Studies on biochemical aspects of bile salt hydrolase from the thermophile, *Brevibacillus borstelensis*

Thesis submitted to the University of Pune for the Degree of
Doctor of Philosophy in Biotechnology

By

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MAY 2009

..... Dedicated to my mentor

DECLARATION BY RESEARCH GUIDE

This is to certify that the work incorporated in the thesis entitled, '**Studies on biochemical aspects of bile salt hydrolase from the thermophile *Brevibacillus borstelensis***', submitted by Ms. Nidumukkala Sridevi, for the Degree of ***Doctor of Philosophy***, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune-411008, India. Materials obtained from other sources have been duly acknowledged.

Dr. (Mrs). Asmita. A. Prabhune

(Research Supervisor)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Studies on biochemical aspects of bile salt hydrolase from the thermophile *Brevibacillus borstelensis***", submitted for the Degree of **Doctor of Philosophy** to the University of Pune, has been carried out by me under the supervision of Dr. Asmita. A. Prabhune at Division of Biochemical Sciences, National Chemical Laboratory, Pune 411008, India. This work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Nidumukkala Sridevi

(Research Scholar)

Acknowledgements

I would like to sincerely thank people who helped me either directly or indirectly in this thesis. Scientific research is a group activity. It is not possible without the help and suggestions of people.

I have no words to express my deep gratitude and sincere thanks to my research guide Dr. Mrs. Asmita Prabhune, who has always filled positive energy in me with her constant encouragement in my research work. Her inspiring guidance and constant motivation helped me to achieve goals in research. I am grateful to her attitude for giving me liberty in the work I have undertaken. Without her persuation and interest, it would not have been possible for me to gain confidence that I have today. I salute her supporting attitude that has always led me to think and work independently.

I am thankful to Dr. Mrs. Archana Pundle for invaluable suggestions, constant support and help offered by her during my work.

I am also thankful to Dr. C.G. Suresh and Dr. J.K. Pal who has constantly observed the progress of my research and has given very important and timely suggestions during my assessments.

I would like to thank University Grants Commission for providing me financial assistance during my research work. I am grateful to the Director, National Chemical Laboratory, Pune and the Head, Division of Biochemical Sciences for providing facilities to carry out my work. I am thankful to all scientific and non-scientific staff members of Biochemical Sciences Division for their help.

I do not have words to mention the impact on me of Dr. Mala Rao, who has always appreciated my efforts in research.

It is my immense pleasure to express thanks to my labmates, Ambrish, Atul, Sharath, Sachin, Umesh, Sulabha madam, Ashwini, Asmita, Nupur, Reetika, Kasturi, Jayshree, Siddharth, Avinash, and Aparna for their help and co-operation in my work and also for making fun filled environment in lab. Special thanks are due for Ambrish, who helped me in the final stage of my thesis.

I also would like to thank all my friends in hostel, Manaswini, Shraeddha, Ashwini, Smita, Uma, Poorva, Meera, Maitri, Gitali, Maansi, Nitasha,

Priyanka and Gauri for their help and immense support during my stay in hostel. Our conversations in mess hall have been refreshing entertainment.

I specially would like to thank my friends, Bubhan, Sashidhar, Urvashi, Imraan, Rohtas, Purnima, Tonima, Viku, Pankaj, Pandey, Reeti, Atul, Gayatri, Nishant, Abhishek, Noor, Rishi, Somesh, Kannan for always being there whenever I needed them.

Friendship with Shailesh and Sneha is always memorable for their jolly companionship.

I owe special thanks to Mr. Chinmay Kumar Mantri and Mr. Sameer Srivastava for thei timely help and suggestions about my work. They stood by me like a rock during my tough times and also they are a source of inspiration for me to complete my work successfully.

No words could express my special gratefulness to my family members. Without their love, patience, sacrifice, support and faith in this long journey, this work would not have been possible.

N. Sridevi

Contents

ABBREVIATIONS

SBA-15	Santa Barbara amorphous number 15
MCM-41	Mobile composition of matter number 41
TEOS	Tetra ethoxysilicate
BET	Braunauer-Emmett-Teller
BJH	Barrett-Joyner Halenda
CP-MAS NMR	Cross Polarization Magic angle spinning- Nuclear magnetic resonance
DTT	Dithiothreitol
ppm	Parts per million
h	hour
O.D	Optical density
S.D	Standard deviation
BSH	Bile salt hydrolase
PGA	Penicillin G acylase
APTES	3-amino propyl triethoxy silane
P123	Pluronic 123 triblock co-polymer
FTIR	Fourier transform infrared spectroscopy
XRD	X-ray diffraction
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
Am-SBA-15	Amino functionalized mesoporous silica
PGA-Am-SBA-15	Penicillin G acylase immobilized amino functionalized mesoporous silica
BSH-Am-SBA-15	Bile salt hydrolase immobilized amino functionalized mesoporous silica
DCA	Deoxycholic acid
GDCA	Glycodeoxycholic acid
GCA	Glycocholic acid
GChDCA	Glycochenodeoxycholic acid

TDCA	Taurodeoxycholic acid
TCA	Taurocholic acid
TChDCA	Taurochenodeoxycholic acid
rDNA	Ribosomal DNA
BLAST	Basic Local Alignment Search Tool
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction
Ntn	N-terminal nucleophile
ANBA	5-amino 2-nitro benzoic acid
TRIS	Tris (hydroxymethyl) amino methane
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
HPLC	High performance Liquid chromatography
CDW	Cell dry weight

ABSTRACT

Chapter 1: Introduction.

The ***first chapter*** is general introduction of the THESIS and it gives brief review of literature on bile salt hydrolases and bile salt hydrolase producing microorganisms. This chapter deals with Ntn hydrolase super family members, their structural similarities, catalytic behavior and mechanism. Characteristics of bile salt hydrolases, produced by various microorganisms and the bile salt tolerance mechanism are discussed in this chapter.

Chapter 2: Isolation, identification of the thermophile, *Brevibacillus borstelensis* and optimization of fermentation conditions for the production of bile salt hydrolase.

The ***second chapter*** describes the isolation of bile salt hydrolase producing thermophile from hot water springs in Maharashtra, India. Based on phenotypic analysis and 16S rDNA sequencing, the isolate was identified as *Brevibacillus borstelensis.* Standardization of fermentation conditions for the optimum production of bile salt hydrolase from *Brevibacillus borstelensis* was studied. Optimization of fermentation conditions resulted in 2.9-fold enhancement in the enzyme production. The enzyme production was enhanced when sodium glutamate medium was used as growth medium. Peak in enzyme production was attained after 12 h fermentation period with this medium. Cell bound bile salt hydrolase activity was optimum when the initial pH of the medium was 6.0 and incubated at temperature (55°C).

Chapter 3: Purification and characterization of bile salt hydrolase from *Brevibacillus borstelensis*.

The ***third chapter*** gives details about the purification of intracellular bile salt hydrolase from newly identified thermophilic source, *Brevibacillus borstelensis*. The enzyme was purified to homogeneity from this thermophilic source by Q-Sepharose chromatography and its enzymatic properties were characterized. The sub-unit molecular mass of the purified enzyme was estimated to be 28 kDa by SDS- PAGE and, 28.2 kDa by MALDI-TOF analysis. The native molecular mass was estimated to be 56 kDa by gel filtration

chromatography, indicating the protein as homodimer. The pH and temperature optima for the enzyme catalysis were 9.0 and 60°C, respectively. Even though BSH from *Brevibacillus* sp. hydrolyzed all of the six major human bile salts, the enzyme preferred glycine conjugated substrates with apparent K_M and k_{cat} values of 3.08 μM and 6.32 $\times 10^2$ s^{-1}, respectively for glycodeoxycholic acid. The NH_2-terminal sequence of the purified enzyme was determined and it did not show any homology with other bacterial bile salt hydrolases.

Chapter 4: Structural features of Ntn hydrolases immobilized on APTES functionalized mesoporous silica.

The ***Chapter four*** is divided into two sections: A and B.

Section A: Structural features of bile salt hydrolase encapsulated in APTES functionalized mesoporous silica.

This section deals with preparation and functionalization of mesoporous silica i.e. SBA-15 and its application in the immobilization of bile salt hydrolase. Bile salt hydrolase (BSH; EC.3.5.1.24) was immobilized on mesoporous SBA-15 by cross linking method. Optimum bile salt hydrolase activity was observed when 0.1% of glutaraldehyde was used as cross linking agent. Maximum loading of the enzyme was observed at pH 6.5 and the loading capacity of immobilized BSH is 4.1 mg protein per gram of SBA-15. Characteristics of free enzyme such as pH and temperature profile, pH and temperature stability were studied and compared with immobilized enzyme. Physicochemical characterization of the immobilized enzyme by nitrogen adsorption, powder X-ray diffraction (XRD), transmission electron microscopy (TEM) and ^{29}Si CP-MAS NMR indicated that the characteristic hexagonal features and the original pore structure of the parent SBA-15 is retained after immobilization reaction.

Section B: Structural features of penicillin G acylase adsorption on APTES functionalized mesoporous silica

This section deals with immobilization of penicillin G acylase (PGA) which belongs to Ntn hydrolase superfamily. The enzyme was immobilized on amino-functionalized

mesoporous silica by adsorption method. The effect of PGA immobilization on amino-functionalized SBA-15 materials and the effect of silica as host matrix on enzyme kinetics were studied. Maximum loading of the enzyme was observed at pH 7.8 and, the loading capacity of immobilized PGA is 68 mg protein per gram of fuctionalized SBA-15. Physicochemical characterization of the immobilized enzyme by nitrogen adsorption, powder XRD and TEM methods indicated that the characteristic hexagonal features and the original pore structure of the parent SBA-15 is retained after immobilization reaction. Thermal stability of penicillin G acylase was enhanced after immobilization reaction. This enhanced stability is attributed to the protective nature of the cage itself and to the rigidity of the SiO_2 matrix, which reduces the freedom of peptide-chain refolding of molecular motions that occur in denaturation processes.

Chapter 5: *In vivo* cholesterol reduction studies

The ***fifth chapter*** deals with optimization of fermentation conditions for the maximum production of bile salt hydrolase from *Lactobacillus buchneri* ATCC 4005. Furthermore, effect of various carbon and nitrogen sources on the production of bile salt hydrolase from the bacterial culture is described in the chapter. Optimization of culture conditions resulted in 2.9-fold enhancement in enzyme production. The effect of oral administration of the immobilized bile salt hydrolase enzyme from the FDA (Food and Drug Administration) approved organism *Lactobacillus buchneri* ATCC 4005 on serum cholesterol, triglyceride, high density lipoprotein levels and its application in the therapeutic treatment of hypercholesteremia were assessed. Bile salt hydrolase was isolated from *L. buchneri* ATCC 4005 and immobilized in 0.5% w/v low acyl gellan gum gel with 64.5% efficiency. The immobilized enzyme was orally delivered in wistar rats, induced with hypercholesteremia by triton X-100. The serum cholesterol and triglycerides were reduced by 50% and 15% respectively, in the group fed with immobilized enzyme 10 U/kg dose whereas administration of 20 U/kg immobilized enzyme resulted in reduction of serum cholesterol by 58% and triglycerides by 45% respectively. The results indicate that bile salt hydrolase has potential cholesterol lowering property and oral administration of the immobilized enzyme is an alternative pharmacological approach to reduce serum cholesterol levels.

Chapter 6: Summary and conclusions

The ***sixth chapter*** summarizes the work presented in the THESIS and emphasizes possible future research in this area. Further studies remained to be done to elucidate the three dimensional structure and this may provide insight into the development of a genetically engineered BSH. Also, importance of the enzyme for *in vivo* studies is highlighted in this chapter.

Chapter 1

Introduction

1.1. Introduction

Cholesterol is an essential component of cell membrane and also involved in the synthesis of hormones and steroids; however, elevated levels of blood cholesterol is a risk factor for cardiovascular diseases. The results from several epidemiological and clinical studies indicate a positive correlation between elevated total serum cholesterol levels and occurrence of coronary heart disease (Pereira and Gibson, 2002). Coronary heart disease is one of the major causes of death and disability in many countries. According to a recent survey from World Health Organization, globally cardiovascular diseases are the number one cause of death and are projected to remain so. An estimated 17.5 million people died from cardiovascular disease in 2005, representing 30% of all global deaths. Of these deaths, 7.6 million were due to heart attacks and 5.7 million, due to stroke. About 80% of these deaths occurred in low- and middle-income countries. If current trends are allowed to continue, by 2015 an estimated 20 million people will die from cardiovascular diseased strokes (World Health Organisation Fact sheet N°317, 2007).

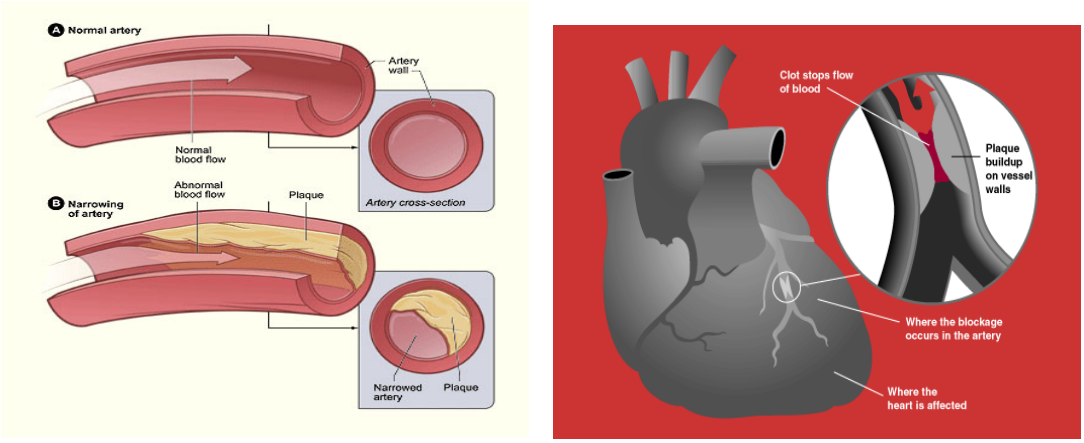

Fig. 1.1: Deposition of excess cholesterol inside the artery

Various approaches have been used to alleviate this issue. Recent modalities for lowering blood cholesterol level involve dietary management, behavior modification, regular exercise and drug therapy (Lichtenstein, 1998; Ornish and Denke, 1994). Pharmacologic agents such as fibrates, nicotinic acids, bile acid sequestrants are also available for treatment of high cholesterol. Although these drugs effectively reduce cholesterol levels, these are expensive and known to have side effects (Bliznakov, 2002; Willman, 2001).

Hence there is a requirement for more natural methods for the treatment of hypercholesteremia. Enzymatic deconjugation of bile salts has been related to a reduction of serum cholesterol levels in mammals (Klaver and Van der Meer, 1993; Pereira *et al.,* 2003). The corresponding enzymes responsible for bile salt deconjugation are called "bile salt hydrolases".

1.1.1. Bile salt hydrolase

Bile salt hydrolase (BSH) (cholylglycine hydrolase, EC 3.5.1.24) catalyzes the hydrolysis of amide bond in conjugated bile salts resulting in the release of free amino acids.

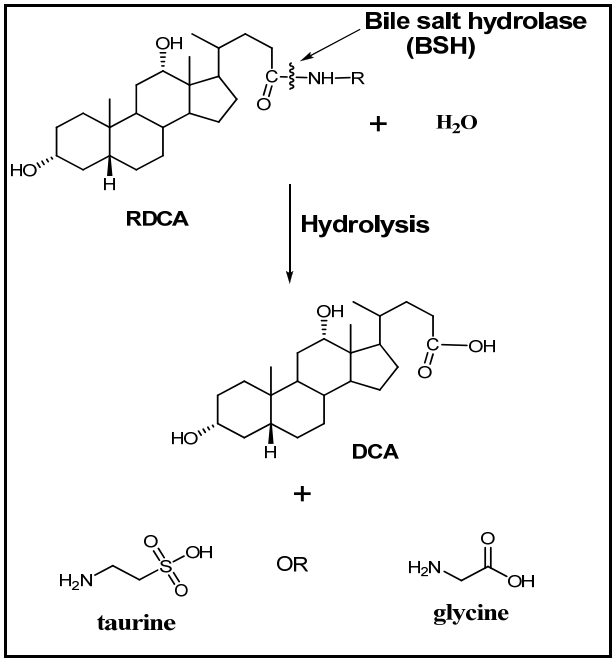

Fig. 1.2: Hydrolysis of conjugated bile salts by bile salt hydrolase (BSH) enzyme. 'R' indicates the amino acid glycine or taurine. RDCA: Glyco-or Tauro-deoxycholic acid, DCA: Deoxycholic acid.

1.1.2. Source and distribution of bile salt hydrolase among microorganisms

Bile salt hydrolase activity has been widely detected in several bacterial genera of the autochthonous gastrointestinal microbiota of animals including mice, rats, humans,

chickens, and swine (Savage *et al*., 1977). Deconjugation has been observed mainly in bacterial species of gastrointestinal microorganisms including *Lactobacillus sp*. strain 100-100 (Lundeen and Savage, 1990), *Lactobacillus acidophilus* strains (Corzo and Gilliland, 1999a), *Bifidobacterium longum*, BB536 (Grill *et al*., 1995), *Clostridium perfringes* (Gopal-Srivastava and Hylemon 1988), *Bacteroides vulgatus* (Kawamoto *et al*., 1989) and *Bacteroides fragilis ssp. fragilis* (Stellwag and Hylemon, 1976). In addition to autochthonous intestinal microbiota, BSH activity was also reported from an enteropathogenic strain of *Listeria monocytogenes* (Dussurget *et al.*, 2002) and a bile adapted strain of *Xanthomonas maltophila* (Dean *et al*., 2002).

Table 1.1: Summary of bile salt hydrolase (BSH) activity among various microorganisms

Organisms	Strains tested	Origin	BSH positive (%)	BSH negative (%)	Reference
Lactobacillus	66	Human or animal	46(70%)	20(30%)	Dashkevicz & Feighner (1989)
Bifidobacterium	44	Feces or intestine	43(98%)	1(2%)	Tanaka *et al.* (1999)
	18	Other sources	15 (83%)	3(17%)	”
Lactobacillus	39	Feces or intestine	23(59%)	16(41%)	”
	105	Other sources	28(27%)	77(73%)	”
Lactobacillus	30	Feces or intestine	21(70%)	9(30%)	Moser and Savage(2001)
	19	Other sources	7 (37%)	12(63%)	”
Enterococcus	117	Cheese	72(62%)	45(38%)	Franz *et al*. (2001)
E. faecium	11	Cheese	9(82%)	2(18%)	Saavedra *et al*. (2003)
L. reuteri	6	Pig feces	5(83%)	1(15%)	Rodriguez *et al*. (2003)

Table 1.2: Landmarks in bile salt hydrolase research

Year	**Contribution**	**Reference**
1967	First partial purification of bile salt hydrolase	Nair *et al.,*
1974	Reduction in serum cholesterol after consumption of fermented milk containing *Lactobacillus*	Mann and Spoerry
1988	Production of bile salt hydrolase antibody	Gopal-Srivastava and Hylemon
1989	Bile salt hydrolase assay	Dashkevicz and Feighner
1992	Cloning and heterologous expression of bile salt hydrolase gene	Christiaens *et al*.
2000	Site directed mutagenesis of bile salt hydrolase gene	Tanaka *et al*.,
2002	Production of bile salt hydrolase from pathogenic source, *Listeria monocytogenes*	Dussurget *et al*.,
2003	Multiple bile salt hydrolase loci	Kleerebezem *et al*.,
2004	Microencapsulation studies	Jones *et al*.,
2005	Bile salt hydrolase structure determination	Rossocha *et al*.,

1.2. BSH as a new member of N-terminal nucleophile (Ntn)-hydrolase superfamily

Branningan *et al*. (1995) recognized a new protein structural superfamily called Ntn hydrolases (N-terminal nucleophile). The typical fold of this superfamily consisted of four layered catalytically active αββα core structure (Oinonen and Rouvinen, 2000). This core was formed by two antiparallel β-sheets packed against each other and these β-sheets were covered by a layer of antiparallel α-helices on one side (Artymiyuk, 1995).

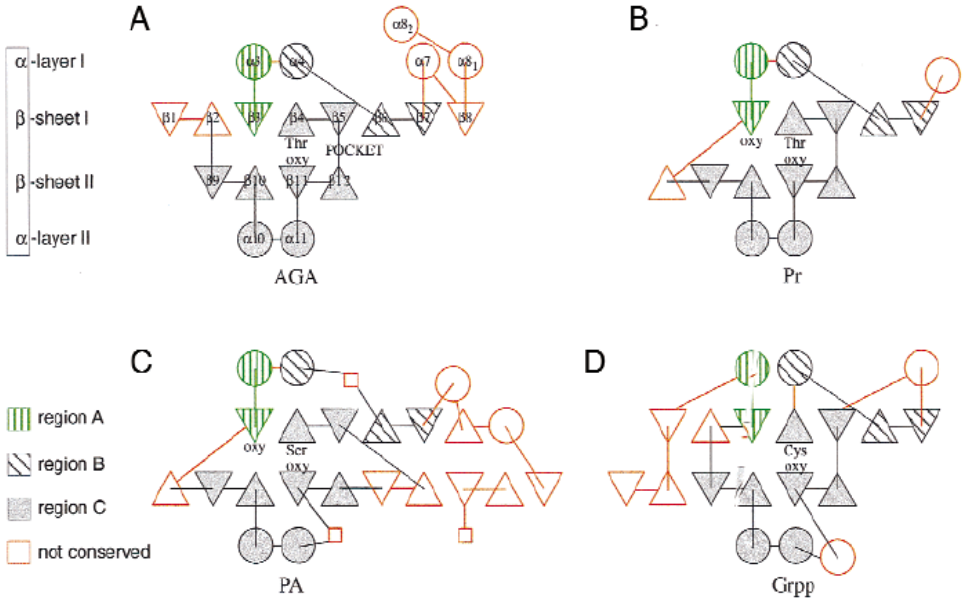

Fig.1.3: Topological diagram of the Ntn-hydrolases: (A) Aspartyl glucosaminidase from human, (B) Proteasome from *S. cerevisiae*, (C) Penicillin acylase from *E. coli*. (D) Glutamine phosphoribosyl pyrophosphate amido-transferase. Circles represent α-helices and triangles represent β-strands. The secondary structural elements are indicated according to the AGA's structure. Apex up means that the strand is viewed from the N-terminus, which means the strand direction, runs down into the plane. The topological positions of the catalytically active N-terminal (Thr, Cys, or Ser) and the oxyanion hole have been indicated in the scheme.

Ntn hydrolases go through the post-translational processes, which lead to an autocatalytically activated enzyme (Xu *et al*., 1999). They all catalyzed the amide bond hydrolysis, but they were different in their substrate specificities. This family contains

enzymes of markedly varying size and complexity, with a wide range of substrate and operating in very different biological contexts; it includes penicillin G acylase with a catalytic serine, the aspartylglucosaminidase, proteasome with threonine and penicillin V acylase with cysteine. Inspite of this variety, the simple presence of N-terminal serine, threonine or cysteine in an enzyme with a nucleophilic mechanism may be sufficient to place it in the Ntn hydrolase family (Aronson, 1996). The enzymes shared similar catalytic residues and therefore probably catalyzed substrate hydrolysis in a similar way (Branningan *et al*., 1995; Duggleby *et al*., 1995).

Since the bile salt hydrolases have extensive sequence similarity to penicillin V acylase (PVA) and typically have an N-terminal methionine, followed directly by cysteine, Suresh *et al*. (1999) assigned BSH enzymes as a member of Ntn hydrolase family. A homology analysis of four of the five amino acids at the active site were conserved in BSH, while Tyr-82 was replaced by Asn-81 in BSH. Since the catalytically important part of Tyr-82 in PVA was replaced with NH-group of peptide bond, it has been proposed by Tanaka *et al*. (2000) that Tyr-82 could be replaced easily by another amino acid if the NH group was kept in the right position for (BSH Asn-81). Recently the crystal structure of BSH from *Bifidobacterium longum* and *Clostridium perfringens* were determined (Kumar *et al*., 2006, Rossocha *et al*., 2005). The crystal structures of BSH from these organisms revealed close relationship with penicillin V acylase from *Bacillus sphaericus*. The overall structure of BSH from *Bifidobacterium longum* confirms the characteristic Ntn hydrolase fold comprised of a four-layered αββα core structure and the approximate monomer dimensions were 75 × 38 × 44 Å. The crystal structure of *Clostridium perfringens* BSH also revealed close structural and catalytic similarity to Ntn-hydrolases due to its overall topography with the characteristic αββα fold of Ntn hydrolases and the exposure of Cys-2 active site residue at the N-terminal end resulted from post translational processing. The appropriate dimensions of the monomer were 40 × 50 × 55 Å. Crystal structure of BSH from *Clostridium perfringens* and *Bifidobacterium longum* revealed that Cys-1 is essential for BSH catalysis. Replacement of the Cys-1 with the nucleophilic amino acids serine or threonine which have a hydroxyl group instead of a thiol group abolished BSH activity in *Bifidobacterium bifidum* BSH (Kim *et al*., 2004a),

and exchange of Cys-1 with alanine resulted in an inactive protein in *Bifidobacterium longum* BSH (Tanaka *et al*., 2000). Other amino acids that are reported to play an important role in BSH catalysis include Asp-20, Tyr-82, Asn-175, and Arg-228.

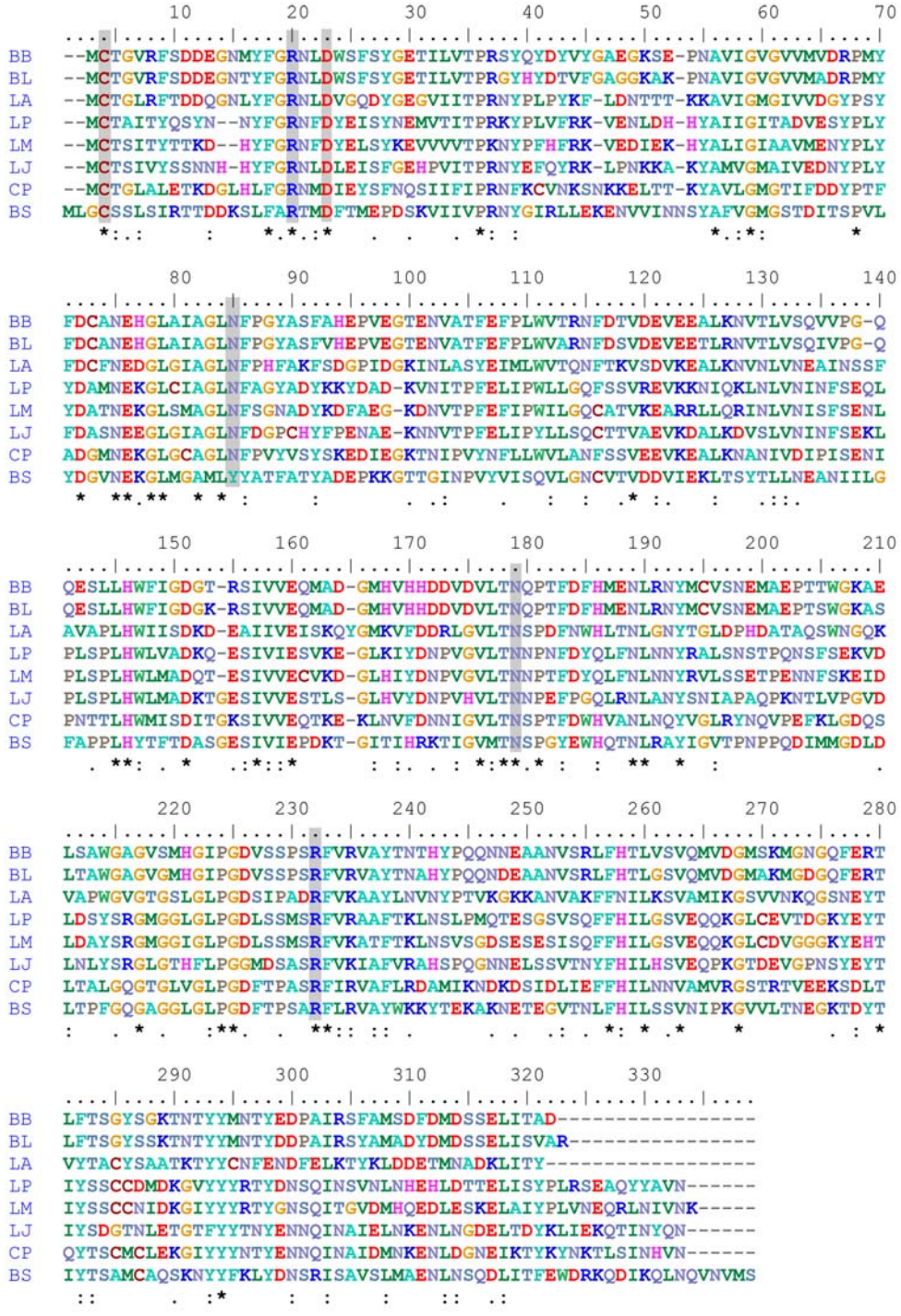

Fig. 1.4: Multiple alignment of BSH from different microorganisms. Protein sequences were obtained from the National Center for Biotechnology Information (NCBI data

bank). Alignments were performed by using CLUSTALW program. Amino acids involved in catalysis are Cys-1, Asp-21, Tyr-82, Asn-175, and Arg-228. Identical amino acids are marked by an asterisk, conserved substitutions are marked by two dots, and semi conserved substitutions are marked by a single dot. BB, *Bifidobacterium bifidum* (AAT11513; Kim *et al*., 2004a); BL, *Bifidobacterium longum* (AAF67801; Tanaka *et al*., 1999); CP, *Clostridium perfringens* (P54965; Coleman and Hudson, 1995); LA, *Lactobacillus acidophilus* (AAD03709; Elkins *et al*., 2001); LJ, *Lactobacillus johnsonii* (AAG22541; Elkins *et al*., 2001); LP, *Lactobacillus plantarum* (AAB24746; Christiaens *et al.,* 1992); LM, *Listeria monocytogenes* (CAD00145; Dussurget, 2002); and BS, *Bacillus sphaericus* (P12256; Olsson and Uhlen, 1986).

1.3. The role of bile salt hydrolase in the physiology of gastrointestinal system

The major *in vivo* role of bile is to act as a biological detergent to emulsify and solubilize fats. This also confers potent antimicrobial properties to bile and gives it an important role in body's physiochemical defence system. To understand the role of bile salt hydrolase in gastrointestinal system we need to acquaint with the bile acid circulation in the gastrointestinal system.

1.3.1. Enterohepatic circulation

Bile acids are C_{24} steroids with a carboxyl group at the end of the side chain (Fig. 1.5) and they are end products of cholesterol metabolism in humans and animals. After synthesis from cholesterol in liver, the steroid nucleus of the bile acids is conjugated with an amide bond at the carboxyl C_{24} position to one of two amino acids glycine or taurine (Hoffmann, 1977). The reaction is catalyzed by the amino acid N-acyltransferase enzyme (Johnson *et al*., 1990). The main deciding factor for the ratio of glycine to taurine conjugated bile acids in humans is the relative abundance of glycine and taurine and may have no functional and regulatory consequences. Conjugated bile salts are impermeable to cell membranes because of their enhanced solubility and amphipathicity as a result of conjugation. The oxygen atoms bonded to sulfur of taurocholic acid or to the terminal carbon of glycocholic acid will be ionized at physiological pH. The ionization of oxygen atom together with the planar structure of the bile acid and the hydroxyl groups present in

the rings render it highly amphipathic. Conjugation of cholic acid with glycine reduces pKa of cholic acid from 6.4 to 4.4 units ensuring that the bile acid gets completely ionized and becomes highly soluble (Hofmann and Mysels, 1992).

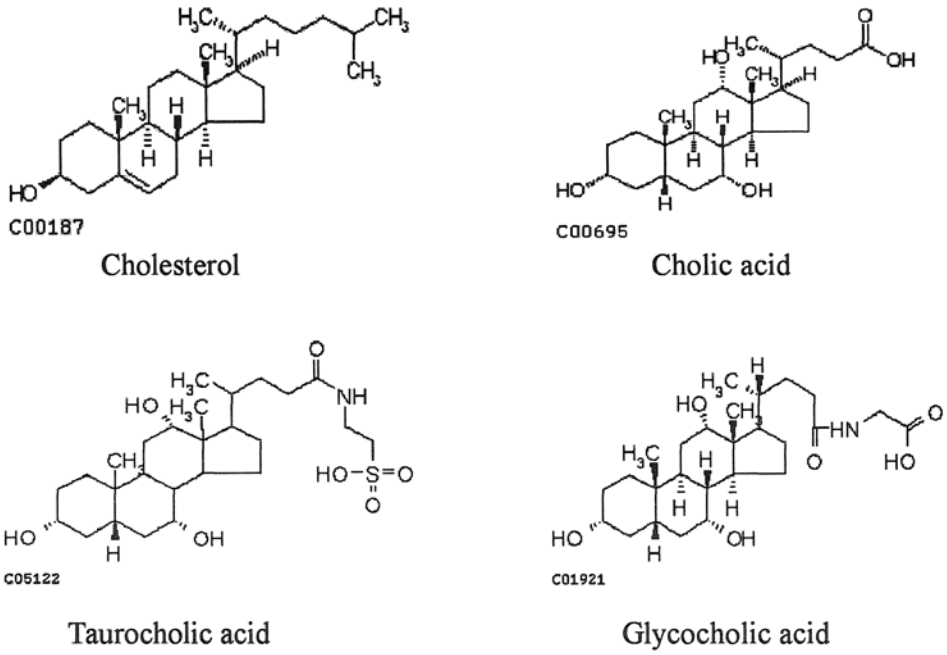

Fig. 1.5: Structure of cholesterol, free cholic acid, and conjugated cholic acid with taurine or glycine.

The gallbladder stores and concentrates bile during fasting state. Upon lipid intake the bile salts are secreted via the common bile duct into the duodenum, where they are intimately associated with dietary lipids and various digestive products (Johnson, 1998). Conjugated bile acids are not absorbed during digestion in the proximal small intestine because they are relatively bulky, ionized molecules and resistant to deamidation by pancreatic and mucosal carboxypeptidases. Instead the conjugated bile acids pass to the distal ileum, where they are efficiently absorbed by an active transport system, which is called as ileum bile acid transporter (IBAT) and the members of the ATP binding cassette (ABC) family of transporters (Lack and Weiner, 1966). After absorption, the mixture of bile salts is partly returned to the liver by hepatic portal circulation in the process known as enterohepatic circulation (Fig 1.6). Roughly 600 to 800 ml bile is produced everyday. The bile acid pool is approximately 1.5 to 4 g in size, whereas the daily, total circulating

bile acid pool is 17 to 40 g. The entire bile acid pool recirculates 6 to 15 times per day, and approximately 0.2 to 0.5 g is lost in the feces, which is repleted by *de novo* bile acid synthesis.

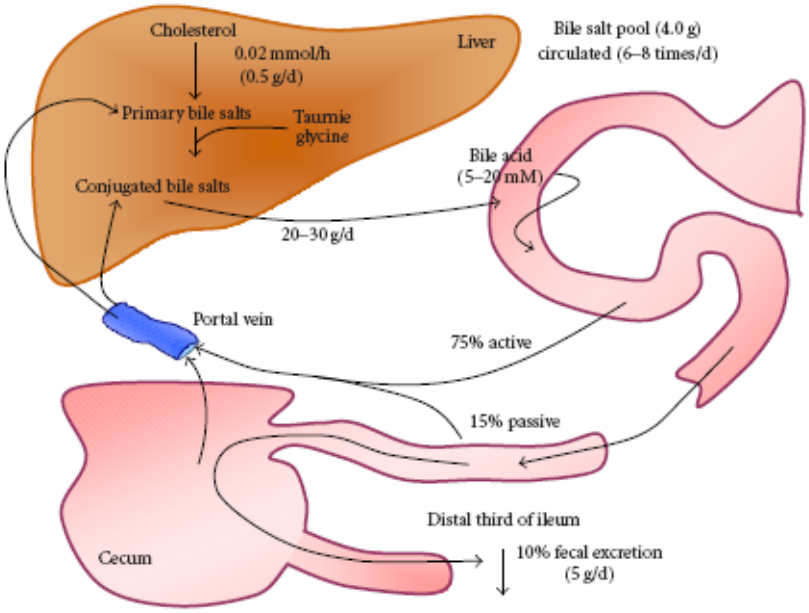

Fig. 1.6: Enterohepatic circulation of bile acids

The hydrolysis of conjugated bile acids in the distal ileum by bacterial enzyme, BSH results in the production of free bile acids, which are less efficiently reabsorbed than their conjugated counterparts. This leads to majority of free bile acids passing into large intestine or cecum, where the primary bile acids are converted into secondary bile acids. Enhanced loss of free bile acids may result in an increased requirement for cholesterol as a precursor for the *de novo* synthesis of bile salts to maintain bile salt homeostasis. There, bile salt hydrolysis by BSH enzyme could lead to reduction in serum cholesterol levels.

1.4. Bile salt hydrolase activity and its implication for BSH-producing bacterial cell

The most essential question still remains unanswered about the advantages and evolutionary importance of BSH gene for the bacteria expressing it in the human gastrointestinal tract. Many studies were conducted and several hypotheses have been proposed to answer this.

1.4.1. Nutritional role

The amino acids liberated from bile salt deconjugation could potentially be used as carbon, nitrogen, and energy sources, since glycine may be metabolized to ammonia and carbon dioxide, and taurine may be metabolized to ammonia, carbon dioxide, and sulfate. Bile salt deconjugation may therefore confer a nutritional advantage on hydrolytic strains. In support of this hypothesis, Huijghebaert *et al.* (1982) and Van Eldere *et al.* (1996) observed that certain BSH-positive strains of *Clostridium* utilized the released taurine as an electron acceptor and growth rates improved in the presence of taurine-conjugated bile salts.

1.4.2. Alteration of membrane characteristics

The composition, fluidity, permeability, hydrophobicity, and net charge of bacterial membranes all determine the extent of damage by these host defenses (Peschel, 2002). It has been proposed that BSHs facilitate incorporation of cholesterol or bile into bacterial membranes (Taranto *et al*., 1997, Dambekodi and Gilliland, 1998, Taranto *et al.,* 2003). This incorporation may increase the tensile strength of the membranes (Boggs, 1987) or may change their fluidity or charge. Cell surface modifications that may result from BSH activity could potentially offer protection against perturbation of the structure and integrity of bacterial membranes by the immune system, and such resistance mechanisms may be important in establishing persistent infections.

1.4.3. Bile detoxification

Bile salt deconjugation might be a detoxification reaction and BSH enzymes play a vital role in bile tolerance and consequently in survival in gastrointestinal tract. Although many investigators have negated this hypothesis and proposed that there is no correlation between bile tolerance and BSH activity (Taranto *et al*., (1996), Moser and Savage (2001), Ahn *et al*. (2003). Studies by three independent groups using wild type and BSH-mutant pairs provide a link between bile salt hydrolysis and bile tolerance. Grill *et al*. (2000) observed that *L. amylovorous* BSH-mutant with decreased bile salt hydrolase activity displayed decreased growth rates in presence of bile salts. Dussurget *et al*. (2002)

showed that deletion of BSH in *L. monocytogenes* decreased the MICs for both bile and bile salts. As the protonated form of bile salts are thought to exhibit toxicity through intracellular acidification in a manner similar to organic acids, BSH positive cells may protect themselves through the formation of weaker deconjugated counterparts. This could help counteract the drop in pH by recapturing and exporting the co-transported proton (Fig. 1.8). These investigations with BSH mutants strongly suggest a role for BSH enzyme in bile tolerance, and particularly tolerance to glyco-conjugated bile salts. It is possible that the contradictory studies may have used inappropriate experimental conditions; for example, using taurine-conjugated bile acids to detect BSH activity even though the majority of BSHs show a preference for glycine-conjugated bile acids. Also the bile tolerance of strains was often assayed by growth in lower concentration of bile where BSH activity may not be important. The deconjugated bile acids resulting from bile salt hydrolysis have greater inhibitory effects on bacteria than conjugated bile acids *in vitro*. However, it is possible that they are precipitated at low pHs in the intestine caused by the fermentation products of lactic acid bacteria. BSH positive strains may be associated with 7α-dehydroxylating bacteria that will dehydroxylate unconjugated bile acids (De Boever and Verstraete, 1999).

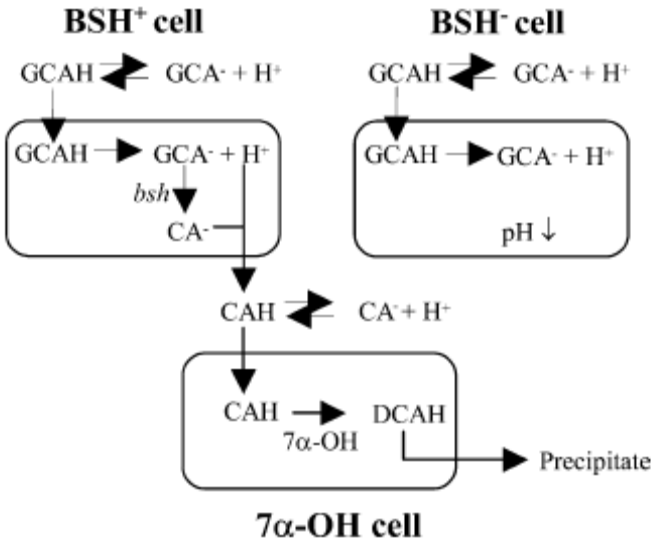

Fig. 1.8: Schematic representation of the hypothesis that bile salt hydrolysis is a detoxification mechanism. Bile salt hydrolases converts the protonated-conjugated bile

salts that enter cells to their weaker unconjugated counterparts which may recapture co-transported protons thereby preventing the excessive expenditure of ATP to maintain pH homeostasis. A large energy burden may be put on BSH negative cells since they cannot form unconjugated bile salts that trap the protons. 7α-dehydroxylating bacteria may dehydroxylate unconjugated bile acids and the resulting molecules precipitate at moderately acidic pH. Abbreviations: BSH–bile salt hydrolase, 7α-OH–7α-dehydroxylating, GCAH– glycocholic acid, CAH–cholic acid, DCAH–deoxycholic acid.

1.5. Methods of measurement for bile salt hydrolase activity

1.5.1. Qualitative assays for bile salt deconjugation

Among the qualitative assays for the presence of bile salt hydrolase from selected strains, thin layer chromatography has been selected as one of the simplest methods (Aries and Hill, 1970). After the growth of selected strains in a proper broth medium containing conjugated bile salts, conjugated and free bile acids were extracted by acidification to pH 1.0 with 6 M HCl followed by extraction with ethyl acetate (Stellwag and Hylemon, 1976). Conjugated and free bile acids could be separated in a solvent system and identified by comparing their mobilities to the mobility of known standard following development by spraying plates with phosphomolybdic acid and heating at 150°C for 10 min.

Dashkevicz and Feighner (1989) developed a differential medium for BSH active strains, based on the unique characteristics of free bile acids that are different from those of conjugated counterparts. They prepared MRS agar plate with 0.5% sodium salt of taurocholic acid (TCA), taurochenodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TCDCA) as a source of deconjugated bile salts. Among the bile salts tested, incorporation of TDCA into the medium was the best method for the selection of BSH positive strains because copious amounts of deoxycholic acid precipitated around active colonies and diffused into the surrounding medium. They proposed that this white precipitation was related to their pKa values. The taurine conjugates exhibit an apparent pKa of 1.9 in acqueous solution, whereas the pKa of unconjugated species is approximately 5.0 (Fini and Roda, 1987). Therefore at pH values achievable by acidic

fermentative metabolism, unconjugated bile acids would be protonated and hence precipitated, while taurine conjugates get completely ionized and remain in solution.

BSH activity staining method was also developed on the basis of precipitation phenomenon described by Grill and Schneider (1997). After the non-denaturing polyacrylamide gel electrophoresis, BSH activity was detected by washing the gel with sodium acetate buffer (pH 4.5) at 10°C and then incubating the gel in a reaction mixture with phosphate buffer, pH 4.5, 10 mM β-mercaptoethanol and 10 mM TDCA. BSH activity in the resulted in the form of white precipitate of deoxycholic acid at the position of the enzyme (Grill *et al*., 2000).

1.5.2. Quantitative assays for bile salt deconjugation

For the quantitative assays for BSH activity, like other enzyme assays, it can be achieved by measuring the disappearance of the substrate or the formation of the enzymatic products. Aries and Hill (1970) have measured the amount of bile conjugates and free bile acids using spectrophotometry assay. After enzymatic reaction followed by TLC separation in proper solvent conditions, areas corresponding to substrate and product were recovered by scraping and centrifugation and the absorbance of supernatant read at 385 nm. Walker and Gilliland (1993) used a modified spectrophotometric assay for the quantification of BSH activity. After growing selected bacterial cell in a growth medium containing Taurocholic acid (TCA), free cholic acid was extracted with ethyl acetate under acidic conditions and the absorbance at 660 nm was read against a reagent blank and compared with a standard curve to determine the concentration of free cholic acid.

Alternatively, BSH activity can be quantified by measuring released amounts of amino acid moiety from conjugated bile salts by BSH activity. Concentrations of glycine and taurine were determined spectrophotometrically at 570 nm by modification of ninhydrin reaction (Tanaka *et al*., 2000). For a continuous spectrophotometric assay of BSH activity, a chromogenic substrate has been developed (Kirby *et al*., 1995). Instead of amino acid group in natural bile salts, a chromophore 5-amino 2-nitro benzoic acid (ANBA) was chemically conjugated with cholic acid. Using this synthetic substrate, BSH

activity can be assayed spectrophotometrically at 420 nm which is directly related with the amounts of ANBA released from the substrate.

As a radiochemical assay, BSH activity has been quantified by using carboxy- ^{14}C Taurocholic acid (TCA) as substrate and measuring carboxy- ^{14}C Cholic acid (CA) as product of such enzymatic activity after an ethyl acetate extraction under acidic conditions. Radioactivity was measured in a liquid scintillation counter. Counts per minute were corrected with an external standard channel ratio and a ^{14}C quench curve (Feighner and Dashkevicz, 1988; Dashkevicz and Feighner, 1989; Lundeen and Savage, 1990).

Since bile salt hydrolases have activity on conjugated bile salts, enzymatic activity can be measured by the enzymatic deconjugation of sodium taurocholate or sodium glycocholate to sodium cholate. These conjugated and deconjugated free bile acids can be detected by HPLC using a C18 column or cartridge with a suitable mobile phase (Klaver and Van der meer, 1993; De Smet *et al*., 1994). Therefore bile salt hydrolase activity can be quantified by measuring the disappearance of sodium taurocholate or sodium glycocholate or the formation of cholic acid by using HPLC methods of analysis. Since radiochemical assays are quite expensive and spectrophotometric assays may not be very accurate because of the possible interference of other substances that absorb light at the wavelength of the assay, Corzo and Gilliland (1999b) proposed that HPLC assays offer the best approach. They measured the BSH activity of *L. acidophilus* based on the disappearance of sodium taurocholate or glycocholate from the reaction mixture using HPLC and suggested that HPLC assay had the advantage of accuracy and precision.

1.6. Biochemical characteristics of bile salt hydrolases

Nair *et al*. (1967) partially purified, for the first time, the bile salt hydrolyzing enzyme from *Clostridium perfringes*, and this enzyme is termed cholylglycine hydrolase (CGH) and is now commercially available (Sigma. Co.). They reported that the enzyme was intracellular and the optimum pH was between 5.6 and 5.8. It was inhibited by

iodoacetate and p-chloromercuri-benzoate, suggesting the importance of sulfhydryly group as one of the active sites on enzyme.

Aries and Hill (1970) studied the optimal activity of BSH in two strains each of *Enterococcus, Bacteroides, Bifidobacterium* and *Clostridium*. They observed the irreversible loss of BSH activity in the absence of reducing agent or in the presence of oxygen. Most of the BSH enzymes from above stated sources had a pH optimum between 5.0 and 6.0.

Stellwag and Hylemon (1976) have purified a high molecular weight BSH from *B. fragilis ssp. fragilis* ATCC 25285. They reported that the native molecular mass was 250 kD and the subunit size was 32 kD, suggesting that octameric structure (32 × 8 = 256 kD) of the purified enzyme. The enzyme had a pH optimum between 4.2 and 4.6 and showed wide range of substrate specificity for different bile salts.

Masuda (1981) studied the BSH from strains of *Bacteroides* and *Clostridium.* They reported that BSH was produced constitutively and not sensitive to oxygen, which was not the case for the BSH from other strains (Aries and Hill 1970a, Gilliland and Speck, 1977).

Gopal-Srivastava and Hylemon (1988) purified BSH from *C. perfringes* MCV 815. The subunit size as measured by SDS-PAGE was 56 kD and the native size as determined by nondenaturing PAGE, was 250 kD, suggesting a tetramer. The pH optimum was in the range of 5.8 to 6.4. By using rabbit polyclonal antibody, it was demonstrated that cell crude extract contained a single form of enzyme.

Lundeen and Savage (1990, 1992a, 1992b) reported that *Lactobacillus sp.*100-100 have a unique bile salt hydrolase system, the intracellular activity of the organism was regulated by a factor induced by conjugated bile salts. They proposed that the factor facilitated the entry of conjugated bile salts into the cell followed by enzyme action. The pH optima for the isozymes of above said enzyme was between 4.2 and 4.5. The molecular weights

were 115, 105, 95 and 80 kD and the enzymes were trimers with two peptide units of 42 and 38 kD, respectively.

Grill *et al*. (1995) purified the BSH from *B. longum* BB536, which was capable of hydrolyzing taurine and glycine conjugates of cholate, deoxycholate, chenodeoxycholate. The optimum pH was between 5.5 and 6.5 and the optimum temperature from 35°C to 42°C. Moreover, the enzyme was probably a hexamer with a native and subunit molecular mass 250 kD and 40 kD respectively.

Corzo and Gilliland (1999a) purified BSH from three strains of *L. acidophilus.* They reported that the molecular mass of the enzyme was 126 kD and the optimum pH was not the same for each conjugated bile salts ranging from 3.5 to 5.0 on taurocholate and 3.5 to 5.5 on glycocholate.

Tanaka *et al.* (2000) have reported BSH from *B. longum* SBT2928; the enzyme was reported to be sensitive to oxygen during sonication and enzyme assay, and thus the addition of 10 mM DTT was proven to be very effective to keep the activity in purification steps. The enzyme was strongly inhibited by five SH enzyme inhibitors, suggesting that a cysteine residue(s) with a free SH group play an important role in the enzyme activity. The enzyme had a native molecular mass of 125 ~ 130 kD and a subunit molecular weight of 35 kD, indicating that the enzyme was a homotetramer. The summary of purification studies on BSH from various microorganisms is shown in the Table 1.3.

1.6.1. Substrate specificity

It is not clear whether bile salt hydrolases recognize the bile acids on cholate group or amino acid groups (glycine/taurine). It may be possible that bile salt hydrolases have evolved to recognize both groups for the enzyme catalysis. Kinetic data from available literature, however, suggest that these enzymes recognize their substrates predominantly at the amino acid moieties and not at the cholate moieties (Tanaka *et al*., 2000, Kim *et al*., 2004b). Recognition of the cholate group has also been reported in the literature. A study

by Moser and Savage (2001) revealed that *L. buchneri* JCM1069 expressed taurodeoxycholic acid hydrolase activity but not taurocholic acid hydrolase activity. Taurodeoxycholic acid and taurocholic acid both have taurine as their amino acid moiety but differ at the 7 α-position of their steroid moieties. In addition, inactivation of BSH-A of *L. acidophilus* NCFM reduces the strain's ability to hydrolyze bile salts containing chenodeoxycholic as the steroid moiety, e.g., TCDCA and GCDCA (Mc Auliffe *et al*. 2005).

Most bile salt hydrolases efficiently hydrolyze glycine-conjugated bile salts than taurine-conjugated bile salts (Coleman and Hudson, 1995, Tanaka *et al.,* 2000). Sequence alignments reveal that although the amino acid residues at active site are strictly conserved in bile salt hydrolases, the residues for substrate recognition are not particularly conserved. Future structure analyses of bile salt hydrolases from various species will undoubtedly discover key residues of the active site and substrate binding pocket and provide information on the substrate selectivity of BSH enzymes (Kumar *et al*., 2006, Rossocha *et al*., 2005).

Table 1.3: Summary of purification studies for BSH from various microorganisms

Organisms	Molecular weight		Optimum pH	Location	Reference
	Native	Subunit			
C. perfringens ATCC 19574			5.6 ~ 5.8	Intracellular	Nair *et al*. (1967)
E. faecalis			<4.8	Intracellular	Aries and Hill (1970)
C. welchii			5.0 ~ 6.0	Intracellular	„
Bifidobacterium			5.0 ~ 6.0	Extracellular	„
B. fragilis $NCTC_{9343}$			5.0 ~ 6.0	Intracellular	„
B. fragilis ssp. fragilis ATCC 25285	32.5 kD	250 kD	4.2 ~ 4.5	Intracellular	Stellwag and Hylemon (1976)
B. fragilis 2536			4.5 ~ 5.0	Intracellular	Masuda *et al*. (1981)
C. perfringens PB 6K	56 kD	250 kD	4.5 ~ 5.0	Intracellular	„
B. vulgatus	36 kD	140 kD	5.6 ~ 6.4	Intracellular	Kawamoto *et al*. (1989)
L. acidophilus sp. 100-100	42 kD 38 kD			Intracellular	Lundeen and Savage (1990)
B. longum BB536	40 kD	250 kD	5.5 ~ 6.5	Intracellular	Grill *et al.* (1995)
L. acidophilus O16		126 kD	5.0 ~ 6.0	Intracellular	Corzo and Gilliland (1999b)
L. acidophilus L1		126 kD	3.5 ~ 4.5	Intracellular	„
L. acidophilus ATCC 43121		126 kD	3.5 ~ 4.5	Intracellular	„
B. longum SBT 2928	37.3 kD	130 kD	5.0 ~7.0		Tanaka *et al.* (2000)
Xanthomonas maltophila CBS 827.97	52 kD	100 kD	7.9 ~ 8.5	Intracellular	Dean *et al.* (2002)

1.7. Cloning and characterization of *bsh* genes

For the screening of *bsh* gene from the genomic library, a selective medium has been developed based on the principle of Dashkevicz and Feighner (1989), Christiaens *et al.* (1992) developed a differential medium for BSH-active *E. coli* clones using Luria bertani (LB) medium with a modification. Glucose (1%) was added for the acidification of the medium during the growth of *E. coli* and Calcium chloride (0.035%) was used since Ca^{+2} can enhance the precipitation of deoxycholic acid. Due to hydrolysis of TDCA and medium acidification, BSH-active clones can be easily detected by the formation of white precipitate around colonies. The first cloning experiment of *bsh* was carried out by Christiaens *et al*. (1992), who cloned the gene from the genomic library of *L. plantarum* 80. They identified a single open reading frame (ORF) of 972 nucleotides bounded by an ATG start codon and a translational termination codon TAA. At a distance of 10 bp from the start codon, a typical Shine-Dalgarno sequence 5'-AGGAGG-3' was found. Potential promoter sequence elements were indicated at a distance of 120 (-10 element) and 144 (-35 element) nucleotides upstream of the start codon. They reported that *bsh* gene was transcribed as a monocistronic unit, which was further confirmed by Northern blot analysis.

Coleman and Hudson (1995) screened the *bsh* gene from the genomic library of *Clostridium perfringes* 13. They identified a 990-bp ORF with a coding potential for 329-residue polypeptide. A potential Shine Dalgarno sequence was found with a spacing of 11 bases to the putative starting codon. They reported the BSH enzyme as tetramer based on the native molecular mass of 147 kD measured by gel filtration chromatography and the subunit size of 36 kD measured by SDS-PAGE as well as the calculated molecular mass based on analysis of the ORF (37,185 kD). The BSH from another strain of *Clostridium*, *C. perfringes* MCV 815, had a subunit size of 56 kD (Gopal-Srivastava and Hylemon, 1988), along with its N-terminal sequence dissimilarity between them. This supports the possibility that they are derived from a different protein family from the penicillin amidase-BSH cluster (Coleman and Hudson, 1995).

Elkins and Savage (1998) cloned the *bsh* gene along with two ORFs encoding bile acid related functions (transporter) and also three cluster ORFs as a part of coordinately regulated operon. Further studies of the molecular structure of the *bsh* gene revealed three ORFs and three genes, *cbsT1, cbsT2* and *cbsHβ* that probably constitute a BSH operon with a putative promoter sequence identified upstream of *cbsT1*. The same genetic organization was obtained from a human isolate, *L. acidophilus* KS-13. The organism has *cbsT1*, *cbsT2*, *cbsHβ* genes that are 84, 85 and 87% identical in DNA sequence to those of *L. johnsonii* 100-100.

Tanaka *et al*. (2000) cloned the *bsh* gene from *B. longum* SBT 2928 for the first time in a member of the genus *Bifidobacterium*. They confirmed the importance of –SH group at the N-terminal end by the fact that Cys1Ala exchange by site directed mutagenesis resulted in an inactive protein. Dussurget *et al*. (2002) studied the *bsh* gene encoding a functional intracellular enzyme in a pathogenic strain of *Listeria monocytogenes*.

1.7.1. Horizontal transfer of *bsh* genes

Multiple BSH homologs were observed in several bacterial strains associated with gastrointestinal tract. Since variability in BSH phenotypes has been observed within isolates of some species (Corzo and Gilliland, 1999a, Franz *et al.,* 2001, Tanaka *et al.,* 1999), it has been speculated that *bsh* genes may have been acquired horizontally (Elkins *et al.,* 2001). Comparison of the *bsh* genes and surrounding sequences of *L. acidophilus* strain KS-13 and *L. johnsonii* 100-100 by Elkins *et al*. (2001) revealed little or no synteny flanking this locus. It was also noted that *L. johnsonii* 100-100 encodes a group II intron protein (maturase *mat*) downstream of *bsh*. In addition to reverse transcriptase activity, these proteins can function as maturases and endonucleases and facilitate movement and splicing of cDNA into the genome. Sequencing of the entire genome of *L. acidophilus* NCFM revealed that this strain possesses two *bsh* genes (*bsh-A* and *bsh-B*). The predicted sequence of the BSH enzymes encoded by these loci share a higher level of similarity to BSH enzymes from other *Lactobacillus* species than to each other, suggesting that they may have been acquired from different sources (Mc Auliffe *et al.,* 2005).

The *L. monocytogenes bsh* gene is absent from the genome of the nonpathogenic strain *L. innocua*, whereas flanking regions have the same organization in both (Dussurget *et al.,* 2002). In addition, the G + C content of the gene is lower than that of neighboring genes (36% versus 38% to 41%) but similar to the G + C content of the *L. plantarum bsh*, and the encoded protein shows most homology to the BSHs of lactobacilli (67% identity to the BSH of *L. plantarum*). These observations, together with the fact that *L. monocytogenes* shares the same microenvironments as lactobacilli during its life cycle (intestine, decaying vegetation, food, and vegetables) strongly suggest that the listerial gene may have been acquired from lactobacilli. It has also been noted that the *bsh* gene in *B. longum* SBT2928 is flanked by inverted repeats (Tanaka *et al.,* 2000), which may have played a role in the horizontal transfer of the gene. However, a study by Kim *et al.* (2004a) revealed that *bsh* genes were highly conserved within all bifidobacterial strains tested, and their G + C content reflected the overall G + C content of the genome.

Table 1.4: Genetic information of *bsh* genes from various microorganisms

Organisms (accession no) [1]	**G+C %**	**ORF**	**Theoretical**		**Reference**
			pI	**Mol.wt**	
L. plantarum 80 (A24002)	33.44	975 bp	5.12	37.08	Christiaens *et al*. (1992)
C. perfringes 13 (U20191)	27.50	990 bp	5.26	37.18	Colemon and Hudson (1995)
L. johnsonii 100-100 (AF09471)	38.38	951 bp	4.88	34.914	Elkins and Savage (1998)
L. acidophilus Ks13 (AF091248)	37.12	951 bp	5.29	35.055	Savage and Moser (1999)
B. longum SBT 2928 (AF148138)	59.34	954 bp	4.71	35.155	Tanaka *et al*. (2000)
L. gasseri ADH (AF30558)	33.23	978 bp	4.76	36.132	Russel and Klaenhammer (2001)
L. johnsonii 100-100 (AF297873)	33.94	981 bp	5.05	36.666	Elkins *et al*. (2001)
L. monocytogenes EDG-e (NP_465591)	36.40	978 bp	5.02	36.848	Dussurget *et al*. (2002)
Organisms (derived genomic sequences)					
L. plantarum WCFS NP_786739	33.44	975 bp	5.12	37.042	Kleerebezem *et al*. (2003)
L. gasseri L gas_3 (ZP_00046148)	33.74	978 bp	4.85	36.270	NCBI (2003a)
E. faecalis V583 (NP_814249)	30.67	975 bp	4.90	36.932	Paulsen *et al*. (2003)
E. faecium FAIR-E 345 (AY260046)	30.56	975 bp	4.97	36.645	Wijaya *et al*. (2003)
C. perfringens str 13 (NP_561625)	27.47	990 bp	5.26	37.185	Shimizu *et al*. (2002)
B. longum NCC 2705 (NP_695975)	59.54	954 bp	4.71	35.125	Schell *et al*. (2002)

1: Gen Bank Accession Number

1.8. Applied aspects

1.8.1. Bile salt hydrolase and hypocholesterolemic effect

Potential hypocholesterolemic pharmaceuticals and foods products are continuously being developed in order to control serum cholesterol levels in hypercholesterolemic patients. These pharmaceuticals are mostly based on the interruption of enterohepatic circulation (EHC) of bile salts. Recently, many studies have been carried out to elucidate the cholesterol lowering effect of probiotics with bile salt hydrolase activity with the same concept based on the interruption of EHC. So far this theory has been one of the successful tools for explaining the hypercholesterolemic mechanisms of probiotics.

Even though the hypocholesterolemic mechanism of probiotics has not yet been fully understood, it is an established fact that cholesterol and bile salt metabolism is closely linked. De Smet *et al*. (1994) suggested that the consumption of BSH-active strains, or cultured products containing them might bring about a lowering of serum cholesterol levels through an interaction with hosts bile salt mechanism. The proposed mechanism of hypocholesterolemic effect is comparable with that of bile salt sequestrants, which bind bile salts and prevent them from being reabsorbed (Suckling *et al*., 1991).

Recently, the hypocholesterolemic effects of probiotics, which showed high BSH activities from *in vitro* trails, have been confirmed in human (Anderson and Gilliland, 1999) as well as in animals (De Rodas *et al*., 1996; Taranto *et al*., 1998; De Smet *et al*., 1998). It has also been reported that it reduces serum cholesterol levels by 22% to 33% (Jones *et al*., 2004; Lim *et al*., 2004). Even a small reduction in serum cholesterol of 1% can reduce risk of coronary heart disease by 2-3% (Gilliland *et al*., 1985; Manson *et al*., 1992). Moreover, considering that a number of commercial probiotic strain exhibit BSH activities, further studies will be needed to determine whether the BSH activity of the probiotics is beneficial or detrimental to the host.

1.8.2. Immobilization studies

Although oral administration of free bacteria with bile salt hydrolase activity has promising effect on cholesterol reduction, it has several limiting factors. For example of those free bacteria ingested, only 1% survives in the gastrointestinal transit, limiting the overall therapeutic effect (De Smet *et al.,* 1994). Also, oral administration of live bacterial cells can cause a host immune response and can be retained in the intestine, replacing natural flora (Taranto *et al.,* 2000, Chin *et al.,* 2000). Administration of encapsulated bacteria have several advantages to free bacteria in such context.

Jones *et al.* (2004) studied the potential of artificial cell microencapsulated genetically engineered *Lactobacillus plantarum* 80 cells (pCBH1) for oral delivery applications In their study, the authors encapsulated *L. plantarum* 80 in calcium alginate and coated with 1% solution of poly-L-lysine and low viscosity alginate. The authors claimed that microencapsulated *L. plantarum* 80 can effectively breakdown the conjugated bile acids, glycodeoxycholic acid (GDCA) and taurodeoxycholic acid (TDCA) with bile salt hydrolase activities of 0.19 and 0.08 μmol DCA/mg CDW/h respectively.

Investigation of oral formulations in the GI environment is crucial before further testing in animal models to understand the potentials and limitations of delivery formulations. With this aim, Martoni *et al.* (2007) studied the potential of microencapsulated live BSH active cells in a computer-controlled dynamic human GI model. The apparatus consisted of a series of bioreactors, which mimics the gradual transit of ingested food products and therapeutics through the human digestive tract in which *in vivo* conditions with regard to pH, temperature, bacteria, enzyme types and activities, volume, agitation, agitation and food particles are closely simulated. The microcapsules were found to be adept at protecting the encased cells by maintaining cell viabilities even at less acidic stomach pH conditions (pH 2.0-3.0). Survival enhancement offered by alginate- poly L-lysine-alginate (APA) encapsulation in this study include the cross linked membrane providing physical barriers against the entry of harmful components found in GI tract. Furthermore, the alginate core may offer a buffering capacity thus limiting the hostile effect induced by the low pH in the stomach.

Although genetically modified organisms pose no greater risk than the original unmodified product (Redenbaugh *et al.,* 1995), there remain public and scientific concerns about the safety of gene manipulation technology. There is a possibility that repeated large doses of microorganisms result in transfer of genes to organisms in the environment (Gruzza *et al.,* 1994). To address this problem, Martoni *et al.* (2008) screened different naturally occurring bacteria for bile salt hydrolase activity. The highest bile salt hydrolase producers among the screened candidates were *Lactobacillus reuteri* and *Bifidobacterium longum*. These microorganisms were further microencapsulated in alginate- poly L-lysine-alginate (APA) matrix and subjected to acid and bile challenge in simulated human GI model. Results showed that *L. reuteri* is acid-and bile salt-tolerant and has significant cholesterol lowering properties.

Lambert *et al.* (2008) investigated on the system of whey protein-gum arabic encapsulates using activated whey proteins. Bile salt hydrolase microencapsulates were prepared using fluidized bed coating technique, applying a coat of a mixture of bile salt hydrolase enzyme from *Lactobacillus plantarum* WCFS1 and heat-denatured whey protein onto microcrystalline cellulose cellets. The heat treatment caused whey proteins to cross-link naturally using disulphide bonds. Microencapsulates were subsequently coated with gum arabic enabling reversible formation of a protein-gum arabic coacervate at low pH. Whey protein-gum arabic encapsulates prepared by the above method allowed highly effective protection of the enzyme under gastrointestinal conditions and led to release of enzyme in the proximal small intestine due to the effect of intestinal pH and pancreatin. Although food grade microencapsulates provide a better approach to withstand harsh gastrointestinal tract and to maintain viability of bile salt hydrolase active bacteria for oral delivery applications, further studies are required in terms of storage and shelf life.

1.9. Conclusions

There is a tremendous increase in the number of people suffering from cardiovascular diseases in the developing countries like India. During the past five decades, rates of coronary artery disease among urban populations have risen from 4 per cent to 11 per cent due to modernization and stressful life styles. Cardiovascular diseases are associated with excessive serum cholesterol levels. Currently available medications for the treatment are expensive and have severe side effects. There is a need for continuous research in this area due to increase in the demand for medication and also there is a need to develop more natural method to decrease serum cholesterol concentration in humans. In this regard, bile salt hydrolase can be considered as an alternative therapy for lowering serum cholesterol levels. Bile salt hydrolase, the newly added member of Ntn hydrolase superfamily is also a topic of academic interest. The molecular structure of bile salt hydrolase has been recently determined and the amino acids involved in catalysis were studied. Still, much work has to be carried out in this direction to understand the mechanism of action and also the effect of bile salt hydrolase on humans. Many clinical trials needed to be done using animal and human models before considering bile salt hydrolase as a natural and an alternative approach for the treatment of hypercholesteremia.

Chapter 2

Isolation, identification of the thermophile, *Brevibacillus borstelensis* and optimization of fermentation conditions for the production of bile salt hydrolase

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The work presented in this chapter has been **published**:

Sridevi, N., Prabhune, A.A (2008) **Applied Biochemistry and Biotechnology**, doi: 10.1007/s12010-008-8326-9.

Abstract

A thermophilic microorganism producing intracellular bile salt hydrolase was isolated from hot water springs at Pali, near Konkan, Maharashtra, India. This is the first report of bile salt hydrolase from a thermophilic source. Based on phenotypic analysis and 16S rDNA sequencing, the isolate was identified as *Brevibacillus borstelensis.* Optimization of fermentation conditions resulted in enhancement in the production of bile salt hydrolase from this thermophile. The enzyme production was enhanced when sodium glutamate medium was used as growth medium. Peak in enzyme production was attained after 12 h fermentation period with this medium. Cell bound bile salt hydrolase activity was optimum when the initial pH of the medium was 6.0 and incubated at temperature (55°C). Culture conditions optimization resulted in a 2.9-fold enhancement in enzyme production.

2.1. Introduction

Bile salt hydrolysis is a biologically significant reaction among the bacterial alterations of bile acids. The enzymes responsible for bile salt hydrolysis are Bile salt hydrolases (BSH, EC 3.5.1.24). BSH catalyzes the hydrolysis of glycine and taurine conjugated bile salts to amino acid residues and free bile acids. High blood cholesterol level is a major risk factor for coronary heart disease. Although different drugs are available for the treatment, they are all known to have side effects. The possibility of using bile salt deconjugation to lower serum cholesterol levels in hypercholesterolemic patients and prevent hypercholesterolemia in normal people is of great interest at present (De Smet *et al.,* 1994). The microorganisms exhibiting BSH activity could be beneficial because they have the potential to reduce serum cholesterol (Anderson and Gilliland, 1999; Pereira and Gibson, 2002).

In humans and other mammals, cholesterol balance is achieved by modulating both synthesis and excretion. Cholesterol excretion is mediated by bile acids, the water-soluble amphipathic molecules formed from cholesterol in the liver (Hofmann, 1999). Bile salts play an important role in lipid digestion and absorption in the small intestine. Bile salts circulate back to the liver through a process known as enterohepatic circulation (Russell, 2003). BSH generates less water-soluble bile acids (to be excreted via feces) with its deconjugation property. This drain of bile salts results in a reduction of serum cholesterol because the replacement of bile salts would require the utilization of cholesterol in the body.

As described in the Chapter- I, several bacteria isolated from various sources have been shown to produce bile salt hydrolase activity. It was observed that bile salt hydrolase is mainly produced by bacteria in the gastrointestinal tract (Kim and Lee, 2005). In addition to autochthonus intestinal microbiota, BSH activity was also reported from an enteropathogenic strain of *Listeria monocytogenes* (Dussurget *et al.,* 2002) and bile adapted strain of *Xanthomonas maltophila* (Dean *et al.,* 2002). To the best of our knowledge there are no reports so far regarding the production of bile salt hydrolase from the thermophilic source. To investigate the production of BSH from thermophilic source,

we have isolated a novel thermophilic microorganism from a hot water springs, near Konkan, Maharashtra, India. We report here for the first time, the production of intracellular bile salt hydrolase from a thermophilic source. The present Chapter describes the isolation of a thermophilic bacterium with BSH activity and optimization of growth conditions for the production of BSH.

2.2. Materials and Methods

2.2.1. Materials

Yeast extract, peptone, and sodium chloride were obtained from Hi-media, India. 2% ninhydrin reagent solution, trichloroacetic acid and bile salts (GDCA, GCA, TDCA, TCA, TchDCA, GchDCA) were obtained from Sigma. QIAquick PCR purification kit was obtained from QIAGEN. All other chemicals were commercially available with high purity or analytical grade compounds. All media were prepared in distilled water and buffers, in glass distilled water.

2.2.2. Source and Isolation of thermophile

A combined sediment-water samples were collected from hot water springs, near Konkan, Maharashtra, India. Location and Altitude is 18° 05′ S, 73° 020′ E and 40 mts above the sea level. The temperature at the site of collection was 60°C. These samples were onsite inoculated into tubes containing 10 ml nutrient broth and incubated at 65°C for 96 h with shaking at 200 rpm. Tubes showing growth were sub-cultured for the desired isolation. Isolated colonies on lysogeny broth agar plates containing 0.025% glycodeoxycholic acid were further characterized.

2.2.3. Morphological, biochemical and physiological characterization

Gram staining was performed for the cells grown on nutrient agar. Catalase, oxidase and nitrate reduction tests were carried out as described by Smibert and Krieg, 1981. Motility test was assessed by hanging drop preparation. Sugar utilization experiments were carried out in peptone water base with the addition of 1% (w/v) of glucose, xylose, arabinose and manitol as a sole carbon source at 55°C. The dimensions of the bacterial isolate were

determined by using a Leica stereoscan 440 Electron microscope. This was done by placing a drop of whole cell suspension on silica wafer, then dried and coated with gold.

2.2.4. Bile salt hydrolase assay

The isolates were screened for BSH activity by modified ninhydrin method according to Suresh Kumar *et al.* (2006). Briefly, the cultures were grown in lysogeny broth at 50°C for 12 h at 160 rpm and centrifuged at 12,000 × g at 4°C for 2 min. The cell pellet was washed with potassium phosphate buffer pH 6.5, centrifuged and the wet weight of the pellet was determined respectively. The cell pellet was suspended in 100 µl reaction mixture containing 10 mM potassium phosphate buffer pH 6.5, 10 mM DTT, and 10 mM sodium glycodeoxycholate. The mixture was incubated at 40°C for 30 min and then terminated by adding 100 µl of trichloroacetic acid (15% w/v). The mixture was centrifuged and 50 µl of the supernatant was mixed with 50 µl of 2% ninhydrin reagent solution. The preparation was thoroughly mixed and boiled for 14 min. The absorbance of the cooled sample was recorded at 570 nm. BSH activity per gram of whole cells was determined by using a standard curve prepared with glycine. One unit of BSH activity was defined as the amount of enzyme that liberated 1 µmole of amino acid from the substrate per minute per gram wet weight of cells.

2.2.5. 16S rDNA sequencing

Genomic DNA was isolated and quantified according to Marmur, (1961). The 16S r DNA was amplified from 100 ng of genomic DNA using primers 121 F (5'GGC GGA CGG GTG AGT AAT 3') and 1488 R (5'CGG TTA CCT TGT TAC GAC TTC ACC 3') (Herrera-Cervera *et al*., 1999). Thermal cycling was performed at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 55°C for 30 sec, 72°C for 1.5 min and a final extension step of 72°C for 10 min. The amplified PCR product was purified and cloned in pGEMT easy vector. The nucleotide sequences were determined using Big Dye terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, and an Applied Biosystems 3730 DNA Analyzer. The partial 16S rDNA sequence was searched for highly similar nucleotide sequences using NCBI BLAST program. Retrieved sequences were aligned by CLUSTAL W program and manually edited. The Phylogenetic tree was constructed by

the Neighbor Joining method, using Jukes-Cantor algorithm in Molecular Evolutionary Genetic Analysis (MEGA) suite version 4.0 (Tamura *et al*., 2007). Bootstrap values were calculated for 1000 trees. The 16S rDNA sequence determined in this study has been deposited in the NCBI data bank with the accession number EU 251074.

2.2.6. Optimization of fermentation parameters

Optimization of different nutrient and physical parameters for BSH production was studied by maintaining all factors at constant level except the one being studied. Erlenmeyer flasks (250 ml) containing 50 ml of sterilized media were inoculated with 10% inoculum and shaken at 160 rpm, 55°C for 12 h. Different growth and fermentation parameters were studied on the cell level as follows.

2.2.7. Media optimization

The effect of medium components on growth of the isolate was studied to increase the enzyme production. Erlenmeyer flasks (250 ml) containing 50 ml of sterilized media were inoculated with 10% seed culture and shaken at 160 rpm, 55°C for 12 h. Following different media were used for the optimum production of BSH from the isolated thermophile: **1.** Lysogeny broth: (g l^{-1}) tryptone 10; yeast extract 5; NaCl 10; pH 7.0. **2.** Nutrient broth: (g l^{-1}) peptone 10; beef extract 10; NaCl 5; pH 7.0. **3.** Sodium glutamate: (g l^{-1}) yeast extract 5; peptone 10; NaCl 2; sodium glutamate 5; pH 7.0. **4.** Minimal medium: (g l^{-1}) $Na_2HPO_4.7H_2O$ 12.8; KH_2PO_4 3.1; NaCl 0.5; NH_4Cl 1; $MgSO_4.7H_2O$ 0.2; glucose 4; yeast extract 5; pH 7.0. **5.** MGYP medium: (g l^{-1}) malt extract 3; yeast extract 3; glucose 20; peptone 10; pH 7.0.

2.2.7.1. Time kinetics profile

Cells were grown in sodium glutamate medium at 55°C on a rotary shaker at 160 rpm. 2 ml samples were withdrawn and centrifuged at intervals of 6 h and BSH activity/g cells was determined. BSH production profile and cell growth of the culture was monitored under shake flask conditions up to 72 h.

2.2.7.2. Effect of initial pH

Production of BSH at different pH values was determined by cultivating the isolate in different initial pH values, in the range pH 5.0-9.0 of the selected medium from the media optimization. Cells were grown in 50 ml of the selected medium for 12 h on a rotary shaker at 160 rpm. Cells were harvested after 12 h and the BSH activity/g was determined.

2.2.7.3. Effect of incubation temperature

Influence of temperature on BSH activity was studied by cultivating the isolate at various incubation temperatures ranging from 40°C to 65°C. Cells were grown in selected medium for 12 h on a shaker (160 rpm) at the above mentioned temperature range. BSH activity was determined as activity/g after 12 h incubation time.

2.2.7.4. Effect of carbon source

The effect of different carbon sources on growth and bile salt hydrolase activity produced in shake flasks was assayed by substituting minimal medium with various carbon sources (0.5%). Simple carbon sources, including glucose, xylose, lactose, mannose, fructose, manitol, galactose, glucose and sorbitol were used. Minimal medium without carbon source served as control.

2.2.7.5. Effect of nitrogen source

The influence of various nitrogen sources on cell growth and enzyme yield was also tested. Nitrogen sources (0.6%) such as yeast extract, peptone, tryptone and beef extract supplemented seperately in minimal medium were used. Cells were allowed to grow in this medium for 12 h at 55°C on a rotary shaker. BSH activity/g of cells was determined as described earlier. Minimal medium without nitrogen source was served as control.

2.2.7.6. Substrate specificity

Substrate specificity was determined by measuring the BSH activity under standard assay conditions with six different bile salts (10 mM). Major bile salts such as Glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GChDCA),

taurocholic acid (TCA), taurodeoxycholic acid (TDCA) and taurochenodeoxycholic acid (TChDCA) were used to determine substrate specificity.

2.3. Results and Discussion

2.3.1. Source and Isolation of thermophile

Five sediment-water samples were collected from hot water springs of Konkan areas in India. These samples were used as inocula for screening and enrichments of aerobic, thermophilic microorganisms. Isolate-2, which exhibited BSH activity and growth in presence of bile salt mixture, was selected for further studies (Table 2.1).

Table 2.1: Screening of thermophiles for BSH activity

Thermophile	Growth in presence of 0.025% bile salt mixture	BSH activity
Isolate - 1	–	–
Isolate - 2	+	+
Isolate - 3	–	–
Isolate - 4	–	–
Isolate - 5	–	–

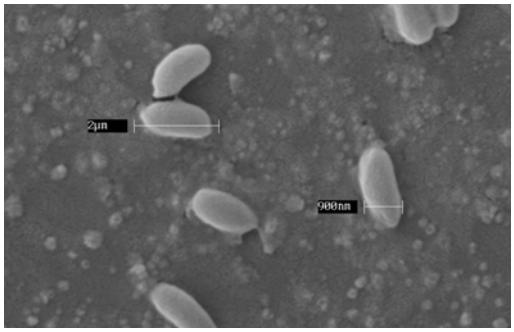

Fig. 2.1: Scanning electron micrograph of thermophilic isolate-2. Bars correspond to measurement of the whole cell dimension. Magnifications of the image is 10 K.

Table 2.2: Morphological, biochemical and physiological characteristics of isolate-2

Characteristics	Presence (+) or absence (-) in isolate:	
Colour of colonies	Brownish white	
Margin	Smooth	
Elevation	Raised	
Opacity	Opaque	
Consistency	Butyrous	
Width (μm)	2	
Length (μm)	0.9	
Gram staining	+	
Motility	-	
Temperature range (^{0}C)	40 – 65	
pH range	5.0-9.0	
Catalase	+	
Oxidase	+	
Nitrate reduction	-	
Indol	-	
Voges-proskauer	-	
Citrate utilisation	-	
Amylase	+	
Protease	+	
Lecithinase	-	
Sugars utilized	Acid	Gas
Arabinose	+	-
Mannitol	+	-
Xylose	+	-
Glucose	+	-
Mannitol	+	-

2.3.2: 16S rDNA sequencing

The isolated thermophile exhibiting BSH activity (isolate-2) was further identified by 16S rDNA sequencing. About 1400 bp sequence of the 16S rDNA was compared to sequences of related bacteria. BLAST analysis showed high nucleotide sequence similarity (99%) with *Brevibacillus borstelensis* DSM 6347T (AB112721). The subsequently constructed phylogenetic tree revealed that the isolate clusters with

representatives of the genus *Brevibacillus* (Fig. 2.4). The phylogenetic analysis, based on the 16S rDNA sequence showed that the isolated thermophile is phylogenetically related to the genus *Brevibacillus*. The threshold for similarity to be considered as the same species is 97%, according to Stackebrandt and Goebel (1994). 16S rDNA analysis of the isolate showed the sequence to be 99% similar to *Brevibacillus borstelensis* type strain DSM 6347 T (AB 112721).

The isolate was Gram positive, aerobic, rod shaped, catalase and oxidase positive. Nitrate reduction tests were negative. Growth of the isolate was observed within a temperature range of 40°C to 65ºC and pH range of 5.0 to 9.0. Acid production was seen in presence of various sugars but formation of gas was not observed. Members of the genus *Brevibacillus* share similar biochemical characteristics (Shida *et al*., 1996). These data suggest that this isolate does belong to this genus and more specifically to *Brevibacillus borstelensis*. The isolated strain of *Brevibacillus* sp constitutes the first report of a thermophilic producer of bile salt hydrolase.

Bile salt deconjugation liberates amino acids such as glycine and taurine, which can be used as carbon source by bacterial strains (Begley *et al*., 2005). The property of deconjugation of bile salts exhibited by gastrointestinal bacteria may be a detoxification mechanism and BSH produced by this bacteria may play a role in bile tolerance and consequently in survival in the gastrointestinal tract. To date BSH activity has not been detected in bacteria isolated from environment in which bile salts are absent (Begley *et al*., 2006). In our present study, we have isolated a thermophile, *Brevibacillus borstelensis* exhibiting BSH activity from hot water springs. The environmental condition of which is quite different from the gut environment, the source for the isolation of BSH positive microorganisms. However it can be presumed that there may be a chance of presence of traces of bile salts in the hot water springs. It can also be postulated that *Brevibacillus borstelensis* must have acquired bile salt hydrolase gene through horizontal gene transfer from the BSH positive organisms dwelling in the same environment of hot water springs. Bile salt hydrolase activity exhibited by *Brevibacillus* sp may also be a

detoxification mechanism to detoxify compounds present in the particular environment. Physiological role of BSH production in the isolated thermophile needs to be explored.

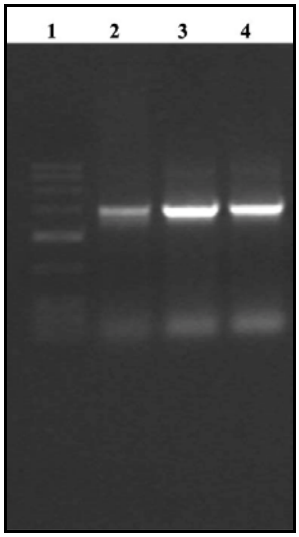

Fig. 2.2: Agarose gel (1%) electrophoresis of PCR amplified product.
Lane 1: Low range DNA ladder (B. Genei), Lane 2, 3, 4: PCR product

Table 2.3: First ten BLAST result hits obtained from NCBI for isolated thermophile 16S rDNA sequence**.**

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU251074.1	Brevibacillus borstelensis isolate NS 1846 16S ribosomal R	2617	2617	100%	0.0	100%
EU714902.1	Brevibacillus borstelensis strain SRDTh1 16S ribosomal RN	2584	2584	100%	0.0	99%
AF378230.1	Brevibacillus borstelensis strain LMG 15536 16S ribosomal	2584	2584	100%	0.0	99%
AB112721.1	Brevibacillus borstelensis gene for 16S rRNA, partial seque	2584	2584	100%	0.0	99%
AB116134.1	Brevibacillus borstelensis gene for 16S ribosomal RNA, pa	2584	2584	100%	0.0	99%
AJ586382.1	Brevibacillus borstelensis partial 16S rRNA gene, strain R-	2584	2584	100%	0.0	99%
FJ529038.1	Brevibacillus sp. ES-SL-1 16S ribosomal RNA gene, partial	2582	2582	99%	0.0	99%
AM910272.1	Bacillus sp. R-30914 partial 16S rRNA gene, strain R-3091	2580	2580	100%	0.0	99%
EU714903.1	Brevibacillus borstelensis strain SRDTh2 16S ribosomal RN	2579	2579	100%	0.0	99%
AY373322.1	Brevibacillus borstelensis strain R-7201 16S ribosomal RN	2579	2579	100%	0.0	99%

Brevibacillus borstelensis isolate NS 1846 16S ribosomal RNA gene, partial sequence

Features Sequence

```
LOCUS       EU251074                1417 bp    DNA     linear   BCT 24-NOV-2007
DEFINITION  Brevibacillus borstelensis isolate NS 1846 16S ribosomal RNA gene,
            partial sequence.
ACCESSION   EU251074
VERSION     EU251074.1  GI:160432053
KEYWORDS    .
SOURCE      Brevibacillus borstelensis
  ORGANISM  Brevibacillus borstelensis
            Bacteria; Firmicutes; Bacillales; Paenibacillaceae; Brevibacillus.
REFERENCE   1  (bases 1 to 1417)
  AUTHORS   Sridevi,N., Deshpandey,N. and Prabhune,A.A.
  TITLE     Brevibacillus borstelensis gene for 16S rRNA, isolate NS1846
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1417)
  AUTHORS   Sridevi,N., Deshpandey,N. and Prabhune,A.A.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-OCT-2007) Biochemical Sciences Division, National
            Chemical Laboratory, Lab No 1846, Pashan Road, Pune, Maharashtra
            411008, India
FEATURES             Location/Qualifiers
     source          1..1417
                     /organism="Brevibacillus borstelensis"
                     /mol_type="genomic DNA"
                     /isolate="NS 1846"
                     /db_xref="taxon:45462"
     rRNA            <1..>1417
                     /product="16S ribosomal RNA"
ORIGIN
        1 ggcggacggg tgagtaatac gtaggcaacc tgcccgtaag ctcgggataa catggggaaa
       61 ctcatgctaa taccggatag ggtcttctct cgcatgagag gagacggaaa ggtggcgcaa
      121 gctaccactt acggatgggc ctgcggcgca ttagctagtt ggtggggtaa cggcctacca
      181 aggcgacgat gcgtagccga cctgagaggg tgaccggcca cactgggact gagacacggc
      241 ccagactcct acgggaggca gcagtaggga attttccaca atggacgaaa gtctgatgga
      301 gcaacgccgc gtgaacgatg aaggtcttcg gattgtaaag ttctgttgtc agagacgaac
      361 aagtaccgtt cgaacagggc ggtaccttga cggtacctga cgagaaagcc acggctaact
      421 acgtgccagc agccgcggta atacgtaggt ggcaagcgtt gtccggaatt attgggcgta
      481 aagcgcgcgc agcggcctat gtaagtctgt gttaaagccc ggggctcaac cccggttcgc
      541 atcggaaact gtgtagcttg agtgcagaag aggaaagcgg tattccacgt gtagcggtga
      601 aatgcgtaga gatgtggagg gacaccagtg gcgaaggcgg ctttctggtc tgtaactgac
      661 gctgaggcgc gaaagcgtgg ggagcaaaca ggattagata ccctggtagt ccacgccgta
      721 aacgatgagt gctaggtgtt gggggtttca ataccctcag tgccgcagct aacgcaataa
      781 gcactccgcc tggggagtac gctcgcaaga gtgaaactca aaggaattga cgggggcccg
      841 cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg aagaacctta ccaggtcttg
      901 acatcccgct gaccgtccta gagatagggc ttcccttcgg ggcagcggtg acaggtggtg
      961 catggttgtc gtcagctcgt gtcgtgagat gttgggttaa gtcccgcaac gagcgcaacc
     1021 cttatcttta gttgccagca ttcagttggg cactctagag agactgccgt cgacaagacg
     1081 gaggaaggcg gggatgacgt caaatcatca tgccccttat gacctgggct acacacgtgc
     1141 tacaatggct ggtacaacgg gaagctagct cgcgagagta tgccaatctc ttaaaaccag
     1201 tctcagttcg gattgcaggc tgcaactcgc ctgcatgaag tcggaatcgc tagtaatcgc
     1261 ggatcagcat gccgcggtga atacgttccc gggccttgta cacaccgccc gtcacaccac
     1321 gggagtttgc aacacccgaa gtcggtgagg taaccgcaag gagccagccg ccgaaggtgg
     1381 ggtagatgac tggggtgaag tcgtaacaag gtaaccg
//
```

Fig. 2.3: 16S rDNA sequence of the isolate-2 (*Brevibacillus borstelensis*)

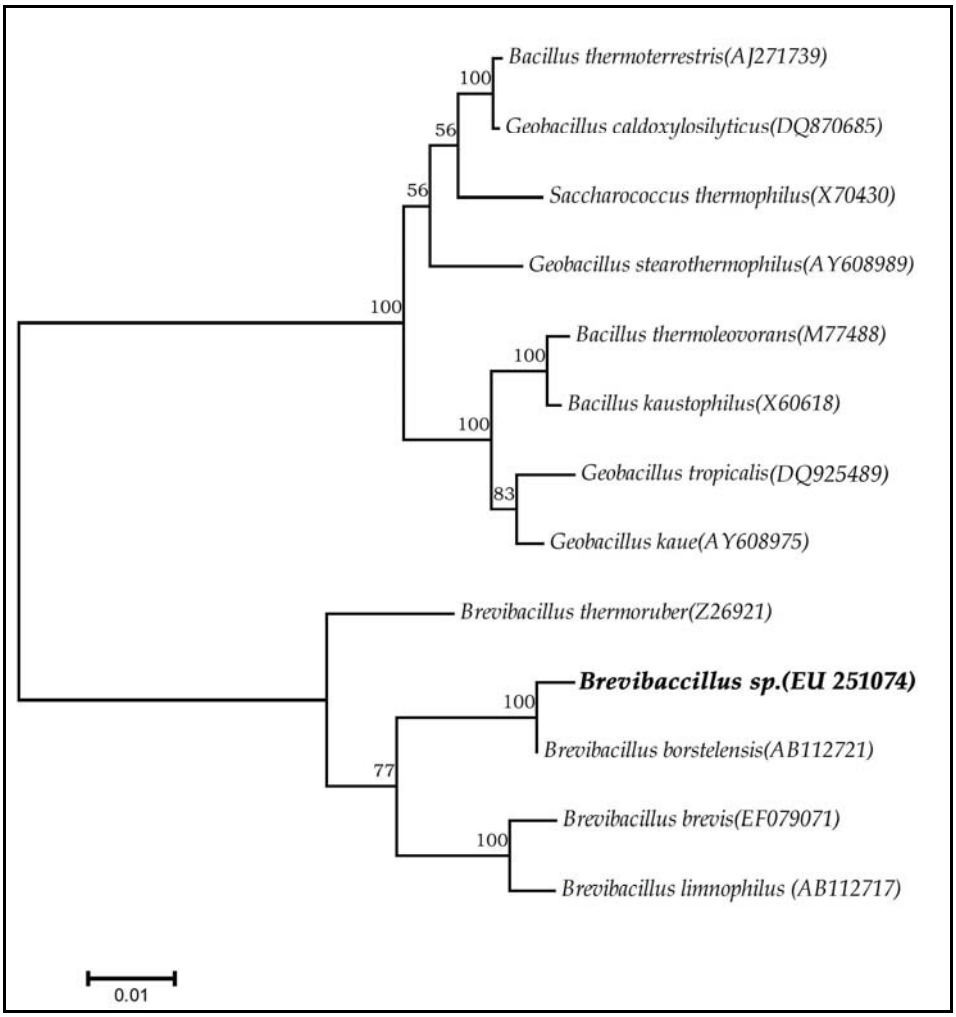

Fig. 2.4: Phylogenetic tree based on 16S rDNA sequences showing the position of isolate among its closely related organisms. Database accession numbers are shown in parentheses after species, strain, or name Values displayed at the nodes (n = 1000) indicate bootstrap values. The tree was constructed by the neighbor-joining method. The scale bar represents 0.01 nucleotide substitution per position.

2.3.3. Optimization of fermentation parameters

Screening of different thermophiles for bile salt hydrolase activity resulted in the identification of *Brevibacillus borstelensis*, which is new thermophilic source of bile salt hydrolase. The production of high titres of any enzyme by optimizing the growth parameters is of prime importance in enzymology. The optimization of various nutritional

and physical parameters to which an organism exposed is known to significantly increase the product yield. Since BSH is a pharmaceutically important enzyme, optimization of its production to increase yield is of great importance. To achieve the maximum production of enzyme from this thermophilic source, culture conditions were optimized.

Among the different media tested for enzyme production, maximum enzyme yield was observed in sodium glutamate medium (Fig. 2.5 a). So this medium was used for the rest of the experiments. Minimal medium and Skimmed milk medium did not support the enzyme production.

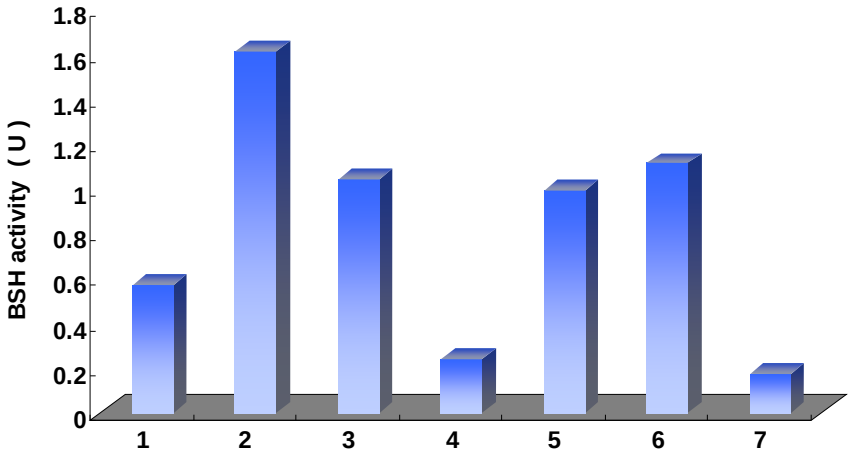

Fig. 2.5 a. Media optimization of bile salt hydrolase. The following different media were used 1) Lysogeny broth 2) Sodium glutamate 3) Nutrient broth 4) Minimal medium 5) EMS 6) MGYP 7) Skimmed milk

2.3.3.1. Time kinetics profile

Time course for growth of *Brevibacillus borstelensis* and BSH production was observed in shake flask conditions (Fig. 2.5 b). In effect, the maximal growth was attained after 24 h and slowed down later. The enzyme activity appeared to be maximum after 12 h fermentation period and decreases sharply between 24 to 72 h. Optimum production of BSH from *Brevibacillus borstelensis* was observed in the exponential phase of cell growth, which is in contrast to the optimum BSH production time for the mesophilic sources (Nguyen *et al.,* 2007; Lundeen and Savage, 1990).

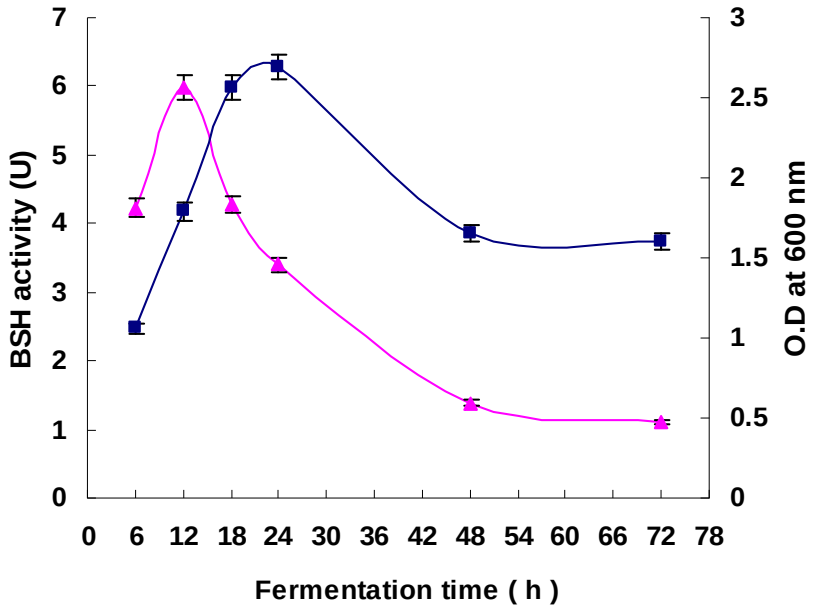

Fig. 2.5 b: Effect of fermentation time on BSH production from *Brevibacillus borstelensis* at 55°C, pH 6.0 and 160 rpm in sodium glutamate medium. Samples were removed at different intervals and assayed for growth (■) and BSH activity (▲) simultaneously

2.3.3.2. Effect of initial pH

The initial pH of the medium has been reported to strongly influence many enzymatic systems. The pH affects the ionization and therefore the binding and interaction of a myriad of molecular processes and this includes very basic things such as nutrient availability. Similarly, pH also affects the solubility of many substances that bacteria need (Sharma *et al*., 2006). Slightly acidic pH favors the BSH production from *Brevibacillus sp*, which is similar to the intestine of healthy humans (Kim *et al*., 2004b). The activity profile of BSH from *Brevibacillus sp* was determined in sodium glutamate medium adjusted to different pH values ranging from 5.0 to 9.0. BSH activity was detected to be optimum in pH 6.0; there was no considerable change in the enzyme production after this pH (Fig. 2.5 c).

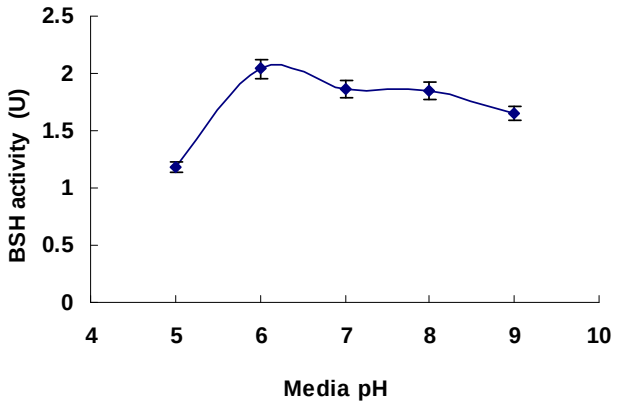

Fig. 2.5 c: Effect of initial pH of the culture medium on BSH activity of *Brevibacillus borstelensis.*

2.3.3.3. Effect of incubation temperature

Temperature profile for BSH activity from the isolate showed that BSH activity was maximum at 55°C. When incubated at different temperatures ranging from 30°C to 75°C, the culture failed to grow below 40°C and above 65°C. The enzyme activity was almost stable from 50°C to 55°C temperature range (Fig. 2.5 d). Optimum production of BSH from mesophilic source is usually observed at human physiological temperature i.e. 37°C.

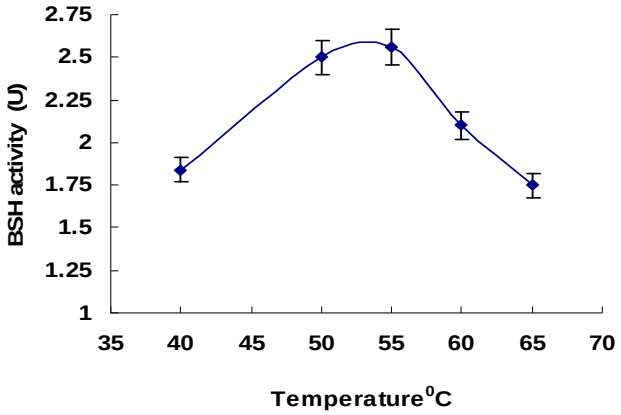

Fig. 2.5 d: Effect of incubation temperature on BSH activity of *Brevibacillus borstelensis.*

2.3.3.4. Effect of carbon source

Influence of various carbon sources were tested for the production of BSH by *Brevibacillus borstelensis* and the results are depicted in Fig. 2.6. Among all sugars, lactose was found to be the most suitable substrate for BSH activity. Supplementation of lactose increased BSH activity by 1.7 fold as compared to control. Mannose and mannitol does not have considerable effect on activity. The other carbon sources had inhibitory effect on BSH activity.

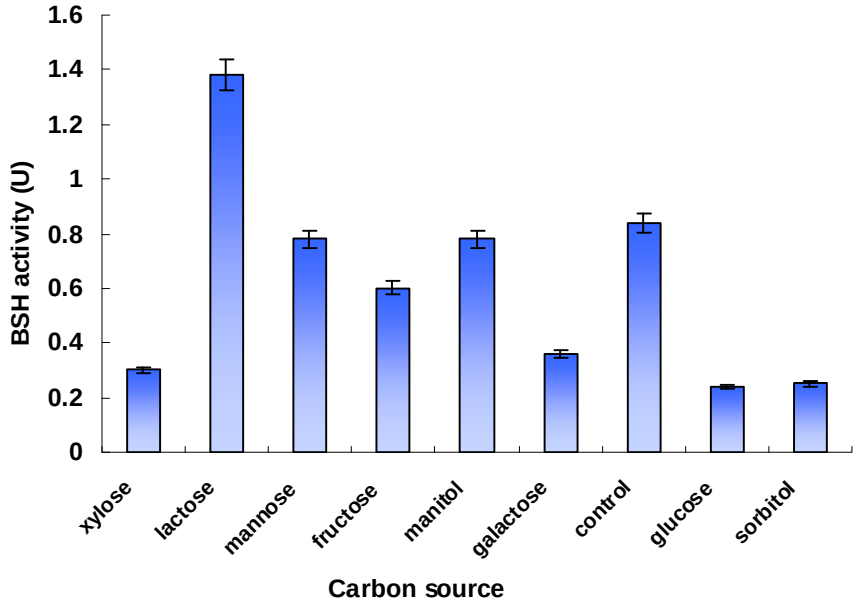

Fig. 2.6: Effect of carbon source on BSH production during cultivation of *Brevibacillus borstelensis.*

2.3.3.5. Effect of nitrogen source

The effect of nitrogen sources on BSH production from *Brevibacillus borstelensis* is depicted in Fig. 2.7. Presence of nitrogen source in the production medium has a significant effect on BSH activity. The best nitrogen source for BSH production by *Brevibacillus borstelensis* was yeast extract, followed by peptone and tryptone. BSH activity was increased 2.9-fold when yeast extract was used as nitrogen source.

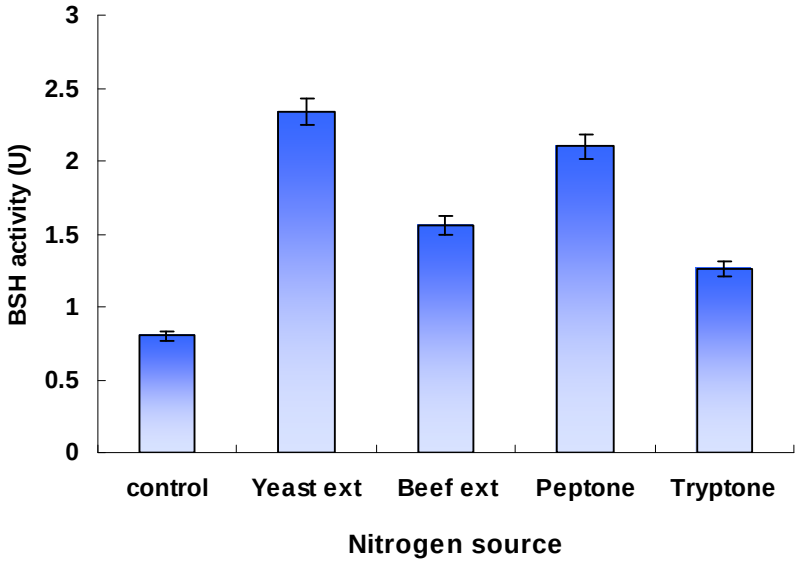

Fig. 2.7: Effect of nitrogen source on BSH production during cultivation of *Brevibacillus borstelensis.*

2.3.3.6. Substrate specificity

Bile salt hydrolase from *Brevibacillus borstelensis* showed a broad substrate range for six major human bile salts. This enzyme showed highest activity with glycodeoxycholic acid (GDCA) and exhibited preference for glycine-conjugated bile salts over taurine-conjugated forms (Fig. 2.8). This property of substrate specificity is similar to the reported BSH from mesophilic bacteria such as *B. longum*, *B. bifidum* (Tanaka *et al*., 2000, Kim *et al.,* 2004b).

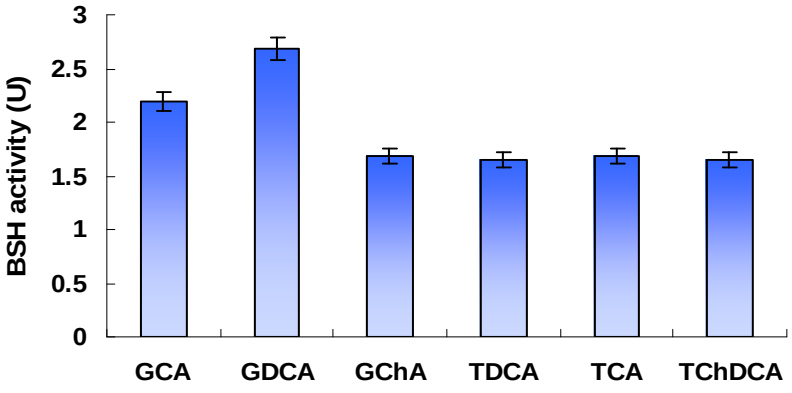

Fig. 2.8: Substrate specificity of bile salt hydrolase from *Brevibacillus borstelensis.*

In summary, the present chapter describes the isolation and identification of *Brevibacillus borstelensis*, which is the first thermophilic producer of bile salt hydrolase. Optimization of cultural parameters resulted in 2.9-fold increase in BSH production from the thermophilic source, *Brevibacillus borstelensis*. Physiological role of BSH production in the isolated thermophile is not known. We presumed that *Brevibacillus borstelensis* must have acquired BSH gene through horizontal gene transfer or BSH activity exhibited by this microorganism play an important role in detoxification of compounds in the surrounding environment.

Chapter 3

Purification and characterization of bile salt hydrolase from *Brevibacillus borstelensis*

The work presented in this chapter has been **published**:

Sridevi, N., Srivastava, S., Khan, B. M., Prabhune, A. A (2009) **Extremophiles**, 13: 363-70.

Abstract

Bile salt hydrolase (BSH) from *Brevibacillus borstelensis,* was purified to homogeneity using Q-sepharose chromatography and its enzymatic properties were characterized. The sub-unit molecular mass of the purified enzyme was estimated to be 28 kDa by SDS-PAGE and, 28.2 kD by MALDI-TOF analysis. The native molecular mass was estimated to be 56 kDa by gel filtration chromatography, indicating the protein to be a homodimer. The pH and temperature optimum for the enzyme catalysis were 9.0 and 60°C, respectively. Even though BSH from *Brevibacillus* sp. hydrolyzed all of the six major human bile salts, the enzyme preferred glycine conjugated substrates with apparent K_M and k_{cat} values of 3.08 μM and 6.32 $\times 10^2$ s^{-1}, respectively for glycodeoxycholic acid. The NH_2-terminal sequence of the purified enzyme was determined and it did not show any homology with other bacterial bile salt hydrolases. To our knowledge, this is the first report describing the purification of BSH to homogeneity from a thermophilic source.

3.1. Introduction

Elevated levels of cholesterol are a risk factor for cardiovascular diseases. The reduction in serum cholesterol could be an important health benefit, as a 1% reduction in serum cholesterol is associated with an estimated reduction of 2-3% in the risk of coronary artery diseases (Manson *et al.,* 1992). Exploring new methods to reduce serum cholesterol is of great importance at present. Enzymatic deconjugation of bile salts has been related to a reduction of serum cholesterol levels in mammals (Klaver and Van der Meer, 1993; Pereira *et al.,* 2003). Deconjugation is catalyzed by bile salt hydrolases, the enzymes which hydrolyze the amide bond and liberate the glycine/taurine moiety from the steroid core of bile salts. The resulting acids are termed as free bile acids or deconjugated bile acids, which are excreted via feces. Bacteria with BSH activity may effectively reduce serum cholesterol by enhancing the excretion of free bile acids, with a consequent increase in the synthesis of bile salts from serum cholesterol; or by decreasing the solubility of cholesterol, and thus reducing its uptake from the gut.

Many reports are available for the production of bile salt hydrolases from mesophilic source. A large number of bacterial strains possessing de-conjugation activity have been isolated from intestine and feces of human and other mammals and also from fermented milk products (Kim and Lee, 2005). Inspite of this wide distribution and high activity of BSH among mesophilic microorganisms, there are no reports so far regarding the isolation and purification of BSH from the thermophilic source with an optimum growth temperature range 40°C to 65°C. As described in Chapter II, screening for BSH producing thermophiles in our laboratory led to the identification of thermophilic bacteria isolated from hot water springs in Pali, Maharashtra, India. This thermophile was identified as *Brevibacillus borstelensis* based on 16S rDNA analysis and is a source of intracellular BSH (Sridevi and Prabhune, 2008). BSH enzymes have been purified from several gastrointestinal microorganisms, belonging to the genus *Lactobacillus* and *Bifidobacterium* and the biochemical characteristics were extensively studied (Stellwag and Hylemon, 1976, Ludeen and Savage, 1990, Kim *et al.,* 2004b, Tanaka *et al.,* 2000). BSH is also purified and characterized from a pathogenic bacterium *C. perfringes* and a bile adapted strain *X. maltophila* (Dean *et al.,* 2002).

In the present Chapter, studies on purification of the intracellular bile salt hydrolase from *Brevibacillus borstelensis* and its biochemical characterization such as molecular weight, N-terminal sequence, kinetic parameters, substrate specificity, effect of different metal ions and amino acid modifying reagents were described.

3.2. Materials and methods

3.2.1. Materials

Q-sepharose, Sephacry S-200, glycodeoxycholic acid, glycochenodeoxycholic acid, glycocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, taurocholic acid, 2% ninhydrin reagent solution, trichloro acetic acid, native molecular weight marker kit (Sigma MW-GF-200 Kit) were purchased from Sigma. Low molecular weight calibration kit for SDS electrophoresis was obtained from Amersham Biosciences. All other chemicals used were of analytic grade.

3.2.2. Growth conditions of *Brevibacillus borstelensis*

Brevibacillus borstelensis was grown under optimal conditions for production of bile salt hydrolase as described in Chapter II. Briefly, a series of 500 ml Erlenmeyer flasks containing 125 ml of sodium glutamate medium (sodium glutamate 0.5%, yeast extract 0.5%, peptone 1.0% and sodium chloride 0.2%) were seeded with 10% inoculum and incubated for 12 h at 55°C and 180 rpm. Cells in a log phase culture were harvested by centrifugation at 7,000 × g for 30 min at 4°C. The harvested cells were used for purification of enzyme.

3.2.3. Bile salt hydrolase assay and protein assay

Bile salt hydrolase activity was determined by measuring the amount of amino acids resulting from the hydrolysis of amide bond of bile salts using ninhydrin assay (Suresh Kumar *et al.,* 2006). Briefly, the enzyme sample was incubated in a reaction mixture containing 1mM sodium glycodeoxycholate, 100 mM potassium phosphate buffer, pH 6.5 at 40°C for 10 min. An aliquot of 25 µl was removed and mixed immediately with 25 µl of 15% (w/v) trichloroacetic acid. The sample was centrifuged at 15,000 × *g* for 2 min and the supernatant was mixed with an equal volume of 2% ninhydrin reagent solution

before boiling for 15 min. The absorption was recorded at 570 nm and the amount of product formed was estimated from a calibration curve. One unit of BSH activity is defined as the amount of enzyme that liberates 1 μmol of the amino acid from substrate per min. Protein concentration was determined in accordance with the method of Lowry *et al.,* (1951) with BSA as a standard.

3.2.4. Purification of bile salt hydrolase

Preparation of cell free extract

Harvested cells from batch fermentation were washed twice in 20 mM Tris buffer pH 8.0, resuspended in the same buffer in the proportion of 1:2 and disrupted by sonication in ice bath for 1 min at 80 amplitude with 0.5 sec pulse on and 0.8 sec pulse off using Branson Sonifier. Cell debris was removed by centrifugation at 15,000 × g for 30 min and the supernatant was stored at -20°C till required further.

Ion exchange chromatography

The cell-free extract was subjected to anion exchange chromatography using Q-sepharose column (2 × 6 cm) pre-equilibrated with 20 mM Tris buffer pH 8.0. The column was washed with equilibration buffer and the unbound protein was collected as fractions (2 ml). Flow rate was 10 mlh^{-1}. Fractions with BSH activity were pooled, concentrated using speed vac (LABCONCO). Concentrated sample was then dialysed against 20 mM Tris buffer, pH 8.0 and rechromatographed as above, on another freshly packed, equilibrated Q-sepharose column (1.5 × 5 cm) and eluted with 20 mM phosphate buffer, pH 8.0. Active fractions were pooled, concentrated and stored in aliquots at -20°C to avoid loss of activity due to freezing & thawing.

3.2.5. Characterization of bile salt hydrolase

3.2.5.1. Molecular weight estimation

SDS PAGE

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE, 12%) was carried out by the method of Laemmli (1970) to check the purity at each step of purification and to

determine the molecular mass of the purified enzyme. SDS-PAGE was carried out in slab gels using Tarsons SDS-PAGE apparatus with 1 mm spacers. The enzyme was dissociated by heating in a boiling water bath in presence of SDS-PAGE loading dye. Cell-free extraxt, fractions from Q-sepharose column were loaded on the gel, and electrophoresed by applying voltage 100 V for 1.5 h. Silver staining was used to visualize protein bands on the gels (Morrissey, 1981) and the molecular weight was estimated based on concurrently electrophoresed marker proteins (Amersham Biosciences). The Molecular weight markers used for the SDS-PAGE were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa) and α lactalbumin (14.4 kDa). The precise molecular weight of the subunit was determined using a MALDI-TOF mass spectrometer (Applied Biosciences).

Molecular weight estimation by MALDI- TOF

Mass spectral analysis was recorded using a time-of-flight delayed extraction MALDI Mass spectrometer (Applied Biosciences). 5 µl of sample was mixed in a microcentrifuge tube with 35 µl of matrix solution. The matrix solution (15 mg/ml sinapic acid) was prepared in 30% acetonitrile (ACN). About 10 µl of the mixture was applied to a stainless steel sample holder and introduced into the mass spectrometer after drying. The spectrum was obtained in the reflectron mode by scanning 5 laser shots with an ion source voltage of 19 kV, ion source 2 of 16.27 kv, 100 ns delay and the low mass gate at *m/z* 600.

Gel filtration chromatography

The molecular weight of native protein was calculated according to Andrews (1964). Gel filtration was carried out using a Sephacryl S-200 column (100 × 1 cm) at a flow rate of 0.1 ml min^{-1} with 50 mM Tris HCl buffer at pH 8.0, containing 150 mM NaCl. The relative molecular weight (Mr) was determined by using protein standards under similar conditions (Sigma MW-GF-200 Kit) which includes β-amylase 200 kDa, alcohol dehydrogenase 150 kDa, bovine serum albumin 66 kDa, carbonic anhydrase 29 kDa and cytochrome *c* 12.4 kDa.

3.2.5.2. Substrate specificity

Substrate specificity of the *Brevibacillus* BSH was determined with six major bile salts namely, glycodeoxycholic acid, glycocholic acid, glychenodeoxycholic acid, taurodeoxycholic acid, taurocholic acid and taurochenodeoxycholic acid. The amount of amino acid released due to enzymatic reaction was determined by modified ninhydrin assay as described in Chapter II.

3.2.5.3. Effect of temperature and pH on BSH activity and stability

To ascertain the pH optimum of the enzyme, 50 μg of the enzyme was measured for BSH activity at different pH values under standard assay conditions as described in section 3.2.3. The following buffers were used: 100 mM acetate buffer (pH 3.0-6.0), phosphate buffer (pH 6.0-8.0), Tris-HCl (pH 8.0-9.0), carbonate-bicarbonate buffer (pH 9.0-11.0).

pH stability of the enzyme was examined in the range of pH 3.0 to 11.0 by using buffers mentioned above. Enzyme was incubated at 25°C for 1 h and the residual activity was determined under standard assay conditions.

For the determination of the optimum temperature of BSH activity, 50 μg of the enzyme was assayed over the temperature range of 30-80°C in 100 mM Tris buffer, pH 9.0 for 10 min. For thermal stability, 50 μg of the enzyme was incubated at different temperatures ranging from 40-80°C for 1 h in 100 mM potassium phosphate buffer, pH 7.0 and residual activity was estimated under standard assay conditions as described earlier.

3.2.5.4. Kinetic studies

The effect of substrate concentration on the reaction rate was determined using glycodeoxycholic acid by standard enzyme assay. The Michaelis Menten constant (K_M) and maximum velocity for the reaction (V_{max}) were calculated from Lineweaver-Burk Plot. Catalytic turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_M) of purified enzyme were calculated using glycodeoxycholic acid as a substrate.

3.2.5.5. Effect of various metal ions and amino acid modifying reagents on BSH activity

The effect of different metal ions on BSH activity was assessed using range of metal salts. The enzyme was incubated with various metal salts with effective concentration of 10 mM for 1 h at 30°C and the residual enzyme activity was determined by standard assay method. Resulting enzyme activities were compared to those of the standard enzyme reaction carried out in controlled condition.

Various potential amino acid modifying reagents such as *N*-bromosuccinamide, phenylymethylsulphonyl fluoride, 5, 5´- dithiobis- (2-nitrobenzoic acid), phenyl glyoxal, N-Acetylimidazole, Woodwards reagent K, citraconic anhydride, trinitrobenzene sulphonic acid were tested for their effect on BSH activity. For this, 50 μg of homogenous preparation of the enzyme was incubated with various concentrations of above-mentioned reagents at 30°C for 30 min and relative BSH activity was measured. The percent residual enzyme activity was determined by standard enzyme assay with reference to the activity of the enzyme in a reaction without the addition of modifying reagent as supplement.

3.2.5.6. Determination of the N-terminal amino acid sequence

To determine N-terminal sequence, the purified enzyme from Q-sepharose chromatography was concentrated and applied to 15% SDS-PAGE. After separation, the proteins were blotted onto a polyvinylidene fluoride membrane and stained with Coomassie brilliant blue R-250. BSH band was cut out and used for N-terminal amino acid sequencing, which was performed using Applied Biosystems Procise 494 protein with standard reagents and methods recommended by manufacturer. The analysis was carried out at Faculty of Biological Sciences, University of Leeds, UK.

3.3. Results and Discussion

3.3.1. Purification

The purification procedure is summarized in Table 3.1. It was observed that protein was purified to homogeneity using ion exchange chromatography on Q-sepharose. The most

common protein purification approach is to let the protein of interest adsorb to the column matrix and allow the contaminants to pass through. However, in some cases the vice versa may be a better approach i.e., to let the protein of interest flow through and to get the contaminants adsorbed. We have adapted the second condition in this purification step. BSH with positive charge, eluted as flow through whereas contaminant proteins with negative charge remain bound to column. This step minimized the contamination to maximum extent with 83% yield and a 12-fold increase in specific activity. The pooled active fractions were concentrated using speedvac (LABCONCO). Extra band of non-specific proteins observed in this step was purified further by rechromatography on another freshly packed and preequilibrated Q-sepharose column, as described above. BSH active fractions were detected for homogeneity on a 12% SDS PAGE (Fig. 3.1a). As a result of purification using this procedure 58% yield was obtained and the protein was purified 13-fold. The enzyme was electrophoretically homogenous and the molecular weight of the enzyme was estimated to be 28 kD from SDS PAGE analysis. For a more precise molecular mass determination a MALDI-TOF analysis was performed (Fig. 3.1b). The purified fraction gave a major peak with a molecular mass of 28.2 kD. The molecular weight (M_r) of the pure native enzyme determined to be approximately 56 kDa ± 1 kD by gel filtration chromatography (Fig. 3.1c), suggesting that the enzyme is a homodimeric protein.

Bile salt hydrolase with different native molecular weights and structures have been reported previously. The native enzymes are octamers in *B. fragilis* (Stellwag and Hylemon, 1976), hexamers in *B. longum* BB536 (Grill *et al.,* 1995), tetramers in *C. perfringens* (Gopal-Srivastava and Hylemon, 1988), *B. longum* SBT2928 (Tanaka *et al.,* 2000), or trimers in *L. johnsonii* (Lundeen and Savage, 1990). Dean *et al.,* (2002) reported a dimeric bile salt hydrolase from bile adapted strain *X. maltophila* with a native and subunit molecular weight 100 and 52 kDa respectively. We are reporting here another dimeric enzyme with smallest sub-unit (28 kD), not documented so far.

Table 3.1: Purification summary of bile salt hydrolase from *Brevibacillus borstelensis*.

Steps of purification	**Total activity (U)**	**Total protein mg**	**Specific activity (U/mg)**	**Fold purification**	**Yield (%)**
Sonicate	158	152	1.03	1.0	100
Q-Sepharose Chromatography (I)	132	10.8	12.2	11.8	83
Q-Sepharose Chromatography (II)	91	6.6	13.3	13.8	58

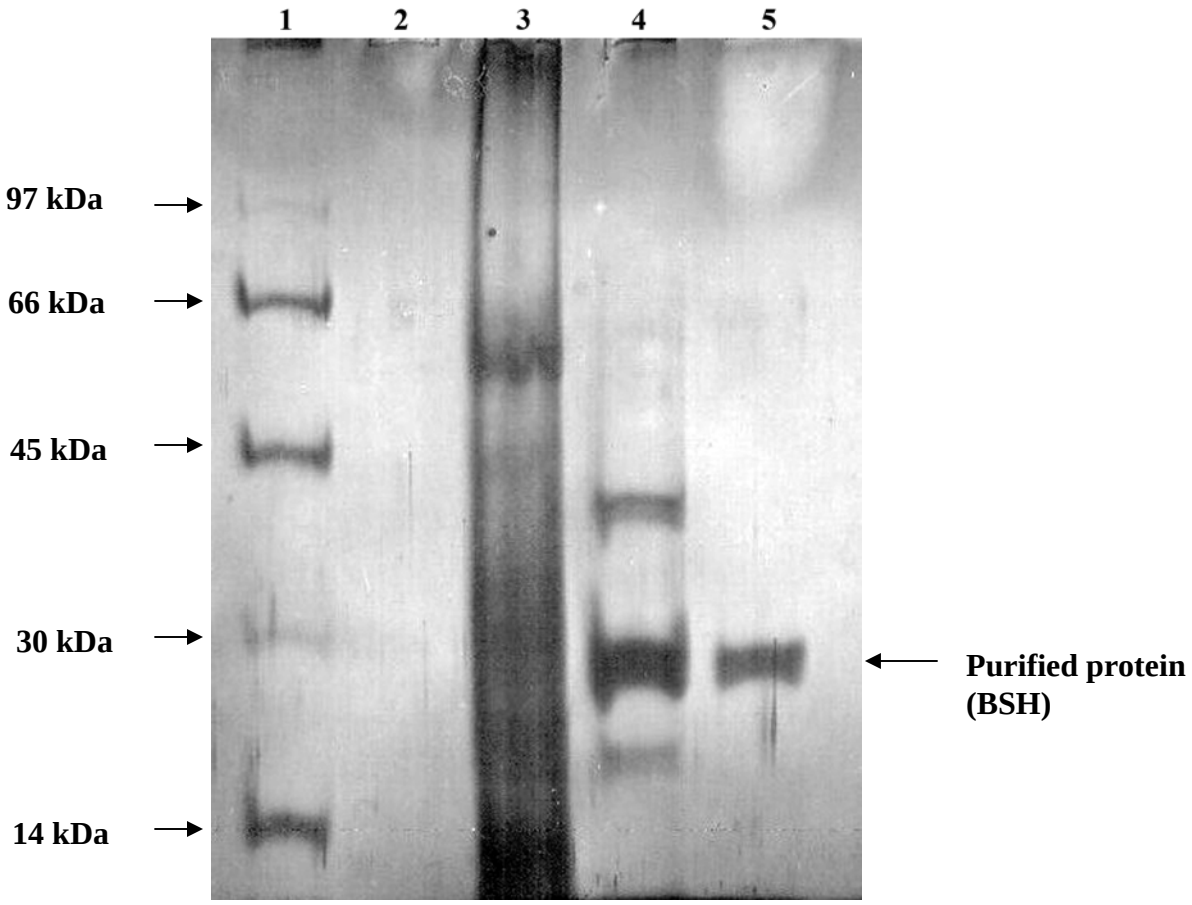

Fig. 3.1a: Profile of *Brevibacillus* BSH in 12% SDS PAGE stained with silver nitrate. lane 1, molecular weight marker; lane 2, fraction after sonication lane 3; fraction after Q sepharose I and lane 4; fraction after Q-sepharose II.

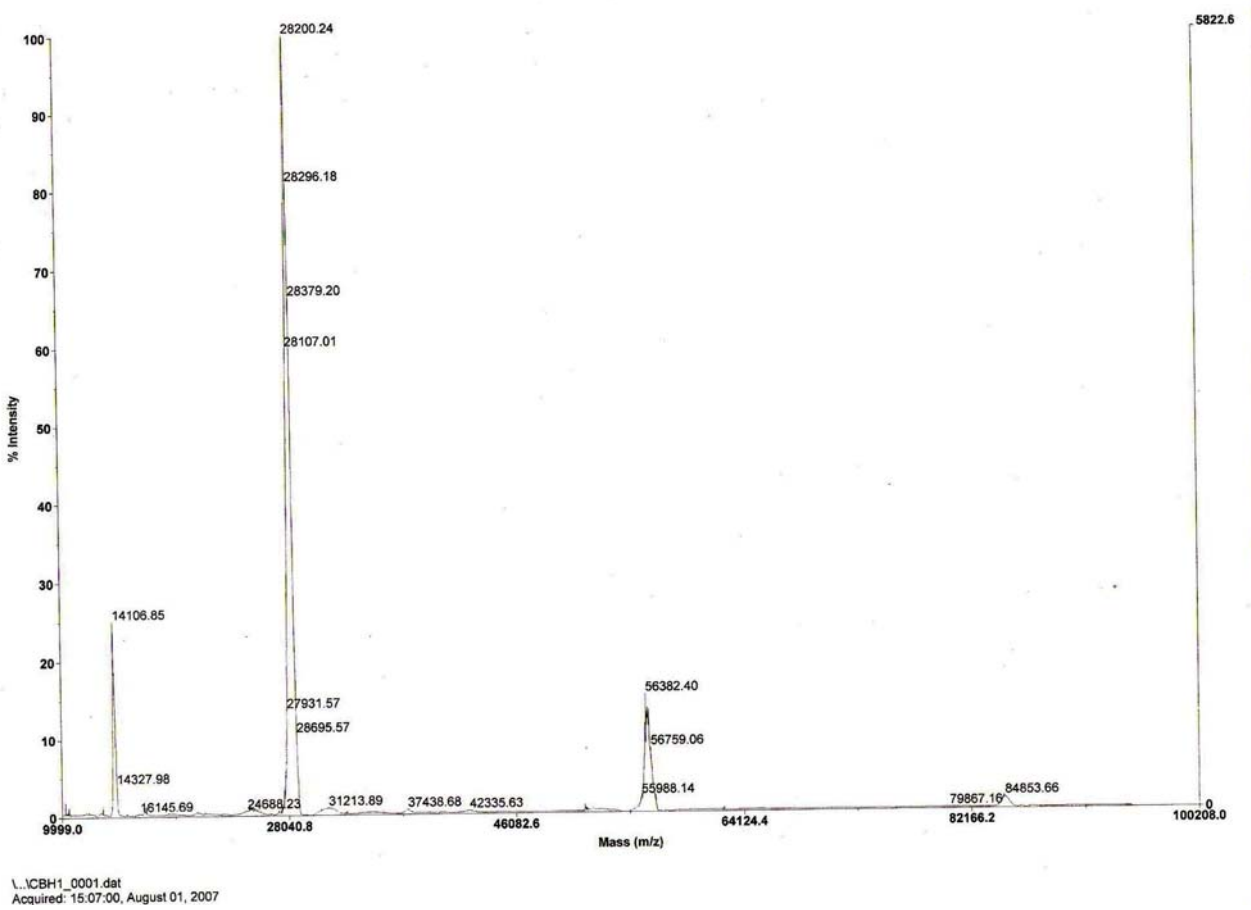

Fig. 3.1b: Matrix-assisted laser desorption ionization / time-of-flight mass spectrometry (MALDI-TOF) spectrum of purified BSH.

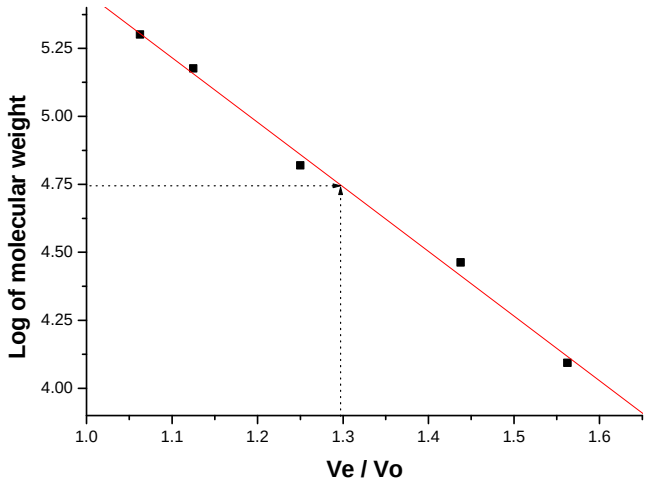

Fig. 3.1c: Molecular Weight estimation of purified *Brevibacillus* BSH by gel filtration on Sephacryl S- 200.

3.3.2. Substrate specificity

Brevibacillus BSH showed a broad substrate range for six major bile salts. The enzyme was active towards glycine and taurine conjugated substrates. The highest levels of activity were observed with glycodeoxycholic acid (defined as 100% activity) and with glycine conjugated bile acids (Fig. 3.2). Substrate specificity of *Brevibacillus* BSH is similar to the other bile salt hydrolases (Tanaka *et al.,* 2000, Kim *et al.,* 2004b). The enzyme showed relatively less activity (50%) with taurine conjugated substrates compared to glycine conjugated substrates.

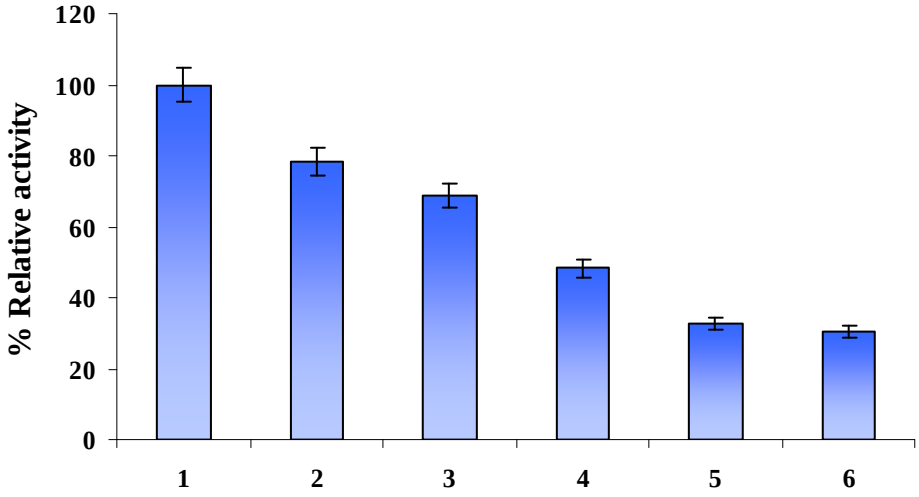

Fig. 3.2: Substrate specificity of BSH from *Brevibacillus* sp. 1 - glycodeoxycholic acid, 2 - glycocholic acid, 3 - Glycochenodeoxycholic acid, 4 - Taurocholic acid, 5 - Taurodeoxycholic acid, 6 - Taurochenodeoxycholic acid.

3.3.3. Effect of pH and temperature on BSH activity and stability

Studies on the effect of pH on enzyme catalysis revealed that the enzyme was active in broad range of pH i.e., pH 3.0 to 11.0 (Fig. 3.3a). Maximum activity of the enzyme was observed at pH 9.0, in contrast to the pH optimum reported for the other enteric bile salt hydrolases. Slightly acidic pH optimum, in the range of 3.5 to 7.0 was observed for BSH activity in such bacteria. Only exception is the one which was purified from *X. maltophila* with a pH optimum range 7.9 to 8.5 (Dean *et al.,* 2002, Kim and Lee, 2005). *Brevibacillus* BSH showed pH stability over the range of 3.0 to 11.0 and the stability optimum was observed at pH 8.0 (Fig. 3.3b). Tanaka *et al.,* in 2000 have reported that BSH purified from *B. longum* is stable in the pH range 4.0 to 8.0 and rapidly inactivated on either side of this range. The purified enzyme from the *Brevibacillus* sp. has potential applications for *in vivo* reduction of cholesterol levels due to its broad range of pH stability. The enzyme was presumed to withstand pH conditions of the stomach (1.0 to 2.0) since more than 70% of the activity was still retained at pH 3.0. When the effect of temperature on BSH activity was studied, the enzyme was active in the range of 30°C to 80°C with optimum temperature at 60°C (Fig. 3.3c) and differs significantly from the

temperature optima (30°C to 45°C) described for other bile salt hydrolases (Tanaka *et al.*, 2000). The enzyme is very stable at 60°C and remained active for more than 1h (Fig. 3.3d). Enzyme retained 100% activity when incubated at 30°C for 12 h whereas there was slight loss of activity observed after 24 h incubation, indicating very good shelf life of this enzyme. Temperature stability studies conclude that the enzyme is stable over a broad temperature range.

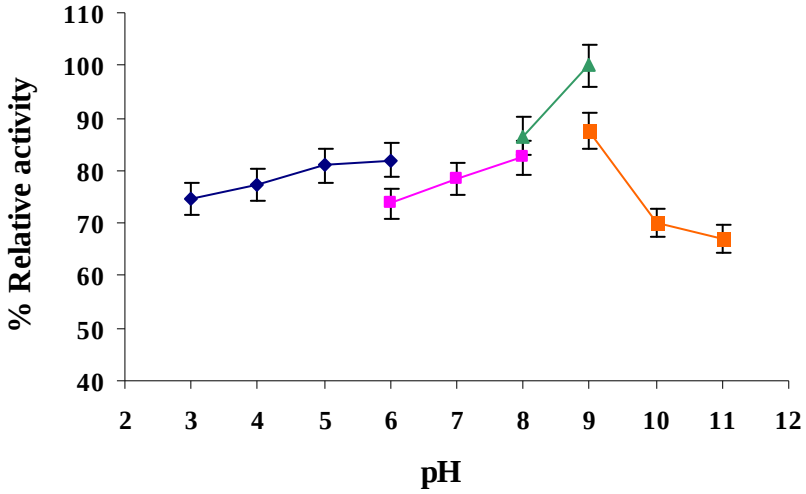

Fig. 3.3a: Effect of pH on BSH activity of *Brevibacillus sp.* The effect of the pH on the enzyme activity was examined in the pH range of 3.0 to 11.0 with the following 100 mM buffers under the standard assay conditions: *symbols in blue*, acetate buffer (pH 3.0 to 6.0); *symbols in pink*, potassium phosphate buffer (pH 6.0 to 8.0); *symbols in green*, tris buffer (pH 8.0 to 9.0); *symbols in orange*, carbonate-bicarbonate buffer (pH 9.0-11.0). Relative activity is expressed as percentage of the maximum activity.

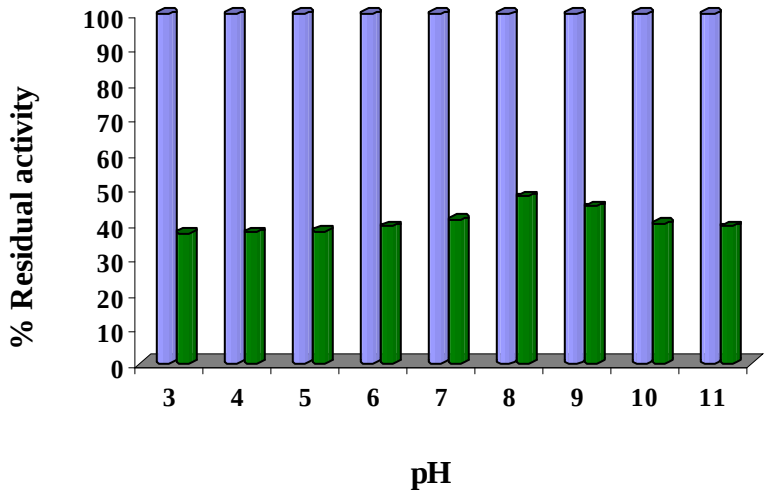

Fig. 3.3b: pH stability of *Brevibacillus* BSH. 50 µg of the enzyme was pre-incubated in buffers at different pH values at 25°C for 1h and the residual activity was determined under standard assay conditions. Symbols: *symbols in purple*: bile salt hydrolase activity at zero hour of incubation; *symbols in green*: bile salt hydrolase activity after one hour of incubation**.**

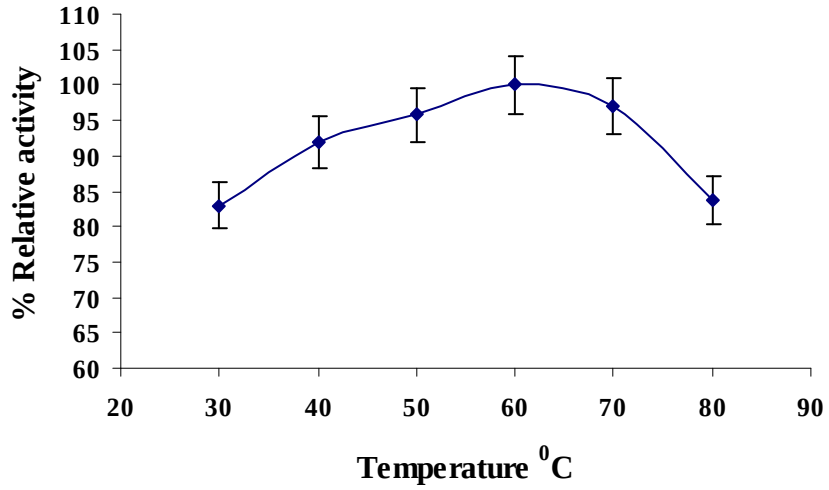

Fig. 3.3c: Effect of temperature on BSH activity of *Brevibacillus sp*.

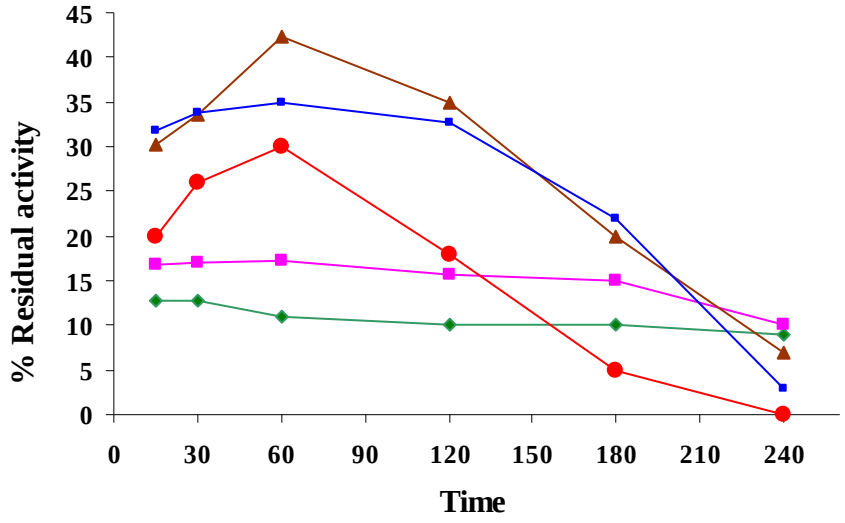

Fig. 3.3d: Thermostability of *Brevibacillus* BSH. Purified enzyme was incubated at different temperatures (40°C to 80°C) for a period of one hour and the residual enzyme activity was assessed under standard conditions. Symbols: *Symbols in green*, 40°C, *symbols in pink*, 50°C, *symbols in brown*, 60°C, *symbols in blue*, 70°C, *symbols in red,* 80°C.

3.3.4. Kinetic studies

The substrate saturation curve of *Brevibacillus* BSH against glycodeoxycholic acid followed Michaelis-Menten kinetics (Fig.3.4). The apparent values of K_M and k_{cat} were calculated from a Lineweaver-Burk plot, and were approximately 3.08 μM and 6.32 x 10^2 s^{-1}, respectively. The catalytic efficiency ($kcat/K_M$) was calculated to be 0.2 x 10^3 M^{-1} s^{-1}. K_M value of the purified enzyme against glycodeoxycholic acid is significantly less in comparision with BSH purified from *B. longum* with an optimal growth temperature 37°C (Tanaka *et al.*, 2000, Suresh Kumar *et al.*, 2006) indicating high substrate affinity of BSH from *Brevibacillus* sp.

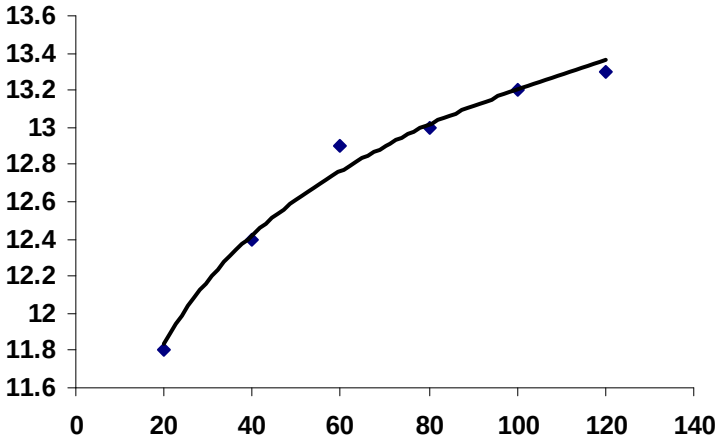

Fig.3.4: Michaelis-Menten kinetics of bile salt hydrolase for the hydrolysis of glycodeoxycholic acid. Substrate (sodium salt of glycodeoxy cholic acid) was added to initiate the reaction. Reaction mixtures contained 0.02 mg of enzyme. Initial rates of hydrolysis were measured over a time course of 10 min.

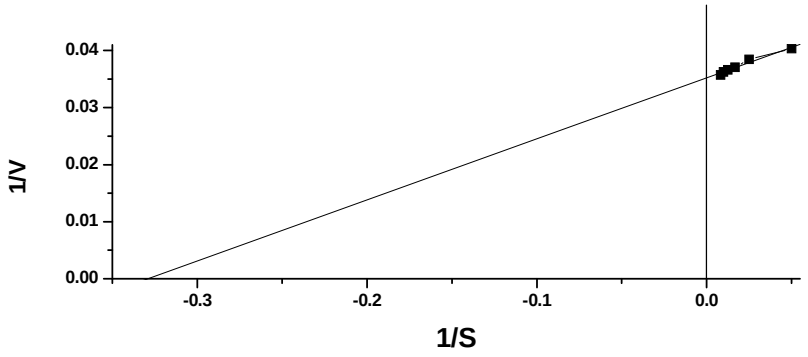

Fig. 3.5: Lineweaver-Burk plot of *Brevibacillus* BSH

3.3.5. Effect of metal ions and amino acid modifying reagents on BSH activity

The effect of metal ions is summarized in Table 3.2. BSH activity was strongly inhibited by Cu^{++} and Hg^{++}. Complete inhibition was observed with Cu^{++}. Complete inactivation of BSH activity by Cu^{++} was also observed in BSH from *X. maltophila* and *B. longum* (Dean *et al.,* 2002, Tanaka *et al.,* 2000). Moderate inhibition was observed with Ba^{++}, Ag^{++},

Zn^{++}, and little inhibition (19%) was observed with metal ion chelator (EDTA). The enzyme activity was enhanced by 14.7% with Fe^{++}.

Effect of specific amino acid modifying agents on BSH activity was studied and the results are summarized in the Table 3.3. Trinitrobenzene sulphonic acid and citraconic anhydride strongly inhibited the enzyme activity at a concentration of 25 mM, with residual BSH activity of 30% and 33% respectively. Inhibition of BSH activity with trinitrobenzene sulphonic acid and citraconic anhydride can be predicted as involvement of Lysine in the catalytic activity of enzyme. There was no inhibition observed with modifying agents such as *N*- acetylimidazole and phenylmethylsulfonyl fluoride. About 50% inhibition was observed with Woodward's reagent K and phenylglyoxal. Suresh Kumar *et al.*, (2006) observed that chemical modification of BSH from *Bifidobacterium longum* with cysteine modifier 5, 5′- dithiobis- (2-nitrobenzoic acid) lowered the activity by 78%. Interestingly, in our study it was observed that *Brevibacillus* BSH was inhibited only by 35% with 5, 5′- dithiobis- (2-nitrobenzoic acid) under identical conditions.

Table 3.2: Influence of metal ions on the BSH activity.
Activity without metal ion was considered as 100%.

Metal ions	**% Residual activity**
$CuSO_4$	0
$BaCl_2$	78.4
$AgNO_3$	77.3
$HgCl_2$	43.2
$CoCl_2$	90.9
$NiSO_4$	92.0
$MnSO_4$	95.5
$ZnSO_4$	76.1
$MgSO_4$	87.5
$FeSO_4$	114.7
EDTA	80.6

Table 3.3: Effect of amino acid modifying reagents on BSH activity. Activity without inhibitor was considered as 100%.

Amino acid modifier	Possible residue modified	Concentration	% Residual activity
Phenyl methyl sulphonyl fluoride	Serine	1 mM	100%
5, 5´- dithiobis- 2-nitrobenzoic acid	Cysteine	1 mM	65%
Woodwards reagent k	Aspartic acid Glutamic acid	2 mM	53.6%
N-Bromosuccinamide	Tryptophan	50 µM	63.6%
N-Acetyl imidazole	Histidine, Tyrosine	10 mM	100%
Citraconic anhydride	Lysine	25 mM	33.3%
Trinitrobenzene sulphonic acid	Lysine	25 mM	30.9%
Phenyl glyoxal	Arginine	3 mM	57.4%

3.3.6. N-terminal amino acid sequencing

N-terminal amino acid sequencing of the purified protein resulted in the following sequence:Lys-Asn-Thr-Asp-Leu-Lys-Gln-Glu-Asn-Lys-Lys-Phe-Glu-Ser-Arg-Leu (KNTDLKQENKKFES). A protein homology comparison revealed that this sequence exhibited no homology to the N-terminal amino acid sequences of the BSH of various lactobacilli and other gastrointestinal microorganisms. Bile salt hydrolases belongs to N-terminal nucleophile (Ntn) hydrolase super family. Members of this family are synthesized as preproteins and go through post-translational processes, which lead to an autocatalytically activated enzyme. This process is thought to generate a new N-terminal residue, which is designed to act as a nucleophile. The N-terminal nucleophile, cysteine is highly conserved in all BSH enzymes (Kim *et al.,* 2004b, Coleman and Hudson, 1995,

Christiaens *et al.,* 1992). The crystal structure of BSH from *C. perfringes* and *B. longum* revealed that the catalytic site possessed a nucleophilic residue cysteine, which is thought to be crucial for catalysis and is central to the mechanism of catalysis/substrate hydrolysis (Rosoccha *et al.,* 2005, Suresh Kumar *et al.,* 2006). The importance of the -SH group was confirmed by the fact that replacement of Cys with other potential nucleophilic residues such as Ser or Thr resulted in the loss of BSH activity (Tanaka *et al.,* 2000, Kim *et al.,* 2004b). It was observed by Dean *et al.,* (2002) that BSH purified from bile adapted strain, *X. maltophila* has alanine as N-terminal residue. In our study, on the basis of N-terminal analysis, we observed a lysine residue at the N-terminus of the enzyme instead of cysteine indicates that there has been appreciable divergence in these enzymes. However, the exact location and role of cysteine in enzyme catalysis will be revealed only after the resolution of three dimensional structure of the *Brevibacillus* BSH. Thermozymes adapt different mechanisms for their thermostability property, one of such mechanisms include small structural modifications that are achieved with the exchange of some amino acids (Li *et al.,* 2005). The significant difference in the amino acid sequence may be due to an adaptation mechanism to improve the resistance to irreversible loss of activity (Iwakura *et al.,* 1995).

In summary, the present Chapter describes purification and characterization of BSH from a novel thermophilic source, *Brevibacillus* sp. To the best of our knowledge this is the first report regarding the purification and characterization of BSH from the thermophilic source. The purified enzyme is the smallest dimeric enzyme and the N-terminal sequence of this protein is distinguishably different from the reported BSH from other sources. The characterization of the enzyme reveals that there was appreciable divergence in the properties of the enzyme when compared with mesophilic proteins and also some properties of the enzyme such as high thermostability and substrate affinity have industrial applications.

Chapter 4

Structural features of Ntn hydrolases immobilized on APTES functionalized mesoporous silica

Chapter 4 A

Structural features of bile salt hydrolase encapsulated in APTES functionalized mesoporous silica

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Sridevi N., Shah P., Prabhune A., Ramaswamy V (2009) (ready to communicate to Applied Catalysis A).

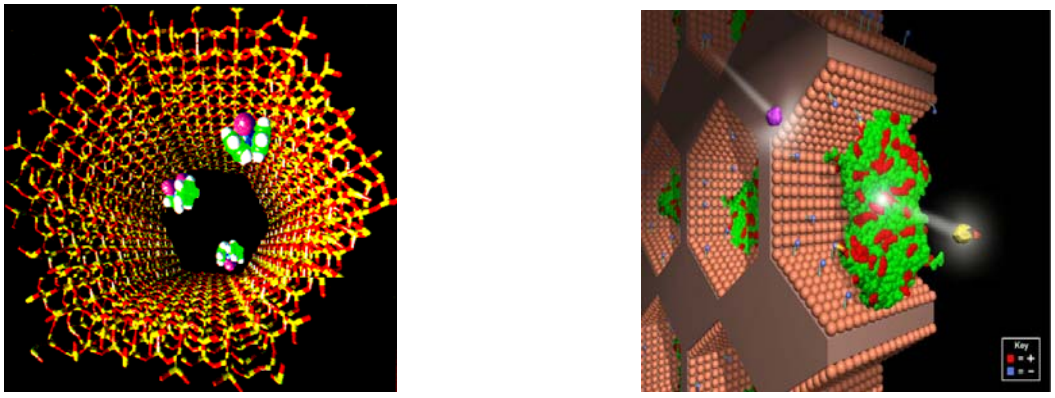
Abstract

Mesoporous silica with larger pore size and hydrothermal stability is a promising matrix for the immobilization of any biological material. The present study focuses on immobilization of bile salt hydrolase on mesoporous silica SBA-15. Bile salt hydrolase was immobilized on mesoporous SBA-15 by cross linking method. Optimum bile salt hydrolase activity was observed when 0.1% of glutaraldehyde was used as cross linking agent. The maximum loading of the enzyme was observed at pH 6.5 and the loading capacity of immobilized BSH is 4.1 mg protein per gram of SBA-15. Characteristics of cross-linked immobilized BSH enzyme such as pH, temperature optima, and pH, temperature stability were compared with the free enzyme. Physicochemical characterization of the immobilized enzyme by nitrogen adsorption, powder XRD, ^{29}Si CP-MAS NMR and TEM methods indicated that the characteristic hexagonal features and the original pore structure of the parent SBA-15 is retained after immobilization reaction.

4.1.1. Introduction

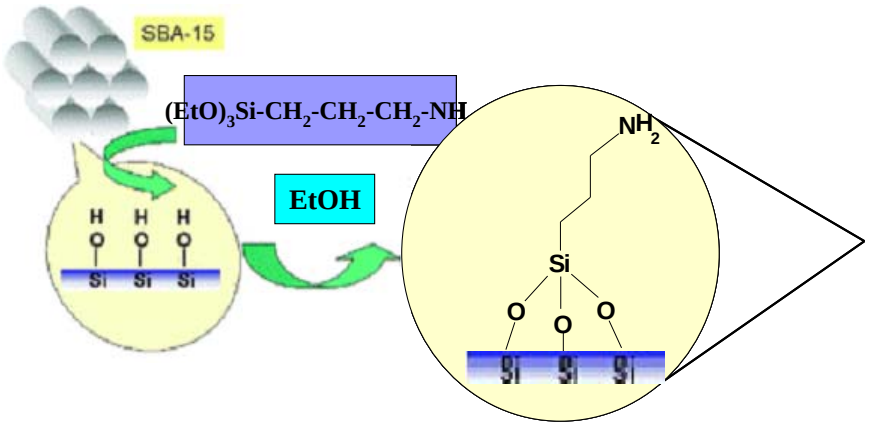
Inorganic materials are excessively studied as subjects for immobilization for its superiority over organic materials. The surface properties and structures of the inorganic support are more stable and can be controlled more easily (Xue *et al.,* 2004). Among all the inorganic supports, the mesoporous molecular sieves (such as M41s, SBA-15) are the promising carrier materials for the immobilization of enzymes. The silica matrices offer a number of advantages over conventional organic polymers for immobilization owing to their high surface area, average pore size, higher mechanical strength and enhanced thermal stability. Besides this they are non-toxic, and highly resistant against microbial attacks and organic solvents (Tischer and Wedekind, 1999). The mesoporous molecular sieves possess large numbers of large pores that are more accessible for anchoring the enzyme at high density and facilitate the transport of substrate and product (Chong and Zhao, 2004). Mesoporous silicas were first explored to immobilize enzyme by Díaz and Balkus in 1996. Hydrogen bonded interaction between hydroxyl groups of MCM-48 and carbonyl or amino groups in enzyme play an important role in immobilization process.

Mesoporous silica materials also have wide range of applications for drug delivery purposes. A lot of investigations have been done in which mesoporous materials employed for drug delivery purpose. Vallet-Regi *et al.* (2001) reported MCM-41 as a novel drug delivery system. The structure of MCM-41 with cylindrical mesopores and free silanol groups facilitated the controlled adsorption and liberation of variety of pharmaceutical compounds (Cavallaro *et al.,* 2004, Qu *et al.,* 2006). Other mesoporous materials used for drug delivery purpose include SBA-15, SBA-16, SBA-1, SBA-6 (Wang, 2009). Pure SBA-15 material has only silanol groups on the surface, which simply form weak intermolecular hydrogen bonds with biological molecules/drugs. Hence they are not strong enough to hold the drugs and allow them to release in a sustainable manner. Functionalization of mesoporous materials with amino groups has profound impact in both adsorption and drug delivery purpose. Song *et al.* (2005) reported that the functionalization of SBA-15 with amine groups enhanced the loading of ibuprofen and bovine serum albumin. In our study, bile salt hydrolase was immobilized on amino functionalized SBA-15 for drug delivery purpose.



(a) **(b)**

Fig. 4.1.1 (a & b): Model of enzyme immobilization on mesoporous silica.



(c)

Fig. 4.1.1 c: Model of mesoporous silica functionalization with 3-aminopropyltriethoxysilane.

4.1.2. Materials and Methods

4.1.2.1. Materials

Glycodeoxycholic acid (GDCA), 2% ninhydrin reagent solution, and trichloroacetic acid were obtained from Sigma. All other reagents and chemical used were purchased from standard commercial sources with highest quality available. All other chemicals used were of analytical grade. Mesoporous silica was synthesized at the Division of Catalysis; NCL, Pune. All buffers were essentially prepared in glass-distilled water.

4.1.2.2. Synthesis of SBA-15

SBA-15 molecular sieves were prepared by employing an optimized procedure reported in the literature (Shah *et al.,* 2007). SBA-15 was prepared by using fixed water to HCl molar ratio of 796 (70 ml of 0.07 M HCl). The molar gel composition was 1 TEOS: 0.016 P123: 0.46 HCl: 127 H_2O. In a typical synthesis, 4 g of P123 was added to 30 ml of water and stirred for few hours until a clear solution was obtained. A required amount of HCl was added and the solution was stirred at 40°C for another 2 h. Then, 9 g of tetraethyl orthosilicate was added and the resulting mixture was stirred for 24 h at 40°C. The mixture was then transferred into an autoclave, aged for 48 h at 100°C. The resultant solid was filtered, washed, dried at room temperature and calcined at 550°C.

4.1.2.3. Functionalization of SBA-15

2.0 g of calcined SBA-15 was suspended in 30 ml of toluene. 4 mM of 3-amino propyltriethoxysilane was added per gram of silica support and the reaction mixture was refluxed for 2 h. The white solid was filtered off and dried under vacuum.

4.1.2.4. Immobilization of bile salt hydrolase with the functionalized SBA-15

Partially purified enzyme from Q-sepharose Step- I, as described in Chapter III, was used for immobilization study. BSH enzyme was immobilized on amino functionalized SBA-15 by cross-linking method using glutaraldehyde as cross-linking agent. About 0.5 g of matrix was suspended in aqueous solution containing BSH enzyme (17.89 U), 100 mM potassium phosphate buffer pH 6.5 and various concentrations of glutaraldehyde ranging from 0.025 to 1%. The reaction mixture was stirred for 24 h at 5-10°C followed by

centrifugation at 19,000 × g for 2 min. The immobilized matrix was washed with 100 mM potassium phosphate buffer pH 6.5. The amount of enzyme immobilized on SBA-15 was measured by determining the difference in the protein concentration of the enzyme before and after the immobilization reaction by Micro Lowry method (Lowry *et al.*, 1951). BSH activity of free and immobilized enzymes was determined as described in Chapter III. Amino functionalized SBA-15 is designated as Am-SBA-15 and BSH immobilized amino functionalized SBA-15 sample is designated as BSH-Am-SBA-15.

4.1.2.5. Determination of the pH and temperature optima for free and immobilized BSH enzymes

Optimum pH for free and immobilized BSH was determined by assaying the enzyme activity in buffers of different pHs. BSH activity of free and immobilized enzymes was determined in the pH range 3.0 to 11.0 under standard assay conditions as described in Chapter III. Buffers used for different pH values are as follows: 100 mM citrate phosphate buffer (pH 3.0); 100 mM sodium acetate buffer (pH 4.0 to 5.0); 100 mM potassium phosphate buffer (pH 6.0 to 7.0); 100 mM Tris buffer (pH 8.0 to 9.0) and 100 mM sodium carbonate and bicarbonate buffer (pH 10.0 to 11.0). The temperature optimum was determined in 100 mM tris buffer at pH 9.0 under standard assay conditions.

4.1.2.6. Determination of the pH and temperature stability for free and immobilized BSH enzymes

The effect of pH on the catalytic activity of BSH, in free as well as immobilized enzyme was evaluated in the pH range of 3.0 to 11.0 by using the buffers mentioned above. Samples were incubated at the different pH for 1 h at room temperature and the residual activity was determined by standard enzyme assay. The temperature stability of the immobilized BSH enzyme was determined at temperatures ranging from 40°C to 80°C in the increment of 10°C and compared with that of free enzyme. Both immobilized and free BSH enzymes were incubated at different temperatures for 1 h, and the residual activity was determined by the standard assay.

4.1.2.7. Characterization

The powder XRD pattern of immobilized BSH enzyme samples were taken from X'Pert Pro (M/s P analytical) diffractometer using Co-Kα radiation and proportional counter as detector. A divergent slit of 1/8° in the primary optics and an anti scatter slit of 1/4° in the secondary optics were employed to measure the data in the low angle region. The BET surface area of the samples was determined by N_2 adsorption at 77K by using Autosorb-1 instrument. The specific surface area, S_{BET}, was determined from the linear part of the BET equation (p/p_0 = 0.05-0.31). TEM image of the samples were recorded on a JEOL Model 1200EX microscope operating at 100 kV. Immobilized BSH enzyme sample was dispersed in isopropyl alcohol and deposited on a Cu grid of 400-mesh size for air drying. The FTIR spectra were recorded in the 400–4000 cm^{-1} region in diffuse reflectance mode (spectral resolution = 4 cm^{-1}; number of scans = 100) using FT-IR spectroscopy (Shimadzu 8201 PC spectrophotometer). ^{29}Si CP-MAS experiment were performed on a Bruker MSL-300 NMR spectrometer at the Larmor frequencies of 59.595 MHz for ^{29}Si respectively. The chemical shift values (in ppm) were calculated with TMS as reference for ^{29}Si measurements.

4.1.3. Results and Discussion

4.1.3.1. Enzyme adsorption and surface properties

The binding reaction must be performed under conditions that do not cause loss of enzyme activity and at the same time the active site of the enzyme must be unaffected by the reagents used. Cross-linking of bile salt hydrolase with SBA-15 was carried out with different glutaraldehyde concentrations. During cross-linking, glutaraldehyde reacted with the amino groups of functionalized SBA-15. Inter and intramolecular cross-linking helps to resist unfolding at higher temperatures and in unfavorable environments. Aldehyde functional supports are a popular choice for protein immobilization because an imine bond can form easily between the surface aldehyde groups and the amine groups on the protein molecules. Formation of covalent bonds with functionalized surface ensures strong binding and negligible leaching into the surrounding solution. The effect of degree of cross-linking indicate that increase in glutaraldehyde concentration prevent

leakage of enzyme but showed a steep decrease in enzyme activity after 0.1% glutaraldehyde concentration as shown in Fig. 4.1.2.

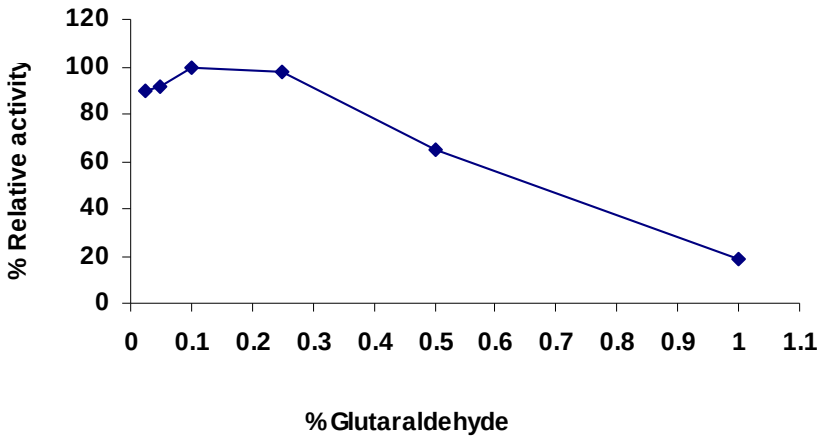


Fig. 4.1.2: The activity of the immobilized BSH-Am-SBA-15 sample as a function of the degree of crosslinking.

So the degree of cross-linking should be the minimum possible to ensure sufficient stability without loss of activity. Cross-linking carried out with 0.1% glutaraldehyde concentration showed the highest activity; hence it was used in all further experiments.

We have used two experimental techniques to probe the presence of enzyme immobilized on SBA-15, nitrogen porosimetry and TEM. All the samples gave typical irreversible type IV adsorption isotherm with an H1 hysteresis loop. Loading of BSH enzyme in Am-SBA-15 resulted in reduction of surface area from 605 m^2g^{-1} to 188 m^2g^{-1}, which is ~69% reduction in total surface area. The pore volume is reduced from 1.25 to 0.44 cm^3g^{-1}, which is 65% reduction of the specific pore volume of the parent SBA-15. This is consistent with the decrease in effective mesoporous diameter (from 6.7 to 6.5 nm), which indicates a considerable fraction of the enzyme is located inside the mesopores. Incorporation of enzyme does not affect the original pore structure of the parent SBA-15. Nitrogen adsorption measurements were carried out at different solution pH values ranging from 4.0, 6.5, 9.0 and 11.0 to observe the textural changes in SBA-15 after

immobilization. N_2 adsorption/desorption isotherms of BSH-Am-SBA-15 samples at various pH values is shown in Fig. 4.1.3. The isoelectric point pI, of BSH is ~ 8.3 and hence, the protein is positively charged at a pH below pI and negatively charged at a pH above pI. At pH 6.5, silica surface (isoelectric point ~ 3.0 - 4.0) has negative charge and the enzyme will posses overall positive charge, due to this electrostatic interaction, maximum amount of enzyme was immobilized. Purely electrostatic interactions are considered to be the driving mechanism for diffusion of the enzyme inside the pores of SBA-15 channels. It is therefore clear that the surface coverage decreases with increasing proton concentration (at pH 4.0) as a result of increased repulsion between molecules, which shows that pH has a strong effect on the BSH adsorption. It may be appropriate to assume the area per molecule for BSH at pH 4.0 is higher, which increases its surface coverage. This is supported by the significant decrease in surface area (166 m^2g^{-1}) upon adsorption of 3.9 mg BSH at pH 4.0. It is also reasonable to assume that BSH molecules get trapped at the mesopore openings or block the mesopore openings and thereby allowing limited access to nitrogen molecules. The lower adsorption capacity at a solution pH of 9.0 and 11.0 is due to the strong electrostatic repulsion between the protein and the adsorbent surface.

Table 4.1.1: Physicochemical characteristics of immobilized BSH at various pH

pH	**BSH activity (U)**	**Protein loaded (mg) per gram of support**	**S_{BET} m^2g^{-1}**
4.0	13	3.9	166
6.5	25	4.1	188
9.0	18	1.6	200
11.0	11	1.5	210

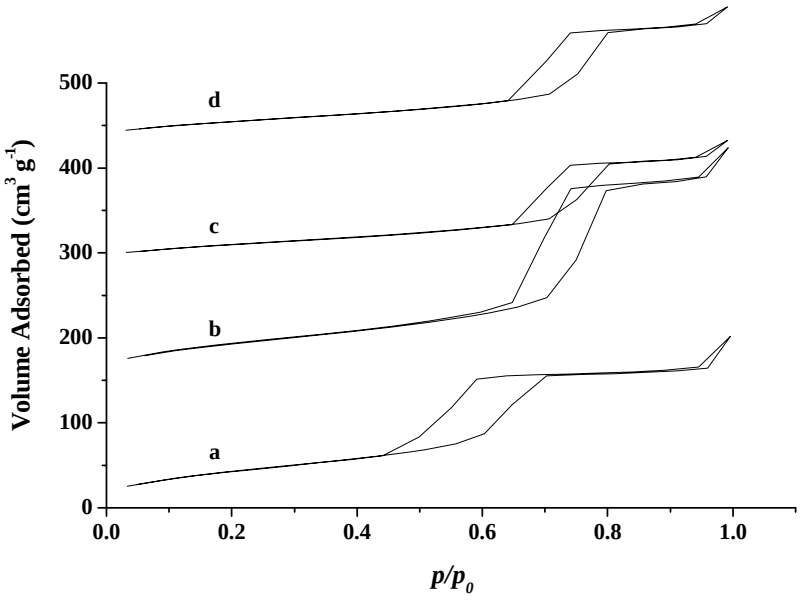


Fig. 4.1.3: Nitrogen adsorption/desorption isotherms of BSH on Am-SBA-15 at various pH values: (a) 4.0, (b) 6.5, (c) 9.0 and (d) 11.0 samples.

4.1.3.2. Transmission electron microscopy

The TEM images shown in Fig. 4.1.4 further justify that structural ordering and long-range order is maintained in the BSH immobilized SBA-15 samples. The well-ordered hexagonal arrays of mesopores and straight lattice fringes can be seen from the images viewed along and perpendicular to the pore axis, confirming the existence of a 2D hexagonal structure of a *p6mm* symmetry.

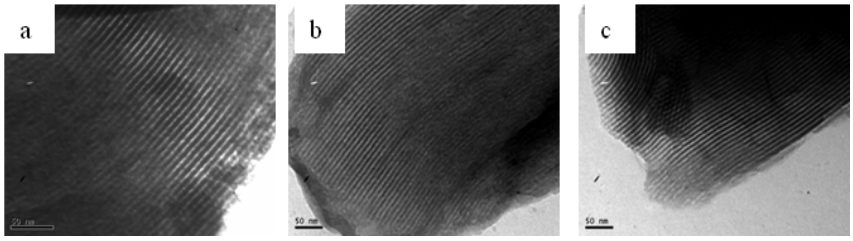


Fig. 4.1.4: Transmission electron micrographs of (a) Am-SBA-15, (b & c) BSH-Am-SBA-15

4.1.3.3. ^{29}Si CP-MAS NMR spectroscopy

The solid-state ^{29}Si CP-MAS NMR spectra of two samples namely Am-SBA-15 and BSH-Am-SBA-15 samples are displayed in Fig. 4.1.5.

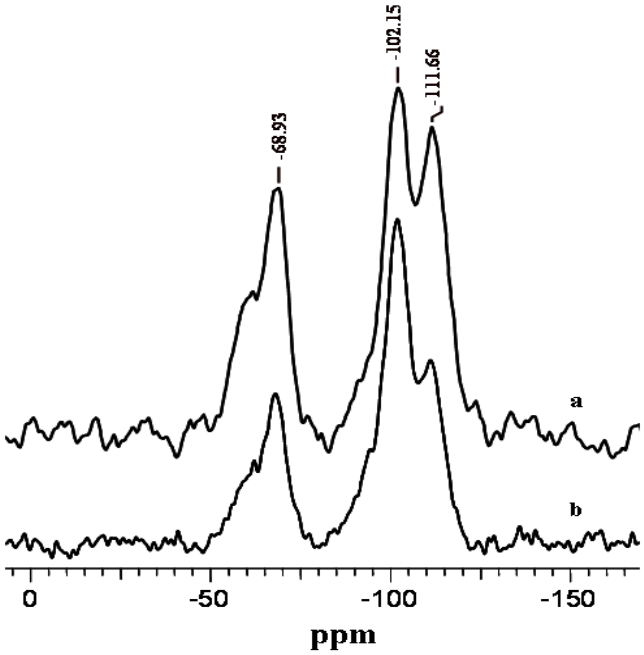


Fig. 4.1.5: ^{29}Si CP-MAS NMR spectra of (a) Am-SBA-15, (b) BSH-Am-SBA-15 samples.

The solid state ^{29}Si CP-MAS NMR spectrum showed Q_2, Q_3 and Q_4 silicon resonances between −90 and −110 ppm associated with the silica framework and T_1, T_2 and T_3 resonances between −40 and −60 ppm corresponding to one, two and three methoxy groups from the silane reacting with the surface. The prominent magnetic signals of amino functionalized sample can be assigned to the presence of a Si-C bond of T_3 [R´ Si (OSi) ^{3}] site which is observed at -68 ppm. This suggests the organic moiety is covalently bound to the silica surface. ^{29}Si CP MAS NMR spectrum also shows the lines with chemical shift at -102.15 ppm and -111.66 ppm, which has been attributed to the presence of Q_3 [$Si(OSi)_3(OH)$] and Q_4 [$Si(OSi)_4$] environment. The BSH immobilized samples also show similar behavior which indicates that the structure of the support is remained intact after immobilization and subsequent treatment conditions.

4.1.3.4. Infrared spectroscopy

Fig. 4.1.6 depicts the FTIR spectrum of SBA-15, Am-SBA-15 and BSH-Am-SBA-15 immobilized samples respectively. In Am-SBA-15, the occurrence of strong absorption bands at 2943 cm^{-1} is assigned to the NH_2 stretching vibration, indicating the formation of the open-chain products containing terminal amine groups. The band at 1311 cm^{-1} is characteristic absorption bands of CH_2-CH_3 groups and the band at 1049 cm^{-1} is due to C-N group. The appearance of these bands shows that the reaction between 3-amino-propyl trimethoxysilane and silanol groups on the SBA-15 silica surface has occurred. The IR spectra of the BSH-Am-SBA-15 sample show the broad band centered at 3500–3400 cm^{-1} due to the stretching and bending modes of water. In addition, weak bands are detectable at 870–810 cm^{-1} which may be assigned to rocking or wagging modes of water molecules. The IR spectra of the BSH-Am-SBA-15 sample shows the occurrence of strong absorption band at 1651 $cm^{-1}$and 1599 cm^{-1} attributed to C=N stretching mode. In the IR spectra, the absence of C=O and NH_2 peaks and appearance of C=N (azomethine) peak indicate that the expected imino compound was formed by condensation of terminal amine groups with glutaraldehyde and further also with C=O group of glutaraldehyde and the amino groups on the surface of the protein.

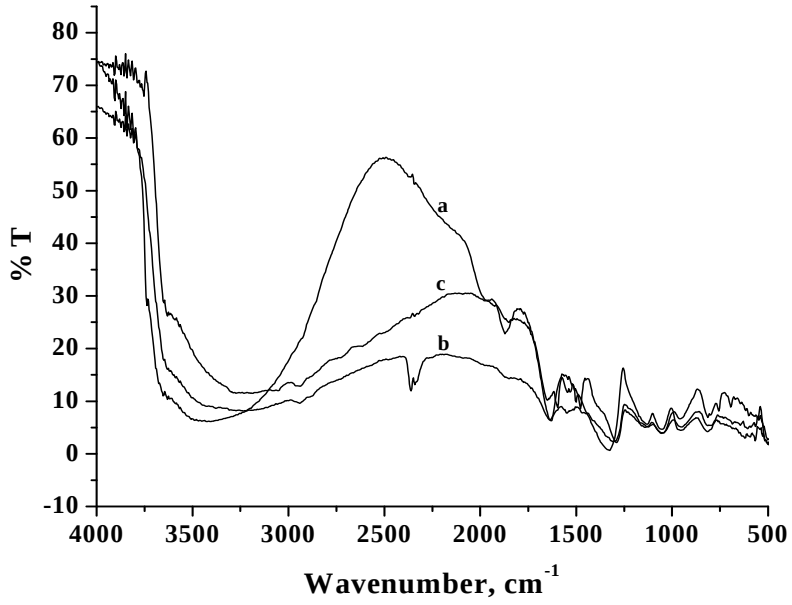


Fig. 4.1.6: FTIR spectrum of (a) SBA-15, (b) Am-SBA-15, (c) BSH-Am-SBA-15 samples.

4.1.3.5. pH and temperature profiles of free and immobilized enzymes

pH and temperature profiles of free and immobilized enzymes were studied. The optimum pH for the enzymatic activity of free enzyme was observed at pH 9.0 while that for the immobilized enzyme was found to be pH 8.0. The pH optimum of bile salt hydrolase was shifted to lower pH after immobilization (Fig. 4.1.7 a). Shift in pH from 9.0 to 8.0 could be due to the loss of charges present on the surface of the molecule during treatment with glutaraldehyde. On the basis of the preceding arguments and the observation, we suggest that the immobilized enzyme experience a different (local) pH in pores of matrix than in the buffer solution and therefore shift in the pH optima was observed (Gao *et al.,* 2006).

The effect of temperature on the enzyme activity of free and immobilized BSH enzymes is depicted in Fig. 4.1.7 b. Maximum BSH activity for free enzyme was observed at 50°C, whereas for immobilized enzyme, it was observed to be 70°C. The optimum temperature for enzyme activity shifted to a higher temperature after immobilization. Immobilized enzyme maintained good catalytic activity at high temperature range; this may be due to the stability of mesoporous materials, which prevents the denaturation of enzyme at higher temperature.

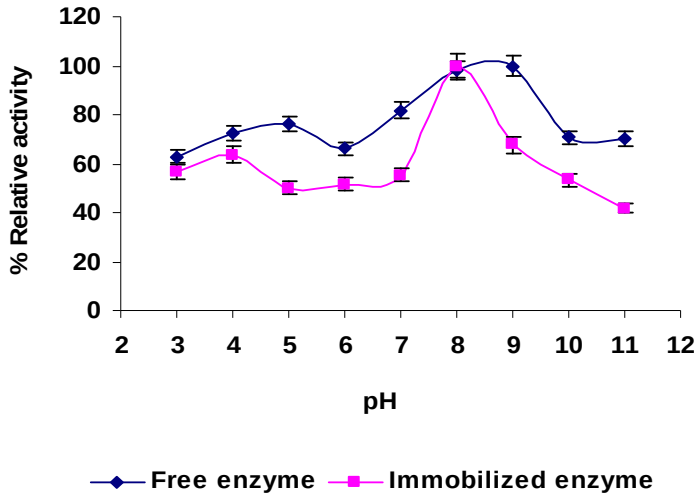


Fig. 4.1.7 a: pH profile of free and immobilized BSH enzymes.

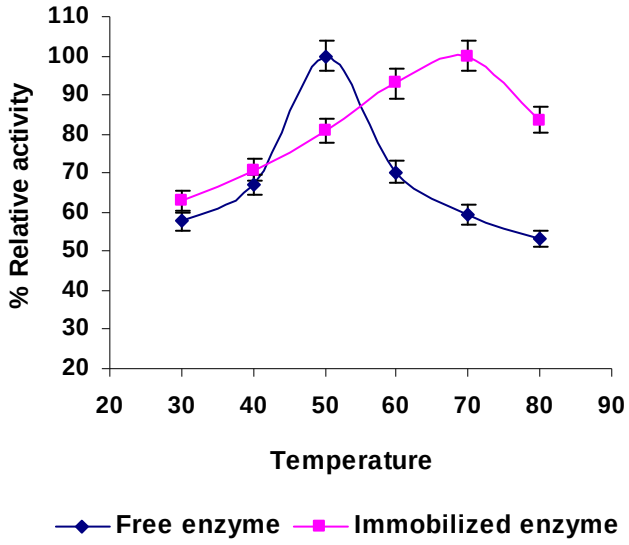


Fig. 4.1.7 b: Temperature profile of free and immobilized BSH enzymes.

4.1.3.6. pH and temperature stabilities of free and immobilized BSH enzymes

pH stability of free and immobilized enzymes was determined in the pH range 3.0 to 11.0. Both free and immobilized enzymes were more stable at pH 8.0 (Fig. 4.1.8a). At higher pH, free enzyme was less stable compared to immobilized enzyme. Significant difference in enzyme activity and stability was not observed in the neutral range of pH but on the extreme side of pH, the catalytic activity and stability of the immobilized enzyme was enhanced compared to free enzyme.

Thermal stability of free and the immobilized BSH enzyme were investigated over the temperature range 40°C to 80°C. Free enzyme was more stable at 50°C after a period of 1 h incubation whereas maximum stability for immobilized enzyme was observed at 60°C. Enzyme activity of the immobilized enzyme enhanced nearly 38% in comparison with free enzyme after incubation for 1h at 40°C. Immobilized enzyme maintained stability upto 60°C but at higher temperatures the enzyme activity decreased about 15% compared to free enzyme (Fig. 4.1.8b).

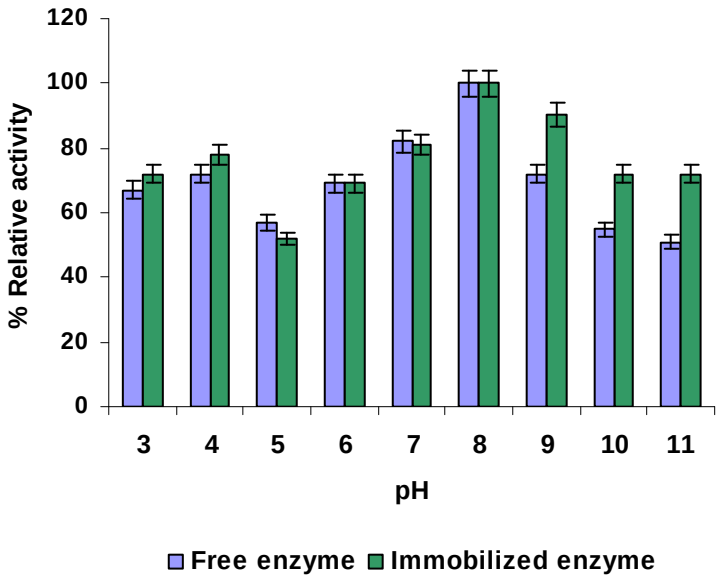


Fig. 4.1.8 a: pH stability of free and immobilized BSH enzymes.

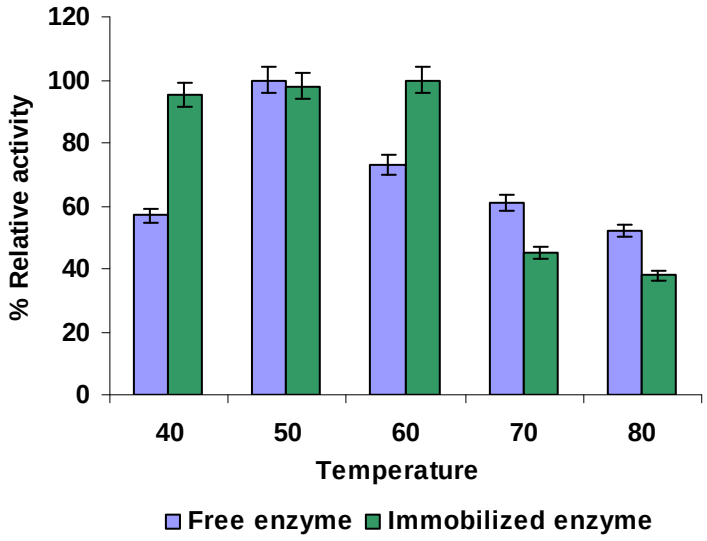


Fig. 4.1.8 b: Temperature stability of free and immobilized BSH enzymes.

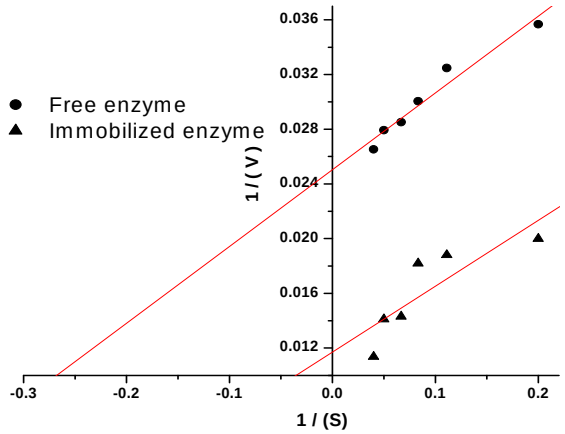


Fig. 4.1.9: Lineweaver-Burk plot of free and immobilized BSH enzymes.

Bile salt hydrolase was immobilized on functionalized SBA-15 with 57% efficiency. The values of the Michaelis constant (K_M) of the immobilized enzymes (4.1 μM) were higher than those of the free enzymes (2.2 μM), indicating the presence of partitioning and diffusional effects in the pores of the SBA-15 matrix (Fig. 4.1.9).

In summary, amino functionalized mesoporous SBA-15 was used as drug delivery matrix for loading of bile salt hydrolase enzyme. This is the first report of bile salt hydrolase immobilization on mesoporous silica. Immobilization of the enzyme did not affect the enzyme activity and about 4.1 mg of enzyme was loaded per gram of mesoporous silica at pH 6.5. Decrease in surface area and pore volume of amino functionalized SBA-15 due to immobilization indicated that the BSH enzyme is incorporated insides the pores of matrix. Physicochemical characterization of the immobilized indicated that the well ordered hexagonal pore structure of SBA-15 is maintained after immobilization. Shift in the pH optima probably due to the maintainence of local pH inside the pores of matrix, affecting the enzyme activity. Enhancement in pH stability at the extreme side of pH may favor the use of immobilized enzyme for drug delivery purpose to withstand extreme pH conditions in the gastrointestinal conditions.

Chapter 4 B

Structural features of Penicillin acylase adsorption on APTES functionalized mesoporous silica

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Part of the work presented in this chapter has been **published:**

Shah, P., Sridevi, N., Prabhune, A., Ramaswamy, V (2008) **Microporous and Mesoporous Materials,** 116:157-165.

Shah, P., Sridevi, N., Prabhune, A., Ramaswamy, V (2007) **Studies in surface science and catalysis,** 170 (B):1891-1898.

Abstract

Penicillin G acylase (PGA), a prominent member of Ntn hydrolase family was immobilized on amino-functionalized mesoporous silica by adsorption method. Penicillin G acylase has tremendous biomedical applications due to its key role in the synthesis of semi synthetic penicillins. The effect of PGA immobilization on amino-functionalized SBA-15 materials and the effect of silica as host matrix on enzyme kinetics were studied. The maximum loading of the enzyme was observed at pH 7.8 and the loading capacity of immobilized PGA is 68 mg protein per gram of functionalized SBA-15. Physicochemical characterization of the immobilized enzyme by nitrogen adsorption, powder XRD and TEM methods indicated that the characteristic hexagonal features and the original pore structure of the parent SBA-15 is retained after immobilization reaction. Thermal stability of penicillin G acylase was enhanced due to immobilization. This enhanced stability is attributed to the protective nature of the cage itself and to the rigidity of the SiO_2 matrix, which reduces the freedom of peptide-chain refolding of molecular motions that occur in denaturation processes.

4.2.1. Introduction

Penicillin G acylase (PGA; EC 3.5.1.11) is one of the key pharmaceutical enzymes in the production of semi synthetic β lactam antibiotics. PGA belongs to the structural superfamily of N-terminal nucleophile hydrolases that share a common fold around the active site bearing a catalytic serine, cysteine, or threonine at the N-terminal position (Duggleby *et al.,* 1995). Functionally, PGA acts on the side chains of penicillins, cephalosporins to produce antibiotic intermediates such as 6-amino penicillanic acid (6-APA) and 7-amino desacetoxy cephalosporanic acid (7-ADCA), leaving behind phenyl acetic acid (PAA) as a common by-product (Fig. 4.2.1) (Arroyo *et al.,* 2003). These antibiotic intermediates are the building blocks of semi-synthetic penicillins and cephalosporins (Elander, 2003).

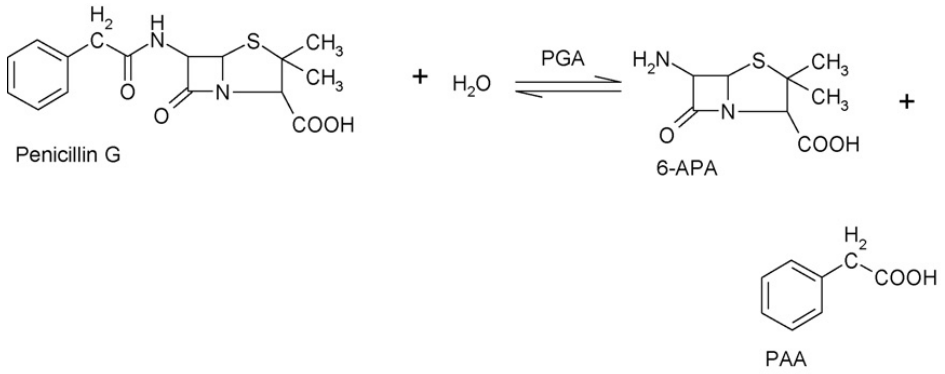

Fig. 4.2.1: Reaction mechanism of Penicillin G acylase

The cost of 6-APA has direct impact on the profitability of semi synthetic penicillin production. Immobilized penicillin G acylase accounts for 88% of the world wide 6-APA production (Rajendran and Gunasekaran, 2004). An efficient recovery and reuse of penicillin G acylase is a prerequisite for its economic industrial applications. Although other methods have been used for hydrolysis of penicillin to 6-APA, use of immobilized enzyme is considered superior to all methods. PGA has been immobilized on various matrices, ranging from natural polymers to synthetic polymers. Numerous matrices, including Eupergit C (Katchalski-Katzir and Kraemer, 2000) amberlite XAD-7, (Bianchi *et al.,* 1996) aldehyde-agarose, (Guisán, 1988) gelatine, (Norouzian *et al.,* 2002) concanavalin A, (Mislovicˇová *et al.,* 2004) layered double hydroxides (LDH), (Ren *et*

al., 2002) and magnetic microparticles of polymers (Bozhinova *et al.,* 2004) have been studied as the supports, aiming at a high activity per unit volume and an operational stability in its applications, and the efficient transfers.

Mesoporous MCM-41, MCM-48, SBA-15, and other types of mesoporous silica materials have been studied as the support for the immobilization of PGA (He *et al.,* 2000, Yiu *et al.,* 2001). Surface modification of mesoporous silica with organo-functionalities can enhance the interactions of enzyme with the support materials. A novel composite support PEI/SiO_2 in which Polyethyleneimine grafted onto the surfaces of silica gel particles was also used for the immobilization of penicillin G acylase (Gao *et al*., 2006). In recent study hollow silica nanotubes have been used for the immobilization of penicillin acylase enzyme (Gui Xiao *et al*., 2006). In this study, we have used amino functionalized mesoporous silica for the immobilization of penicillin acylase enzyme. The amino functionalized SBA-15 provides a large number of amino groups for covalent cross-linking of enzyme.

4.2.2. Materials and Methods

4.2.2.1. Materials

Commercially available penicillin G acylase (PGA; 3.5.1.11) was kind gift from KDL biotech Ltd (India). Sodium salt of penicillin G and 6-APA were kind gifts from Hindustan Antibiotics Ltd (India). All other reagents and chemicals used were from standard commercial sources and of the highest quality available. All buffers were essentially prepared in glass-distilled water.

4.2.2.2. Immobilization procedure

Mesoporous silica was synthesized as described in Chapter IV-A. Immobilization of PGA was performed as per following procedure. About 0.5 g of the support solid, SBA-15 was suspended in an aqueous solution of PGA (2130 U) prepared in potassium phosphate buffer (0.05 M, pH 7.8). The mixture was kept with slow stirring at 10°C for 12 h. The reaction mixture was centrifuged after 12 h and SBA-15 was given a wash with potassium phosphate buffer. The amount of PGA immobilized on SBA-15 was measured

in supernatant by determining the difference in the protein concentration of the enzyme before and after the immobilization reaction. Protein concentration was determined by Micro Lowry method (Lowry *et al.,* 1951). Enzyme activity in the immobilized system was measured as described in pencillin G acylase assay. Amino functionalized SBA-15 is designated as Am-SBA-15 and PGA immobilized amino functionalized SBA-15 sample is designated as PGA-Am-SBA-15.

4.2.2.3. Penicillin G acylase assay

Penicillin G acylase activity of free and immobilized enzymes were determined as described by Suresh Kumar *et al.,* 2004. Briefly, free and immobilized enzymes were suspended respectively in a reaction mixture containing 50 mM potassium phosphate buffer, pH 7.8 and 20 mg/ml Potassium penicillin G. The reaction mixtures were incubated at 40°C for 10 min and the 6-aminopenicillanic acid (6-APA) formed in the reaction was estimated spectrophotometrically using p-dimethyl amino benzaldehyde. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μ mole of 6-APA per min under assay conditions.

4.2.2.4. Penicillin G acylase adsorption at various pH

In each adsorption experiment, 0.25 g of the Am-SBA-15 adsorbent was suspended in 0.2 ml of the PGA solution in 100 mM of respective buffer solutions at different pH (pH 4.0, 6.0, 7.8 and 10.0). The amount of PGA adsorbed was calculated as described in the section 4.2.2.2.

4.2.2.5. pH and temperature profiles of free and immobilized PGA enzymes

The effect of pH on the catalytic activity of both free as well as immobilized PGA was evaluated by measuring initial rates of reaction in different buffers of same ionic strength but of different pH. This was done by assaying the enzyme activity at 40°C in 50 mM strength of buffer solutions with different pH ranging from 4.0 to 10.0. The optimal temperature for the enzyme activity of the immobilized enzyme was determined in a temperature range of 30°C to 80°C and compared with that of free enzyme under similar conditions.

4.2.2.6. pH and temperature stabilities of free and immobilized PGA enzymes

The stability of immobilized PGA enzyme was compared with that of free enzyme at different pH values by using 50 mM strength buffers of different pH ranging from 4.0 to 10.0. Samples were incubated at different pH for 1 h at room temperature and the residual activity of the enzyme was determined by standard PGA assay as described before. In addition, the thermostability of both free and immobilized enzymes were also compared after incubation at different temperatures ranging from 30°C to 80°C for 1 h and the residual activity was determined by the standard assay of PGA as described earlier.

4.2.2.7. Characterization

The powder XRD patterns of SBA-15, Am-SBA-15 and PGA-Am-SBA-15 samples were taken from X, Pert Pro (M/s Panalytical) diffractometer using Cu-Kα radiation and proportional counter as detector. A divergent slit of $1/8^0$ in the primary optics and an antiscatter slit of $1/4^0$ in the secondary optics were employed to measure the data in the low angle region. The BET surface area of the samples was determined by N_2 adsorption at 77K by using Autosorb-1 instrument. The sample was weighed and degassed at room temperature for 75 min, prior to nitrogen gas adsorption. Samples were not heated during degassing to prevent protein denaturation. Scanning electron micrographs of the samples were recorded on a JEOL Model 1200 EX microscope operating at 100 kV. Samples were dispersed in isopropyl alcohol, deposited on a Cu grid and dried. The IR spectra were recorded in diffuse reluctance mode (spectral resolution = 4 cm^{-1}; number of scans =100) using FT-IR spectroscopy (Shimadzu 8201 PC spectrophotometer in the region 400-4000 cm^{-1}).

4.2.3. Results and Discussion

4.2.3.1. Enzyme adsorption and surface properties

Amino functionalized (APTES) mesoporous silica SBA-15 molecular sieves have been prepared and used for the immobilization of PGA. By incorporating the organosilanes on the surfaces of SBA-15 materials, the carboxylic groups on the enzyme surface interacts with amino functionalized SBA-15 by the formation of covalent bond, thus increasing the adsorption rate and amount of PGA enzyme. Surface functionalization also increases in

the operational stability of immobilized enzyme. The surface density of binding sites together with the volumetric surface area sterically available to the enzyme, determine the maximum binding capacity. It can be observed that almost 65% of PGA gets loaded on silica matrix. The optimum pH at which PGA adsorbs on SBA-15 was determined by performing the immobilization process at various pH values (4.0, 6.0, 7.8 and 10.0). Fig. 4.2.2 shows the adsorption isotherms of PGA adsorbed onto Am-SBA-15 at different solution pH values ranging from 4.0 to 10.0. The net positive charge on PGA increases from pH 10.0 to pH 4.0. So the electrostatic attraction between Am-SBA-15 and PGA become less significant at lower pH and hence the surface coverage is expected to decrease with increasing proton concentration as result of increased repulsion between molecules. It is reasonable to assume that the area per molecule of PGA at pH 4.0 is higher, which could be due to an increase number of positive charges on the protein, thereby increasing its surface coverage. Therefore at a solution of pH 4.0, the PGA molecules are larger and occupy more space than in a solution with a pH near the pI. (Fig. 4.2.2). Most of the mesopores of SBA-15 (pores size 66 A°) are only few A° smaller or larger than PGA molecules (70 × 50 × 55 A°), it is reasonable to imagine that protein molecules can partially enter the pores and block the pore openings and thereby allowing limited access to nitrogen molecules. This is likely due to the significant decrease in surface area (from 605 m^2g^{-1} to 175 m^2g^{-1}) upon adsorption of 24.9 mg PGA at pH 4.0 (Table 4.2.1). While at a solution pH of 6.0, 7.8 and 10.0, the adsorption of PGA is 25.2, 34.0 and 22.8 mg, respectively. The lower adsorption capacities at a solution of pH 4.0 and 10.0 as compared to 6.0 and 7.8 are due to the strong electrostatic repulsion between the protein and the adsorbent surface. The isoelectric point (pI) of PGA is around 8.1 and hence the protein is positively charged at a pH below pI and negatively charged at a pH above pI. At a pH near the isoelectric point, the net charge of the protein is zero and hence the lateral interactions between the protein molecules are reduced, thus leading to higher amount of adsorption at a solution pH close to the pI. It is interesting to note that the PGA adsorption on the surface at pH 7.8 is 34 mg, which is more than that of other pH values. This shows that pH has a strong effect on the PGA adsorption. The negatively charged Am-SBA-15 (isoelectric point ~3) surface may attract

the polar and positively charged regions of the PGA molecules, thus leading to higher amount of adsorption at a solution pH close to the pI.

Table 4.2.1: Physicochemical characteristics of immobilized PGA at various pH.

Samples	pH	Protein loaded (mg)	Total SA m^2g^{-1}
PGA-Am-SBA-15	4.0	24.9	175
	6.0	25.2	219
	7.8	34.0	256
	10.0	22.8	271

PGA-Am-SBA-15 = PGA immobilized SBA-15

SA = Surface area

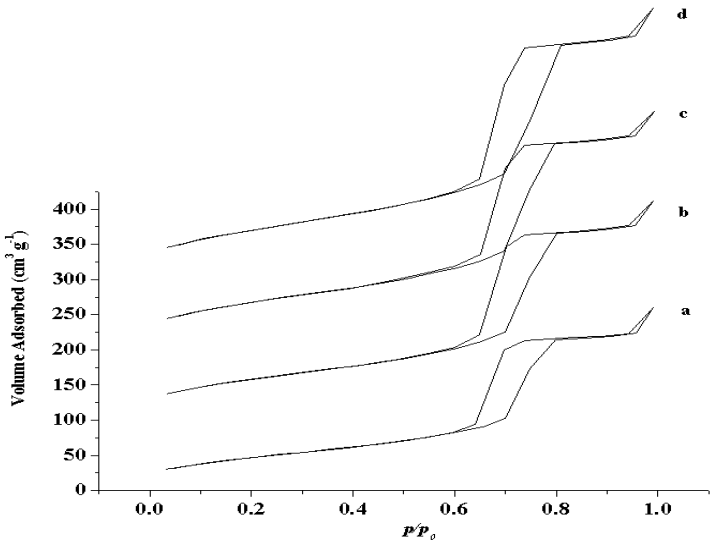

Fig. 4.2.2: Nitrogen adsorption/desorption isotherms of PGA on Am-SBA-15 at various pH values: (a) 4.0, (b) 6.0, (c) 7.8 and (d) 10 samples**.**

4.2.3.2. Structure of mesoporous materials

The nature of the support also has considerable effect on an enzyme's expressed activity and apparent kinetics. The structure of the support material used for immobilization must be stable after immobilization reaction. The structural stability of SBA-15 material after immobilization process was determined with X-ray diffraction (XRD). Typical XRD patterns of SBA-15, Am-SBA-15, and PGA-Am-SBA-15 in the range from $0.5° < 2\theta < 5°$ are shown in Fig. 4.2.3. They exhibit very similar patterns with well-resolved peaks at 0.8° (2 θ) and two peaks at 1.6° and 1.7° (2 θ) due to (10), (11) and (20) Bragg reflections, respectively indicates a good mesoscopic structure of SBA-15 are maintained in PGA-Am-SBA-15 samples. The powder X-ray diffraction patterns also reveal that these materials have a good long range order of mesostructure. A strong decrease in XRD peak intensity is observed after PGA adsorption onto Am-SBA-15 at pH 7.8. Larger contrast in density between the silica walls and the open pores relative to that between the silica walls and the PGA molecule may be responsible for the observed decrease in intensity.

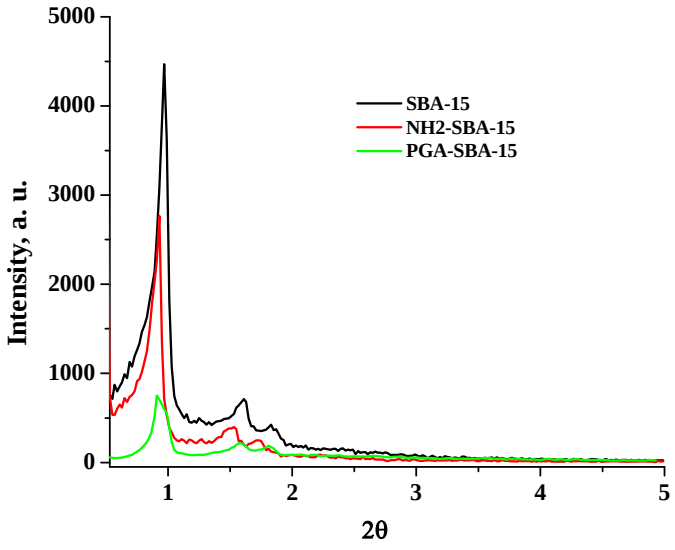

Fig. 4.2.3: Powder XRD patterns in the 2 θ region of 0-5° of SBA-15, Am-SBA-15 and PGA-Am-SBA-15 samples

4.2.3.3. Porosity and surface area

Nitrogen adsorption-desorption isotherms from the experiment carried out at 77 K to observe the textural changes in SBA-15, Am-SBA-15 and PGA-Am-SBA-15 are shown in Fig. 4.2.4 a, b and c respectively. All the three samples gave typical irreversible type IV adsorption isotherm with an H1 hysteresis loop, which is a typical feature of mesoporous materials. The sorption data have been used to get information about the mesoporosity, the total (BET) surface area according to BJH model. Post synthesis modification of mesoporous materials leads to decrease in mesoporous area and with an increase in concentration of the amine-functionalized moiety in SBA-15 samples. Table 4.2.2 presents a summary of the physicochemical characteristics of SBA-15 prior to and after PGA adsorption. The immobilized PGA enzyme samples show isotherms with similar inflections, which tend towards lower p/p_0 range. Larger decrease in specific surface area is observed from 814 m^2g^{-1} to 256 m^2g^{-1} for the immobilized PGA samples. Since SBA-15 is typically known to have considerable micropores, which originate from the corona of the silica wall, it will be interesting to see the changes that pore structure undergoes as a result of immobilized PGA enzyme in the silica matrix. Incorporation of enzyme does not affect the original pore structure of the parent SBA-15. This indicates that PGA molecules can be packed inside the mesopores without affecting the structural integrity of the parent SBA-15 materials.

Table 4.2.2: Physicochemical characteristics of SBA-15 and immobilized PGA

Samples	**Total SA m^2g^{-1}**
SBA-15	814
Am-SBA-15	605
PGA-Am-SBA-15	256

Am-SBA-15 = Amino functionalized SBA-15

PGA-Am-SBA-15 = PGA immobilized SBA-15

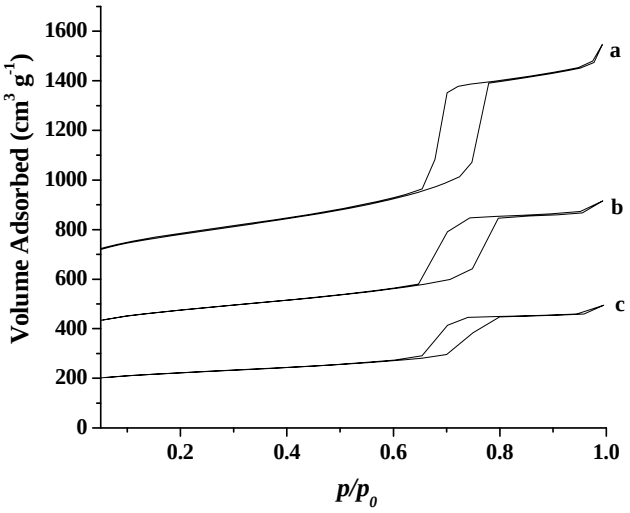

Fig. 4.2.4: Nitrogen adsorption/desorption isotherms of (a) SBA-15, (b) Am-SBA-15, (c) PGA-Am-SBA-15 samples.

4.2.3.4. Infrared spectroscopy

Fig. 4.2.5 shows the FTIR spectrum of SBA-15, Am-SBA-15 and PGA-Am- SBA-15 samples, respectively. In Am-SBA-15, the occurrence of strong absorption bands at 2943 cm^{-1} is assigned to the NH_2 stretching vibration, indicating the formation of the open-chain products containing terminal amine groups. The band at 1311 cm^{-1} is characteristic absorption bands of CH_2 groups and the band at 1049 cm^{-1} is due to C-N group. The appearance of these bands shows that the reaction is occurring between 3-amino propyl trimethoxy silane and silanol groups on the SBA-15 silica surface. The IR spectra of the PGA-Am-SBA-15 sample show the occurrence of strong absorption band at 1651 and 1599 cm^{-1} attributable to C=N stretching modes indicating the expected imino compound was formed by condensation of terminal amine groups with the C=O groups on the surface of the protein.

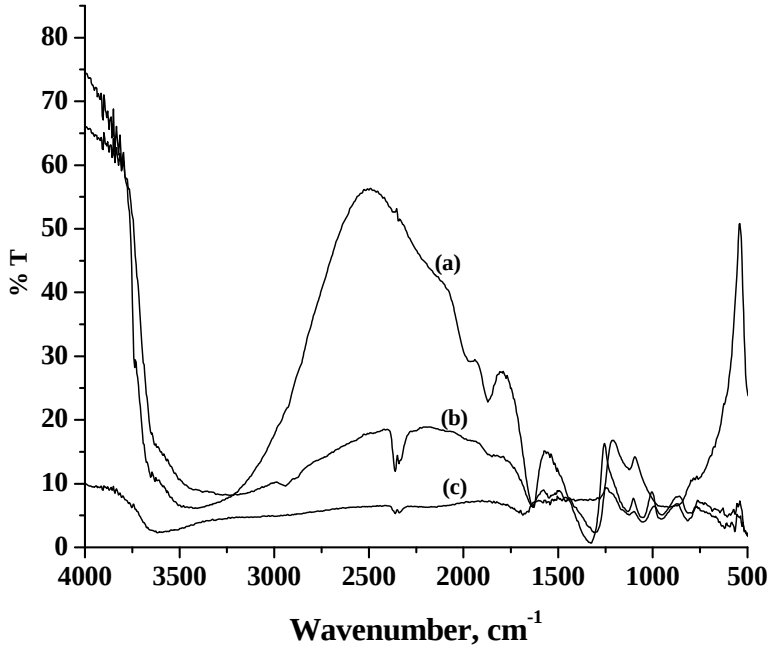

Fig. 4.2.5: FTIR spectrum of (a) SBA-15, (b) Am-SBA-15, (c) PGA-Am-SBA-15 samples.

4.2.3.5. Scanning and transmission electron microscopy

Scanning electron micrographs of the SBA-15 and PGA-Am-SBA-15 are shown in Fig. 4.2.6. Penicillin acylase appeared as white colored dot like structures attached on the plain surface of SBA-15 matrix. SEM images clearly indicate that PGA is adsorbed on mesoporous silica.

Transmission electron micrographs of Am-SBA-15 and PGA-Am-SBA-15 (Fig. 4.2.7 a-c) show the hexagonal array of uniform channels. A well-ordered hexagonal array of mesopores further justify the good mesostructure of both the functionalized and immobilized samples, when the electron beam is parallel to the main axis of these cylinders as shown in Fig. 4.2.7 a & b. The hexagonal array of the cylindrical pores is viewed from the side resulting in a striped image. TEM further confirms that structural ordering is maintained after immobilization of PGA on the SBA-15 materials and the immobilized PGA enzyme appeared as black objects between the walls in Fig. 4.2.7 (b & c). From the well-ordered hexagonal array of mesopores, the two dimensional hexagonal (*p6mm*) mesostructure can be confirmed.

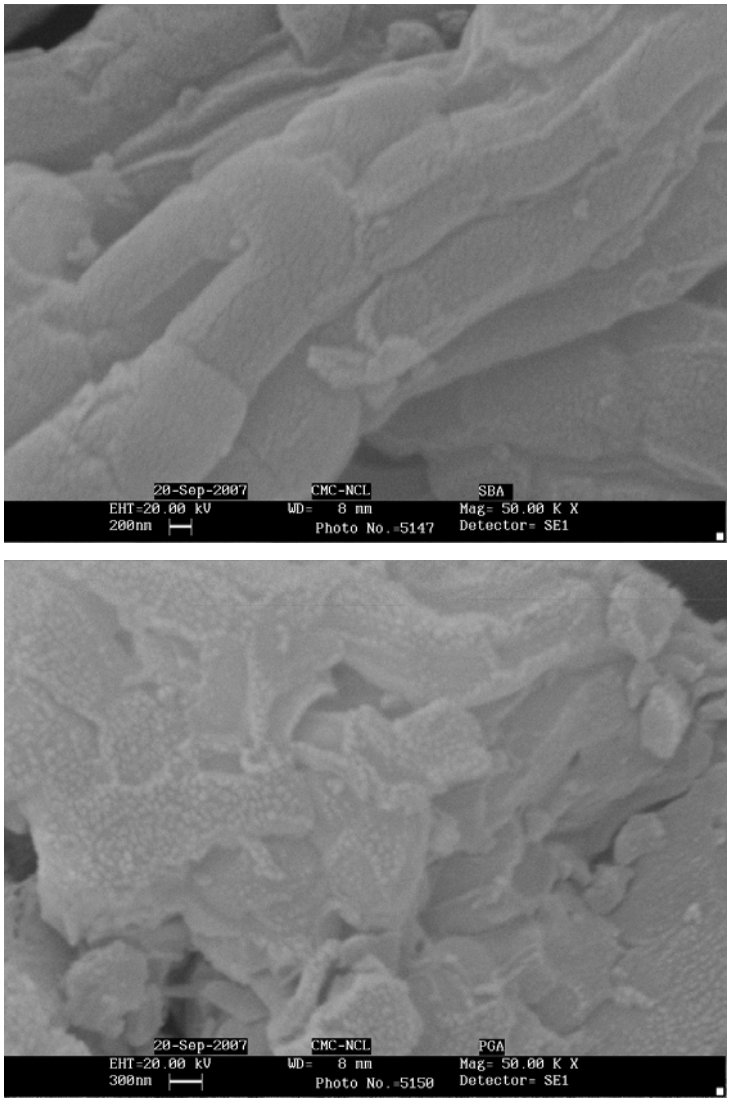

Fig. 4.2.6: SEM pictures of (a) Am-SBA-15 (b) PGA-Am-SBA-15 samples**.**

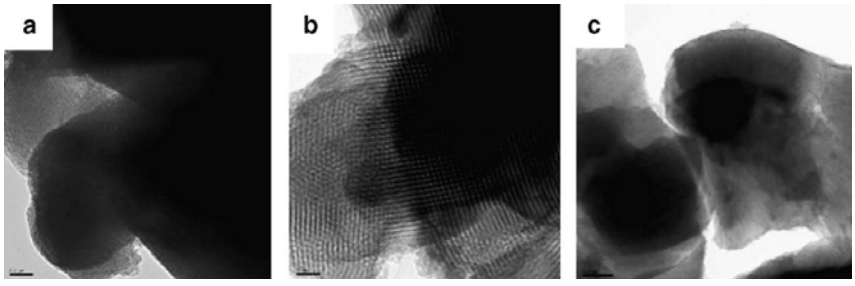

Fig. 4.2.7: TEM images of (a) Am-SBA-15, (b & c) PGA-Am-SBA-15

4.2.3.6. pH and temperature profiles of free and immobilized PGA enzymes

The catalytic properties for the hydrolysis of potassium penicillin G by immobilized and free enzyme were studied under various conditions. The optimum pH for the enzymatic activity of immobilized PGA was 9.0 and the maximum activity for the catalysis of free PGA was found at pH 8.0. The optimum pH for an immobilized enzyme is shifted to a higher or lower pH depending upon surface charges of the support (Mohy Eldin *et al.,* 2000, Liu *et al.,* 2001). As shown in Fig. 4.2.8a, the pH optima curves shifted to higher pH values and the shift is approximately +1 pH unit when enzyme is in the immobilized form. This shift in optimum pH towards alkaline range favors penicillin G hydrolysis. Maintenance of alkaline pH prevents the acidification of reaction mixture due to the accumulation of phenylacetic acid, a byproduct formed during penicillin G hydrolysis (Sudhakaran *et al.,* 1992).

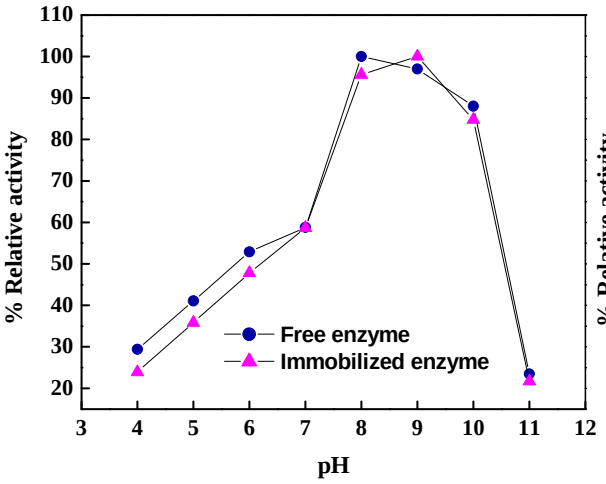

Fig. 4.2.8 a: pH profile of free and immobilized enzymes

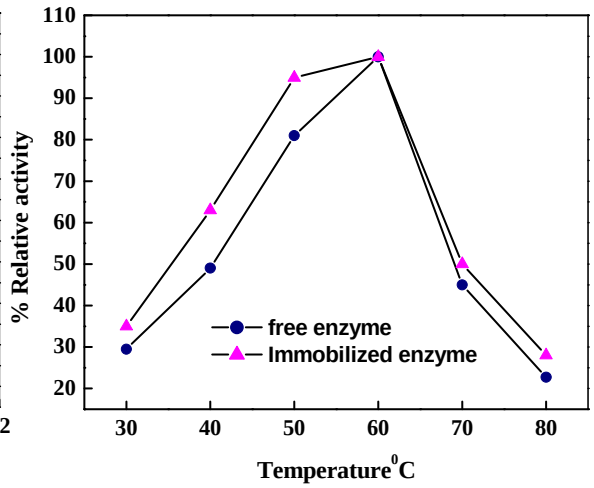

Fig. 4.2.8 b: Temperature profile of free and immobilized enzymes

The temperature profile for the catalytic activity of free and immobilized PGA enzymes is depicted in the Fig. 4.2.8 b. Optimum enzyme activity for both free and immobilized enzymes was observed at 60°C. Slight broadening of temperature activity profile in the case of immobilized enzyme indicates that the immobilized maintained higher enzyme activity compared to free enzyme.

4.2.3.7. pH and temperature stabilities of free and immobilized PGA enzymes

The pH stability for both free and immobilized PGA enzymes was examined in the pH range 4.0 to 10.0. Free enzyme is more stable at pH 9.0 whereas the stability of immobilized system is optimum at pH 8.0 (Fig. 4.2.8c). We investigated the thermostability of free and the immobilized PGA enzyme to determine whether the matrix could protect the immobilized enzyme from thermal denaturation (Fig. 4.2.8d). Free enzyme is very sensitive to high temperature, increase in temperature results in denaturation of the enzyme which leads to a decrease in effective concentration of the enzyme and hence a decrease in reaction rate. Temperature stability data indicates immobilized enzyme is more stable than free enzyme. Increase in temperature stability of immobilized enzyme is attributed due to protective cage structure of SBA-15 material which prevents thermal denaturation of the enzyme.

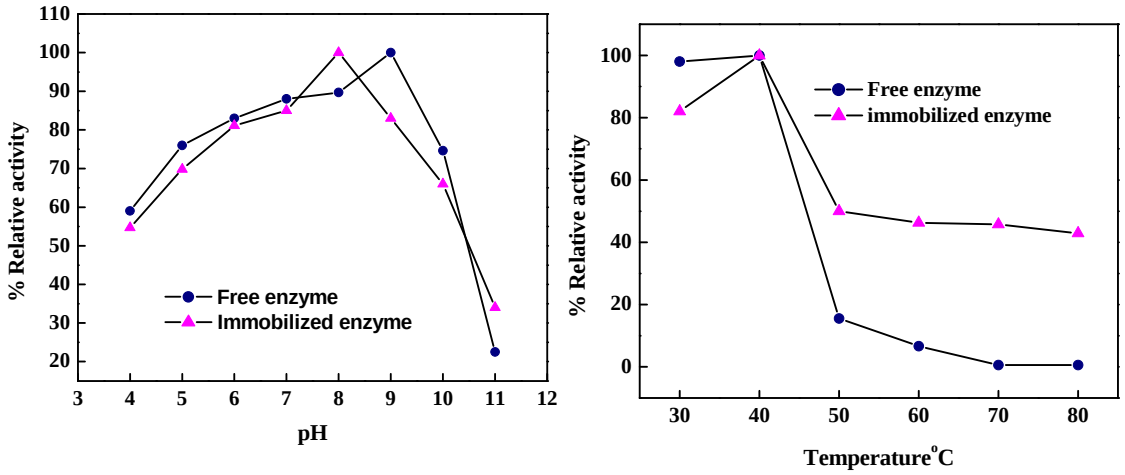

Fig. 4.2.8 c & d: pH and temperature stabilities of free and immobilized PGA.

The operational stability of the immobilized PGA in repeated cycles of use revealed that immobilized PGA retained about 60% of its activity after nine cycles of use (Fig. 4.2.9 a). Compared to free soluble enzymes, immobilized PGA enzyme has the additional advantage of being easy to recover and recycle. Reusability of the immobilized PGA has potential commercial applications.

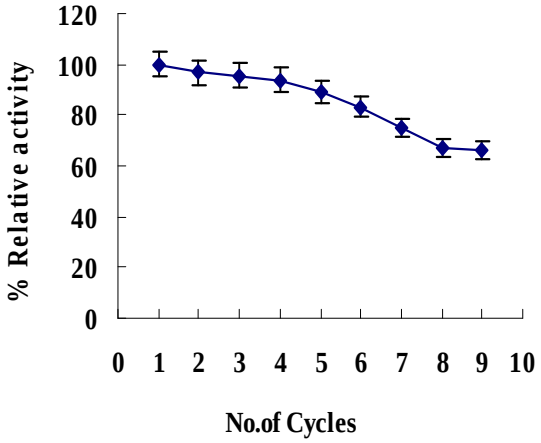

Fig. 4.2.9 a: Reusability of immobilized PGA

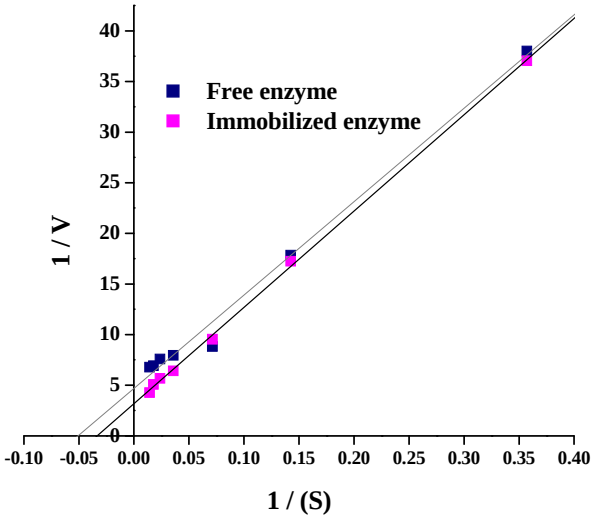

Fig. 4.2.9 b: LB plot of free and immobilized PGA

Kinetic properties of PGA immobilized on mesoporous SBA-15 were compared with free enzyme in solution. K_M values of free and immobilized PGA towards penicillin G were 20.0 μM and 32.3 μM respectively. There is no significant difference in the K_M values of free and immobilized PGA enzymes. This indicates that partitioning and diffusional effect is less in the pores of the SBA-15 in immobilized PGA.

In summary, the present chapter describes the immobilization of industrially important enzyme penicillin G acylase on amino-functionalized mesoporous silica. Interaction between carboxylic groups of protein and amino groups of mesoporous silica play an important role in this reaction. Characterization of the immobilized enzyme revealed that the hexagonal pore structure of the SBA-15 material was maintained after immobilization. Enhanced thermal stability and reusability of the immobilized penicillin acylase have industrial applications.

Chapter 5

In vivo cholesterol reduction studies

The work present in this chapter has been **published**:

Sridevi, N., Vishwe Pradnya., Prabhune Asmita (2009) **Food Research International,** 42:516-520.

Abstract

Food and Drug Administration (FDA) approved probiotic microorganism, *Lactobacillus buchneri* ATCC 4005 was used for *in vivo* cholesterol reduction studies. Culture conditions of this source were optimized for the maximum production of bile salt hydrolase, which resulted in 2.9-fold enhancement in activity. Bile salt hydrolase (BSH; EC 3.5.1.24) was isolated from *L. buchneri* and this partially purified enzyme was immobilized in 0.5% gellan gum gel. The immobilized enzyme was orally delivered in wistar rats, induced with hypercholesteremia by triton X-100. The effect of oral administration of the immobilized bile salt hydrolase enzyme on serum cholesterol, triglyceride, high density lipoprotein levels and its application in the therapeutic treatment of hypercholesteremia was assessed. Serum cholesterol and triglycerides concentrations were reduced by 50% and 15% respectively, in the group fed with immobilized enzyme 10 U/kg dose whereas administration of 20 U/kg immobilized enzyme resulted in reduction of serum cholesterol by 58% and triglycerides by 45% respectively. These results indicate that bile salt hydrolase has potential cholesterol lowering property and oral administration of the immobilized enzyme is an alternative pharmacological approach to reduce serum cholesterol levels.

5.1. Introduction

Coronary heart disease is one of the major causes of death and disability in many countries. The results from several epidemiological and clinical studies indicate a positive correlation between elevated total serum cholesterol levels and occurrence of coronary heart disease (Pereira and Gibson, 2002). Elevated levels of serum cholesterol are also a risk factor for the development of atherosclerotic vascular disease. Increase in cholesterol levels cause functional injury to the endothelium before morphological lesions develop. The primary strategy for reducing atherosclerotic vascular disease includes reducing cholesterol plasma levels and thereby preventing the deposition of plaque inside the artery wall (Lang and Binggeli, 2002). Drug therapy for hypercholesterolemia include fibrates, statins and bile acid sequestrants, however the undesirable side effects of these compounds have caused concerns about their therapeutic use. Hence, there is requirement of a more natural method to decrease serum cholesterol concentration in humans.

Probiotic cultures of *Lactobacilli* have potential health benefits. Several studies indicate consumption of certain cultured dairy products could lower total plasma cholesterol and low-density lipoprotein cholesterol. Mann and Spoerry (1974) discovered that consumption of fermented milk containing *Lactobacillus* strains has cholesterol lowering effect. Hypocholesterolemic effect of *Lactobacillus* was observed in humans (Agerbck *et al*., 1995), mice (Chiu *et al.,* 2006; Liong and Shah, 2005) and pigs (De Rodas *et al*., 1996; Gilliland *et al*., 1985). Therefore, the potential hypocholesterolemic activity of *Lactobacillus* has been a focus of interest.

The proposed mechanism for cholesterol lowering of *Lactobacillus* is comparable with that of a cholestyramine treatment which, like other bile salt sequestrants such as colestipol, binds to bile salts and prevents them from being reabsorbed. Thus, a decreased amount of bile salts would return to the liver, resulting in a loss of feedback inhibition of bile salt synthesis and an increased conversion of cholesterol to bile salts. The ingestion of lactic acid bacteria containing active bile salt hydrolase results in deconjugation of free bile salts, which are less water-soluble and are more easily excreted via faeces. Thus this

therapy might be regarded as a ‘biological’ alternative to common medical or surgical interventions to treat hypercholesterolemia.

Effect of *Lactobacillus buchneri* ATCC 4005 on serum cholesterol concentrations has not been reported previously in animal subjects. We are reporting here for the first time cholesterol lowering effect of *L. buchneri.* This study is designed to assess the impact of oral administration of bile salt hydrolase on serum cholesterol levels. The main objective of the present study is to validate the hypothesis that oral administration of bile salt hydrolase enzyme might lower serum cholesterol level in rats. The study also aimed to evaluate the effect of bile salt hydrolase on total cholesterol, triglycerides, and high density-lipoprotein (HDL) levels when fed orally.

5.2. Materials and Methods

5.2.1. Materials

Glycodeoxycholicacid, 2% ninhydrin reagent solution, trichloroacetic acid were purchased from Sigma. Low acyl gellan gum was kind gift from CP Kelco Ltd, India. Cholesterol estimation kit was obtained from Accurex Biomedical Pvt. Ltd., India. All other chemicals used were commercially available high purity or analytic grade compounds. All media were prepared in distilled water and all the buffers were prepared in glass distilled water.

5.2.2. Microorganisms

Cultures used in the present study were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune. The following cultures were screened for bile salt hydrolase activity: *Lactobacillus plantarum* (ATCC 8014, NCIM, 2083), *Lactobacillus buchneri* (ATCC 4005, NCIM 2357), *Lactobacillus delbrueckii* (NCIM 2025), *Lactobacillus helveticus* (ATCC 8018, NCIM 2126), *Lactobacillus viridescens* (NCIM 2167), *Lactobacillus jugurti* (NCIM 2366), *Streptococcus faecalis* (ATCC 29212, NCIM 5025).

5.2.3. Screening of the cultures for BSH activity

All the cultures obtained were screened for the production of bile salt hydrolase by modified ninhydrin method as described earlier. Briefly, the cultures were grown in MRS medium for 72 h at 37°C under anaerobic conditions. (Composition of MRS media is given in Table 5.1.). Cell bound activity of the cultures were determined as described in the section 2.2.4 of Chapter II**.** Cultures were routinely maintained on MRS stabs or plates by periodic subculture at 37°C under anaerobic conditions. The lactobacilli with measurable bile salt hydrolase activity were sub cultured at least three times in MRS broth under anaerobic conditions before further use.

Table 5.1: Composition of MRS medium

Name of the ingredient	Quantity (gram/lit)
Protease peptone	10.00
Beef extract	10.00
Yeast extract	05.00
Dextrose	20.00
Polysorbate 80	01.00
Ammonium citrate	02.00
Sodium acetate	05.00
Magnesium sulphate	00.10
Di-potassium hydrogen phosphate	02.00

5.2.4. Effect of culture conditions on BSH production from *Lactobacillus buchneri*

Among the different cultures tested, *Lactobacillus buchneri* showed maximum BSH activity for glycodeoxycholic acid and was used for further experiments. Culture conditions were optimized to increase the production of bile salt hydrolase from *L. buchneri*.

Batch fermentation was carried out in Erlenmeyer flasks (250 ml) containing 100 ml of sterilized MRS media, inoculated with 1% (v/v) of seed culture and incubated at 37°C for 72 h under anaerobic conditions.

5.2.4.1. Effect of different carbon sources on bile salt hydrolase activity

Effect of different carbon sources on activity of bile salt hydrolase was evaluated by incorporating 1% w/v carbon sources in the MRS media, which include lactose, maltose, sucrose, fructose, and glucose. Erlenmeyer flasks (250 ml) containing 100 ml of above mentioned medium were inoculated with 1% seed culture and incubated at 37°C for 72 h under anaerobic conditions. The cells were harvested from the culture broth by centrifugation at 19,000 × g for 2 min and used to determine BSH activity and cell biomass. MRS medium without carbon source served as control.

5.2.4.2. Effect of different nitrogen sources on bile salt hydrolase activity

Influence of different combinations of nitrogen sources was studied for the optimum enzyme yield. Erlenmeyer flask (250 ml) containing 100 ml of MRS media with different combinations of nitrogen sources was inoculated with 1% seed culture and incubated at 37°C for 72 h under anaerobic conditions. The results were compared with growth and BSH activity of nitrogen unsupplemented media.

5.2.4.3. Effect of pH on bile salt hydrolase activity

To study the effect of initial pH of medium, flasks containing 100 ml of MRS medium was adjusted to the pH range 4.0 to 11.0, prior to sterilization. The pH adjusted flasks were inoculated with 1% seed culture and incubated at 37°C for 72 h under anaerobic conditions. The cells were harvested from the fermentation medium by centrifugation at 19,000 × g for 2 min to determine cell-bound bile salt hydrolase activity as well as cell biomass.

5.2.4.4. Effect of temperature on bile salt hydrolase activity

Erlenmeyer flasks containing 100 ml of MRS medium with pH 7.0 were inoculated with 1% (v/v) seed culture and incubated in the temperature range of 30°C to 60°C for 72 h

under anaerobic conditions. The culture broth was centrifuged at 19,000 × g for 2 min and assayed for bile salt hydrolase activity and determination of cell biomass.

5.2.4.5. Time course for production of bile salt hydrolase

Kinetics of *L. buchneri* growth and bile salt hydrolase production were investigated in MRS medium at 37°C by inoculating the medium with 1% seed culture. For optimal fermentation time, samples were removed at various time intervals for bile salt hydrolase activity. Culture broth was centrifuged at 19,000 × g for 2 min and assayed for bile salt hydrolase activity with known amount of cells. Bile salt hydrolase production profile and cell growth of the culture was monitored for 120 h.

5.2.5. Preparation of cell free extract

L. buchneri was grown under optimal conditions for the production of bile salt hydrolase. One litre of MRS medium was inoculated with 1% inoculum and incubated for 72 h at 37 °C under anaerobic conditions. Cells in a stationary phase culture were sedimented by centrifugation for 10 min at 6000 ×g and 10°C, washed and suspended in 10 mM potassium phosphate buffer, pH 6.5. Cell suspension was disrupted by sonication in ice bath for 1 min at 80 amplitude with 0.5 sec pulse on and 0.8 sec pulse off using Branson Sonifier. Cell debris was removed by centrifugation at 19,000 ×g for 30 min and the clear supernatant containing enzyme was used for further studies.

5.2.6. Immobilization of bile salt hydrolase

Bile salt hydrolase was immobilized using gellan gum by the method of retrogradation. Gellan gum is exocellular, anionic, tetra polysaccharide with a molecular formula $[C_{23}H_{37}O_{18}\text{-}M^{+}]n$. It has a gelling temperature of 55°C and the melting temperature can be modified to either below or above 100ºC depending on the types and acylation. Deacylated gellan gum is having linear chain structure based on repeating glucose, rhamnose and glucoronic acid units in the ratio of 2:1:1. Gellan gum (Kelcogel F – Low acyl) was used in the present study. 0.5% (w/v) of polymer was dispersed in double distilled preheated water (80^{o}C) by gentle stirring. The temperature was then raised to 90^{o}C to achieve complete hydration of the polymer.

Bile salt hydrolase (51.3 U) was mixed with 0.5% (w/v) preheated and cooled gellan solution. The mixture was allowed to form gel and further subjected to freeze-drying under dust free conditions. The immobilized enzyme obtained was stored under dry conditions at 4°C and used for further studies. A placebo gel prepared by similar method, without the enzyme served as control. The activity of the enzyme was calculated using the enzyme assay method as described in Chapter II.

5.2.7. *In vivo* evaluation of bile salt hydrolase

The male albino wistar rats ranging from 200-220 gm in weight were obtained from National Toxicology Centre, Pune, India. These animals were split into four experimental groups each one consisting of six rats and allocated in polypropylene cages containing paddy husk as bedding. Animals were housed at temperature 24°C and relative humidity of 45-55%. A 12:12 light/dark cycle was followed. The experimental procedures and research protocol used in this study were reviewed and approved by Institutional Animal Ethics Committee (IAEC) constituted as per the guidelines of committee for purpose on animals (CPCSEA), India.

All the animal groups were deprived of food for 8 h prior to evaluate the cholesterol reducing effect of bile salt hydrolase. The formulation administered to different animal groups is given in Table 5.2. Rats were induced with hypercholesterolemia through a single intravenous injection of triton X-100 (200 mg/kg). Blood samples were collected into sterile tubes at 0 h and 24 h after triton X-100 injection by retro-orbital venous plexus. The obtained serum samples were centrifuged at 4000 $\times g$ and analyzed for cholesterol, high density lipoprotein (HDL) cholesterol, and triglycerides by using enzymatic kit.

Table 5.2: Formulations administered to four different animal groups (n = 6).

Group	Formulation
Control	0.5% gellan gum with normal saline
Triton control / HL	200 mg/kg triton X-100
HL with 10 U/kg BSH enzyme	200 mg/kg triton X-100 and 10 U/ kg immobilized BSH enzyme
HL with 20 U/kg BSH enzyme	200 mg/kg triton X-100 and 20 U/ kg immobilized BSH enzyme

HL = Hyperlipidemic

5.2.7.1. Statistical analysis

The following variables were taken into consideration: concentration of total cholesterol, concentration of HDL and concentration of triglycerides. The means per rat of each of these variables were measured by one-way ANOVA. The results obtained for each treatment was then evaluated by Dunnet's Test.

5.3. Results and Discussion

5.3.1. Effect of culture conditions on BSH production from *Lactobacillus buchneri*

Bile salt hydrolase activity is commonly expressed by gastrointestinal bacteria in response to the toxic effects of bile in the intestine and is distributed in several genera, including *Bacteroides*, *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacillus* (Begley *et al*., 2006). Characterization of BSH has been done in many species of lactobacilli including *Lactobacillus acidophilus* (Corzo and Gilliland, 1999a; Ahn *et al*., 2003), *Lactobacillus johnsonii* (Elkins and Savage, 1998), *Lactobacillus gasseri* (Usman and Hosono, 1999), and *Lactobacillus plantarum* (De Smet *et al*., 1994). In the present study, various microorganisms were screened for bile salt hydrolase production, as a result of which *Lactobacillus buchneri* was found to be a good producer of bile salt hydrolase (Fig. 5.1). Despite several reports on BSH production from the genus *Lactobacillus*, the characteristics of BSH from *L. buchneri* have not been elucidated yet. In the present study, we have optimized the production of BSH from *L. buchneri* and further isolated and analyzed the enzyme for its therapeutic potential in the treatment of

hypercholesterolemia. Different nutritional and physical parameters are optimized to improve the enzyme yield.

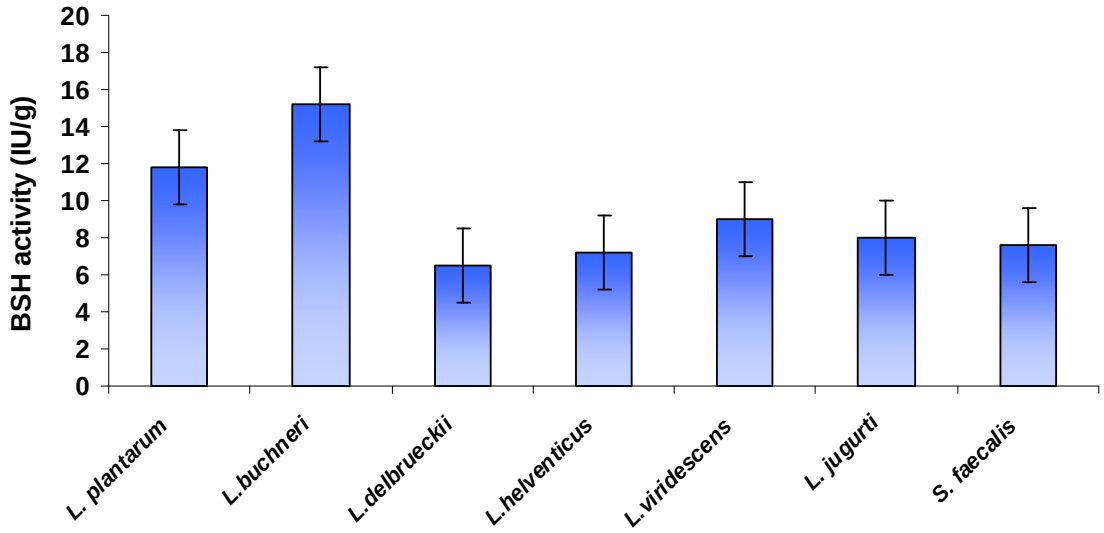

Fig. 5.1: Screening of different bacterial species for bile salt hydrolase activity

5.3.1.1. Effect of carbon source on BSH production from *L. buchneri*

The effect of various carbon sources on BSH production from *L. buchneri* was studied and the results are depicted in Fig. 5.2 a. Among the different carbon sources tested, the enzyme activity was highly influenced by sucrose, followed by maltose producing 15.5 U/g and 12.8 U/g BSH enzyme respectively. Bile salt hydrolase production was enhanced nearly two-fold compared to the control when sucrose was used as carbon source.

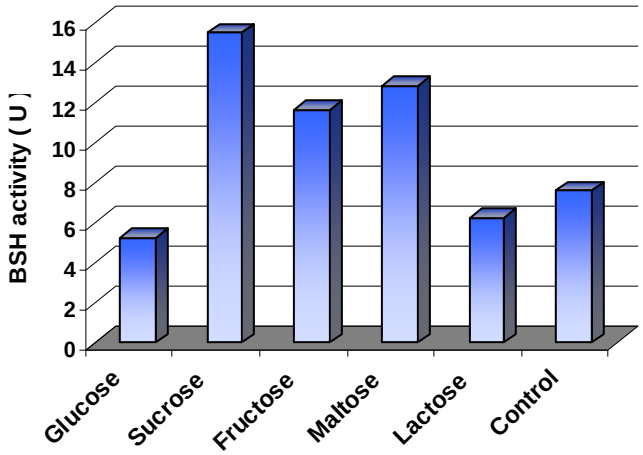

Fig. 5.2 a: Effect of carbon sources on bile salt hydrolase activity of *L. buchneri*.

5.3.1.2. Effect of nitrogen source on BSH production from *L. buchneri*

Different combinations of nitrogen sources were tested to study their effect on BSH production from *L. buchneri*. Among the different combinations of nitrogen sources tested, a combination of 1.25% of each peptone and yeast extract increased the enzyme activity by 2.9-fold (Fig. 5.2 b).

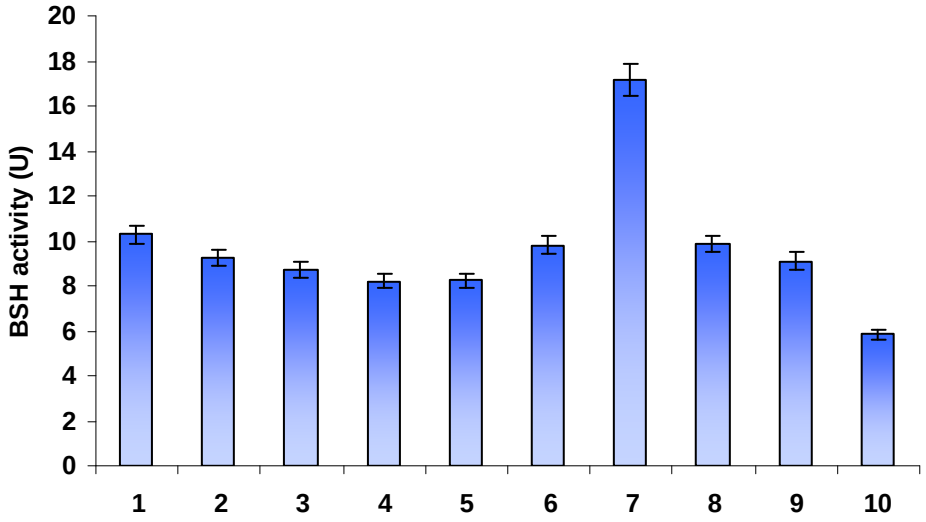

Fig. 5.2 b: Influence of different nitrogen sources on bile salt hydrolase activity of *L. buchneri*. **1**. Beef extract (1%) + Yeast extract (0.5 %) + Tryptone (1%) **2.** Beef extract (1%) + Yeast extract (0.5%) + Malt extract (1%) **3.** Peptone (1%) + Beef extract (0.5%) + Tryptone (1%) **4**. Peptone (1%) + Beef extract (0.5%) + Malt extract (1%) **5.** Peptone (1%) + Yeast extract (0.5%) + Tryptone (1%) **6.** Peptone (1%) + Yeast extract (0.5%) + Malt extract (1%) **7.** Peptone (1.25%) + yeast extract (1.25%) **8.** Peptone (1.25%) + Beef extract (1.25%) **9.** Beef extract (1.25%) + Yeast extract (1.25%) **10**. Control.

5.3.1.3. Effect of initial pH of media

The effect of initial pH of medium, on BSH production from *L. buchneri* is depicted in Fig. 5.2 c. The enzyme production was observed in the wide range of pH i.e. pH 3.0 to 11.0 but the optimum enzyme production was observed in MRS medium adjusted to pH 7.0. However, on acidic side of this pH, drastic decrease in enzyme production was observed. Specific activity also followed the similar pattern and maximum specific

activity was observed at pH 7.0. Optimum production of BSH from *L. buchneri* near human physiological pH favors the application of organism as potential probiotic.

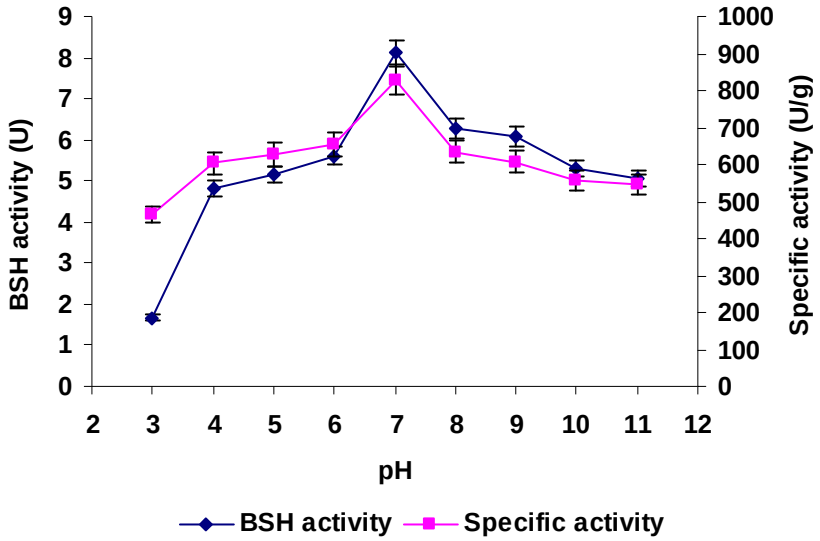

Fig. 5.2 c: Effect of initial pH on BSH production from *L. buchneri*

5.3.1.4. Effect of temperature on BSH production from *L. buchneri*

Effect of temperature on BSH production from *L. buchneri* is depicted in Fig. 5.2 d. The optimum temperature for BSH production was 37°C, which is also the optimum condition for the growth of *L. buchneri*. However, the production drastically decreased with increase in temperature of incubation.

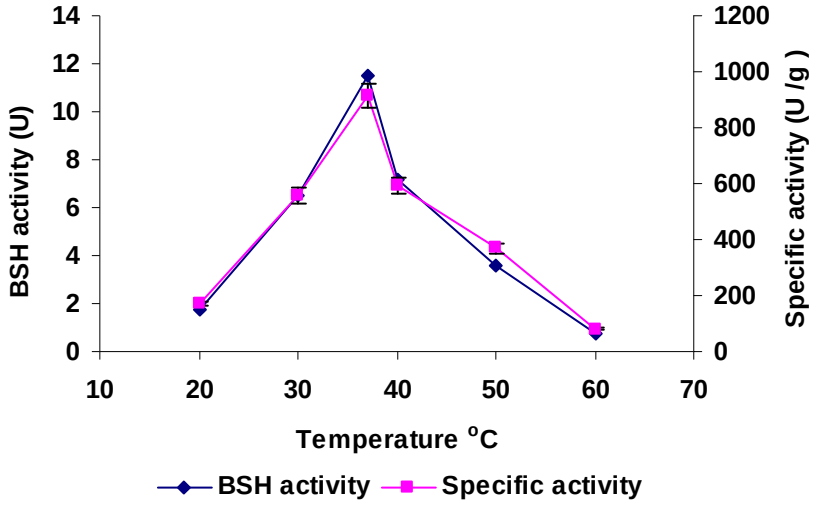

Fig. 5.2 d: Effect of temperature on BSH production from *L. buchneri*

5.3.1.5. Effect of incubation period

Time profile for the growth of *L. buchneri* and BSH production from the organism is depicted in the Fig. 5.2 e. Optimal BSH activity was detected after an incubation period of 72 h during stationary growth phase of culture. The results are similar to the previous findings for other *Lactobacillus* species (Nguyen *et al*., 2007).

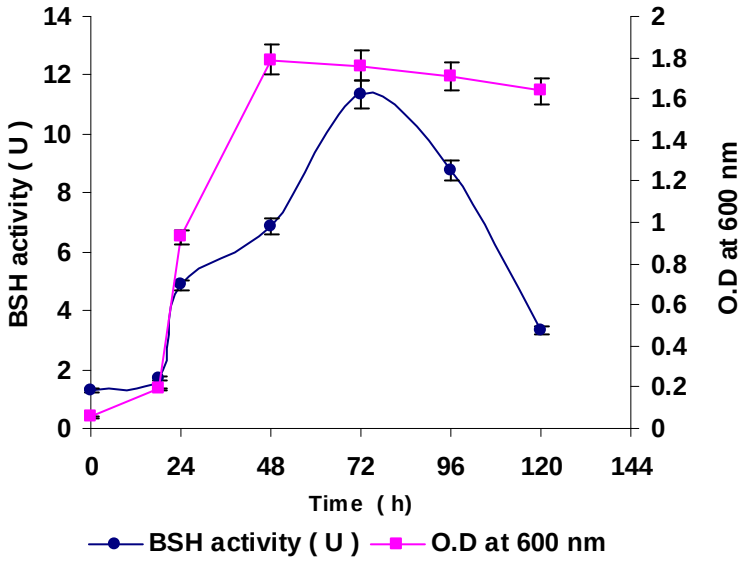

Fig. 5.2 e: Time course for the production of bile salt hydrolase from *L. buchneri*

5.3.2. *In vivo* evaluation of bile salt hydrolase

Elevated levels of the serum cholesterol are the route cause of many diseases such as cardiovascular diseases, peripheral vascular diseases, atherosclerosis etc. Several mechanisms have been proposed to explain the cholesterol lowering effects of *Lactobacillus* sp. One of the hypothesis proposed is the cholesterol lowering property of *Lactobacillus* is due to incorporation of cholesterol into the cellular membranes of bacteria from the medium during its growth period (Gilliland *et al*., 1985) where as the other one include, the main mechanism for the *in vitro* removal of cholesterol is linked to the bile salt hydrolase activity of the cells, releasing unconjugated bile acids that induce the cholesterol precipitation (Taranto *et al*., 1997). Some strains of *Lactobacillus* secrete bile salt hydrolase enzyme, which hydrolyzes conjugated bile acids to release free bile acids thereby increasing the demand of cholesterol for *de novo* synthesis of bile salts. Although various mechanisms were proposed to understand the cholesterol reducing effect of *Lactobacillus* sp. the precise mechanisms has to be determined yet. In our study, the effect of bile salt hydrolase on *in vivo* reduction of serum cholesterol was evaluated. Hypercholesterolemia was induced in the rats using triton X-100, which increased the cholesterol levels by 47% and serum triglycerides by 88% compared with values of control group after 24 h (Table 5.3). Triton-induced hypercholesterolemia may be considered a simple and rapid test for a preliminary evaluation of compounds potentially active on serum cholesterol (Paoletti, 1962). Systemic administration of triton X-100 (200 mg/kg) resulted in a biphasic elevation of plasma cholesterol and triglycerides. Concentration of serum cholesterol levels increased after 24 h in phase I. The mechanism of the triton X-100 induced hypercholesterolemia in phase-I was thought to be due to increased hepatic synthesis of cholesterol and the induced hypercholesterolemia decreased nearly to control levels within next 24 h in phase II.

Table 5.3: Lipid profile of control (n = 6) and triton control (treated) group (n = 6) after twenty four hours.

	Control	Triton control
Total cholesterol, mg/dl	69.21	105.90
Triglycerides, mg/dl	93.15	195.81
HDL, mg/dl	54.97	19.37

The activity of the free enzyme and immobilized enzyme was observed to be 51.29 U and 33.09 U, respectively. Immobilization efficiency was calculated to be 64.51%. Effect of bile salt hydrolase on total cholesterol in different animal groups is shown in Table 5.4. Cholesterol concentration in triton control group increased by 47% after 24 h due to the administration of triton X-100 compared with values of control group (that received normal saline). The total cholesterol concentration of the animal group treated with enzyme dose 10 U/kg and 20 U/kg were 51.972 mg/dl and 44.31 mg/dl, respectively. A reduction in 50% and 58% was observed after 24 h in the groups fed with 10 U/kg and 20 U/kg respectively. The concentration of serum triglycerides in triton control group increased from 103.644 mg/dl to 195.814 mg/dl in the duration of 24 h with an increase of 92.14 mg/dl due to the administration of triton X-100 (200 mg/kg) (Table 5.5). Serum triglycerides concentration were lowered about 15% and 45% respectively, after 24 h in the animal groups treated with triton X-100 and a dose 10 U/kg and 20 U/kg bile salt hydrolase enzyme, respectively. Triton interacts preferentially with HDL, changing the size and density of lipoprotein. Triton has high affinity for HDL, at low concentrations, it acts on surface components of the HDL particle and at higher concentrations, penetrates the particle, causing structural disruption (Yamamoto *et al*., 1984). In our study, the HDL values of animal groups treated with triton X-100 reduced significantly in comparison with control group treated only with normal saline (Table 5.6). HDL concentration in triton control group reduced from 72.918 mg/dl to 19.37 mg/dl after 24 h. Reduction in HDL concentration after 24 h was 53% in group treated with enzyme dose 10 U/kg and the reduction was 71% in the 20 U/kg respectively. Significant difference in HDL concentration between triton control group and other enzyme treated groups was not

observed. Reduction of HDL levels was also observed by Seok *et al*., (2004) in rats treated with triton WR-1339.

In many studies, *Lactobacillus* culture was administered as live microbial supplements in animals to observe the cholesterol lowering effect (De Smet *et al*., 1998). The duration for this effect ranges from 3 to 4 weeks. Colonization of live bacteria is an impact factor in such studies because of the prevailing harsh gastrointestinal conditions. In the present study we have administered immobilized bile salt hydrolase enzyme to prevent the problem of colonization and also the enzyme was immobilized in gellan gum fluid gel to with-stand the drastic changes in the intestinal (*in vivo*) environment. For the first time we have checked the direct effect of bile salt hydrolase enzyme on reduction of serum cholesterol levels.

Reduction in serum cholesterol levels by 3-4% is clinically significant. Since every 1% reduction in serum cholesterol levels leads to a 2-3% reduction in estimated risk for coronary heart disease (Manson *et al*., 1992). Nguyen *et al*., in 2007 have reported that mice fed with *L. plantarum* PH04 had 7% lower total cholesterol and 10% lower serum triglycerides compared to the control mice. In our study we observed 50% reduction in serum cholesterol and 15% reduction in triglycerides in wistar rats fed with 10 U/kg dose of immobilized bile salt hydrolase enzyme and 50% reduction in serum cholesterol and 45% reduction in triglycerides in wistar rats fed with 20 U/kg dose of immobilized bile salt hydrolase.

In summary, the current studies indicate that optimization of culture conditions resulted in 2.9-fold increase in the production of bile salt hydrolase from *L. buchneri* and the isolated enzyme has the potential to reduce serum cholesterol concentrations and could reduce the risk for coronary heart disease. However further studies are required for the better understanding of mechanism of action and the magnitude of the effect from bile salt hydrolase intake.

Table 5.4: Effect of bile salt hydrolase on total serum cholesterol**.**

Group	Zero hour		Twenty four hours	
	Mean	SD	Mean	SD
Triton control	71.99	7.37	105.90	11.43
Test I (10 U/kg)	63.35	5.87	51.97	10.19
Test II (10 U/kg)	67.68	5.95	44.31	10.55

Table 5.5: Effect of bile salt hydrolase on serum triglyceride levels**.**

Group	Zero hour		Twenty four hours	
	Mean	SD	Mean	SD
Triton control	103.64	13.88	195.81	16.27
Test I (10 U/kg)	70.51	8.92	167.25	25.67
Test II (10 U/kg)	82.42	17.89	108.64	18.12

Table 5.6: Effect of bile salt hydrolase on HDL cholesterol.

Group	Zero hour		Twenty four hours	
	Mean	SD	Mean	SD
Triton control	72.91	2.54	19.37	3.60
Test I (10 U/kg)	53.10	6.77	24.59	4.03
Test II (10 U/kg)	61.64	5.83	17.65	5.30

Chapter 6

Summary and Conclusions

6.1. Conclusions

Bile salt hydrolase (BSH; EC.3.5.1.24) is a member of Ntn hydrolase structural super family of proteins. This enzyme plays an important role in cholesterol catabolism and involved in reducing serum cholesterol levels. The exciting structural similarities and the evolutionary link among the members of Ntn hydrolase superfamily are the core reasons for increasing research in this field. The present THESIS emphasizes studies on bile salt hydrolase from a thermophilic source, *Brevibacillus borstelensis*. Optimization of fermentation conditions for the production of bile salt hydrolase, purification, characterization, and immobilization of this enzyme has been studied in detail. *In vivo* application studies were done with immobilized bile salt hydrolase from FDA approved *L. buchneri* and its effect on cholesterol reduction was evaluated. Penicillin acylase, an another member of Ntn hydrolase family, was immobilized on a novel support and detailed characterization was studied.

6.2. Scientific and possible outcomes of the work

Bile salt hydrolase is mostly produced by microorganisms belonging to the genus *Lactobacillus* and *Bifidobacterium*. The enzyme is commonly produced by mesophilic bacteria. Interest in the study of bile salt hydrolase from thermophilic source driven us for the isolation of BSH from thermophilic environment (hot water springs). Screening of bile salt hydrolase producing bacteria from thermophilic environment in our laboratory led to the identification of a thermophilic bacteria, with optimum growth in the temperature range of 45-65°C. This thermophile produces bile salt hydrolase intracellularly. The thermophilic bacterium was identified to be *Brevibacillus borstelensis* by 16S rDNA sequencing. **This is the first report of bile salt hydrolase from a thermophilic source.**

Bacteria with BSH activity are usually isolated from the environment in which bile salts are present. BSH activity is a positive trait for bacteria to survive in the gastrointestinal environment because BSH activity is involved with bile tolerance mechanism. In our present study BSH active *Brevibacillus borstelensis* was isolated from hot water springs, whose environment is quite different from the gastrointestinal environment. We do not

rule out the chance of fecal contamination at the site of sample collection but there is still a chance that this bacterium is a normal inhabitant of this ecosystem and may be a part of the carbon and nitrogen biogeocycles. *Brevibacillus borstelensis* may have been acquired *bsh* gene through horizontal gene transfer. Detailed investigations are required in this direction to support this hypothesis to identify the source of BSH gene in this thermophilic bacterium.

To study the characteristics of purified bile salt hydrolase enzyme from this thermophilic source, we have purified the enzyme to homogeneity and characterized the purified protein. The characteristics of purified enzyme were significantly different from the bile salt hydrolase purified from the other mesophilic sources. The wide range pH stability of this enzyme favors the application of this enzyme for *in vivo* cholesterol reduction studies. N-terminal sequence of the protein is distinguishably different from the other reported bile salt hydrolases. This difference in the N-terminal sequence indicates that there is a probability of divergence in the BSH family of proteins. Since *Brevibacillus borstelensis* is the sole thermophilic source identified till date for the production of BSH, further work needed to be carried out on tryptophan microenvironment of protein under different conditions like native, denatured and in presence of ligands. Folding and unfolding studies of protein with different chaotropic agents like urea, Guanidine hydrochloride alone or in combination with pH and temperature can be done. Molecular structure of the purified enzyme can be determined through X-ray crystallography, which can reveal the details about the active site and also the amino acids involved in the thermal stability of this protein. In depth knowledge gained from this work can be implemented in protein engineering studies to enhance the stability of other mesophilic proteins.

For the first time, we have studied the characteristics of BSH immobilized on amino-functionalized mesoporous silica. Amino-functionalization using the compound amino propyltriethoxy silane facilitated the immobilization reaction by forming covalent bonds between carboxyl groups of protein and amino groups of mesoporous silica.

Physiochemical characterization of the immobilized enzyme indicated that the structure of the support material is stable after immobilization.

Penicillin acylase, a pharmaceutically important enzyme of the Ntn hydrolase family was also immobilized on amino functionalized mesoporous silica by adsorption method. It was observed that pH has profound effect on enzyme immobilization; maximum loading was obtained at pH 7.8. Thermal stability of penicillin G acylase was enhanced after immobilization reaction. This enhanced stability is attributed to the protective nature of the cage like structure of SBA-15 matrix. Reusability of the immobilized enzymes was studied up to nine cycles. Reusability and enhanced thermal stability of the immobilized enzyme may find applications in pharmaceutical industry.

In application studies, we screened different probiotic strains for BSH activity and *L. buchneri* was found to have maximum production of bile salt hydrolase, among the tested microorganisms. Optimization of fermentation conditions for the production of BSH and *in vivo* cholesterol reduction studies of BSH from FDA approved *L. buchneri* have been reported for the first time. In our study we made an attempt for the first time, the usage of immobilized enzyme instead of whole microorganism for *in vivo* studies. This approach may prevent the problem of colonization in the gastrointestinal tract without disturbing the indigenous microflora, which are the common problems associated with the oral administration of whole cells. Reduction in serum cholesterol levels by 3 to 4% is clinically significant. Since every 1% reduction in serum cholesterol levels leads to a 2 to 3% reduction in estimated risk for coronary heart disease. In our study we observed about 50% reduction in serum cholesterol and 15% reduction in triglycerides in wistar rats when fed with 10 U/kg dose of immobilized bile salt hydrolase and 58% reduction in serum cholesterol and 45% reduction in triglycerides in wistar rats when fed with 20 U/kg dose of immobilized bile salt hydrolase. However further studies are required in this direction, which include the magnitude of immobilized BSH intake, duration required for the effective treatment and also its effect on host after prolonged intake of immobilized BSH. The use of genetically modified organisms (GMOs) for both production and delivery of BSH enzymes in the intestinal tract could offer several advantages but there

may be a risk of transfer of genes to the surrounding environment. Use of immobilized BSH offers an advantage over the use of BSH producing microorganisms as probiotics in that, the risk factors involved with the use of GMO can be avoided.

References

- Agerbck M., Gerdes L.U. & Richelsen B. **(1995)** *Eur. J. Clin. Nutr.* 49:346-352.
- Ahn Y.T., Kim G.B., Lim K.S., Baek Y.J. & Kim H.U. **(2003)** *Int. Dairy. J.* 13:303-311.
- Anderson J.W. & Gilliland S.E. (**1999)** *J. Am. College Nutr.* 18:43-50.
- Andrews P. **(1964)**. *Biochem J.* 91:222-233.
- Russell D.W. **(2003)**. *Annu. Rev. Biochem.* 72:137-174.
- Aries V. & Hill M.J. **(1970)** *Biochim. Biophys. Acta.* 202:526-534.
- Aronson N.N., Jr. **(1996)** *Glycobiology* 6:669-675.
- Arroyo M., De la Mata I., Acebal C. & Castillon P.M. **(2003)** *Appl. Microbiol. Biotechnol.* 60:507-14.
- Artymiuk P.J. **(1995)** *Nature. Struct. Biol.* 2:1035-1037.
- Begley M., Gahan G.M. & Hill C. **(2005)** *FEMS Microbiol Rev.* 29**:**625-651.
- Begley M., Hill C. & Gahan C.G.M. **(2006)** *Appl. Environ. Microbiol.* 72:1729-1738.
- Bianchi D., Golini P., Bortolo R. & Cesti P. **(1996)** *Enzyme Microb. Technol.* 18:592-596.
- Bliznakov E.G. **(2002)** *Biomed. Pharmacother.*56: 56-59.
- Boggs J.M. **(1987)** *Biochim. Biophys. Acta.* 906:353-404.
- Bozhinova D., Galunsky B., Yueping G., Franzreb M., Köter R. & Kasche V. **(2004)** *Biotechnol. Lett.* 26:343-350.
- Branningan J.A., Dodson G., Duggleby H.J., Moody P.C.E., Smith J.L., Tomchick D.R. & Murzin A.G. **(1995)**. *Nature* 378:416-419.
- Calleri E., Massolini G., Lubda D., Temporini C., Loiodice F. & Caccialanza G. **(2004)** *J. Chromatogr. A.*1031:93-100.
- Calleri E., Temporini C., Massolini G. & Caccialanza G. **(2004)** *J. Pharm. Biomed. Anal.* 35:243-258.
- Cavallaro G., Pierro P., Palumbo F.S., Testa F., Pasqua L. & Aiello R. **(2004)** *Drug Del.* 11:41-46.
- Chin J., Turner B., Barchia I. & Mullbacher A. **(2000)** *Immunol. Cell. Biol.* 78:55-66.

- Chiu C.H., Lu, T.Y. & Tseng Y.Y. **(2006)** *Appl. Microbiol. Biotechnol.* 71:238-245.
- Chong A.M. & Zhao X.S. **(2004)** *Catal. Today* 93-95: 293-299.
- Chong A.M. & Zhao X.S. (**2004)** *Appl. Surf. Sci.* 237:398-404.
- Christiaens H., Leer R.J., Pouwels P.H. & Verstraete W. **(1992)** *Appl. Environ. Microbiol.* 58:3792-3798.
- Coleman J.P. & Hudson L.L. **(1995)** *Appl. Environ. Microbiol.* 61:2514-2520.
- Corzo G. & Gilliland S.E. **(1999a)** *J. Dairy Sci.* 82:472-480.
- Corzo G. & Gilliland S.E. (1999b) *J. Dairy Sci.* 82:466-471.
- Dambekodi P.C. & Gilliland S.E. **(1998)** *J. Dairy Sci.* 81:1818-1824.
- Dashkevicz M.P. & Feighner. S.D. (**1989)** *Appl. Environ. Microbiol***.** 55:11-16.
- De Boever P. & Verstraete W. **(1999)** *J. Appl. Microbiol.* 87:345-352.
- De Rodas B.Z., Gilliland S.E. & Maxwell C.V. **(1996)** *J. Dairy Sci.* 79: 2121-2128.
- De Smet I., De Boever P. & Verstraete W. **(1998)** *Br. J. Nutr.* 79:185-194.
- De Smet I., Hoorde I.V., De Saeyer M., Vande W.M. & Verstraete W. **(1994)** *Microb. Ecol. Health. Dis.* 7:315-329.
- Dean M., Cervellati C., Casanova E., Squerzanti M., Lanzara V., Medici A., de Laureto P.P., Bergamini C.M. **(2002)** *Appl. Environ. Microbiol.* 68:3126-3128.
- Diaz J.F. & Balkus K.J.Jr. (**1996)** *J. Mol. Catal. B.* 2:115-126.
- Drasar B.S., Hill M.J. & Shiner M. **(1966)** *Lancet* 1:1237-1238.
- Duggleby H.J., Tolley S.P., Hill C.P., Dodson E.J., Dodson G. & Moody P.C.E. **(1995)** *Nature* 373:264-268.
- Dussurget O., Cabanes D., Dehoux P., Lecuit M., Buchrieser C., Glaser P. & Cossart P. **(2002)** *Mol. Microbiol.* 45:1095-1106.
- Elander R P. **(2003)** *Appl. Microbiol. Biotechnol.* 61:385-392.
- Elkins C.A. & Savage D.C. **(1998)** *J. Bacteriol*. 180:4344-4349.
- Elkins C.A., Moser S.A. & Savage D.C. **(2001)** *Microbiology* 147**:**3403-3412.
- Feighner S.D.& Dashkevicz M.P. **(1988)** *Appl. Environ. Microbiol.* 54:337-342.
- Fini A. & Roda A. **(1987)** *J. Lipid. Res.* 28:755-759.

- Franz C.M.A.P., Sepcht I., Haberer P., Holzapfel W.H. **(2001)** *J. Food Prot.*; 64:725-729.
- Gao B., Wang X. & Shen Y. **(2006)** *Biochem. Eng. J.* 28:140-147.
- Gilliland S.E. & Speck M.L. **(1977)** *Appl. Environ. Microbiol.* 33:15-18.
- Gilliland S.E., Nelson C.R. & Maxwell C. **(1985)** *Appl. Environ. Microbiol*. 49: 377-381.
- Gopal-Srivastava R. & Hylemon P.B. (**1988)** *J. Lipid. Res*. 29:1079-1085.
- Grill J.P. & Schneider F. **(1997)** Microbiologie- Aliments-Nutrition.15: 307-313.
- Grill J.P., Cayuela C., Antonie J.M. & Schneider F. **(2000)** *J. Appl. Microbiol.* 89:553-563.
- Grill J.P., Schneider F., Crociani J. & Ballongue J. **(1995)** *Appl. Environ. Microbiol*. 61**:**2577-2582.
- Gruzza M., Fons M., Ouriet M.F., Duval-Iflah Y. & Ducluzeau R. **(1994)** *Microbial releases* 2:183-189.
- Gui Xiao Q., Tao X., Zhang J.P. & Chen J.F. **(2006)** *J. Mol. Catal.* B 42:14-19.
- Guisán J.M. **(1988)** *Enzyme Microb. Technol.* 10:375-382.
- He J., Li X., Evans D.G., Duan X. & Li C. **(2000)** *J. Mol. Catal. B* 11:45-53.
- Herrera-Cervera J.A., Caballero- Mellado J., Languerre G., Tichy H.V., Reguena N., Amarger N., Romero E.M., Olivares J. & Sanjuan, J. **(1999)** *FEMS Microbiol. Ecology.* 30:87-97.
- Hofmann A F. **(1977)** *Clin. Gastroenterol*. 6:3-24.
- Hofmann A F. **(1999)** *News Physiol. Sci.* 14:24-29.
- Hofmann A.F. & Mysels K.J. **(1992)** *J. Lipid Res.* 33:617-626.
- Huijghebaert S.M., Mertens J.A. & Eyssen H.J. **(1982)** *Appl. Environ. Microbiol*; 43:185-192.
- Iwakura M., Jones B.E., Luo J. & Matthews C.R. **(1995)** *J. Biochem.* 117:480-488.
- Johnson L.R. **(1998)** *Lippincott-Raven, Philadelphia* 465-471.
- Johnson M.R., Barnes S., Sweeny D.J. & Diasio B. **(1990)** *Biochem. Pharmacol*. 40:1241-1246.

- Jones M.L., Chen H., Ouyang W., Metz T. & Prakash S. **(2004)** *J. Biomed. Biotech.* 1:61-69.
- Katchalski-Katzir E. & Kraemer D. **(2000)** *J. Mol. Catal. B.* 10:157-176.
- Kawamoto K., Horibe I. & Uchida K. **(1989)** *J. Biochem.* 106:1049-1053.
- Kim G.B. & Lee B.H. **(2005)** *Asian-Aust. J. Anim. Sci*.18:1505-1512.
- Kim G.B., Miyamoto C.M., Meighen E.A. & Lee B.H. **(2004a)** *Appl. Environ. Microbiol* 70: 5603-5612.
- Kim G.B., Yi S.H. & Lee B.H. **(2004b)** *J. Dairy Sci.* 87:258-266.
- Kirby L.C., Klein R.A. & Coleman J.P. **(1995)** *Lipids* 30:863-867.
- Klaver F.A.M. & Van der Meer R. **(1993)** *Appl. Environ. Microbiol.* 59:1120-1124.
- Kleerebezem M., Boekhorst J., van Kranenburg R., Molenaar D., Kuipers O.P., Leer R., Tarchini R., Peters S.A., Sandbrink H.M., Fiers M.W.E.J., Stiekema W., Klein Lankhorst Bron R.M., Hoffer P.A., Nierop S.M., Groot M.N., Kerkhoven R., de Vries M., Ursing B., de Vos W.M. & R. J. Siezen. **(2003)** *Proc. Natl. Acad. Sci.* USA. 100:1990-1995.
- Lack L. & Weiner I M. **(1966)** *Am. J. Physiol.* 210:1142-1152.
- Laemmli U.K. **(1970)** *Nature* 227:680-685.
- Lambert J.M., Weinbreck F. & Kleerebezem M. **(2008)** *J. Agric. Food. Chem.* 56:8360-8364.
- Lang G.N. & Binggeli C. **(2002)**. *Circulation* 105:1399-1402.
- Li W.F. Zhou X.X. & Lu P. **(2005)** *Biotechnol. Adv.* 23: 271-281.
- Lichtenstein A.H. **(1998)** *N. Engl. J. Med*. 339:1552-1553.
- Lim H.J., Kim S.Y. & Lee W.K. **(2004)** *J. Vet. Sci.* 5:391-395.
- Liong M.T. & Shah N.P. **(2005)** *Int. Dairy J.* 15:391-398.
- Liu J.G., Cong W., Wang S. & Ouyang F. **(2001)** *React. Funct. Polym.* 48:75-84.
- Lowry O.H., Rosebrough N.J., Farr A.L. & Randall R.J. **(1951)** *J. Biol. Chem.*193:265-275.
- Lundeen S. & Savage D.C. **(1990)** *J. Bacteriol*. 172: 4171- 4177.
- Lundeen S. & Savage D.C. **(1992a)** *FEMS Microbiol. Lett*. 94:121-126.

- Lundeen S. & Savage D.C. **(1992b)** *J. Bacteriol*. 174:7217-7220.
- MacDonald I.A., Williams C.N., Sutherland J.D. & MacDonald A.C. **(1983)** *Annal. Biochem*. 135:349-54.
- Mann G.V. & Spoerry A. **(1974)** *Am. J. Clin. Nutr*. 27: 464-469.
- Manson J.E., Tosteson H., Ridker P.M., Satterfield S., Hebert P. & O'Connor G.T. **(1992)** *N. Engl. J. Med*; 326:1406-1416.
- Marmur J. **(1961)** *J. Mol. Biol*. 5:109-118.
- Martoni C., Bhathena J., Jones M.L., Urbanska A.M., Chen H. & Prakash S. **(2007)** *J. Biomed. Biotechnol.* doi:10.1155/2007/13684.
- Martoni C., Bhathena J., Malgorzata U. & Prakash S. **(2008)** *Appl. Microbiol. Biotechnol* 81:225-233.
- Masuda N. **(1981)** *Microbiol. Immunol*. 25:1-11.
- Mc Auliffe O., Cano R. J. & Klaenhammer T. R. **(2005)** *Appl. Environ. Microbiol.* 71:4925-4929.
- Mislovicˇová D., Masárová J., Vikartovská A., Gemeiner P. & Michalková E. **(2004)** *J. Biotechnol.* 110:11-19.
- Mohy Eldin M.S., Bencivenga U., Rossi S., Canciglia P., Gaeta F.S., Tramper J. & Mita D.G. **(2000)** *J. Mol. Catal. B* 8:233-244.
- Morrissey J.H. **(1981*)*** *Anal. Biochem.* 117: 307-10.
- Moser S.A. & Savage D.C. **(2001)** *Appl. Environ. Microbiol.* 67:3476-3480.
- Nair P.P., Gordon M. & Reback J. **(1967)** *J. Biol. Chem*. 242:7-11.
- NCBI Microbial Genomes Annotation Project **(2003)** Direct Submission to the GenBank/EMBL/DDBJ. Accession number NZ_AAAO02000005.
- Nguyen T.D.T., Kang J.H. & Lee M.S. **(2007)** *Int. J. Food. Microbiol.* 113:358-361.
- Norouzian D., Javadpour S., Moazami N. & Akbarzadeh A. **(2002)** *Enzyme Microb. Technol.* 30:26-29.
- Oinonen C. & Rouvinene J. **(2000)** *Protein Sci.* 9:2329-2337.
- Olsson A. & Uhlen M. **(1986)** *Gene* 45**:**175-181.
- Ornish D. & Denke M. **(1994)** *J.Cardiovasc. Risk.* 1: 283-286.

- Paoletti R. **(1962)** *Am. J. Clin. Nutr.* 10: 277-284.
- Paulsen I.T., Banerjei L., Myers G.S., Nelson K.E., Seshadri R., Read T.D., Fouts D.E., Eisen J.A., Gill S.R., Heidelberg J.F., Tettelin H., Dodson R.J., Umayam L., Brinkac L., Beanan M., Daugherty S., DeBoy R.T., Durkin S., Kolonay J., Madupu R., Nelson W., Vamathevan J., Tran B., Upton J., Hansen T., Shetty J., Khouri H., Utterback T., Radune D. & C. M. Fraser. **(2003)** *Science* 299:2071-2074.
- Pereira D.I., McCartney A.L. & Gibson G.R. **(2003)** *Appl. Environ. Microbiol.* 69**:**4743-4752.
- Pereira D.I.A. & Gibson G.R. **(2002)** *Appl. Environ. Microbiol*. 68: 4689-4693.
- Peschel A. **(2002)** *Trends Microbiol.* 10:79-86.
- Qu F.P., Zhu G.S., Huang S.Y., Li S.G. & Qiu S.L. **(2006)** *Chemphyschem.* 7: 400-406.
- Rajendran J. & Gunasekaran P. **(2004)** *J. Biosci. Bioeng.* 97:1-13.
- Redenbaugh K., Hiatt W., Martineau B. & Emlay D. **(1995)** *American Chem. Soc*. 605:72-87.
- Ren L., He J., Zhang S., Evans D.G. & Duan X. **(2002)** *J. Mol. Catal. B.* 18:3-11.
- Rodriguez E., Arques JL., Rodriguez R., Nunez M. & Medina M. **(2003)** *Lett. Appl. Microbiol.* 37 : 259-263.
- Rossocha M., Schultz-Heienbrok R., von Moeller H., Coleman J.P. & Saenger W. **(2005)** *Biochemistry* 44:5739-5748.
- Russell, D.W. **(2003)**. *Annu. Rev. Biochem.* 72:137-174.
- Russell W.M. & Klaenhammer T.R. **(2001)** *Appl. Environ. Microbiol*. 67:1253-1261.
- Saavedra L., Taranto M.P., Sesma F. & de Valdez G.F. **(2003)** *Int. J. Food Microbiol.* 88: 241-245.
- Savage D.C., Lundeen S.G. & Conor L.T.O. **(1977)** *Ann. Rev. Microbiol.*31:107-133.
- Savage D.C. & Moser S.A. **(1999)** Direct submission to the Genbank/EMBL/ DDBJ. Accession no. AF091248.

- Schell M.A., Karmirantzou M., Snel B., Vilanova D., Berger B., Pessi G., Zwahlen M.C., Desiere F., Bork P., Delley M., Pridmore R.D. & Arigoni F. **(2002)** *Proc. Natl. Acad. Sci*. 99:14422-14427.
- Seok S.H., Park J.H., Cho S.A., Choi S.A. & Park J.H. **(2004)** *J. Ethnopharmacol.* 91:231-235.
- Shah P., Ramaswamy A.V., Lazar K. & Ramaswamy V. **(2007)** *Micropor. Mesopor. Mater.*100:210-226.
- Sharma A., Adhikari S. & Satyanarayana T. **(2006)** *World J. Microbiol. Biotechnol.* 23: 483-490.
- Shida O., Takagi H., Kadowaki K. & Komagata K. **(1996)** *Int. J. Syst. Bacteriol.* 46:939-946.
- Shimizu T., Ohtani K., Hirakawa H., Ohshima K., Yamashita A., Shiba T.,Ogasawara N., Hattori M., Kuhara S. & Hayashi H. **(2002)** *Proc. Natl. Acad. Sci.* 99:996-1001.
- Smibert R.M. & Krieg N.R. **(1981)**. Systematics: general characterization, *In* Gerhardt P. , Murray R.G.E., Costilow R.N., Nester E.W., Wood N.A., Krieg N.R., & Phillips G.B. (ed.), *Manual of methods for general bacteriology Am. Soc. Microbiol. Washington, D.C.* p. 409-443.
- Song S.W., Hidajat K. & Kawi S. **(2005)** *Langmuir* 21:9568-9575.
- Sridevi N. & Prabhune A.A **(2008)** *Appl. Biochem. Biotechnol.* doi: 10.1007/s12010-008-8326-9.
- Stackebrandt E. & Goebel B.M. **(1994)** *Int. J. Syst. Bacteriol.* 44**:**846-849.
- Stellwag E.J. & Hylemon P.B. **(1976)** *Biochim. Biophys. Acta.* 452**:**165-176.
- Suckling K.E., Benson G.M., Bond B., Gee A., Glen A., Haynes D. & Jackson B. **(1991)** *Athrosclerosis* 89: 183-190.
- Sudhakaran V.K., Deshpande B.S., Shewake J.G. & Naik S.R. **(1992)** 14:913-918.
- Suresh C.G., Pundle A.V., SivaRaman H., Rao K.N., Branningan J.A., McVey C.E., Verma C.S., Dauter Z., Dodson E.J., Dodson G.G. **(1999)** *Nature Struct Biol* 6:414-416.

- Suresh Kumar R., Branningan J.A., Prabhune A.A., Pundle A.V., Dodson G.G., Dodson E.J. & Suresh C.G. **(2006)** *J. Biol. Chem.* 43:32516-32525.
- Suresh Kumar R., Suresh C.G., Pundle A. & Prabhune A. **(2004)** *Biotech. lett.* 26:1601-1606.
- Tamura K., Dudley J., Nei M. & Kumar S. (2007) *Molecular Biology and Evolution* 24: 1596-1599.
- Tanaka H., Doesburg K., Iwasaki T. & Mierau I. **(1999)** *J. Dairy Sci.* 82:2530-2535.
- Tanaka H., Hashiba H., Kok J. & Mierau I. **(2000)** *Appl. Environ. Microbiol.* 66:2502-2512.
- Taranto M.P. & Font de Valdez G. **(1999)** *Biotechnol. Lett.* 21: 935-938.
- Taranto M.P., Fernandez Murga M.L., Lorca G. & Font de Valdez G. **(2003)** *J. Appl. Microbiol.* 95:86–91.
- Taranto M.P., Medici M., Perdigon G., Ruiz Holgado A.P. & Valdez G.F. **(1998)** *J. Dairy Sci.* 81:2336-2340.
- Taranto M.P., Medici M., Perdigon G., Ruiz Holgado A.P. & Valdez G.F. **(2000)** *J. Dairy Sci.* 83:401-403.
- Taranto M.P.F., Sesma A.P., Holgado R. & Font de Valdez G. **(1997)** *Biotechnol. Lett.* 19:845-847.
- Tischer W. & Wedekind F. **(1999)** *Top. Curr. Chem.* 200:95-126.
- Toranto M.P., De Llano D.G., Rodriguez A., De Ruiz Holgado A.P. & Font de Valdez G. **(1996)** *Milchwissenschaft* 51:383-385.
- Usman & Hosono A. **(1999)** *J. Dairy. Sci.* 82:243-248.
- Vallet-Regi M., Ramila A., del Real R.P. & Perez Pariente J. **(2001)** *Chem. Mater.* 13:308-311.
- Van Eldere J., Celis P., De Pauw G., Lesaffre E. & Eyssen H. **(1996)** *Appl. Environ. Microbiol.* 62:656-661.
- Walker D.K. & Gilliland S.E. **(1993)** *J.Dairy Sci*.76:956-961.
- Wang S. **(2009)** *Micropor. Mesopor. Mater.* 117:1-9.

- Wijaya A., Herrmann A., Abriouel H., Specht I., Yousif N.M.K., Holzapfel W.H. & Franz C.M.A.P. **(2003)** Direct submission to the Genbank/EMBL/DDBJ. Accession number AY260046.
- Willman D. **(2001)** *New YorkTimes Online*. September 14.
- World health Organization fact sheet N°317**(2007).**
- Xu Q., Buckley D., Guan C. & Guo H.C. **(1999)** *Cell* 98: 651-661.
- Xue P., Lu G., Guo Y., Wang Y. & Guo Y. **(2004)** *J. Mol. Cat. B* 30:75-81.
- Yamamoto K., Shen B., Zarins C. & Scanu A.M. **(1984)** *Atherosclerosis* 4:418-434.
- Yiu H.H.P., Wright P.A. & Botting N.P. **(2001)** *J. Mol. Cat. B* 15: 81-92.

List of Publications

- **Sridevi, N.**, Vishwe Pradnya., Prabhune Asmita. **(2009)** Hypocholesteremic effect of bile salt hydrolase from *Lactobacillus buchneri* ATCC 4005. **Food Research International 42:516-520**.

- **Sridevi, N.**, Srivastava, S., Khan, B. M., Prabhune, A. A. **(2009)** Characterization of the smallest dimeric bile salt hydrolase from a thermophile *Brevibacillus sp.* **Extremophiles 13:363-70.**

- **Sridevi, N.**, Prabhune, A. A. **(2008)** *Brevibacillus sp*: A Novel Thermophilic Source for the production of Bile salt hydrolase. **Applied Biochemistry and Biotechnology doi: 10.1007/s12010-008-8326-9**.

- Shah, P., **Sridevi, N**., Prabhune, A., Ramaswamy, V **(2008)** Structural features of Penicillin acylase adsorption on APTES functionalized SBA-15. **Microporous and Mesoporous Materials 116:157-165**.

- Shah, P., **Sridevi, N**., Prabhune, A., and Ramaswamy, V **(2007)** Immobilisation of Ntn hydrolases on APTES functionalized SBA-15. **Studies in surface science and catalysis 170 (B):1891-1898**.

- Structural features of bile salt hydrolase immobilized on APTES SBA-15 **(2009)** **N.Sridevi**, Pallavi shah, Asmita Prabhune and Veda Ramaswamy (ready to communicate to **Applied catalysis A**)

Awards and honors

- Received best research paper (poster) award for the paper entitled “Isolation, Identification of a *Brevibacillus sp:* A novel thermophilic source for the production of bile salt hydrolase” presented in the International conference, **New Horizons in Biotechnology** (**NHBT-2007**) Trivandrum, Nov 26-29, 2007.

Abstracts accepted in National/International Conferences

- Hypocholesteremic effect of bile salt hydrolase isolated from *Lactobacillus buchneri* ATCC 4005. N.Sridevi, Pradnya Vishwe, Asmita Prabhune. **International Congress on Bioprocesses in Food Industries (ICBF-2008)**, Hyderabad, Nov 6-8, 2008.

- Isolation, Identification of a *Brevibacillus sp:* A novel thermophilic source for the production of bile salt hydrolase. N. Sridevi, Asmita Prabhune **International conference on New Horizons in Biotechnology (NHBT-2007)**, Trivandrum, Nov 26-29, 2007.

- Organo functionalised Mesoporous silica for the Immobilization of Penicillin acylase. N.Sridevi, Pallavi shah,Veda Ramaswamy and Asmita Prabhune.**75th Annual Meeting of the SBC (I)**, New Delhi, Dec 8-11, 2006.

- Biochemical Insights into Bile salt hydrolase from a thermophile *Geobacillus caldoxylosilyticus*. N.Sridevi, Asmita Prabhune **Thermophiles-2007**, Bergen, Norway, Sep 24-27, 2007.

- *Geobacillus caldoxylosilyticus:* A novel thermophile producing bile salt hydrolase and penicillin V acylase. Sridevi Nidumukkala, Archana.V.pundle, Neelima M.Deshpande and Asmita Prabhune **Thermophiles-2005**, Gold coast, Australia, Sep 18-22, 2005.