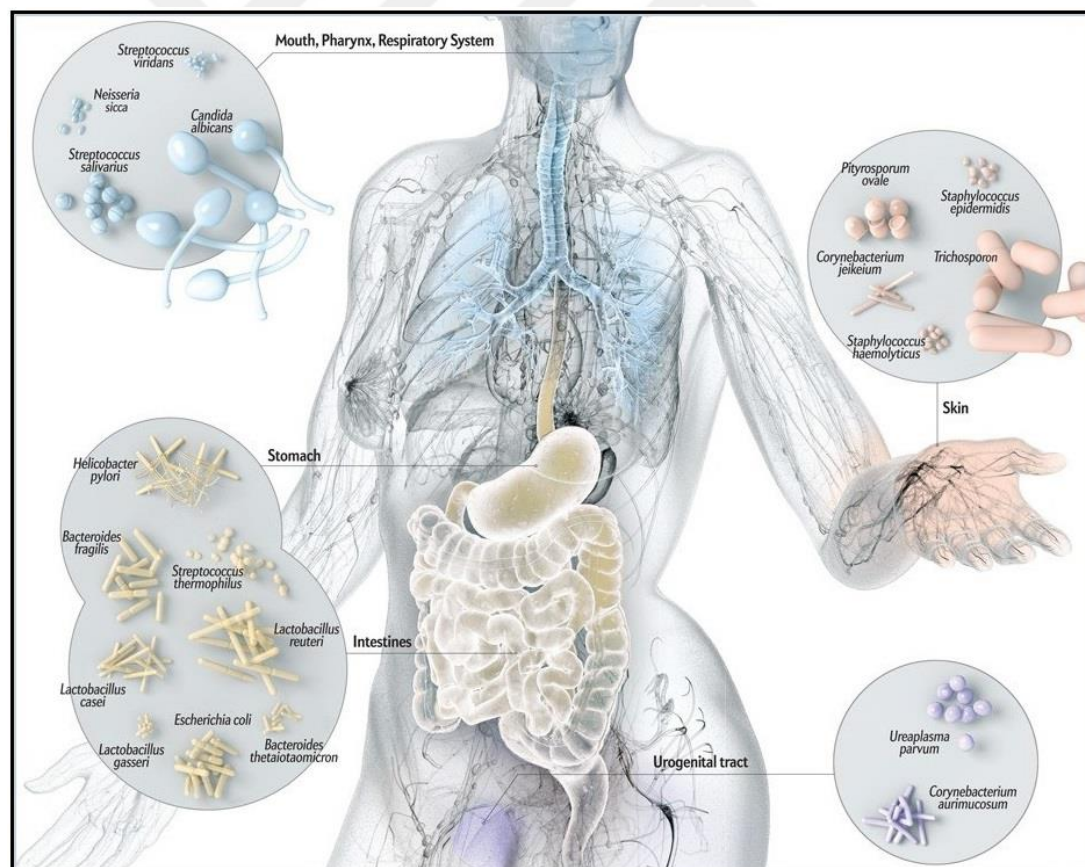


Figure 1.1. The human gastrointestinal tract. (Adapted from The Working Hypothesis Blog: the Case Against being Germophobe by Wudan, 2013)

Most of these bacteria are regarded as beneficial since they help fight the harmful bacteria, hasten the digestion process and perhaps even improve the immune function. These gut bacteria are either commensal (a person's innate, colonising microorganisms) or transient (microbes that are just fleeting). Probiotics are considered as transient even though some of them may belong to a normally commensal species. Some, but not all, probiotics are able to replicate and persist in the gut at least temporarily, but they disappear a few days after cessation of their intake.

Another term less known by the general public is prebiotics. These prebiotic are specific plant fiber that nourishes the good bacteria already in our gut. Where probiotics add the good bacteria into the host's gut, prebiotics only act as a fertilizer for the autochthonous bacteria that's already present there. And since they can not be digested by the body itself, they are only used to augment the growth of the good microbes in the gut. These, in turn, will provide the health benefits. (Jackson, 2016).

While probiotics have already demonstrated their effectiveness in managing certain gastrointestinal diseases, they sometimes work in parallel with prebiotics to form synbiotics. Even though the combination operates independently throughout the small and large intestine, the separate effects synergistically boost the overall health of the host's gut (Pineiro et al., 2008).

1.2 The Selection Criteria for Probiotics

The worldwide interest in probiotics is driven by the newly manufactured products found in the market and by the eagerness of researchers to evaluate the potential preventive and therapeutic health benefits that probiotics-containing products seems to have on the consumers.

The initial step in choosing a probiotic is knowing the taxonomic classification of the microorganism because determining the source, environment and physiological properties of the strain can impact our understanding of the functionality of the probiotics (Morelli, 2007). Probiotics varies from other types of bacteria in that they fit certain criteria. They must not be harmful meaning they

should fit the FDA (Food and Drug Administration) criteria for specific uses or be considered as GRAS (Generally Recognized As Safe); remain alive throughout the processing and shelf life of the food, be able to endure digestion and remain alive in the gut to generate the health benefits they are associated with. Therefore, screening microorganisms with those properties will require both in vitro and in vivo testing (Figure 2) and these standard tests generally include:

Bile and Acid Resistance: in order for the probiotics to exert their effects, they need to survive the journey through the GI tract. They should be capable of sustaining the severe acidic condition of the stomach (pH between 1.5 and 3.0)

Adherence to mucus or human epithelial cells and cell lines: A strong adherence to the mucosal lining of the gut ensures that the probiotics can successfully colonize its new environment. Therefore allowing them to better compete with the pathogens

Ability to reduce pathogen adhesion to surfaces: By limiting the pathogen's ability to settle in the mucosal lining of the gut, probiotics reduce the chances of the pathogen to fully colonize the gut and thereby negate their detrimental effect

Antimicrobial activity against possible pathogens: Probiotics should have the ability to secrete antimicrobial substances like hydrogen peroxides, short chain fatty acids or bacteriocins that can hinder the growth or destroy pathogens.

Effects on immune expression: Besides the ability to minimize the production of certain cytokines, probiotics should as well be able to stimulate certain parts of the immune system. (Shewale et al., 2014)

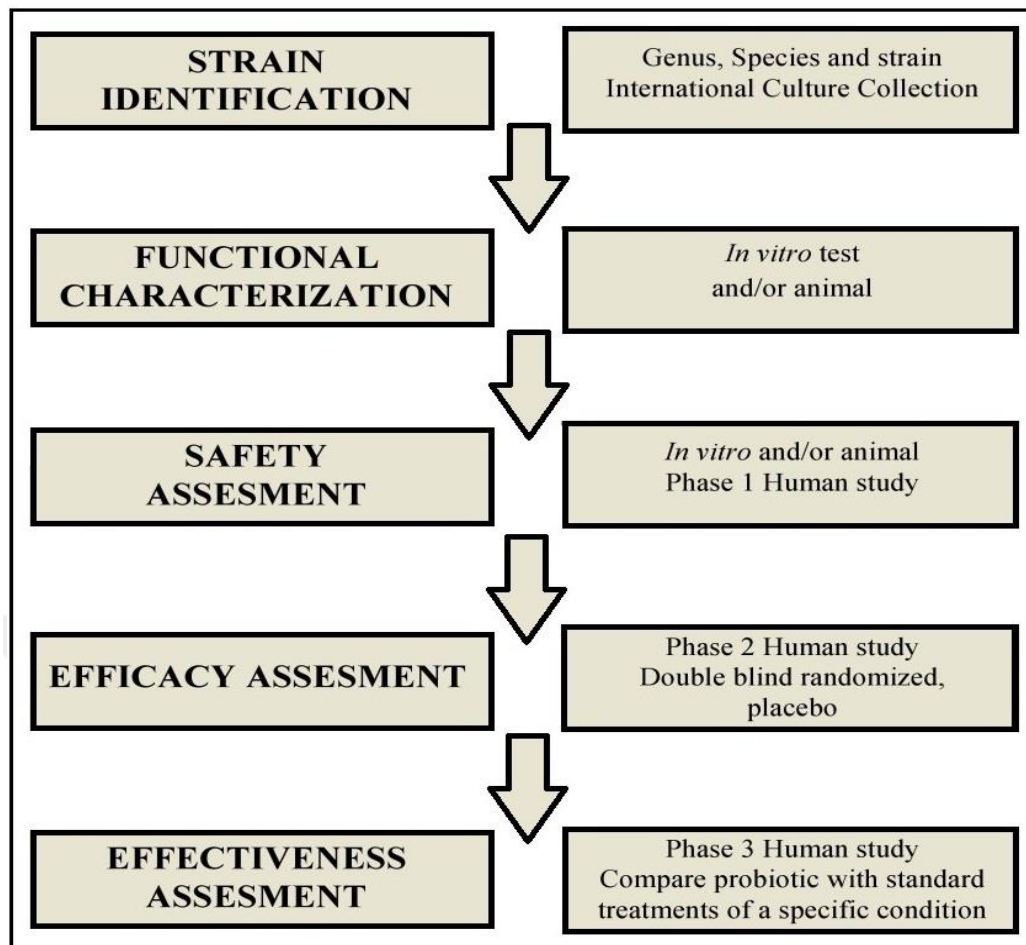


Figure 1.2. Scheme of the guidelines for the evaluation of probiotics for food use. (Adapted from, Collado et al., 2009).

1.3 The Impact of Probiotics on Human Health

Ever since the introduction of the concept of probiotics, there has been a growing interest on their influence in the human nutrition and health. Worldwide researches on this topic have accelerated in the recent years with many scientists trying to understand the nature and structure of said probiotics as well as their mechanism of action in the human body. Some of these health-related effects so far discovered were listed below (Sanders, 2013):

- Management of lactose intolerance
- Improvement of the immune system

- Lowering effect on the blood pressure.
- Prevention of colon cancer
- Prevention of urogenital infections
- Reduction of the plasma cholesterol concentrations
- Reduction of inflammations and allergic symptoms
- Reduction of pathogens like *Helicobacter pylori*

And so many more.

1.3.1 Lactose Intolerance

Surprisingly many non-Caucasians are lactose intolerant after the halt of breastfeeding. They can not metabolize lactose into its main components, glucose and galactose, because of the low levels or lack of a crucial enzyme, the β -galactosidase. So when they drink milk or ingest any type of lactose-containing foods, they will start developing symptoms ranging from abdominal pain, bloating, flatulence to diarrhea. Studies have showed that adding certain cultures like *Bifidobacterium longum*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* to the milk products prior to their ingestion, can allow the patients suffering from lactose intolerance to consume those products without experiencing the usual side effects. (Fooks et al., 1999; Scheinbach, 1998). The noticeable beneficial effects that probiotics can have on lactose intolerance can be explained by two alternatives. One of them would be the lower lactose concentration in the fermented foods that is due to the high lactase activity of the additional bacterial preparations used in the production. While the other one would be the increased lactase active enzyme that enters the small intestine along with the fermented product or with the viable probiotic bacteria (Salminen, et al., 2004).

In addition to the added β -galactosidase effects of the bacteria contained in them, fermented dairy products can significantly improve the lactose digestion by

delaying the gastric emptying of the semi-solid milk products such as yogurt, thereby giving the residual β -galactosidase activity in the small intestine of those lactose intolerant people more time to hydrolyze lactose (de Vrese et al., 2001). So even though probiotics normally promote the lactose digestion less efficiently than do the yogurt cultures, they may, at least reduce the symptoms brought about by the undigested lactose alleviating some of the pain of those lactose intolerant people.

1.3.2 Improvement of the Immune System

Even though human studies have shown that probiotics can have beneficial effects on their host's immune system (Mombelli and Gismondo 2000), the mechanism by which they operate is not yet fully understood. But it has been demonstrated that probiotic bacteria can greatly affect the immune system in many different ways such as; by stimulating the macrophages, producing cytokines and even increasing the secretion concentrations of immunoglobulin A (IgA) (Scheinbach, 1998; Dugas et al., 1999). It has been shown that probiotics enhance the humoral immune responses thereby strengthening the intestine's immunologic barrier. For instance, Fooks et al., (1999) for 4 months have feed humans in a clinical trial with 450 g of yogurt per day and by the end of the trial, a significant increase in the production of γ -interferon was observed. Scientists like Mattila-Sandholm and Kauppila (1998) demonstrated that extracts derived from *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 can actually suppress the proliferation of the Lymphocytes in vitro.

Further evidence for the immuno-modulation capability of these two bacterial strains have been observed on a children clinical trial with patients suffering from severe atopic eczema resulting from food allergy: the children fed with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12 showed an alleviation of the clinical symptoms compared to the group that was given the placebo (Saarela et al., 2000). These data show that probiotics can be used as innovative tools to restore the immune system defenses and normalize a gut mucosal dysfunction.

1.3.3 Lowering Effect on the Blood Pressure.

One of the leading cause of cardiovascular disease is high blood pressure, also known as hypertension. High blood pressure is also a risk factor for psoriasis which is a skin disease marked by red, itchy, scaly patches. Leavitt (2015) studied 9 clinical trials involving 543 participants in order to understand the effects of probiotics on blood pressure. The authors of those clinical trials reported that five out of nine of the trials showed a reduction in the level of systolic blood pressure, and two showed a reduction in the level of diastolic blood pressure. For the purpose of the studies, the patients were feed probiotics in dairy products, such as yogurt. According to the American Heart Association, the results of those trials showed that the probiotics can moderately improve the blood pressure and that they were most efficient in patients whose blood pressure was higher than 130/85 where the normal blood pressure should be lower than 120/80.

It is important to understand that the effect of probiotics on the blood pressure can vary due to factors like the type of probiotic strains, the way the clinical trial was designed as well as the initial lipid levels. Therefore, understanding the role of probiotics on lipid profiles may give ideas for new prevention strategies and if usable, these probiotic supplementation may bring about a new way for managing cardiovascular disease risk factors. But nonetheless, further studies are needed to decide the types and amount of probiotics that could have the greatest impact on the blood pressure.

1.3.4 Prevention of Colon Cancer

Colorectal cancer is a major public health problem being the second most diagnosed cancer in developed countries. Even though radiotherapy and chemotherapy have been used as treatments for colon cancer, they are usually accompanied by some serious side effects such as an increased risk for infections, hair loss, nausea, vomiting, diarrhea, and bloody stools (Martenson et al., 2004). Diet supplements of probiotics have now been rigorously studied to reduce the risks of colon cancer as a preventive method instead of a cure. In-vivo and molecular studies

have shown promising outcomes, mostly due to the antimicrobial effects of probiotics against carcinogen-producing microorganisms. The probiotics antimutagenic properties may have also played an important role in altering the tumor differentiation processes (Yu and Li, 2016). Studies have suggested that prebiotics which are indigestible food that could stimulate the growth of probiotics can also have a protective effect against colon cancer, mostly because of the short chain fatty acids production upon their fermentation by the gut bacterial microflora. The combined actions of probiotic and prebiotic known as synbiotic effect, has been found to have the capacity to act in improving the colon carcinogenesis compared to trials where both prebiotics and probiotics were used individually (Liong, 2008).

While these laboratory studies display optimistic results on the anti-tumor effects of probiotics or synbiotics, the benefits should not be overstated before additional results from human subjects are gathered. And even so, more randomized clinical trials should be performed in order for the medical community to fully accept the exploitation of probiotics as an alternative therapy for cancer control.

1.3.5 Prevention of Urogenital Infections

Urinary Tract Infections (UTIs) are bacterial infections that cause inflammation of any part of the urinary tract. The healthy women genital flora is dominated by *Lactobacillus* species that prevent these pathogens from accessing the urinary tract. So it is no surprise that women with recurrent UTIs have low lactobacilli counts (Hanson L, 2011). Furthermore, regular urination is a defense mechanism against the ascension of the infection. The capabilities of probiotic to intercede in the containment of UTIs has long been considered and is now supported by a growing number of clinical evidence for various specific strains. For instance, it was suggested that some probiotic lactobacilli improve the urogenital health by modulating the immune system, reducing the pathogen's ascension, and interfering with the colonization of the urinary tract by the pathogens (Reid et al., 2009). A randomized, double-blind, placebo-controlled trial in 2006 also demonstrated the effectiveness of probiotics by showing that an oral ingestion of a mixture of *Lactobacillus rhamnosus* and *Lactobacillus reuteri* was successful in recovering the

vaginal counts of *Lactobacillus* species following antibiotics and infection by 96% compared to 53% in controls (Anukam and Osazuwa, 2006). These probiotic therapies have been shown to not only be able to restore the urogenital flora to normal, but to also safely maintain it that way.

Even though further trials are required, the encouraging results of the studies stated above have lead to believe that daily probiotic supplementation could be beneficial in maintaining the urinary tract health and definitely prevent any UTIs.

1.3.6 Reduction of the Plasma Cholesterol Concentrations

Even though there are numerous risk factors for coronary heart disease (CHD), hypercholesterolemia is still the leading factor for this illness. Therefore, reducing the cholesterol level of hypercholesterolemic patients is crucial to preventing CHD. Pharmacological drugs can efficiently reduce the cholesterol concentrations in the serum but because of the adverse side effects or contraindications of these drugs, patients usually prefer to go for for alternative therapies like the dietary supplement of probiotics.

Researchers studying the efficiency of probiotics in reducing the plasma cholesterol level usually do not sufficiently address the mechanisms by which the hypocholesterolemic effects of probiotics operate nor do they elaborate on the optimum dose, frequency, and duration required for treatment according to the different probiotic strains. So far, several mechanisms have been proposed, which include the enzymatic deconjugation of bile acids by the bacterial bile-salt hydrolase (Lambert et al., 2008), the assimilation of cholesterol by probiotics (Pereira et al., 2002), the co-precipitation of cholesterol with deconjugated bile (Liong et al., 2006), the cholesterol binding to cell walls of probiotics (Liong et al., 2005), the incorporation of cholesterol into the cellular membranes of probiotics during their growth or the conversion of cholesterol into coprostanol (Lye et al., 2010) and the production of short-chain fatty acids by probiotics upon their fermentation in the presence of prebiotics (De Preter et al., 2007).

Needless to say that more studies are required, not only to define the potent dosage of probiotics or symbiotic necessary to produce the hypocholesterolemic effects, but also to estimate the effects of the degree of symbiosis between probiotics and prebiotics on cholesterol-lowering or removing properties and precisely map their mechanism of action.

1.3.7 Reduction of Inflammations and Allergic Symptoms

Inflammation is the path our immune system uses to tell us to pay attention to our health and it is also considered a crucial step in the healing process. But even so, a chronic inflammation may cause swelling, pain, damage tissues and mean a more dangerous underlying condition. Recently, researchers are studying the potential of probiotics to alleviate inflammation in our gut and provide a better resistance against the harmful bacteria. Probiotics can help regulate inflammation in a different ways: Indirectly, through the maintenance and mending of epithelial barriers, enhancing production of short chain fatty acids with anti-inflammatory properties thereby suppressing the source of pro-inflammatory stimuli. They can also increase the synthesis of antimicrobial peptides that influence the inflammation resolution pathways in the mucosa. And directly, by binding to the innate immune system receptors and stimulating the pathways that affect the production of cytokines associated with inflammation. (Lescheid, 2014).

Probiotics play a major role in the prevention of allergies. For instance, when a non-pathogenic *E. coli* was administered to children between 10 and 20 years of age, scientists noted that the children began to experience significantly fewer allergic reactions. In a parallel study by the same group of researches, expecting mothers were divided into groups receiving either probiotics or a placebo. And after delivery, the children of mothers who received the probiotics continued the treatment for 6 more months. After 4 years, only 26% of the children on probiotics developed eczema while 46% of those without the probiotics developed the condition (Ouweland, 2007). Another study conducted at the Vanderbilt University in Nashville, Tennessee, found that probiotics may actually alleviate the symptoms and

ease the harshness of allergic rhinitis better known as seasonal allergies (Freeman et al., 2015).

It should be noted that the probiotics mechanisms of action may differ from one kind of probiotic to another for the same immune response and may also be regulated by a combination of several events (Walker, 2008).

1.3.8 Reduction of Pathogens like *Helicobacter pylori*

Helicobacter pylori is a highly powerful pathogen that can induce chronic gastritis and ulcer thereby representing the major risk factor for gastric malignancies. Per usual, the antibiotics treatments are nearly 90% effective but they are still very expensive and the negative side effects are numerous without considering the possibility of developing an antibiotic resistance.

Probiotics however can inhibit *H. Pylori* via non-immunological mechanisms or by secreting antimicrobial substances without the added side effects. The acidity of the stomach forms a non-immunological barrier strengthened by the antimicrobial substances produced by the probiotics. And by competing with *H. pylori* for adhesion to the mucosal receptors, probiotics stimulate the mucin production to further stabilize the gut mucosal barrier. It has been reported that some *Lactobacillus* strains like *Lb. gasseri* and *Lb. plantarum* are able to inhibit *H. pylori* adherence to the gastric epithelial cells (Chen et al., 2012). Probiotics can also directly inhibit the growth of *H. pylori* by synthesizing antibacterial substances or other antimicrobial compounds such as hydrogen peroxide and lactic and acetic acid (Vandenbergh, 1993). Studies also show that *L. acidophilus* produces conjugated linoleic acids that interfere with the inflammation resulting from a *H. pylori* infection (Kim et al., 2008). Regardless of the mechanism of action of the probiotics, the patient's genetic background as well as his/her microbiota composition can greatly influence the effectiveness of the probiotic treatment (Perri et al., 2003).

As discussed earlier, the human gastrointestinal tract is home to thousand different bacterial species that cohabit in a complex equilibrium. These bacteria represent 35 to 50% of the total volume content of the human colon and include

species like *Bacteroides*, *Clostridium*, *Eubacterium*, *Escherichia* and many other with *Bifidobacterium* and *Lactobacillus* which show identified beneficial properties. However, many external factors such as the host genetics, diet, age and antibiotic treatments can affect the microbial composition in the gut. The intestine has a primordial role in the host defense against external attacks via the intestinal mucosa, the local immune system, and the interactions with the intestinal microbiota. Therefore, it is easy for the bacteria colonizing the GI tract to alter the host health through an influence on those defense system (Collado et al., 2009).

Because of their massive impact on the human health described above, probiotics are being more often used as therapeutic dietary supplements. The vast majority of the probiotic cultures found in nutritional supplements, pharmaceuticals and functional foods consist of lactic acid bacteria (Del Piano, 2006).

1.4 Lactic Acid Bacteria

The Lactic Acid Bacteria (LAB) are regarded as the major group of probiotic bacteria (Collins et al., 1998; Schrezenmeir and de Vrese, 2001; Tannock, 1998). They consist of a multitude of bacterial genera like *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* which are all within the phylum *Firmicutes* (Ercolini et al., 2001; Jay, 2000; Holzapfel et al., 2001). Even though LAB are usually found in soil, water, fertilizer and silage, many of them reside in the human oral cavity, intestinal tract, and genital tract, and are thought to exert a great influence on these various ecosystems. Members of the LAB are facultative anaerobic non-spore-forming Gram-positive cocci, coccobacilli or rods (Fooks et al., 1999) that lack catalase. They ferment carbohydrates primarily into lactic acid but their metabolic pathways can vary depending on the organism (Jay, 2000). LAB have been granted the GRAS (Generally Regarded as Safe) status and are now among the primary groups of microorganisms used in food industry as fermenting agents. They are responsible for the different taste and texture of fermented products and are used to inhibit food

spoilage by secreting growth-inhibiting substances and large amounts of lactic acid (Salminen et al., 1998).

All these characteristics described above are consequential on their possible selection as probiotics. The *Lactobacillus* and *Bifidobacterium* genera are the most well studied and documented LAB in regard to their potential probiotic properties.

1.4.1 The *Lactobacillus* Genus

The *Lactobacillus* genus is a group of gram-positive bacteria from the *Lactobacillaceae* family. They are commensal residents of the human and animal gastrointestinal tracts, as well as the human mouth and genital tract. The *Lactobacillus* are characterized by a genomic GC content lower than 54mol and by their ability to produce lactic acid as a by-product of carbohydrate metabolism. When glucose is the main source of carbon, many *Lactobacilli* degrade it using either the homofermentative or the heterofermentative pathway depending on the organism. The homofermentative bacteria such as *Lb. acidophilus*, *Lb. casei*, and *Lb. plantarum* will yield lactate as at least 85% of their end metabolic products, whereas the heterofermentative ones like *Lb. brevis* and *Lb. fermentum*, will produce lactate, ethanol and carbon dioxide making lactate 50% of their metabolic byproducts (Caplice and Fitzgerald, 1999; Jay, 2000; Kuipers et al., 2000).

LAB are also part of the microbiota found on mucosal membranes, such as the intestines, mouth, skin, urinary and genital organs of both humans and animals, and they might have a beneficial effect on these ecosystems (Daoud Harzallah, 2013). LAB require a nutritionally rich environments like dairy, meat, vegetable, and cereal plant environments, where fermentation can occur. Some LAB species can even be found in the respiratory, intestinal and genital tracts of both humans and animals. This remarkable ability to colonize such places testifies to the impressive metabolic versatility of these bacteria (Kuipers et al., 2000; Socol, et al., 2010). Nutritional benefits aside, the ingestion of LAB and/or their fermented foods has been known to have many health benefits:

Constipation: Fermented milk products containing *Lb. acidophilus* and *bifidobacteria* were proven to reduce the intestinal transit time which can alleviate the pains of those with constipation, such as the elderly (de Vrese et al. 2001).

Ulcers: Studies show that by competing with *H. Pylori*, *Lb. acidophilus* effectively limit the number of *H. pylori* that can attach to the cell wall thereby reducing the risk for stomach ulcers (Brassart et al., 1995). Another study demonstrated that *Bifidobacteria bifidum* promoted the healing of gastric ulcers in 50% of the patients and eradication of *H. pylori* from the mucous membranes in 30% of the patients (Tanaka, 1995).

Hypocholesterolemic effects: in vitro studies show that cholesterol can precipitate with free bile salts in the presence of *Lb. acidophilus* which could help lower the serum cholesterol level in humans (Liong et al., 2006).

Colon cancer: LAB may help prevent the onset of colon cancer by slowing the growth of experimental cancers. This is accomplished by reducing the levels of β -glucuronidase, nitroreductase, and azoreductase enzymes that convert the pro-carcinogens into carcinogens (Fernandes et al., 1990).

1.4.1.1 *Lactobacillus gasseri*

Lactobacillus gasseri (*Lb. gasseri*) is an autochthonous rod shaped gram-positive anaerobic bacterium commonly found in the GI tract and various other places. Its probiotic activity has been estimated based on the various health benefits it elicits and its adaptability to the human mucosal niches. *Lb. gasseri* is classified as a group B acidophilus complex microorganism because of the visible lack of major surface-layer (S-layer) proteins (Pot et al., 1993; Boot et al., 1996). For instance, *Lb. gasseri* is commonly found in children and constitutes one of the most predominant species involved in early GIT colonization and remains persistent throughout adulthood (Wall et al., 2007). Furthermore, the presence of *Lb. gasseri* in healthy individuals and its absence in patients suffering from any health detriment validates its safety (De Backer et al., 2007; Delgado et al., 2007; Hojo et al., 2007; Hernandez-Rodriguez et al., 2011).

Studies of the genomic sequence of *Lb. gasseri* ATCC 33323 strain revealed that the bacteria does not possess transmissible antibiotic resistance genes or any other unwanted genes beyond those needed to ease its adherence to the mucosal layer or those encoding for toxin and drug efflux systems (Azcarate-Peril et al., 2008). But even so, it cannot be assumed that all strains of *Lb. gasseri* are without transmissible antibiotic resistance genes, hence the necessity for further inquiries.

Lb. gasseri is one of those probiotics you don't hear about often but it is however, extremely powerful. One of the most interesting health benefits of *Lb. gasseri* is its effectiveness for weight loss: a Korean clinical trial revealed that *Lb. gasseri* 2055 to be an effective anti-obesity probiotic for overweight and obese adults. (Kadooka et al., 2010). Weight loss aside, this microorganism has many other beneficial health effects such as immunity boost, anti-inflammatory effects, protection against seasonal allergies and hypocholesterolemic effects. This cholesterol lowering effect of not only *Lb. gasseri* but many other probiotics is exerted through the deconjugation of bile salts by their bile salt hydrolase enzyme and understanding the mechanism behind it has been the main goal of many researchers.

1.5 Cholesterol Lowering Mechanism

1.5.1 Cholesterol

Being an important structural component of the animal cell membranes, cholesterol is an organic modified steroid molecule (sterol) synthesized by all animal cells. It is needed to build and maintain the structural integrity and modulates the fluidity of the animal cell membrane thereby allowing the cells to change shape and move (Hanukoglu, 1992). Within the cells, cholesterol is also a precursor molecule for the synthesis of bile acids, steroid hormones and is used in several biochemical pathways. For instance, it is used for the synthesis of vitamin D and all steroid hormones like cortisol, aldosterone, progesterone, estrogens, testosterone, and many others.

There are two different types of lipoproteins that carry the cholesterol throughout our entire body: low-density lipoproteins (LDL) and high-density lipoproteins (HDL). LDL cholesterol is sometimes referred to as “bad” cholesterol and a high LDL level can cause cholesterol to build up in your arteries which is very dangerous since those arteries are responsible for carrying the blood from your heart to the other components of body providing them oxygen and nutrients. HDL cholesterol on the other hand is called “good” cholesterol because it carries the cholesterol from other parts of your body back to your liver where it is removed from the body by being turned into a different molecule. Therefore, having healthy levels of both types of lipoproteins is important and lowering our cholesterol level might reduce or even stop the cholesterol plaque build up in our arteries (Gary, 2013)

1.5.2 Bile and Bile Salts

Bile is a water-soluble yellow greenish substance produced in the liver by hepatocytes using cholesterol as a precursor that aids the digestion of lipids in the small intestine. It is concentrated and stored in the gallbladder before being released into the duodenum upon ingestion of food (Begley et al., 2006). Bile is made of cholesterol, phospholipids, conjugated bile acids, bile pigments and electrolytes. By emulsifying the dietary lipids, bile acids improve the solubility and digestion of triacylglycerols by lipases, liberating the free fatty acids in the small intestine. The lipid-soluble bile acids are conjugated to either glycine or taurine molecules to form water soluble primary conjugated bile acids, usually called bile salts. Bile salts are hydrophobic on one side and hydrophilic on the other; therefore, they tend to gather around the small drops of lipids to form micelles, with their hydrophobic sides facing the fat molecule and their hydrophilic sides facing outwards. The negative charges on the hydrophilic sides prevent the re-aggregation of the fat droplets (Barrett and Kim, 2012). Bile salts can also act as bactericides, destroying most of the microbes that might be present in the food.

Previous *in vitro* studies all came up with different hypotheses for the mechanisms behind the cholesterol-lowering effects of probiotics. One of these

proposed hypothesis is based on the enzymatic deconjugation of bile acids by the bile-salt hydrolase enzyme of the probiotics.

1.5.3 Bile Salt Hydrolase

Bacterial bile salt hydrolase (BSH) is an N-terminal nucleophilic hydrolase that can recognize its substrate at both the amino acid conjugate or steroid nucleus (Patel et al., 2010). Probiotics like the lactic acid bacteria possess BSH and/or BSH homologs which give them an advantage against the other microorganisms (Begley et al. 2006). Previous studies of different bacterial BSH enzymes revealed that the enzyme is intracellular, sensitive to oxygen, and optimally active at a slightly acidic pH (Pavlović et al., 2012).

The overall function of the BSH is to catalyze the deconjugation of bile salts, but there are several explanations as to how this can possibly benefit the bacteria secreting it. One of the proposed benefits is that BSH-producing strains by deconjugating bile salts, can gain nutritional advantages by using the amino acids liberated from the deconjugation reaction as carbon, nitrogen, and energy sources (Begley et al. 2006). Another proposition is that BSH eases the cholesterol or bile incorporation into the bacterial membrane thereby improving its structural integrity against host immune defenses (Patel et al., 2010). However, the most likely predominant role for BSH activity is that it helps in bile detoxification process (Kim and Lee 2005). Bile is a natural detergent with antimicrobial activity that could dissolve bacterial membranes so naturally the GI bacteria should possess some form of defense mechanism against this threat in order to survive in environments like the GI tract (Begley et al., 2005).

1.5.4 Cholesterol -Lowering Effects of Bile Salt Hydrolase

Even though there are a multitude of mechanisms proposed for the hypocholesterolemic effect that probiotics have on their host, the one that stands out the most is the enzymatic deconjugation of bile acids by the bacterial bile-salt hydrolase enzyme.

Following their excretion into the intestinal lumen, bile salts can be hydrolysed by the bacterial bile salt hydrolase enzyme (BSH) into a glycine or taurine moiety and free bile acids. Usually 95% of these free bile acids will be recycled by the enterohepatic circulation in the terminal ileum and taken up by the liver to be reused in the de novo synthesis of bile salts (Figure 1.5.4a). Because of the pH of the small intestine, most of the bile acids will be ionized and mostly found in their sodium salts forms called “primary conjugated bile salts.” In the lower small intestine and colon, the gut bacteria transform some of the primary bile salts into secondary conjugated bile salts by hydrolyzing the amide bond and liberating the glycine/taurine moiety from the steroid core (Figure 1.3). Once in the proximal and distal ileum, these conjugated bile salts are passively reabsorbed back into the hepatic portal circulation. Since only 95% of the bile acids made it back to the liver, in order to synthesize bile salt de novo, the liver has to tap into the plasma cholesterol reserve to replace the 5% lost in the feces. And by doing so, the cholesterol level of the blood will gradually decrease. This enterohepatic circulation of bile acids takes place 4 to 6 times in a day which allows the recycled usage of each bile salt molecule nearly 20 times during a single digestive phase (Joyce J. Diwan 2012).

The hypocholesterolemic effect can also be attributed to the ability of probiotics to bind cholesterol in the small intestines. For instance, it has been reported that strains of *Lb. gasseri* could remove cholesterol from laboratory media by binding onto cellular surfaces (Usman and Hosono, 1999). This cholesterol-binding ability seemed to be growth and strain specific which was later confirmed when live and growing cells were compared to those that were non- growing (live but suspended in phosphate buffer) and dead (heat-killed). Researchers found that even though the growing cells were able to remove more cholesterol than dead cells,

the heat-killed cells could still remove cholesterol from media, indicating that some cholesterol was bound to the cellular surface.(Kimoto et al., 2002).

Either way, both these mechanism even though different, lead to the reduced cholesterol level of the bacterial host.

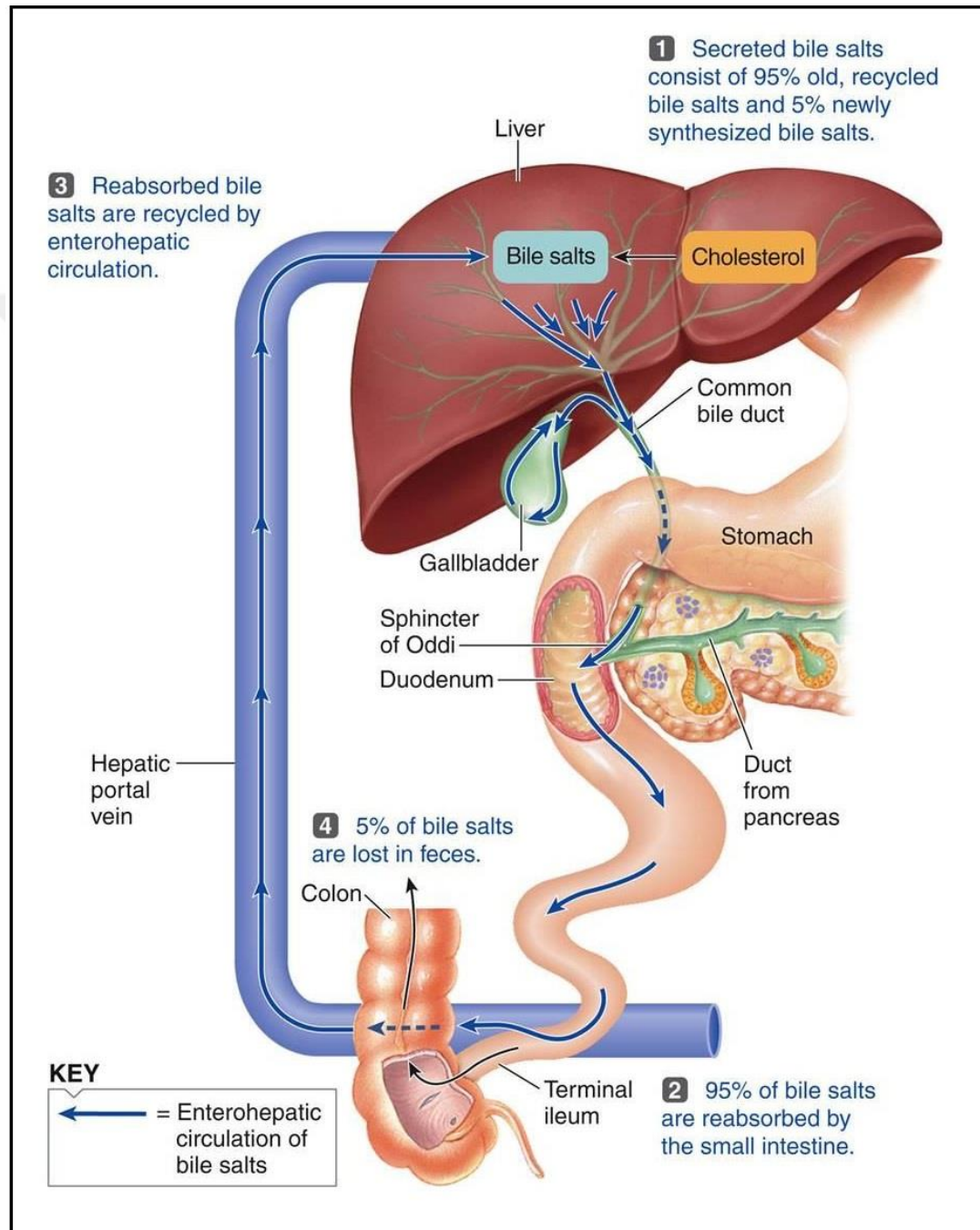


Figure 1. 3. Enterohepatic circulation of bile salts (adapted from www.gestaltreality.com)

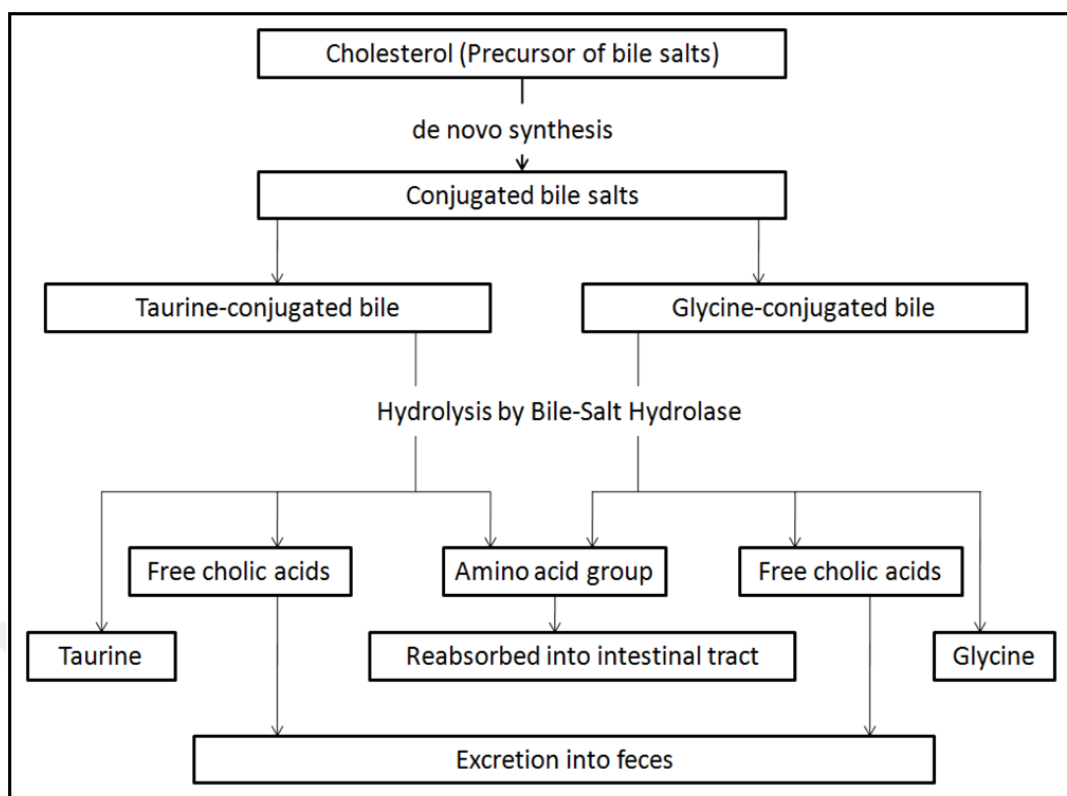


Figure 1. 4. The hypocholesterolemic role of bile salt hydrolase (adapted from Ooi and Liong 2010).

1.5.5 Side Effect of Bile Salt Hydrolase

During the enterohepatic circulation, bile salts come in contact with the facultative and anaerobic bacteria found in the small bowel. The major bile salt modifications by these bacteria consist mainly of the deconjugation and hydroxy group oxidation (Vlahcevic et al, 1996). Deconjugation and $7\alpha/\beta$ dehydroxylation of bile salts increase their hydrophobicity and allowing their recycling through passive absorption across the colonic epithelium. But unfortunately, the increased hydrophobicity of these deconjugated bile salts is also linked to increased toxic and metabolic effects. (McGarr et al., 2005). The secondary bile acids produced by the bacterial enzymatic deconjugation and dehydroxylation of primary bile acids in the intestines can accumulate in the enterohepatic circulation of some individuals to reach high levels and may well take part in the onset of colon cancer, cholesterol gallstones, and other gastrointestinal diseases. (Ridlon et al., 2006).

Bile Acids are involved in the emulsification and digestion of dietary fats but high concentration levels of bile acids can actually harm the cell membrane. In fact, secondary bile acids, particularly lithocholic acid (LCA) constitute an example of toxic endobiotics (Hofmann et al., 2004). It has been shown that high physiologic concentrations of bile acids can induce oxidative/nitrosative stress, DNA damage, apoptosis, and mutation (Payne et al., 2008). But since bile acids are natural part of the luminal contents of the GI tract, the exact mechanism by which these toxic secondary bile acids exert their action is not well understood. Several hypothesis stipulate that they might act through membrane damage, intracellular mitochondrial action or genotoxic effects (McGarr et al., 2005). So far the involvement of bile acids in onset of colon cancer is mainly circumstantial

2. AIM AND SCOPE OF THE STUDY

Lately, the battle against hypercholesterolemia has been intense and new potential hypocholesterolemic drugs and foods products are being manufactured with the intention of regulating the cholesterol levels of hypercholesterolemic patients. All of these drugs mostly act by interrupting the enterohepatic circulation of bile salts. Even though we still do not properly understand the hypocholesterolemic mechanism of probiotics, it is a well-known fact that cholesterol and bile salt metabolism are closely linked. Recently, many studies are trying to elucidate the cholesterol lowering effect of probiotics that display BSH activity. It has also been reported that probiotics, which exhibited BSH activities from their vitro trails can reduce serum cholesterol levels by 22% to 33% (Jones et al., 2004; Lim et al., 2004). Seeing that even a small reduction of 1% in the serum cholesterol level can lower the risk of coronary heart disease by 2% to 3% (Gilliland et al., 1985; Manson et al., 1992). It is no suprise that the alernative use of said probiotics is preferred to the conventional pharmaceutical drugs. Therefore, knowing and understanding the mechanism of action of the BSH enzymes of those probiotic is of extreme importance. Even though *Lactobacillus gasseri* is probably one of the most beneficial probiotics on the market and bile salt resistance one of the criteria for the initial probiotics selection, there is little to no information on the mechanism of action of the catalytic activity and substrate preferences of the BSH enzyme of *Lb. gasseri* which catalyze the deconjugation of bile salts.

The present study aims to detect the substrate specificity of the BSH enzyme of the *Lb. gasseri* ATCC 33323 strain between six different bile acids GCA, GDCA, GCDCA, TCA, TDCA and TCDCA; and to determine the optimum temperature and pH of the BSH enzyme's activity by using different temperatures ranging from 25°C to 65°C and various pHs from 2.5 to 8.0 in the presence of GDCA as a substrate by ninhydrin assay.

And to do so, the *bsh* gene from *Lb. gasseri* ATCC 33323 strain was cloned into *Escherichia coli* XL1-Blues strain, expressed and characterized in *E. coli* BLR(DE3) strain.

3. MATERIALS AND METHODS

3.1 Bacterial growth and genomic isolation

The *Lactobacillus gasseri* strain used in this experiment is the commercially available *Lb. gasseri* ATCC 33323 strain. The streak plate technique was used to isolate the *Lb. gasseri* on a fresh MRS (de MAN, ROGOSA and SHARPE) plate. The streaked plate was then incubated in an anaerobic jar at 37°C. Even though the bacterial growth was slow, we made sure to not exceed the 31 hours growth or we may lose the culture entirely. After 22 hours of incubation, we started the first liquid culture with 4mL of MRS broth and a colony from the previously streaked plate and incubate it alongside a MRS broth control in an anaerobic jar at 37°C. The following day, the overnight culture was retrieved to later on start a second liquid culture with 2% of the previous overnight culture. The pellet of this second culture was then collected by first centrifuging at 4000 rpm at 25°C for 4 minutes and then at 6000 rpm for 3 minutes after re-suspending the pellet in 1 ml of MRS broth. Using the pellet obtained, we proceeded to the genomic DNA isolation using the protocol prescribed on the Vivantis Genomic DNA Isolation Kit (California, United States).

3.2 DNA Amplification by PCR

Our DNA of interest was amplified using species specific primers (Table 3.1) by the Techne TC-3000. The reaction was performed in a 50 µl solution containing 25mM MgCl₂, 25µM of the LBGASF2 (forward) and LBGASR1 (reverse) primers, 5mM dNTP mix, the genomic DNA as a template and the Pfu DNA polymerase enzyme (Thermo) (Table 3.2). The PCR conditions are grouped in Table 3.3. The Pfu DNA polymerase was preferred because of its proofreading activity thereby reducing the chance of unwanted mutations.

Table 3.1. Primers used for amplification of the deired *bsh* gene

Primers	Sequences (5' to 3')	Tm
LBGASF2	5' - gtcgacatgtgtacatcaattttatacag -3'	52°C
LBGASR1	5' - cgcgccgtctaatttatgaaactaatatcttg - 3'	64°C

Table 3.2. PCR Reactants Dosage

Reactants	Quantities
Up (ultra pure) H ₂ O	33 µl
MgCl ₂ (25mM)	4 µl
LBGASF2 (25µM)	2 µl
LBGASR1 (25µM)	2 µl
dNTP mix (5Mm)	2 µl
Template DNA (2ng)	1 µl
Pfu DNA polymerase Buffer (10X)	5 µl
Pfu DNA polymerase Enzyme (2.5 U/µl)	1 µl
TOTAL VOLUME	50 µl

Table 3.3. PCR Reaction Conditions

Cycles	Temperatures	Time
Initial Denaturation	95°C	10 min
Denaturation	94°C	1 min
Annealing	49°C	1 min
Extension	72°C	3 min
Final Extension	72°C	1 min
Number of Cycles	30	

3.3 Purification of the PCR Products

The PCR amplicons were loaded and separated from unwanted products on a 1% agarose gel (Sigma, USA) for extraction and purification. After being separated by electrophoresis at 75 volt for 1 hour, the PCR products were purified using the protocol described in the Thermo Scientific GeneJET Gel Extraction Kit (Massachusetts, USA).

3.4 Preparation of pNMB1 Construct

3.4.1 DNA isolation of the pBluescript Vector

From a previously streaked plate of pBluescript incubated overnight at 37°C, we started a liquid culture with one colony to again incubate overnight for nearly 14 to 16 hours at 37°C and 175 rpm. The pellet of this liquid culture was then collected by first centrifuging at 4000 rpm at 25°C for 4 minutes and then at 6000 rpm for 3 minutes after re-suspending the pellet in 1 ml of LB (Luria-Broth) medium. Using the pellet obtained, we proceeded to the plasmid DNA isolation using the protocol prescribed on the Vivantis plasmid DNA Isolation Kit (California, United States).

3.4.2 Digestion of pBluescript by SmaI Restriction Enzyme

Since the PCR products are blunt ended, the circular pBluescript vector was digested by SmaI restriction enzyme (Fermentas) in reaction conditions in Table 3.4 for future ligation reactions as followed:

Table 3.4. Reaction condions for pBluescript digestion by SmaI

Reactants	Quantities
Up H ₂ O	13 µl
pBluescript DNA isolation	30 µl
Tango Buffer (10X)	5 µl
SmaI Enzyme (10 U/µl)	2 µl
TOTAL VOLUME	50 µl

3.4.3 Insertion of *Lb. gasseri* *bsh* gene into pBluescript vector

The *Lb. gasseri* *bsh* DNA and pBluescript vector DNA were ligated at 22°C for one hour using the T4 DNA ligase enzyme (Fermentas) and its appropriate buffer (Figure 3.1 and Table 3.5). The new construct was designated as pNMB1. PEG (Polyethylene glycol) (Fermentas) was also added to the reaction to improve the ligation efficiency. The exact amount of vector and insert DNA to be used in this ligation reaction was determined by the following formula:

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

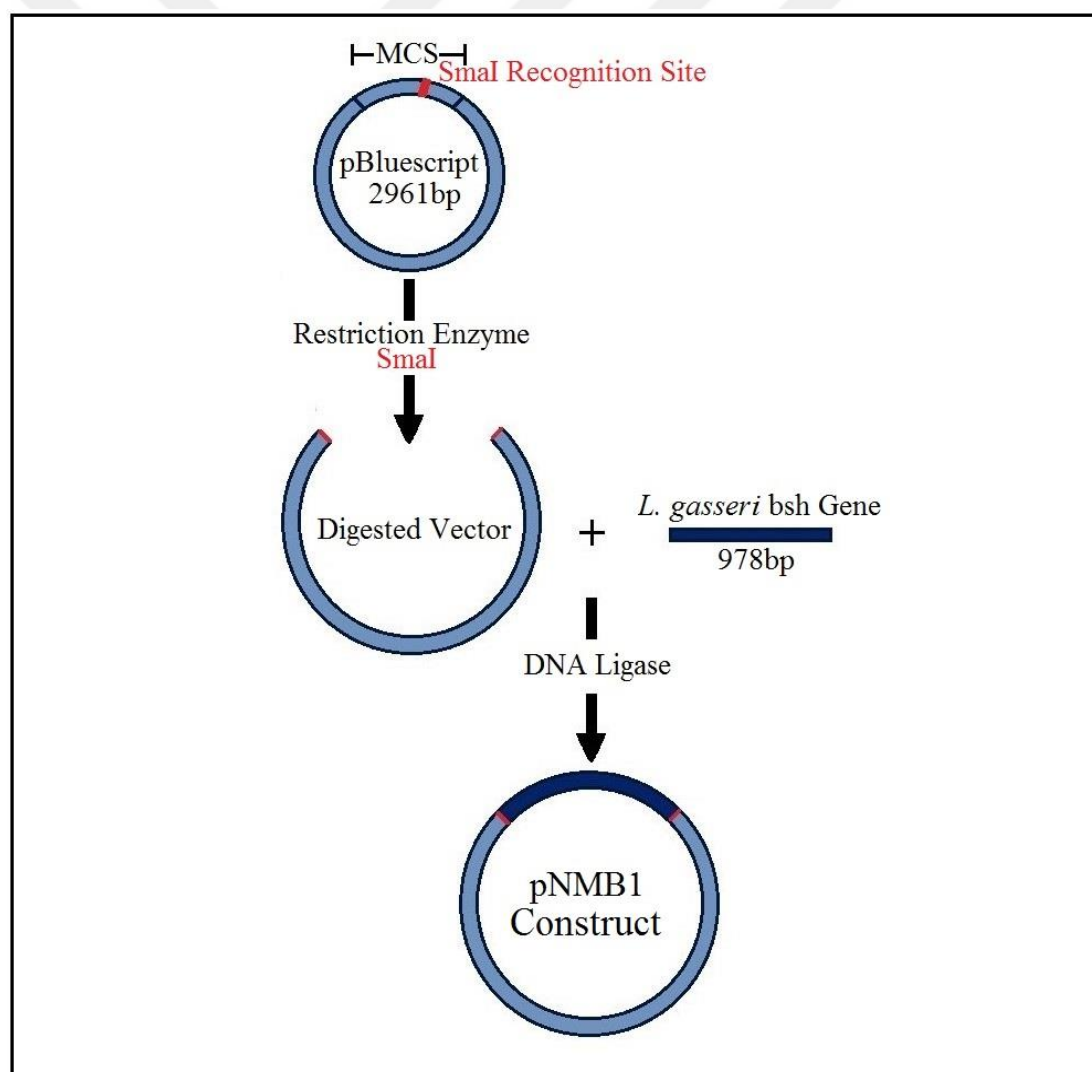


Figure 3.1. Preparation of pNMB1 Construct.

Table 3.5. Ligation of *bsh* DNA to pBluescript vector

Reactants	Quantities
Up H ₂ O	10 µl
pBluescript Vector	1 µl
<i>Lb. gasseri bsh</i> DNA	4 µl
PEG (Polyethylene Glycol) (50% w/v)	2 µl
T4 Ligase Buffer (10X)	2 µl
T4 DNA Ligase Enzyme (5 U/µl)	1 µl
TOTAL VOLUME	20 µl

3.5 Preparation of the XL1-Blue and BLR(DE3) Competent cells

The XL1-Blue and BLR(DE3) competent cells to be used in the transformation reactions were obtained through a two days process. First, a colony of XL1-Blue and BLR(DE3) was inoculated in a 3ml LB medium each and incubated overnight at 37°C and 175rpm for nearly 14-16 hours. Then 1% of this culture was transferred to a 50 ml LB and re-incubated at 37°C at 175rpm for 2-2.5 hours until the optic density (OD) of the culture at 600nm wavelength reached 0.04 - 0.06. Once the desired OD, 40 ml of the culture was separated into 2 sterile falcon tubes of 20ml each. They were centrifuged at 4°C and 4000 rpm for 5 minutes. The pellets were re-suspended in 10 ml of CaCl₂ (100mM, pH: 7.0) and centrifuged at 4°C and 4000rpm for 10 minutes. The newly obtained pellets were re-suspended in 1 ml of CaCl₂ containing 10% glycerol and allocated into 100 µl of culture in different eppendorf tubes and stored at -80°C.

3.6 Transformation of pNMB into *E.coli* XL1-Blue strains

10 µl of the ligation reaction product was transferred into 100 µl of XL1-Blue competent cells. The sample has undergone a heat shock treatment of 45 minutes of incubation on ice then 5 minutes incubation at 37°C and finally 5 minutes incubation on ice. Then, 1 ml of LB was added to the sample and incubated at 37°C and 175

rpm for 1 hour. Later, the sample was centrifuged at 6000 rpm for 3 minutes and the pellet was re-suspended in 100 µl LB. The spread plate technique was used to inoculate a LB agar plate containing IPTG (Isopropyl β-D-1-thiogalactopyranoside), Xgal (5 bromo 4 chloro 3 indolyl β D galactopyranoside) and ampicillin (25mg/ml). The plate was incubated overnight at 37°C and 175rpm for nearly 16-18 hours (Davis et al., 1988). Four random white colonies were selected from transformant LB plates and grown in an overnight culture of 5 ml LB with ampicillin. Then, once the pellet of that liquid culture was collected, we proceeded to the DNA isolation using the protocol prescribed on the Vivantis plasmid DNA Isolation Kit (California, United States).

3.7 DNA Sequencing of *Lb. gasseri* bsh gene

The DNA isolated from XL1-Blue/pNMB1 transformant was sequenced by Macrogen (Seoul, Korea) using the universal primers that are M13/pUC Forward (5'-GTAAAACGACGGCCAGT-3' with 17bp) and M13/pUC Reverse (5'-CAGGAAACAGCTATGAC-3' with 17bp).

3.8 Stock Preparation

Once we know that our insert is in the right orientation, we proceed to start four liquid cultures from the cultured plates of the sample that was sent for sequencing: two for stock storage and two for DNA isolation. After the cultures were incubated overnight at 37°C and 175rpm for nearly 16-18 hours, the liquid cultures were retrieved and the ones for stock preparation were transferred into glass tube to be centrifuged at 25°C at 5000rpm for 4 minutes. Then, each pellet was re-suspended in 4 ml of LB containing 30% glycerol and allocated into 2 different cryogenic tubes and stored at -80°C.

3.9 Preparation of pNMB2 Construct

3.9.1 pET22b Plasmid DNA Isolation

Since pET22b is a low copy plasmid, it was inoculated into a 10 ml of LB containing Ampicillin (25mg/ml) and incubated overnight at 37°C and 175rpm for nearly 14-16 hours. The pellet of this liquid culture was then collected by first centrifuging at 4000 rpm and 25°C for 4 minutes and then at 6000 rpm for 3 minutes after re-suspending the pellet in 1 ml of LB (Luria-Broth) medium. Using the pellet obtained, we proceeded to the plasmid DNA isolation using the protocol prescribed on the Vivantis plasmid DNA Isolation Kit (California, United States).

3.9.2 Digestion of pNMB1 and pET22b DNAs by NotI/SalI Restriction Enzymes

In order to retrieve our *Lb. gasseri bsh* gene stored in the XL1-blue/ pNMB1 transformant and to later ligate it into the pET22b plasmid, transformant and plasmid DNAs were digested by NotI/SalI restriction enzymes (Thermo Scientific) at 37°C for 2 hours (Table 6 and 7).

The digested DNA samples were separated by agarose gel electrophoresis and our desired *Lb. gasseri bsh* gene and the pET22b vector were purified from the gel using the Thermo Scientific GeneJET Gel Extraction Kit (Massachusetts, United States) and its instructions.

Table 3.6. Digestion of pNMB1 DNA by NotI and SalI restriction enzyme

Reactants	Quantities
Up H ₂ O	18 µl
pNMB1 DNA	40 µl
Fast Digest Buffer	7 µl
NotI Enzyme	2.5 µl
SalI Enzyme	2.5 µl
TOTAL VOLUME	70 µl

Table 3.7. Digestion of pET22b DNA by NotI and SalI restriction enzyme

Reactants	Quantities
Up H ₂ O	20 µl
pET22b	30 µl
Fast Digest Buffer	6 µl
NotI Enzyme	2 µl
SalI Enzyme	2 µl
TOTAL VOLUME	60 µl

3.9.3 Insertion of *Lb. gasseri bsh* gene into pET22b Vector

The *Lb. gasseri bsh* DNA was inserted into pET22b expression vector DNA at 22°C for one hour using the T4 DNA ligase enzyme and its appropriate buffer (Figure 3.2 and Table 3.8). The new construct was designated as pNMB2.

Table 3.8. Ligation of *bsh* gene and pET22b vector DNA

Reactants	Quantities
Up H ₂ O	13.75 µl
pET22b Vector	2 µl
<i>Lb. gasseri bsh</i> DNA	1.25 µl
T4 Ligase Buffer (10X)	2 µl
T4 DNA Ligase Enzyme (5 U/µl)	1 µl
TOTAL VOLUME	20 µl

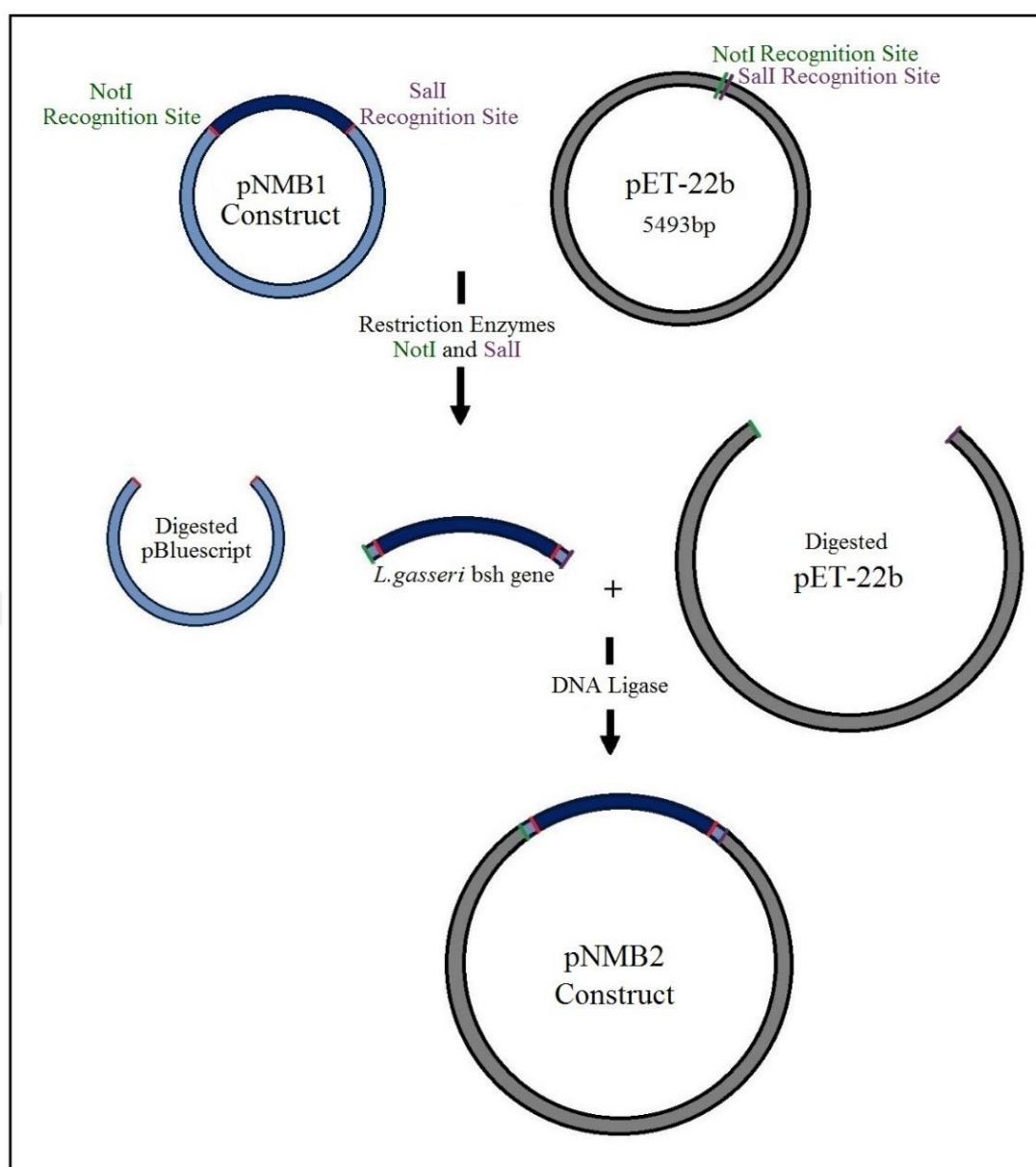


Figure 3.2. Preparation of pNMB2 Construct

3.10 Transformation of pNMB2 into *E.coli* BLR(DE3) Strains

10 μ l of the ligation reaction product was transferred into 100 μ l of BLR(DE3) competent cells. The sample has undergone a heat shock treatment of 45 minutes of incubation on ice then 5 minutes incubation at 37°C and finally 5 minutes incubation on ice. Then, 1 ml of LB was added to the sample and incubated at 37°C and 175 rpm for 1 hour. Later, the sample was centrifuged at 6000 rpm for 3 minutes

and the pellet was re-suspended in 100 µl LB. The spread plate technique was used to inoculate a LB Agar plate containing Ampicillin (25mg/ml). The plate was incubated overnight at 37°C and 175rpm for nearly 16-18 hours. From the overnight plate, 4 random colonies were selected and grown in an overnight culture of 10 ml LB as well as on an Ampicillin containing LB Agar plate. Then once the pellet of that liquid culture was collected, we proceeded to the DNA isolation using the protocol prescribed on the Vivantis plasmid DNA Isolation Kit (California, United States).

3.10.1 Detection of the Transformants that had Desired Insert

Once we determined which one those 4 cultures have taken an insert, the DNAs were isolated from four transformants were digested with NotI/SalI restriction enzymes (Fermentas) in the reaction condition in Table 3.9.

Table 3.9. Digestion Condition of pNMB2 DNA with NotI/SmaI Restriction Enzymes

Reactants	Quantities
Up H ₂ O	3.5 µl
pNMB2 DNA	5 µl
Fast Digest Buffer	1 µl
NotI Enzyme	0.25 µl
SalI Enzyme	0.25 µl
TOTAL VOLUME	10 µl

Once we now know that our pNMB2 transformant has taken only one insert in the right orientation, we proceed to start 2 liquid cultures from the cultured plates of the sample that were used for stock storage. The cultures were incubated overnight at 37°C and 175rpm for nearly 16-18 hours. The liquid cultures were then retrieved and transferred into glass tube to be centrifuged at 25°C and 5000rpm for 4 minutes. Then, each pellet was re-suspended in 4 ml of LB containing 30% glycerol and allocated into 2 different cryogenic tubes and stored at -80°C.

3.11 Determination of the BSH Enzyme Activity by Ninhydrin Assay

3.11.1 Preparation of Cell Extract

The newly obtained transformant with our desired *bsh* gene was inoculated in a 10 ml LB containing Ampicillin and was incubated overnight at 37°C and 175 rpm for nearly 16-18 hours. Five ml of this overnight culture was then transferred into a 250 ml LB supplemented with Ampicillin and 4% Glycerol. This newly prepared culture was then incubated at 37°C and 175 rpm until the OD600 values of culture reached 0.45 - 0.6. When these OD600 values of the culture finally reached 0.5, 0.3mM of Isopropyl β -D-1-thiogalactopyranoside (IPTG) was added and the culture was re-incubated at 37°C and 175 rpm until the OD600 values of the culture reached 1.5 - 3.0. Once those values were observed 100 ml of culture was transferred into 250 ml falcon tubes and centrifuged at 4°C and 8000g for 15 minutes. The pellets were re-suspended each with 2 ml of binding buffer at pH: 7.8 then transferred all into a single tube. Then, lysozymes were added to a final concentration of 1mg/ml and incubated the mixture on a rocking platform for 10 minutes at 4°C. After the incubation, we added Tween20 (Polyethylene glycol sorbitan monolaurate), DNase and RNase to the tube to a final concentration of 1%, 5 μ g/ml and 5 μ g/ml respectively and continued the incubation on the rocking platform for another 10 min at 4°C. Then, to remove the insoluble debris by centrifugation at 13520g and 4°C for 30 minutes. The supernatant was collected through a 0.45 μ m filter into a fresh tube. The protein concentration of the cell extract was then measured by the Lowry method with Bovine Serum Albumin (BSA) as a standard protein.

3.11.2 The Ninhydrin Assay

BSH enzyme activity was determined by Ninhydrin assay. For this reaction, 10 μ l of the cell extract obtained was mixed with 100 μ l of 0.1M sodium phosphate buffer, 50 μ l of 40 mM Dithiothreitol (DTT) and 50 μ l of 40 mM of one of the six different bile salts to be tested: glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA). The final

mixture was incubated at 37°C for 30 minutes. Then, 210 µl of Trichloroacetic acid (TCA) was added (for a ratio of 1:1) and the mixture was centrifuged at 14000 rpm for 15 minutes. 20 µl of the supernatant obtained was transferred into a glass tube containing 80 µl of UP water and 1.9 ml of ninhydrin reagent (0.5 ml of 1% Ninhydrin with 1.2 ml glycerol in 0.2ml of 0.5M sodium citrate buffer) and boiled for 15 minutes. After cooling the tubes, the absorbance of the samples were measured at 570 nm with glycine as a standard. The BSH activity was expressed as 1µmol of amino acids liberated from the substrate per minute per mg of BSH.

3.11.3 Detection of the Optimum pH and Temperature of the BSH

To detect the optimum temperature of BSH enzyme, the Ninhydrin assay was carried out again as described above but at various temperatures ranging from 25°C to 65°C. And likewise, the effects of the pH on the BSH enzyme activity was also determined by performing the Ninhydrin assay at pH values ranging from 2.5 to 8.0 using various 0.1 M sodium phosphate buffer of different pH (2.5 to 8.0).

Both pH and temperature assays were performed using the standard procedure with glycodeoxycholic acid (GDCA) as the substrate. All experiments were run in duplicate.

3.11.4 Partially Purification of the BSH Enzyme

In order to purify the BSH protein, the samples were grown the same way they were in order to obtain a cell extract. But once the supernatant was filtered through a 0.45µm filter, their absorbance were measured at both 600nm and 280nm. Then, this supernatant containing His-tagged BSH protein was purified using the ÄKTAprime plus™ chromatography system with a Nickel chelated column for an affinity purification.

The purified sample was then mixed with a protein loading dye and β-mercaptoethanol and incubated at 37°C for 10 minutes. The mixture was then loaded

on a 12% polyacrylamide gel (PAGE) and electrophoresed for nearly 3 hours at 120 volt according to Laemmli protocol (Laemmli, 1970). The gel was then soaked in tank containing Coomassie Brilliant Blue G250 dye and incubated at room temperature on a rocking platform for 20-30 minutes. Once the dye was removed and the gel soaked in a de-staining solution overnight to efficiently remove any trace of stain left, the gel was then visualised.



4.1 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 DNA Amplification by PCR

The PCR products of the DNA amplification of the bile salt hydrolase gene of the *Lb. gasseri* ATCC 33323 strain were loaded on a 0.8% agarose gel and electrophoresed at 75 volt for one hour. PCR amplicon (1.0 kb) was then visualized after staining of agarose gel with Ethidium bromide on UV-transilluminator (Figure 4.1).

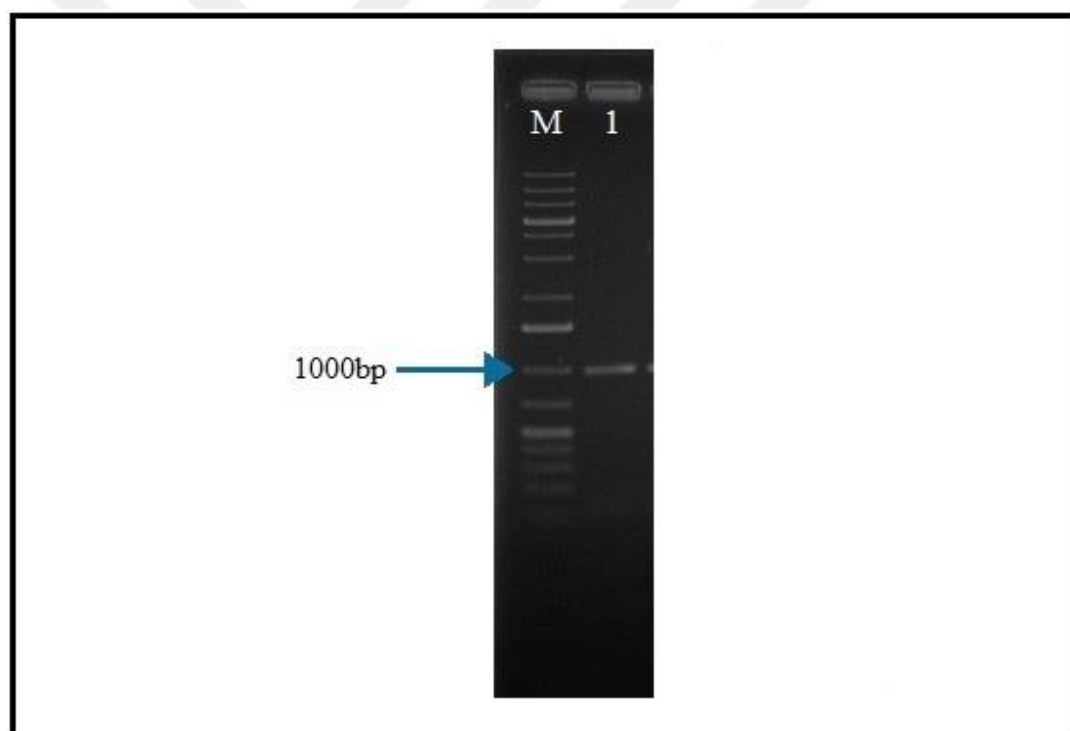


Figure 4.1. The DNA amplification of the bile salt hydrolase gene of *Lactobacillus gasseri* ATCC 33323 strain by PCR. M represents the marker while line 1 is the PCR amplicon, unpurified *bsh* DNA.

4.1.2 Purification of the PCR Products

The purified 1kbp PCR amplicons were loaded and electrophoresed on a 1% agarose gel (Figure 4.2). They were purified using the Thermo Scientific GeneJET Gel Extraction Kit according to its manual (Massachusetts, USA).

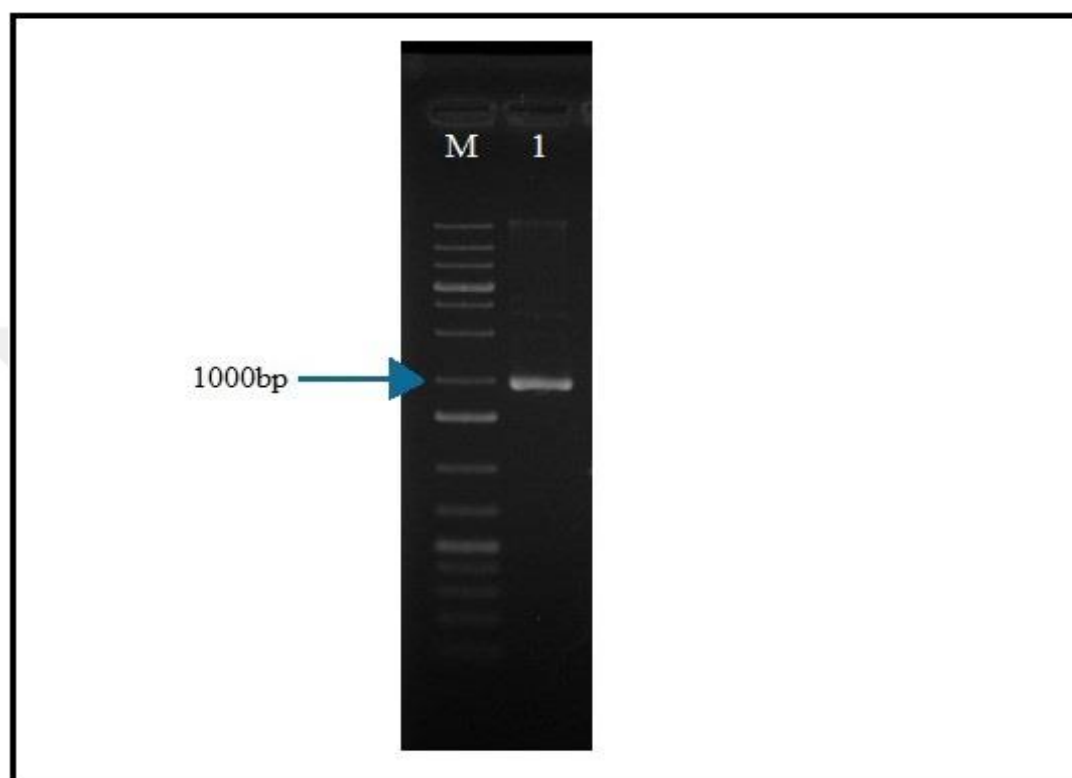


Figure 4.2. Purification of the PCR products. M represents the marker while line 1 is the purified PCR product, *bsh* DNA.

4.1.3 Preparation of pNMB1 Construct

4.1.3.1 DNA Isolation of the pBluescript Vector

The pBluescript DNA isolation was conducted using the protocol prescribed on the Vivantis plasmid DNA Isolation Kit (California, United States). The DNA isolation product was loaded on a 0.8% agarose gel and visualized after staining with Ethidium bromide on UV-transilluminator (Figure 4.3).

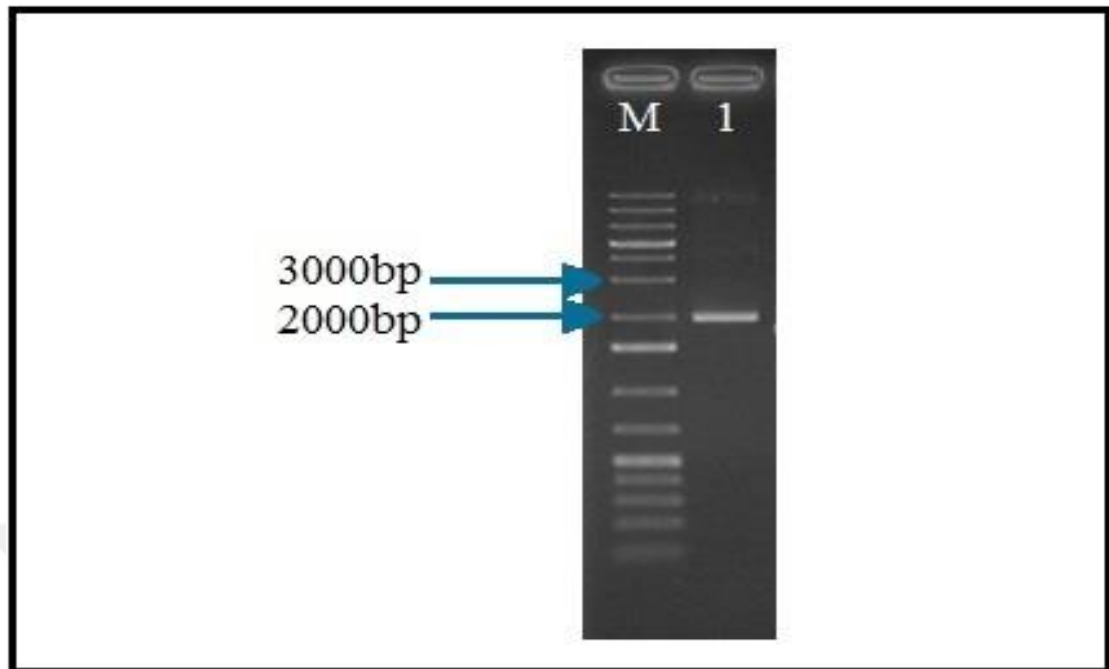


Figure 4.3. DNA isolation of the pBluescript Vector.
M represents the marker and line 1 is circular pBluescript vector DNA

4.1.3.2 Digestion of pBluescript DNA by SmaI

Since the PCR products are blunt ended, we need to digest the pBluescript vector by SmaI restriction enzyme for future ligation. The digestion rendered pBluescript linear resulting in a DNA band slightly equals to 2.9 kbp (Figure 4.4).

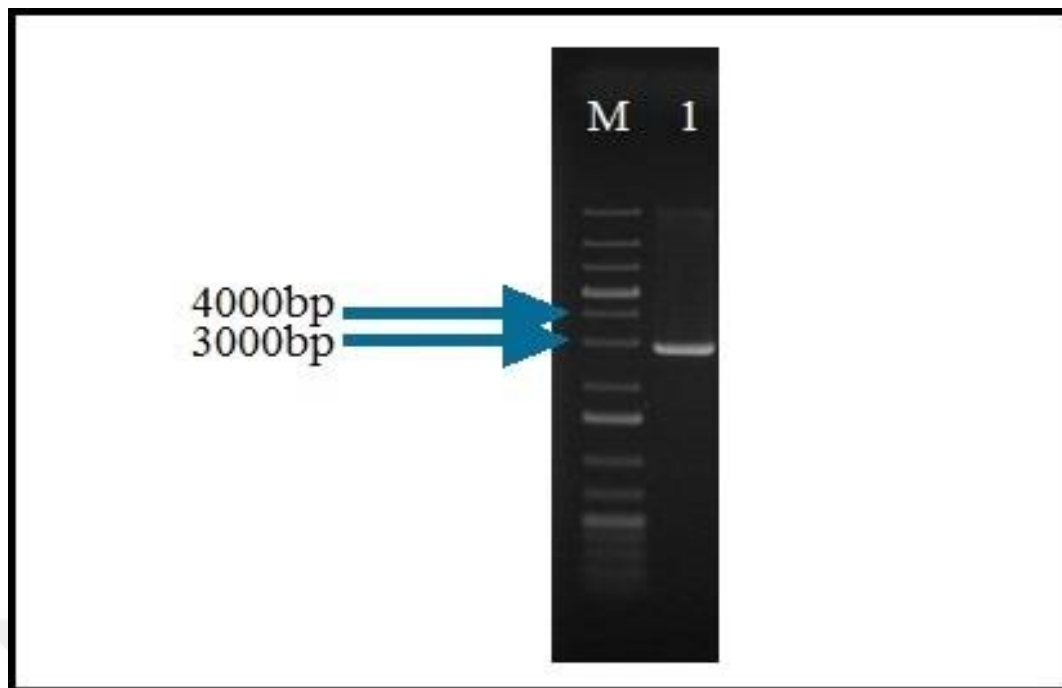


Figure 4.4. Digestion of pBluescript by SmaI restriction enzyme. M represents the marker and line 1 is the linear pBluescript DNA digested by SmaI restriction enzyme

4.1.4 Transformation of pBluescript-*bsh* DNAs into *E. coli* XL1-Blue Strains

Once the ligation reaction was completed, 10 μ l of the reaction product was transferred into 100 μ l of XL1-Blue competent cells treated with CaCl_2 . Four random white colonies from the plate containing IPTG, Xgal and Ampicillin were selected and grown overnight for DNA isolation. The DNA isolation products were loaded on a 0.8% agarose gel to determine which of those randomly selected colonies had taken an insert (Figure 4.5). This agarose gel showed that all construct DNAs isolated from four different transformants had an insert *bsh* DNA except the second construct DNA loaded in Figure 4.5 lane 3.

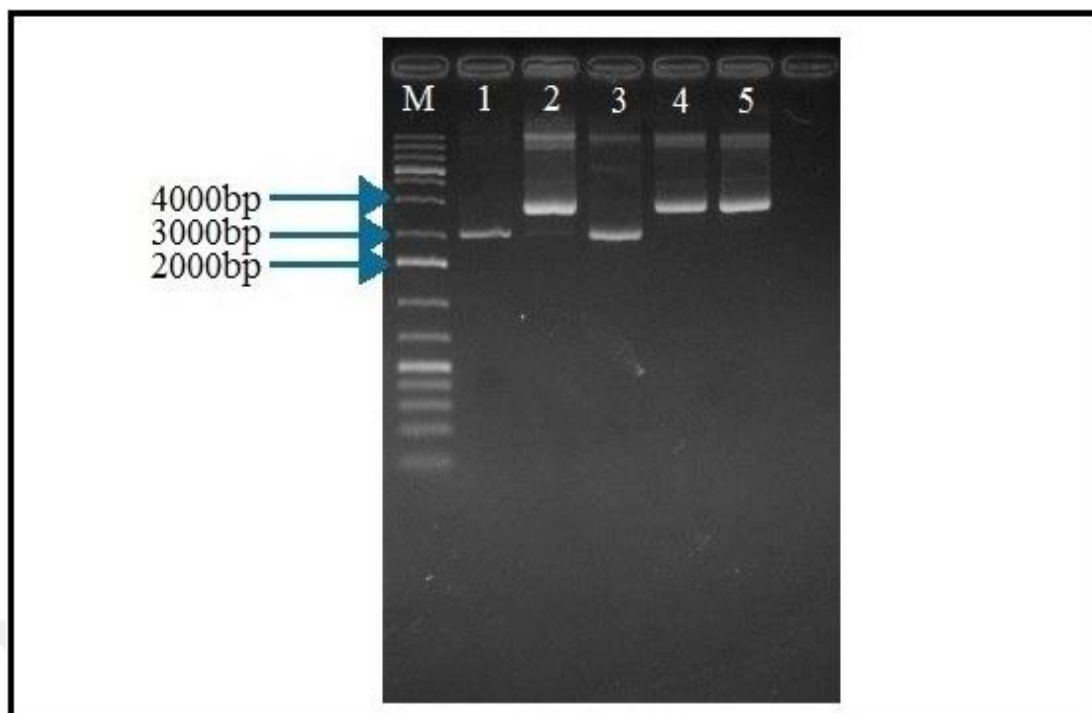


Figure 4.5. Transformation of pBluescript-*bsh* into *E. coli* XL1-Blue strains. M represents the Marker; line 1: Linear pBluescript DNA; line 2: pNMB1A DNA; line 3: pNMB1B DNA; line 4: pNMB1C DNA; line 5: pNMB1D DNA.

4.1.5 DNA Sequencing of *bsh* Gene

The *bsh* gene on pNMB1 construct was sequenced by Macrogen (Seoul, Korea) using the universal primers, M13/pUC Forward (5'-GTAAAACGACGGCCAGT-3') and M13/pUC Reverse (5'-CAGGAAACAGCTATGAC-3'). Using the SIB (Swiss Institute of Bioinformatics) ExPASy sequence translate tool (<http://web.expasy.org/translate/>), the following amino acid sequence was obtained (Figure 4.6).

```

atgtgtacatcaatTTTtatacagTcccaaagaccactatTTTtggtagaaacttagattat
M C T S I L Y S P K D H Y F G R N L D Y
gaaattgcttatggtcaaaaagtggTTattacaccaagaaattatgaatttaagtttgcc
E I A Y G Q K V V I T P R N Y E F K F A
aatttaccagctgaaaaatcacattatgcaatgattggcatagctgctgtagcaaataat
N L P A E K S H Y A M I G I A A V A N N
acccactatattgCGatgCGattaatgaaaagggTTTgggagtagctggtTTtaagtttt
T P L Y C D A I N E K G L G V A G L S F
gctggacaaggaaagtatTTTccagtagtagaagataagaaaaacattgcttcatttgaa
A G Q G K Y F P V V E D K K N I A S F E
TTTatttcttatataattagctacttatgaaactgttgatcaggTTaaagaaaatttgact
F I S Y I L A T Y E T V D Q V K E N L T
gatgttaatatTTTctgatgtgagTTTtctaaaaatacaccagcttcagaacttcattgg
D V N I S D V S F S K N T P A S E L H W
ctagtaggagataagaccggttaagagtatcgTTgttgagtcagatgaaaaaggtttacat
L V G D K T G K S I V V E S D E K G L H
gtttatgataatccagTTaatgctTTgacgaacgcaccgTTattcccgCagcagTTaact
V Y D N P V N A L T N A P L F P Q Q L T
aatttggcaaattatgctgcagTTgttcccgGCCaacctaataatgattTTTtacctgga
N L A N Y A A V V P G Q P N N D F L P G
gttgatcttaagatgtatagccgcagcttaggaactcatcatttacctggtggaatggat
V D L K M Y S R S L G T H H L P G G M D
tcagaatcacgTTTgttaaagtatgTTTgctTTgaatcatgcaccaaagatagtgat
S E S R F V K V C F A L N H A P K D S D
gaggtagaaagtgttactaattTTTccatattTTTgcagTctgTTgaacaggtaaaaggg
E V E S V T N F F H I L Q S V E Q V K G
atggatgaagTTggtcctaataTTTtgaatacaccatgtatactagTTgtatgaatctt
M D E V G P N I F E Y T M Y T S C M N L
gaaaagggTatTTTgtacttcaattgTTatgatgacagtagaattagtgctgtagacatg
E K G I L Y F N C Y D D S R I S A V D M
aacaaggaagatttgagTTcatcagacttgatcgTgtTTgacttgTTcaagaaacaggat
N K E D L S S S D L I V F D L F K K Q D
attagTTTcataaattaa
I S F I N -

```

Figure 4.6. Amino acid sequence of the *Lb. gasseri* *bsh* gene obtained by using the SIB (Swiss Institute of Bioinformatics) ExPASy sequence translate tool (<http://web.expasy.org/translate/>).

The fragments contained a single open reading frame (ORF) of 5 nucleotides flanked by a methionine start codon (ATG) and transitional termination codon (TAA) and encoded 325 amino acid protein with theoretical molecular mass of 36269.92 Da and pI of 4.85 respectively, as predicted by the EXPASY tool available at (<http://swissmodel.expasy.org/> Arnold et al., 2006). Obtained amino acid residues of BSH enzyme from *Lb. gasseri* ATCC 33323 strain were aligned with the amino acid sequences of BSH enzymes belonging to different *Lactobacillus* species such as

Lactobacillus salivarius (GenBank: ACL98203.1), *Lactobacillus plantarum* (GenBank: CCC80500.1), *Clostridium perfringens* (GenBank: EDS79409.1), *Lactobacillus gasseri* (GenBank: ABJ60345.1), *Lactobacillus johnsonii* (GenBank: EGP12391.1), *Lactobacillus acidophilus* (GenBank: ACL98176.1), *Lactobacillus reuteri* (GenBank: ACH81023.1), *Lactobacillus crispatus* (GenBank: WP_023488404.1), *Lactobacillus rhamnosus* (GenBank: AEP69108.1), *Lactobacillus brevis* (GenBank: KIP00124.1), and *Lysinibacillus sphaericus* (GenBank: AMO33264.1) obtained from NCBI GenBank using Jalview 2.8.0b1 program (Figure 4.7). The alignment showed that C2, R16, D19, N170 and R223 amino acids are fully conserved and amino acid motifs around these amino acids are also conserved (Figure 4.7).



			C2	R16 D19		
<i>reuteri</i>	1	--MCTSVIYTA--GYYFGRNLDLEVNML-----GQEVVITPRNK--TLEFREMPENLEHHYAIIGMSIVRVD	60			
<i>plantarum</i>	1	--MCTAITYQS--YNNYFGRNFDYEISY-----NEMVTITPRKY--PLVFRKVENLDHHYAIIGITADVDS	60			
<i>perfringens</i>	1	--MCTAITYTT--KNNYFGRNFDYEISY-----NEKVVITPRNF--PFKFRKVENLDHHYAIIGVATVVD	60			
<i>salivarius</i>	1	--MCTAITLNS--NNNYFGRNLDLDFS-----GEQVITTRAEY--EFKFRKEKAIKNNHSLIGVGIVAND	60			
<i>johnsonii</i>	1	--MCTSIYSP--KNNYFGRNLDYEIAY-----GQKVITPRNY--QLNRYHLPTQDTHYAMIGSVVAND	60			
<i>acidophilus</i>	1	--MCTSIYSP--KNNYFGRNLDYEIAY-----GQKVITPRNY--EFKYREMPSSQMHYAFIGSVVND	60			
<i>crispatus</i>	1	--MCTSIYSP--QDHYFGRNLDLEITF-----GQVVVITPRNY--VFNFRRKMPKMHYAMVGLADAGD	60			
<i>pNMB</i>	1	--MCTSIYSP--KNNYFGRNLDYEIAY-----GQKVITPRNY--EFKFAHLPAEKSHYAMIGLAAVANN	60			
<i>brevis</i>	1	--MCTSLTYENSRGDHLARTMDFAFEL-----GGQPIFMERQ--QEIDGAGTFTTKYGFIGAGRNLSH	61			
<i>sphaericus</i>	1	MLGCSLSIHTTDEKSLARTMDFTMEP-----DSKVIIVPRNYGIRLLEKD NKVINKPFAFVGMSGSTD	66			
<i>rhamnosus</i>	1	--MCSMTIKSLQGIIFWGRMDYNTSFHESFESAGGVPGKIVSLAN---QTLPAQTATWTKYAAVGVGV--DQ	68			
<i>reuteri</i>	61	YELYFDGVNEKGVGMAGLNFDGPAHFFVQEGKDNIAFP---ELVFIILAAASSVAAKLLSNANIANINFS-	130			
<i>plantarum</i>	61	YELYDDAMNEKGLCIAGLNFAGYADKKYDA-DKVNITFP---ELIPWLLGQSSVREYVKKNIQKLNIVNINFS-	130			
<i>perfringens</i>	61	YELYDDATNEKGLSIAGLNFEGMAYGEEVK-GKLNIAFP---EFIPWLLGQCATIKDKVKLLQINFEVNIINFS-	130			
<i>salivarius</i>	61	YELYFDAINEDGLGMAGLNFEGMAYSDALENDKDNITFP---EFIPWLLGQCSVNEARNIVEKINILNLSFS-	131			
<i>johnsonii</i>	61	YELYCDAINKGLGIAGLNFTGPGKFAVDE-SKKNVTSF---ELIPYLLSSCETIEDVKLLSETNITDESFS-	130			
<i>acidophilus</i>	61	YELYCDAINKGLGIAGLNFTGPGKFAVDE-SKKNVTSF---ELIPYLLSSCETIEDVKLLSETNITDESFS-	130			
<i>crispatus</i>	61	YELYFDAAINEKGLGMAGLNYPDNATYDEVA-NKDNIAFP---EFIPWLLGQCATVADAKVLLKINIVNINFS-	130			
<i>pNMB</i>	61	TELYCDAINKGLGVAGLSFAGQKFFPVVE-DKKNIASF---EFISYILATYETDQVKENLTQVNIISDVFS-	130			
<i>brevis</i>	62	Y-IFVDGVNEFSLGAAALYFERYAKQQSAPADKLAIAFP---DVVAWALGNAQSVADLRRELVKHIQLDVPVS-	131			
<i>sphaericus</i>	67	SELYDGVNEKGLGMAGLYYATPATMADEPKESGRINPV---YVVSQVLGSCATINDVVEKLAFTLLNEPNE-	137			
<i>rhamnosus</i>	69	SVALLFDGVNSELGAGD-LQVLVRCWSAESAELAPRLKEIKGEFVTLALITTKNVAAPVRAIASQYGLLDEPFOF	142			
<i>reuteri</i>	131	-DKLQAAPLHMIADKTGASVTVESTAKGLNVDNPGVLTNNPEFFPQQLNLSNYSRVSAPAN-PAVFAAPNVDL	203			
<i>plantarum</i>	131	-EQLPLSPLHMLVADKQ-ESIVIESVKEGLKIYDNPVGLTNNPEFFYQLFNLNNTYRALSNT-PCNSFSEKVDL	202			
<i>perfringens</i>	131	-EKLPLSPLHMLVADKN-ESIVIEMLKTGLKIYDNPVGLTNNPEFFYQLFNLNNTYRALSNT-PCNSFSEKVDL	202			
<i>salivarius</i>	132	-EQLPLAGLHMLIAGRE-KSIVVEVTKSGVHVYDNPVGLTNNPEFFYQMYNKNYRNLISIST-PCNTFSDSVDL	203			
<i>johnsonii</i>	131	-KDLFVTTLHMLMGKSGKSIVIESTETGLHVYDNPVGLTNNPEFFYQMYNKNYRNLISIST-PCNTFSDSVDL	203			
<i>acidophilus</i>	131	-ANYPADLHMLSLDKAKSIAVESTNSGLHIYDNPVGLTNNPEFFDQLIKLSDYADVTPHN-PKNTLVPNVDL	203			
<i>crispatus</i>	131	-DKMQASPLHMLIADKTGVSIVVETDKDGMHVYDNPVGLTNNPEFFYQMYNKNYRNLISIST-PCNTFSDSVDL	203			
<i>pNMB</i>	131	-KNTFASLHMLVGDKTGKSIVVESDEKGLHVYDNPVGLTNNPEFFDQLIKLSDYADVTPHN-PKNTLVPNVDL	203			
<i>brevis</i>	132	-LLGLTTPMHFIPTPTGDTAVLHATSADLHLIDDEVGVMANSPELSWHLLQNLSTYGLTQAARERLQDLRLG-YQL	204			
<i>sphaericus</i>	138	-ILGFAPPLHYTFTPTSTGESIVIEPDKTGITHIRPTIGVMTNSPGYEWHTQNLRAIIGVTPNP-PEDITMGEPFL	210			
<i>rhamnosus</i>	143	GGQGVKIPLHYTFVDPGAGLVIELTGHSAFLYDSVGAMTNSPEYGWHTTNLRNPVSLNDRDYREGADLGDQHL	217			
<i>reuteri</i>	204	PV--YSRGLGTHFLPGGMDSESPFVKATFTKMHAPVGNSEVENITNYFHLQSVQEQKGLDEVAPNT--FEYTYI	274			
<i>plantarum</i>	203	DS--YSRGMGGLGLPGDLSSMSRPFVRAAFTKLSLSMQTESGSVSQFFHILGVSVEQKGLCEVTDGK--YEYTYI	273			
<i>perfringens</i>	203	NS--YSRGMGGLGLPGDLSSMSRPFVRAAFTKLSLSMQTESGSVSQFFHILGVSVEQKGLCEVTDGK--YEYTYI	273			
<i>salivarius</i>	204	KV--DGTGFGGIGLPGDVSPESPVRVAFSKLSSKGTVEEDITQFFHILGTVGEQIKGVNKTSESG--BEYTYI	274			
<i>johnsonii</i>	204	NL--YSRGLGTHHLPGGTDSNSRPFKASVFLAHSKPGKNDENVNINFFHILHSVEQAKGTDVEVDNV--FEYTYI	274			
<i>acidophilus</i>	204	NL--YSRGLGTHHLPGGTDSNSRPFKASVFLAHSKPGKNDENVNINFFHILHSVEQAKGTDVEVDNV--FEYTYI	274			
<i>crispatus</i>	204	NG--YSRGLGSRNLPGGMDSESPFVRVAFKFNAPVKGSEENVNINFFHILHSVEQKGLDQVGPNS--FEYTYI	274			
<i>pNMB</i>	204	KM--YSRGLGTHHLPGGMDSESPFVKCFALNHAKDSDEHESVNTNFFHILQSVQEQKGMDEVGPNH--FEYTYI	274			
<i>brevis</i>	205	PT--QGPFTGALGLPGDYTPSPFVTVFNKHYSEAAADTPSTLTTLQHLIDGVTTIPKGVKLMADGT--SDYTCY	275			
<i>sphaericus</i>	211	TP--FGQAGGLGLPGDFTPSARFLRVAYWKYTTQQAQNETGVNLFHILSSVNIPKGVVMTKEGK--TDYTYI	281			
<i>rhamnosus</i>	218	EPIELGTGYGMFGLPGDYTPSPFVRAMFVSRNLDPFNSN-EGIRVLYNAFKTVLIPQGLGRDPKHSILTDYTCY	291			
<i>reuteri</i>	275	SDGSNLKKGIFYKYTYE NSQINAVDMHKK-----ILKHQ-----	309			
<i>plantarum</i>	274	SSCCDMDKGVYYRYTYD NSQINSVSLNHEHLDITTEL-ISK-PLRSEAYQYAVN-----	324			
<i>perfringens</i>	274	SSCCNIDRGYYKYTYE NSQITAIDMKNKALESSTL-PTY-NLINNQIINYLN-----	324			
<i>salivarius</i>	275	SNCYDLNKKLYTYTYE NRQIVAVTLNKK-DGNRI-VTY-PFERKQIINKLN-----	324			
<i>johnsonii</i>	275	SDCMNLDKGLYFTTYD NNQINAVDMNNEHLDITSDL-ITY-ELFKDQAIKFN-----	325			
<i>acidophilus</i>	275	TDGMNLDKGLYFTTYD NNQINAVDMHKAHLGSEDL-ICY-DLFFKQDI EYMH-----	325			
<i>crispatus</i>	275	SDGTNLDKGLYFTTYD NNQINAVDMHKAHLGSEDL-ICY-DLFFKQDI EYMH-----	325			
<i>pNMB</i>	275	TSCMNLEKGLIFNCTYDSRISAVDMNNEHLDITSDL-IVF-DLFFKQDISFIN-----	325			
<i>brevis</i>	276	RGYACLDPRAYTMEPYD NRELQGLRLTDMNDWDTPVEY-LLDHTPHVKHLN-----	327			
<i>sphaericus</i>	282	TATMCAQSKNYFPLYD NSRISAVSLMNEHLNGKEL-ITF-EWDRKQDIKQLNQANVMS-----	338			
<i>rhamnosus</i>	292	WSGYDLKRAIFVQ-----DANTLTMTTKTLDPNLTEVYDDLVKTEQFNQL-----	338			
			N170			
			R223			

Figure 4.7. BLAST analysis of the BSH enzyme amino acid sequences and multiple sequence alignment by the Jalview tool available at www.jalview.org The Barton Group., 20012. The fully conserved amino acids **C2**, **R16**, **D19**, **N170** and **R223** are shown in red.

	1	2	3	4	5	6	7	8	9	10	11
1	100.00	54.49	54.80	43.65	44.89	44.89	47.08	50.77	28.12	30.22	31.17
2	54.49	100.00	67.28	46.30	48.77	45.37	50.32	50.62	25.56	29.28	31.79
3	54.80	67.28	100.00	49.38	51.85	50.31	53.25	55.56	27.16	28.97	33.64
4	43.65	43.65	49.38	100.00	66.15	64.92	56.63	56.00	24.20	27.33	31.69
5	44.89	48.77	51.85	66.15	100.00	68.92	57.93	54.15	26.11	27.33	29.23
6	44.89	45.37	50.31	64.92	68.92	100.00	61.81	55.08	25.16	28.57	33.85
7	47.08	50.32	53.25	56.63	57.93	61.81	100.00	62.14	27.85	32.03	28.80
8	50.77	50.62	55.56	56.00	54.15	55.08	62.14	100.00	26.11	29.19	32.31
9	28.12	25.56	27.16	24.20	26.11	25.16	27.85	26.11	100.00	33.44	31.23
10	30.22	29.28	28.97	27.33	27.33	28.57	32.03	29.19	33.44	100.00	33.54
11	31.17	31.79	33.64	31.69	29.23	33.85	28.80	32.31	31.23	33.54	100.00

Figure 4.8. BLAST identity percentage analysis of the *Lb. gasseri* bsh DNA sequences and multiple other sequences by the Standard Nucleotide BLAST tool available at blast.ncbi.nlm.nih.gov. The Barton Group 2012. 1: *Lactobacillus salivarius*; 2: *Lactobacillus plantarum*; 3: *Clostridium perfringens*; 4: *Lactobacillus gasseri*; 5: *Lactobacillus johnsonii*; 6: *Lactobacillus acidophilus*; 7: *Lactobacillus reuteri*; 8: *Lactobacillus crispatus*; 9: *Lactobacillus ramnosus*; 10: *Lactobacillus brevis* and 11: *Lysinibacillus sphaericus*.

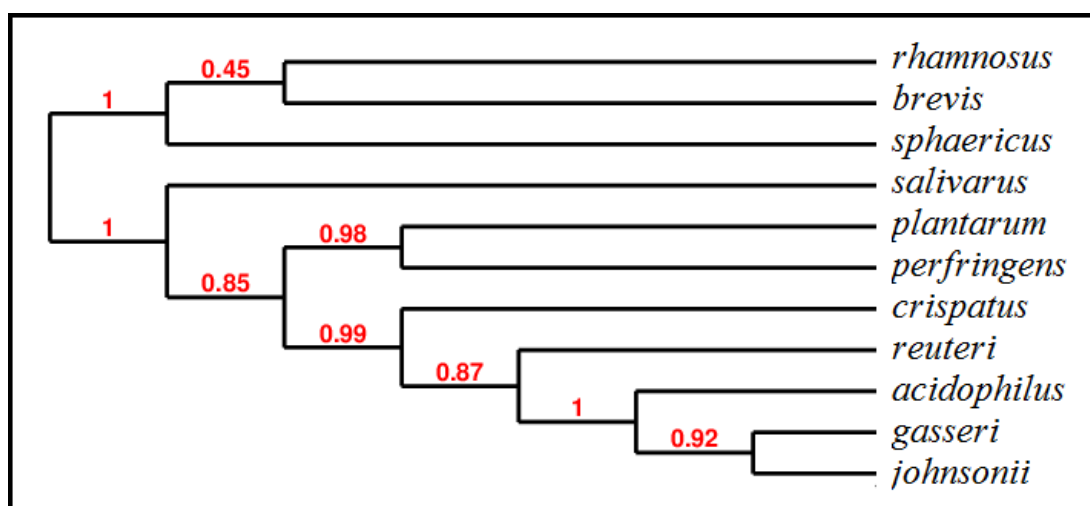


Figure 4.9. "One Click" Mode cladogram generated phylogenetic tree of *Lb. gasseri* bsh DNA sequences and multiple other sequences by MAB: Méthodes et algorithmes pour la bio informatique available at phylogeny.fr

The BLAST identity percentage analysis of the *bsh* nucleotide sequence of *Lb. gasseri* ATCC 33323 strain showed a highest sequence identity of 66.15% and 64.72% with the *bsh* of *Lb. johnsonii* (GenBank: EGP12391.1) and the *bsh* of *Lb. acidophilus* (GenBank: ACL98176.1) respectively. The lowest similarity was recorded with the *bsh* of *Lb. rhamnosus* (GenBank: AEP69108.1) with an identity percentage of 24.20% and *Lb. brevis* (GenBank: KIP00124.1) with an identity percentage of 27.33%.

4.1.6 Preparation of pNMB2 Construct

4.1.6.1 pET22b DNA Isolation

Using the protocol prescribed on the Vivantis plasmid DNA Isolation Kit (California, United States), we proceeded to the DNA isolation of the 5.5 kbp low copy plasmid pET22b (Figure 4.10).

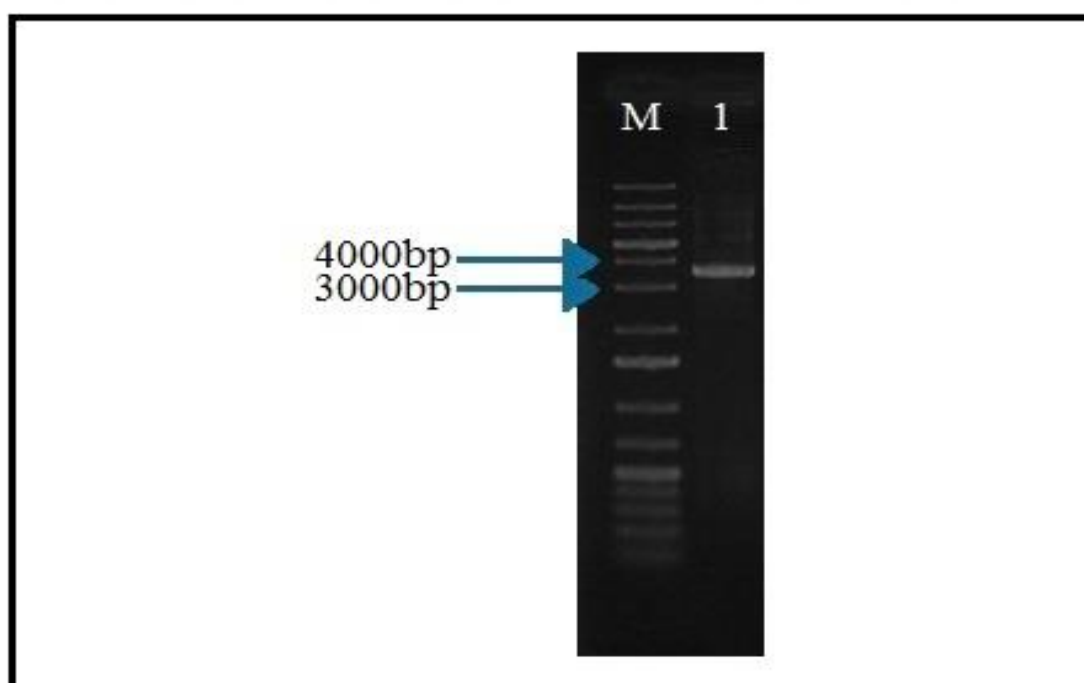


Figure 4.10. pET22b plasmid DNA Isolation. M represents the Marker and line 1 represents the isolated pET22b plasmid DNA.

4.1.6.2 Digestion of pNMB1 and pET22b DNAs by NotI and SalI Restriction Enzymes

In order to retrieve our *bsh* gene stored in the pNMB1 transformant, we proceeded to the digestion of said transformant and plasmid DNAs with NoI and SalI restriction enzymes (Thermo Scientific) at 37°C for 2 hours. The digestion products were then separated by agarose gel electrophoresis (Figure 4.11) and our desired *bsh* gene and the pET22b vector were purified from the 1% agarose gel (Figure 4.11).

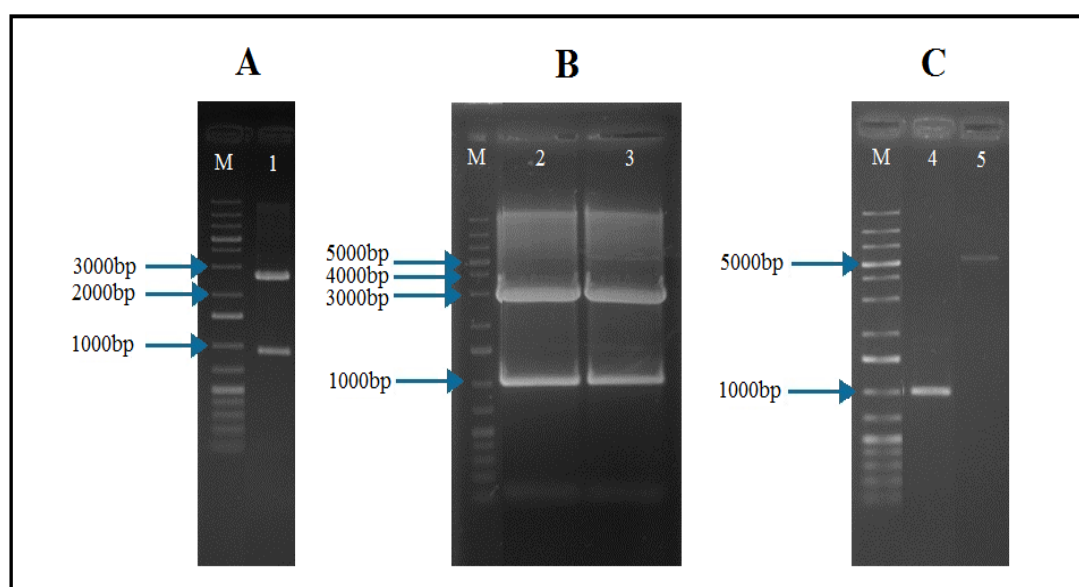


Figure 4.11. Digestion of pNMB1 construct and pET22b DNAs by NotI and SalI. M represents the marker; A-Line 1: Digestion of pNMB1 transformant by NotI and SalI; B-Line 2: Digestion of pNMB1 transformant by NotI and SalI before gel purification; B-Line 3: Digestion of pNMB1 transformant by NotI and SalI before gel purification; C-Line 4: Extraction of the *bsh* form the digested pNMB1 transformant; C-Line 5: Digestion of pET22b by NotI and SalI

4.1.7 Transformation of pNMB1 DNA into *E. coli* BLR(DE3) Strains

Once the insertion of *bsh* DNA into the pET22b expression vector was completed by ligation reaction, ligation reaction product was transferred into BLR(DE3) competent cells. Four random colonies were selected and grown overnight for DNA isolation. The DNA isolation products were loaded on a 0.8% agarose gel to determine which of those randomly selected colonies had taken an insert (Figure 4.12). Lanes 3 and 4 in Figure 4.12 indicated that these clones had *bsh* insert DNA.

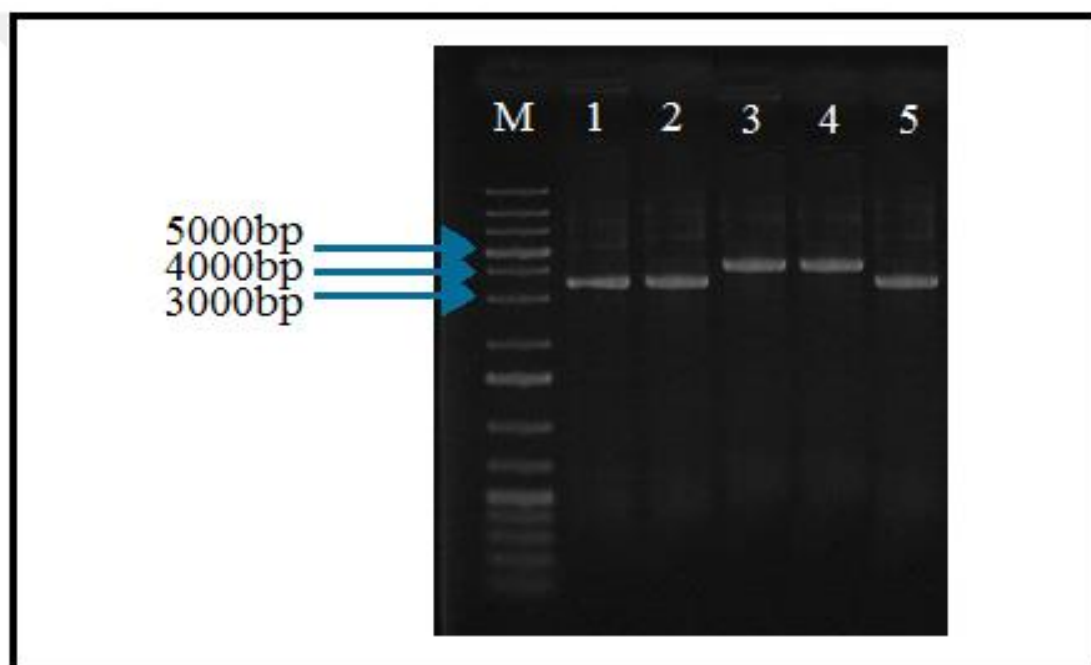


Figure 4.12. Transformation of pNMB1 into *E. coli* BLR(DE3) strains. M represents the marker; Line 1: Uncut pET22b DNA; Line 2: pNMB1A transformant DNA; Line 3: pNMB1B transformant DNA; Line 4: pNMB1C transformant DNA; Line 5: pNMB1D transformant DNA.

4.1.7.1 Detection of the Inserted *bsh* Gene from the Clones

The inserted *bsh* genes were detected by digesting the plasmid DNAs isolated from the transformants with NotI and SalI restriction enzymes. The digested DNA products were loaded on a 0.8% agarose gel (Figure 4.11). Even though the digestion was not totally complete, It was visible that pNMB1/BLR(DE3) transformant DNA in lane 1 in Figure 4.13 had taken an insert.

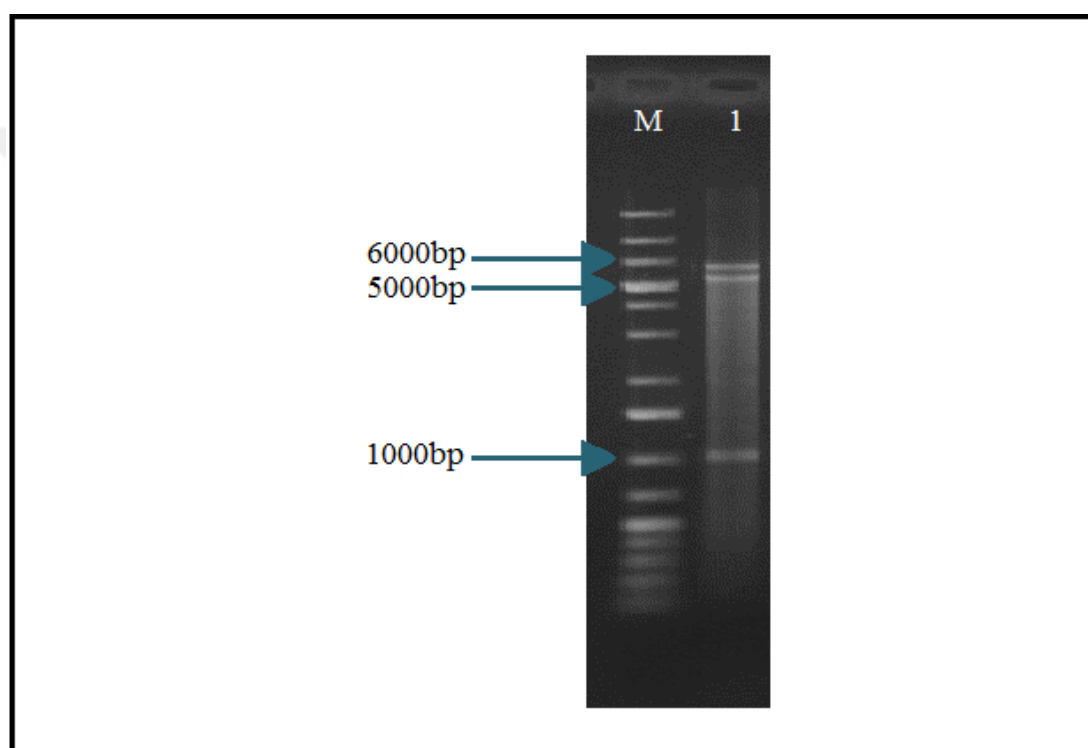


Figure 4.13. Detection of the *bsh* insert by NotI/SalI enzymes digestion test. M represents the marker and lane 1 represents the NotI/SalI digested DNA of pNMB1/BLR(DE3) transformant.

4.1.8 Determination of the Substrate Specificity of BSH Enzyme by Ninhydrin Assay

4.1.8.1 The Ninhydrin Assay

The substrate specificity of the rBSH was determined by ninhydrin assay. In the Ninhydrin assay test, the activity of the BSH enzyme was detected against each one of the six different human bile salts: glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA). Ninhydrin test results with the six major human bile salts indicated that the rBSH enzyme from *Lb. gasseri* ATCC 33323 strain exhibited the highest affinity for glycochenodeoxycholic acid (GCDCA) and higher affinities for glycine- conjugated bile acids than for taurine-conjugated bile acids (Figure 4.14).

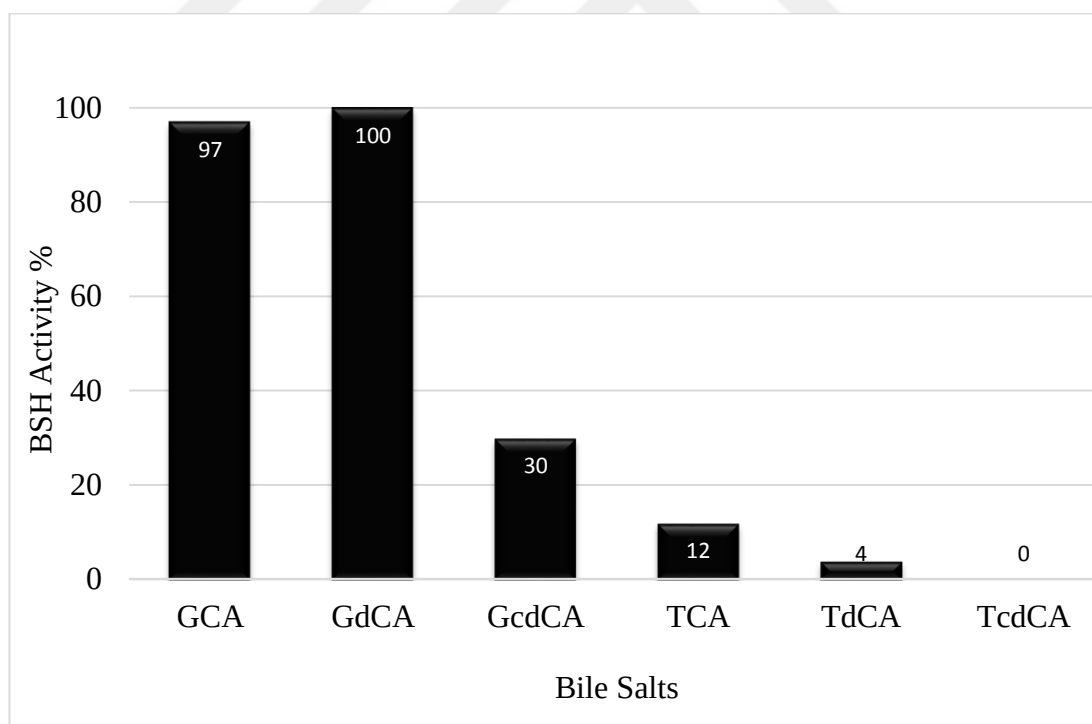


Figure 4.14. Determination of the substrate preference of BSH enzyme by ninhydrin assay. Six major bile salts were used: glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA). Relative activity is defined as the measured bile salt hydrolase activity for each substrate compared with pCON1 as the highest activity and pET22b as the lowest.

4.1.8.2 Optimum pH and Temperature of the wrBSH Enzyme

The optimum pH and temperature of the wild-type recombinant BSH enzymes (wrBSHs) were determined by ninhydrin assay. The effects of various temperatures and pH values on its relative activities were determined using the common human bile salts, glycodeoxycholic acid (GDCA). The maximum hydrolysis activity of wrBSH enzyme occurred at 55°C (Figure 4.15). The activity of rBSH enzyme peaked at pH 4.0 (Figure 4.16).

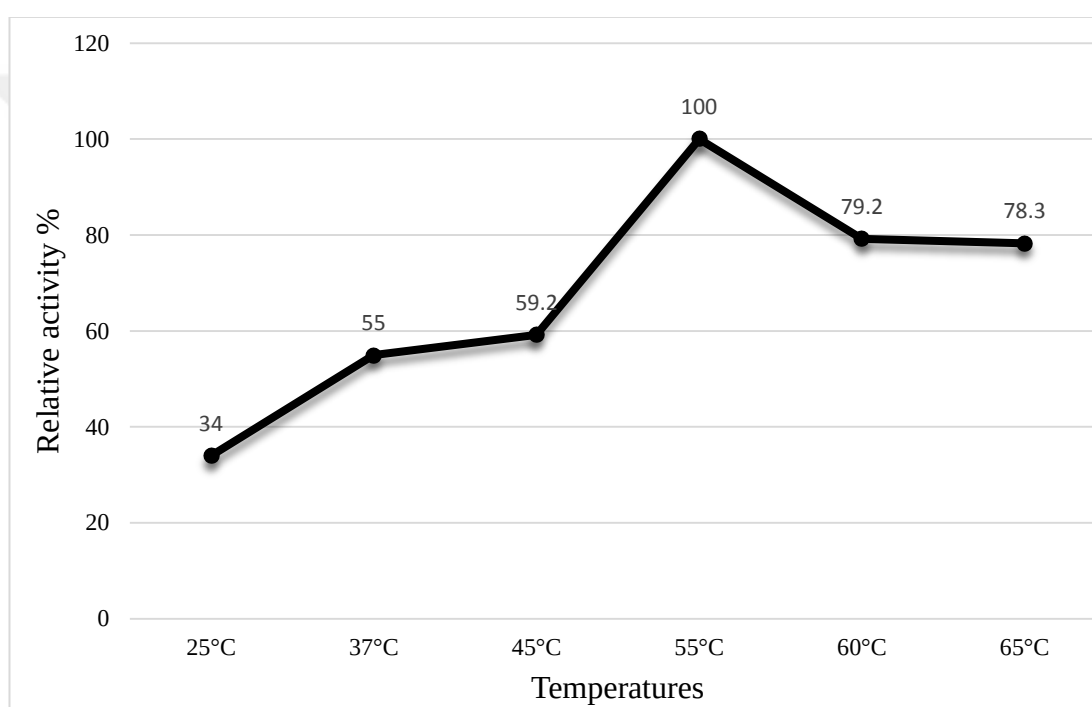


Figure 4.15. Effect of temperature on the wrBSH enzyme activity. One of major bile salts, glycodeoxycholic acid (GDCA), was used for the detection of optimum temperature of BSH enzyme. The relative activity is defined as the measured bile salt hydrolase activity for each substrate compared with PCON1 as the highest activity and pET22b as the lowest.

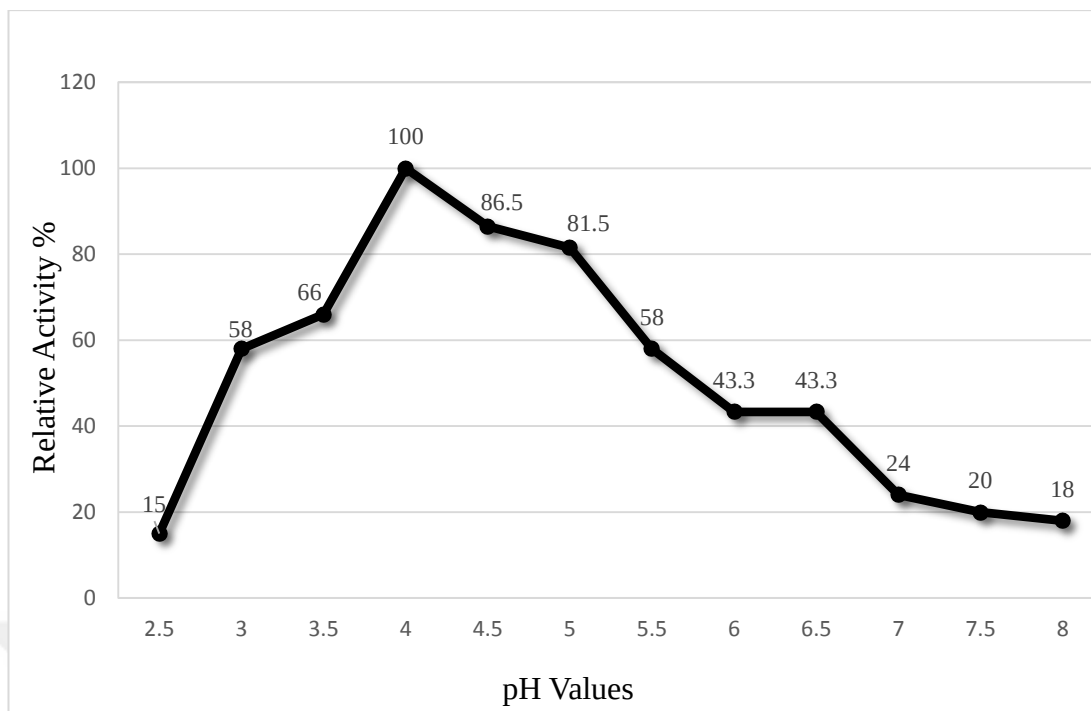


Figure 4.16. Effect of pH on the wrBSH enzyme activity. One of major bile salts, glycodeoxycholic acid (GDCA), was used for the detection of optimum pH of BSH enzyme. The relative activity is defined as the measured bile salt hydrolase activity for each substrate compared with PCON1 as the highest activity and pET22b as the lowest.

4.1.9 Partial Purification of the BSH Enzyme and SDS PAGE Analysis

Over expressed BSH proteins were purified using the AKTA Prime Plus system (GE Healthcare) with Ni^{2+} chelated agarose column. Purified BSH protein of *Lb. gasseri* was visualized by SDS-PAGE. The 37 kDa molecular mass of BSH enzyme, which is consistent with theoretical molecular mass of rBSH proteins obtained by ExPASy program (<http://web.expasy.org/protparam/>), was observed on SDS-PAGE (Figure 4.17).

Number of amino acids: 325					
Molecular weight: 36269.92 Da					
Theoretical pI: 4.85					
Amino acid composition:					
Ala (A)	21	6.5%	Lys (K)	21	6.5%
Arg (R)	5	1.5%	Met (M)	8	2.5%
Asn (N)	24	7.4%	Phe (F)	19	5.8%
Asp (D)	23	7.1%	Pro (P)	15	4.6%
Cys (C)	5	1.5%	Ser (S)	26	8.0%
Gln (Q)	9	2.8%	Thr (T)	14	4.3%
Glu (E)	19	5.8%	Trp (W)	1	0.3%
Gly (G)	19	5.8%	Tyr (Y)	17	5.2%
His (H)	8	2.5%	Val (V)	27	8.3%
Ile (I)	18	5.5%	Pyl (O)	0	0.0%
Leu (L)	26	8.0%	Sec (U)	0	0.0%
Total number of negatively charged residues (Asp + Glu): 42					
Total number of positively charged residues (Arg + Lys): 26					

Figure 4.17. Amino acid composition analysis of the *Lb. gasseri bsh* gene by SIB (Swiss Institute of Bioinformatics) ExPASy ProtParam sequence analysis translate tool (<http://web.expasy.org/protparam/>).

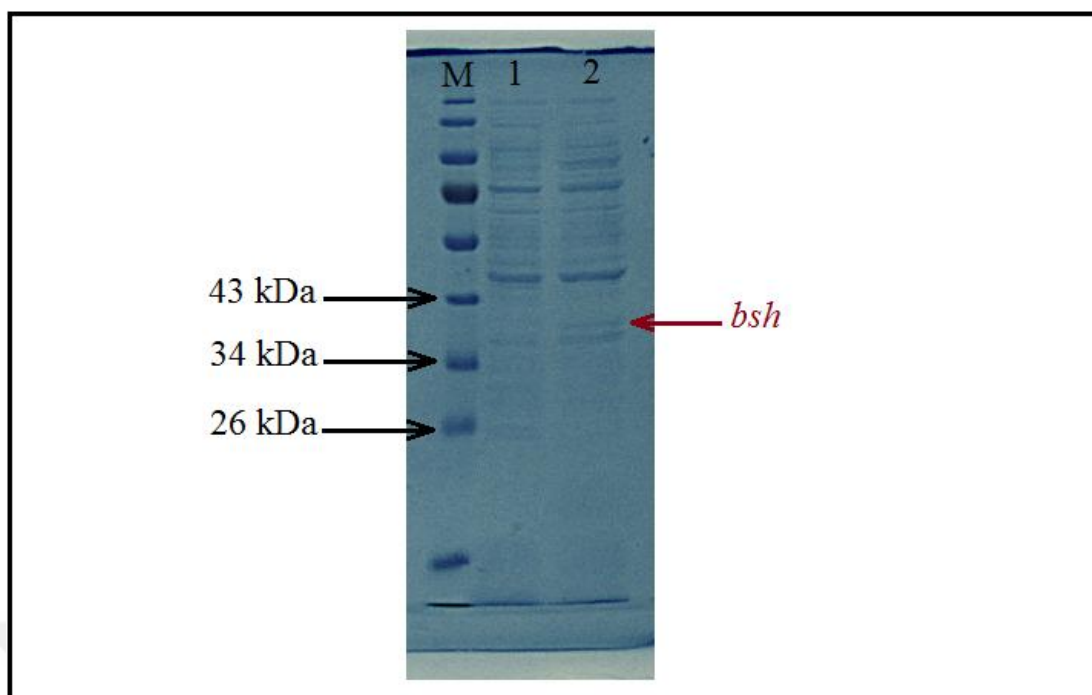


Figure 4.18. SDS-PAGE profile of purified *Lb. gasseri* BSH protein. M represent the protein molecular weight marker; Lane 1: pET22B the empty vector; Lane 2: purified *Lb. gasseri* BSH protein by column chromatography

The SDS profile of our purified *Lb. gasseri* BSH protein displayed a molecular band seated between 34 and 43 kDa of molecular mass (Figure 4.18) which is consistent with the theoretical molecular mass of rBSH proteins of 36.270 kDa obtained by the ExPASy program (<http://web.expasy.org/protparam/>)

4.2 DISCUSSION

Hypercholesterolemia is by far one of the leading factor for coronary heart disease (CHD). The quest for finding ways to reduce the cholesterol level of hypercholesterolemic patients without the expensive cost or the undesired side effects of the proposed pharmacological drugs already on the market has forced researchers to focus on the probiotics and their efficiency in reducing the plasma cholesterol level. So far, several mechanisms have been proposed including the enzymatic deconjugation of bile acids by the bacterial bile salt hydrolase.

The *Lactobacillus* genus is one of the main constituent of the human GI microbiota and is composed of a wide-ranging number of species that play key roles in the maintenance of the gut health (Azcarate-Peril et al., 2008). Amongst those species is the *Lactobacillus gasseri* ATCC 33323 strain which is a very versatile bile salt hydrolase active probiotic with various properties. This *Lb. gasseri* showed its capability to survive in the GI tract (Pedrosa et al., 1995); its ability to stimulate the macrophages (Kirjavainen et al., 1999), and its competence to produce important bacteriocins (Itoh et al, 1995; Sawai et al., 1998). And as such, this probiotic has been extensively used in countless industrial applications.

On the other hand, the secondary bile acids produced by the bacterial enzymatic deconjugation and dehydroxylation of primary bile acids in the intestines can accumulate in the enterohepatic circulation of some individuals to reach high levels and may well take part in the onset of colon cancer, cholesterol gallstones, and other gastrointestinal diseases. (Jason M. Ridlon, et al 2005). Therefore understanding the proper mechanism of action of the catalytic activity and substrate preferences of the BSH enzyme of this probiotic is of vital importance.

In the present study, BLAST analysis of the *bsh* DNA sequences and multiple sequence alignment revealed that BSH in *Lb. gasseri* ATCC 33323 strain belong to the N-terminal nucleophile aminohydrolases also known as the Ntn hydrolase super family, whose members have conserved N-terminal cysteine residue (Cys-2) in the active center (Rossocha et al. 2005), serving as a nucleophile and a proton donor in the process of catalysis (Oh et al. 2008; Kumar et al. 2010). In addition to this, amino acid sequence alignment confirmed that the other four predicted catalytic sites, Arg-

16, Asp-16, Asn-171 and Arg-224, were strictly conserved in all BSH enzymes (Rossocha et al. 2005) and the amino acid motifs around active sites of BSH proteins (Kim et al. 2005; Kumar et al. 2010) are consistent in BSH of *Lb. gasseri* ATCC 33323 strain (Figure 4.16).

Although the BSHs of *Lb. gasseri* ATCC 33323 strain have the same catalytic sites to other lactobacillus species, the capabilities of substrate-hydrolyzing are different. It has been suggested that most evolution occurs to broaden substrate specificity (Jiang et al. 2010). Begley et al. (2006) and Patel et al. (2010) emphasized that substrate specificity may occur at amino acid or steroid cholyl moieties. Generally, BSHs from intestinal lactic acid bacteria have higher affinity to glucoconjugated bile salts (Coleman et al., 1999; Tanaka et al., 2000; Kim et al., 2004). Our ninhydrin results obtained using partially purified rBSH enzyme were also demonstrated that although rBSH enzyme of *Lb. gasseri* ATCC 33323 exhibited its substrate preference towards glycine-conjugated bile substrates and tauro-conjugated cholates, it had a high activity towards GDCA rather than TDCA substrates (Figure 4.12). Our data is consistent with the fact that the majority of the BSH had higher affinity towards glyco-conjugated bile substrates (Kim et al. 2005; Lambert et al. 2008) and prediction of Rossocha et al. (2005) that BSH might recognize substrates predominantly at the amino acid moieties but not at the cholate moieties.

The optimum temperatures and pH values for rBSH of *Lb. gasseri* ATCC 33323 strain were comparable to those of other cloned BSH enzymes, such as those of *Bifidobacterium pseudocatenulatum* and *Bifidobacterium longum subsp* (Jarocki et al. 2014). The BSH enzyme of *Lb. gasseri* ATCC 33323 was able to hydrolyze mainly glyco-conjugated major human bile salts under at pH 4 and at 55°C conditions. These conditions are unfortunately not convenient for lowering the cholesterol in the human intestines. In this respect, *Lb. gasseri* ATCC 33323 strain cannot be a potential probiotic candidate for the reduction of elevated serum cholesterol levels, if in vivo bile salt hydrolysis contributes to cholesterol lowering (Kumar et al. 2012).

5. CONCLUSIONS

In a World where both the prevention and treatment of cardiovascular diseases now include the use of probiotic bacteria with active bile salt hydrolase because of their hypocholesterolemic properties, understanding the mechanism behind this enzyme's catalytic activity and substrate preferences is crucial.

In this study, the *bsh* gene from *Lb. gasseri* ATCC 33323 strain was cloned, expressed and characterized in *Escherichia coli* BLR(DE3) strain. The standard Ninhydrin assay was performed in order to uncover the substrate specificity of the BSH enzyme by testing the enzyme's activity in the presence of six different bile salts (GCA, GDCA, GCDCA, TCA, TDCA and TCDCA). The effects that various pHs and temperature can have on the enzyme's activity were also studied by examining the BSH enzyme's activity at different temperatures and pHs ranging from 25°C to 65°C and 2.5 to 8.0 respectively.

It was showed in this study that the optimum pH and temperature of the BSH enzyme from *Lb. gasseri* ATCC 33323 strain were not convenient with that of the human gastrointestinal system and that the catalytic activity of this BSH enzyme is very low when it is compared to other *Lactobacillus* species such as *Lb. plantarum* B14 (Data was not published). In this respect *Lb. gasseri* ATCC 33323 cannot be a potential probiotic candidate to reduce elevated serum cholesterol levels.

Even though BSH from *Lb. gasseri* ATCC 33323 had strictly conserved catalytic sites and the conserved amino acid motifs around active sites, our findings revealed that rBSH enzyme of *Lb. gasseri* ATCC 33323 strain expressed in *E. coli* BLR (DE3) strain has a clear preference for the glycoconjugated bile salts compared to the tauroconjugated bile salts. Therefore, our findings still support the suggestion that BSH enzymes recognize their substrates predominantly at the amino acid moieties and not at the cholate moieties. But in order to fully understand the BSH enzyme's catalytic activity and substrate preferences, more extensive researches are needed.

To better comprehend the mechanisms for this substrate affinity difference among the BSHs, substitution of some of the partially conserved amino acids by PCR-based site directed mutagenesis can be used. Substitution of the partially conserved amino acids thought to be responsible for the substrate specificity, may allow a prediction of the structures of BSH enzymes of *Lb. gasseri* ATCC 33323 and provide a determination of their substrate specificities.



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APPENDICES

1. APPENDICES

APPENDICE A

BACTERIAL GROWTH MEDIA

- **Liquid MRS (de MAN, ROGOSA and SHARPE) Medium**

To prepare a liquid MRS medium, 52.2g of MRS is dissolved in upH₂O to a final volume of 1L. MRS broth pH does not require adjusting. The preparation is then sterilized at 121°C for 15-20 minutes.

- **Liquid MRS with 30% glycerol**

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

- **MRS Plate**

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

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

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

These contents are then dissolved in 900 ml upH₂O and the pH is adjusted to 7.5 with NaOH.

The final volume is then completed to 1L with upH₂O and sterilized at 121°C for 15-20 minutes.

- **LB Plate**

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

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

- **Ampicillin**

- ✓ 0.25g Ampicillin Sodium Salt
- ✓ 10 ml upH₂O

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

- **LB Plate with ampicillin**

To make an LB plate with ampicillin, 4mL of ampicillin is added for 1L of sterilized liquid LB medium.

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

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

Contents	For 1 Liter
IPTG	3 mL
X-gal	4 mL
Ampicillin	4 Ml

APPENDICE B

SOLUTIONS AND BUFFERS

- **For Genomic DNA Isolation**

➤ **20Mm Tris HCl**

1.576g of Trizma Hydrochloride (Sigma) is dissolved in 10mL of upH₂O. Then, from that solution, 2μL is dissolved in 100mL of upH₂O to get the final concentration of 20mM.

➤ **2mM EDTA**

0.447g of EDTA is dissolved in 30mL of upH₂O. Then, 20μL from that solution is dissolved in 400μL of upH₂O to get the final concentration of 2mM. The pH has to be adjusted to 8.0 or else the EDTA will not completely dissolve.

➤ **Lysis Buffer**

Contents	For 400μL
20mM Tris HCL	8μL
2mM EDTA	20μL
1.2% Tripton X-100	4.8μL
20mg/mL Lysozyme	266.7μL

All this components are added to 100.5μL of upH₂O for a final buffer volume of 400μL.

- **For primer resuspension**
- **TE Buffer**
 - ✓ 3.634g of Trizma base
 - ✓ 30mL upH₂O

The pH is adjusted to 8.0.

- **For Competent Cell Preparation**
- **0.1M CaCl₂**
 - ✓ 1.48g of CaCl₂
 - ✓ 100mL upH₂O

The pH is adjusted to 7.0.

- **0.1M CaCl₂ with 10% glycerol**
 - ✓ 9mL of CaCl₂
 - ✓ 1mL of glycerol

- **10X TAE Buffer**

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

All these components are dissolved in enough upH₂O to make a final buffer volume of 1L.

- **1X TAE Buffer**

- ✓ 100mL of 10X TAE buffer
- ✓ 900mL upH₂O

- **Preparation for Cell Extract**

- **1 M NaH₂PO₄ Buffer**

12g of monobasic sodium phosphate (NaH₂PO₄) are dissolved in 100mL upH₂O. The pH recorded was 3.97. The buffer solution was then sterilized at 121°C for 15-20 minutes.

- **1M Na₂HPO₄ Buffer**

14.2g of dibasic sodium phosphate (Na₂HPO₄) are dissolved in 100 ml ddH₂O and pH measured was 10.02. The buffer solution was then sterilized at 121°C for 15-20 minutes.

- **0.1M Sodium Phosphate Buffer**

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

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

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

- **For Ninhydrine Assay**

- **0.1M NaOH**

- ✓ 0.4g NaOH
- ✓ 100mL upH₂O

- **1% SDS in 0.1M NaOH**

- ✓ 0.05g Sodium Dodecyl Sulfate
- ✓ 5mL of 0.1M NaOH

- **Reagent A**

- ✓ 1g Na₂CO₃ (Sodium carbonate)
- ✓ 50mL of 0.1M NaOH

- **Reagent B**

- ✓ 5mg CuSO₄·5H₂O (Copper II sulphate pentahydrate)
- ✓ 0.5mL of H₂O

- **Reagent C**

- ✓ 10g C₄H₄KNaO₆·4H₂O (Potassium sodium tartarate)
- ✓ 0.5mL of H₂O

- **Reagent D**

- ✓ 500 μ L Reagent B
- ✓ 500 μ L Reagent C

- **Reagent E**

- ✓ 50mL Reagent A
- ✓ 1mL Reagent D

- **1mg/mL BSA**

- ✓ 0.001g BSA (Bovine Serum Albumin)
- ✓ 1mL of H₂O

- **0.2M Glycine Solution**

- ✓ 0.7507g H₂NCH₂COOH (Glycine)
- ✓ 50mL of H₂O

- **0.5 M Sodium Citrate Buffer**

- ✓ 2.941g C₆H₅Na₃O₇·2H₂O (Sodium Citrate)
- ✓ 20mL of H₂O

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

- **15% Trichloroacetic Acid**

- ✓ 7.5 g trichloroacetic acid
- ✓ 50mL of H₂O

The solution was then sterilized by filtration.

- **1% Ninhydrin Solution**

- ✓ 0.1g ninhydrin
- ✓ 10mL of Sodium Citrate Buffer

- **Ninhydrin Reagent**

- ✓ 0.5mL of 1% Ninhydrin Solution
- ✓ 1.2mL glycerol
- ✓ 0.2mL of 0.5M Sodium Citrate Buffer

- **1M DTT**

- ✓ 0.154g $\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$ (Sodium Dithiothreitol)
- ✓ 1mL of H_2O

- **For Purification of BSH Enzymes**

- **Binding Buffer pH: 7.8**

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

These mixture is dissolved in upH₂O for a final volume of 1L. The pH is adjusted with phosphoric acid.

- **Elution Buffer pH: 6.0**

Contents	For 1L
20mM NaPi Buffer	20mL of 1M NaPi
500mM NaCl	29.22g
200mM Imidazole	13.616g

These mixture is dissolved in upH₂O for a final volume of 1L. The pH is adjusted with phosphoric acid.

- **For SDS-PAGE Analysis**

- **Acrylamide-Bis Acrylamide Mixture**

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

- **10% SDS**

✓ 10g Sodium Dodecyl Sulfate
✓ 100mL upH₂O

APPENDICE C

CHEMICALS

Acrylamide (Sigma)
Agar (Merck)
Agarose (Sigma)
Ammonium persulfate [APS] (Sigma)
Ampicillin (Sigma)
Bovine Serum Albumin (Sigma)
Calcium Chloride [minimum 93.0%, granular anhydrous] (Sigma)
Calcium chloride dihydrate [CaCl_2] (Sigma)
Comassie Brilliant Blue G-250 (Fluka)
Copper II sulphate pentahydrate (Sigma)
EDTA [Ethylenediaminetetraacetic acid] (Sigma)
EtBr [Ethidium Bromide] (Sigma)
Glycerol, cell culture tested (Sigma)
Glycine (Merck)
IPTG [isopropyl-beta-D-thiogalactopyranoside] (Thermo Scientific)
Man Rogosa Sharpe (MRS) (Sigma)
N,N'-Methylenabisacrylamide (Sigma)
Ninhydrin (Sigma)
Peptone from casein (Merck)
Potassium Sodium Tartarate (Sigma)
Sodium Carbohydrate (Sigma)
Sodium Chloride [NaCl] (Merck)
Sodium Citrate Dihydrate (Merck)
Sodium Dithiothreitol [DTT] (Aplichem)
Sodium dodecyl sulfate [SDS] (Sigma)
Sodium Hydroxide [NaOH] (Merck)
Sodium Phosphate dibasic (Merck)
Sodium Phosphate monobasic (BDLab)
Tetrachloro acetic acid (Sigma)
Trichloroacetic Acid (Merck)

Trizma Base (Sigma)

Trizma Hydrochloride (Sigma)

Yeast Extract Granulated (Merck)

Tween® 20 (Sigma)

X-Gal (5bromo4chloro3indolylbetaDgalactopyranoside) (Fermentas)



APPENDICE D

ENZYMES AND OTHER CHEMICALS

Not I (Fermentas)

EcoR I (Fermentas)

Fast Digest Pack (Fermentas)

Sal I (Fermentas)

*Sma*I (Fermentas)

Dnase (Sigma)

Rnase (Sigma)

Lysozyme (Fluka)

Polyethylene Glycol (Fermentas)

T4 DNA Ligase (Fermentas)

Page Ruller Prestain Protein Ladder (Thermo Scientific)

Pfu DNA Polymerase (Fermentas)

dNTP Mix (Fermentas)

1 kb Plus DNA Ladder (Fermentas)

6X Loading Dye Solution (Fermentas)

Glycocholic Acid, Sodium Salt (Calbiochem)

Glycodeoxycholic Acid, Sodium Salt (Calbiochem)

Glycochenodeoxycholic Acid, Sodium Salt (Sigma)

Taurocholic Acid, Sodium Salt (Calbiochem)

Taurodeoxycholic Acid, Sodium Salt (Calbiochem)

Taurochenodeoxycholic Acid, Sodium Salt (Sigma)

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

APPENDICE E

EQUIPMENTS USED IN THIS STUDY

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

-20°C deepfreeze (Arçelik)

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

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

+4°C refrigerators (Arçelik)

AKTAprime™ plus (GE Healthcare)

Autoclave (Hirayana)

Centrifuge (Hettich Rotina 38R)

Desktop centrifuge (Hettich Micro 120)

Electrophoresis system (Thermo Scientific)

Imaging system (UVP Photo Doc-It TM)

Micropipettes (Finnipipette)

PCR (Techne, TC 3000)

pH meter (HANNA HI 221)

Power supply (Thermo EC 250-90)

Shaker-heater (IKA RCT basic)

Spektrophotometer (HITACHI U-1900)

UV Transalluminator (UVP)

Vortex (Yellowline TTS2)

Water Purification System (Human Corporation)

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List of Publications :

- 1) Mehmet ÖZTÜRK, Cansu ÖNAL, Zekiye KILIÇSAYMAZ and **Ndeye Mareme BA** (2016) Functional Characterization of Val58Met and Phe129Ile Mutants of bile Salt Hydrolase from *Lactobacillus plantarum* in *E. coli* BLR(DE3). The FEBS Journal Special Issue Volume 283 Supplement 1 September 2016: 41st Federation of European Biochemical Societies (FEBS) of Molecular and Systems Biology for a Better Life.
- 2) **Ndeye Mareme BA**, Zekiye KILIÇSAYMAZ, Cansu ÖNAL and Mehmet ÖZTÜRK (2016). Functional Characterization of Bile Salt Hydrolase from *Lactobacillus gasseri* in *E. coli* BLR(DE3) Strain. International Conference on Biological Sciences (ICBS), Konya, Turkey, October 21-23, 2016.