Production, Purification and Cloning of Bacterial Cholesterol Oxidases and their Application in Medical Bioremediation

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

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In

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By

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Islamabad

2020

DEDICATED

TO

TO MY LOVING PARENTS, HUSBAND AND TEACHERS

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LIST OF ABBREVIATIONS

3,4-DHSA	3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione
3-HSA	3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione
7-KC	7-keto cholesterol
ABCA1	ATP-binding cassette sub-family A member 1
ABCG1	ATP-binding cassette sub-family G member 1
ACC1	Acetyl CoA carboxylase 1
AD	Androsta-4-ene-3,17-dione
ADA	Adenosine deaminase
ADD	Androsta-1,4-diene-3,17-dione
AMD	Age-related macular degeneration
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CETP	Cholesteryl ester transfer protein
CFS	Cell free supernatant
ChO	Cholesterol oxidase enzyme
ChO	Cholesterol oxidase gene
CVDs	Cardiovascular diseases
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acids
dNTPs	Deoxyribonucleotide phoshate
DOHNAA	9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acids
DSAO	1,17-dioxo-2,3-seco-adrostan-3-oic acid
E3	Estriol
EE2	17β-ethinyl estradiol
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ERT	Enzyme replacement therapy
FAD	Flavin adenine dinucleotide
FAS	Fatty acid synthase
FASTA	Fast alignment sequence tool

GC	Gas chromatography
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HMGCR	3-hydroxy-3-methyle-glutaryl-coenzyme A reductase
HMGCS	Hydroxyl-3-methyleglutaryl (HMG)-CoA synthase
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IDL	Intermediate density lipoprotein
IDT	Integrated DNA technology
IEDB	Immune epitope database
IMVIC	Indole production, methyl red, voges-Proskauer reaction and citrate utilization
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
kDa	Kilodalton
LB	Luria-Bertani
LDL	Low density lipoprotein
LP	Lignin peroxidase
Lp (a)	Lipoprotein (a)
MnP	Manganese peroxidase
mRNA	Messenger ribonucleic acid
MR-VP	Methyl red-Voges proskauer
MS	Mass spectrometry
MSM	Mineral salt medium
mTORC1	Molecular target of rapamycin complex 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Nonalcoholic fatty liver diseases
NASH	Nonalcoholic steatohepatitis
NCBI	National center for Biotechnology information
NFT	Neurofibrillary tangles
NOS	Nitric oxide synthase
NPC	Niemann-Pick type C
NPCSC	Niemann-Pick C1 signaling complex

nTreg	Natural T-regulatory
PCB	Poly chlorinated biphenyl
PCR	Polymerase chain reaction
PCSK9	Proprotein convertase subtilisin/kexin type 9
PEG	Polyethylene glycol
pH	Potential of hydrogen ions
PPS	Phosphate buffer saline
PsChO	Pimelobacter simplex cholesterol oxidase
QC-PCR	Quantitative competitive polymerase chain reaction
RNA	Ribonucleic acid
RORγ	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
SB	Super broth
SCD1	Stearoyl-CoA desaturase 1
SCID	Severe combined immune deficiency disease
SDAT	2,5-seco-3,4-dinorandrost-2,5,17-trione
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SRB	Sulforhodamine B
SR-B1	Class B, Type 1 scavenger receptor
SREBP2	Sterol regulatory element-binding protein 2
TB	Terrific broth
TCA	Trichloro acetic acid
TLC	Thin layer chromatography
TSI	Triple sugar iron
US EPA	United States environmental protection agency
US FDA	United states Food and drug administration
VLDL	Very low density lipoprotein
WHO	World health organization
7α-OHC	7α hydroxy cholesterol

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Acknowledgments

I am grateful to Almighty Allah, the most merciful and beneficent; His blessings enabled me to achieve my goals. Tremulous veneration is for His Holy Prophet Hazrat Muhammad (PBUHS.A.W.A.W.) who is forever torch of guidance and knowledge for humanity.

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Hasina Wali

ABSTRACT

In living cells, the recycling process is hampered by accumulation of metabolic waste, resulting to triggers the aging process. The accumulated recalcitrant compounds are difficult to be degraded by endogenous system and have deleterious effect on cell leading to progression of several diseases. Some aging storage diseases (ASD) such as atherosclerosis and cancer are affiliated with accumulation of cholesterol and 7-ketocholesterol (7-KC). There are several therapies for reversing this accumulation and recently focus is on a new approach in which human's natural catabolic machinery is boosted with foreign enzymes. Removal of these compounds by exogenous enzymes of microbial origin to relief the disease associated conditions is termed as medical bioremediation.

No doubt there are proven challenges to use the foreign enzymes in therapeutic trials such as delivery, efficacy, and immunogenicity problems. To overcome all that there is a need to engineer the therapeutic proteins with desired characteristics. The goal of this research is to screen and isolate the cholesterol and 7-KC degrading bacteria from diverse environmental samples and identification and purification of candidate enzyme involved in the degradation and have therapeutic potential.

Total of twelve bacterial strains were isolated with capability to utilize the cholesterol as a source of energy in M9 cholesterol agar plate from different environmental samples. They were characterized taxonomically by morphological, biochemical and 16S rRNA sequencing and were identified as *Bacillus subtilis* LSB, *Bacillus safensis* W1, *Stenotrophomonas pavanii* GS2, *Psychrobacter sp*. GS3, *Moretella sp*. GS6, *Vibrio sp*. GS7*, Moretella sp.* GS9, *Shewanella pealeana* GSS, *Klebsiella sp*. WS, *Vibrio sp*. DS and *Psychrobacter sp.* UP.

Bacterial isolates were tested for their degradation potential in shake flask experiments. Two of the bacterial strains *B. safensis* W1 and *B. pumilus* W8 were selected for degradation optimization studies due to their best growth in the presence of cholesterol. Optimum pH, temperature and cholesterol concentration was found to be 7, 35°C and 1g/L respectively for both the strains. It was also observed that $MgSO_4$ and $CaCl_2$ favors the cholesterol degradation. Later, under optimized conditions degradation potential of all the strains were tested for cholesterol and some selected strains for 7-keto cholesterol and their reduction was analyzed by HPLC. The results indicated 58-86% cholesterol reduction, with

maximum reduction by *B. pumilus* W8. While 7-KC degradation was 70, 90, 86 and 92% by *B. safensis* W1, *B. pumilus* W8, *Moretella sp*. GS6 and *S. pealeana* GSS, respectively. Furthermore, cholesterol metabolites generated by some of the isolates including *B. subtilis* LSB, *B. safensis* W1, *Psychrobacter sp.* UP, *Vibrio sp*. DS, *Psychrobacter sp*. GS3 and *Moretella sp*. GS6 were detected on GCMS. Several significant peaks were detected and identified as 7-oxo-cholesterol, 2-dodecene-1-yl-Succinic anhydride, 26,27-Dinorergosta-5,23-dien-3-ol and some of common fatty acids including hexadecenoic acid and octadecanoic acid.

All the isolates were screened for production of cholesterol oxidase ChO and found positive. Two isolates *B. safensis* W1 and *B. subtilis* LSB showing promising results for cholesterol oxidase production (0.140 and 0.336 U/mL activity respectively) were selected for further studies. Extracellular flavin adenine dinucleotide (FAD) containing ChO was purified from both of the selected isolates by passing through Diethylaminoethyl sepharose (DEAE- Sepharose) column. Cholesterol oxidase with a molecular weight of 58 kDa and 22 kDa was purified from *B. safensis* W1 and *B. subtilis* LSB, respectively.

The *ChO* gene from *Chromobacterium* DS1 strain was synthesized by Integrated DNA technology (IDT) and cloned in pET21 vector. Expression of recombinant cholesterol oxidase was carried out in *E. coli* strain *BLR* and *origami* in terrific broth (TB) and super broth (SB) at three different post induction temperatures (20, 30, 37°C). Good expression was seen in TB media at low temperature (20°C) and longer incubation (16 hours). Protein solubility was enhanced by using 0.1mM of Isopropyl β-d-1-thiogalactopyranoside (IPTG as inducer. Recombinant protein (Molecular weight 58 kDa) was purified at room temperature (25°C) by passing through NpuC trapped affinity resin from *BLR* and *Origami* with activity of 26 U/mg and 33 U/mg respectively. Finally, cholesterol oxidase was engineered with one amino acid change by replacing the threonine with phenylalanine at position one and significant effect on elusion profile was seen with pronounced increased activity (41 U/mg).

To understand the biochemical properties of recombinant cholesterol oxidase effect of several factors such as pH (3, 5, 7, 9, 11), temperature (20-100°C), solvents (methanol, ethanol, isopropanol, dimethyl sulfoxide (DMSO), ethyl acetate, acetone) and detergents (tween 80, triton-x-100, sodium cholate and sodium dodecyl sulphate (SDS) were evaluated. Protein was more active at pH 6 followed by 7 and active in broad range of

temperature (40-80℃) with optimum activity at 60℃. Moreover, protein was found to be active in the presence of all the solvents tested except of ethyl acetate and acetone where maximum activity was seen in the presence of methanol and tween 80.

The recombinant cholesterol oxidase sequence when analyzed on immune epitope database (IEDB) for prediction of B and T cells epitopes showed potential immunogenic response and could be pegylated to be used in therapeutic trials. Recently it has been reported that cholesterol play an important role in development of cancer and cholesterol lowering agents could be used as anticancer therapy. In view of these reports when the activity of purified ChO against various cancer cells including colon carcinoma (HT-29), prostate cancer (DU-145) and breast cancer (MCF-7) cells was tested, result showed maximum cell inhibition of all the tested cell types with 13U /mL enzyme concentration. Maximum inhibition was recorded of DU-145 cells by recombinant ChO (96.6%), followed by HT-29 and MCF-7. Purified cholesterol oxidase of *Bacillus safensis* W1 and *Bacillus subtilis* LSB also showed inhibition from 53-92.2% with tested cell lines.

From present research work it is concluded that bacterial isolates from environmental samples can degrade cholesterol and 7-keto cholesterol. The potential therapeutic enzyme cholesterol oxidase has been identified and successfully purified and cloned as model enzyme that can pave the way towards exogenous enzymatic therapy termed as medical bioremediation. Furthermore, cell toxicity of ChO has been found against HT-29, DU-145 and MCF-7 cancer cell lines. The cytotoxic potential of the cloned and purified enzymes comparable with Taxol as control showed high potential and further research is needed to reduce the immunogenicity and increase the safe delivery towards the targeted sites. In future this enzyme may become first choice of therapeutics for diseases considered the first (cardiovascular diseases) and second (cancer) leading cause of death worldwide.

INTRODUCTION

Steroids are diverse group of organic compounds having a core of tetracyclic fused ring which gives them hydrophobicity. Steroids have various functional groups at different positions which affect their distribution in the membrane and the spatial arrangement of cholesterol in membrane is due to aliphatic tail at C-17 and a hydroxyl group at C-3 atom (Figure 1.1). This specific position of functional group gives the ability to steroid for the interaction of aliphatic tails with lipids while the hydrogen bond is formed by hydroxyl group interaction with polar head of the lipids. In synthesis and pharmacology an important role has been played by chemistry of steroidal skeleton (Kumar *et al.,* 2016; Lednicer, 2011). Steroids have vital role in different functions of the cells and involved in number of metabolic pathways. The structure of biological membranes has been modulated by several steroidal compounds for instance β-sitosterol in plants, ergosterol in fungi and cholesterol in animals. Cholesterol is an important steroidal compound involved in different cellular functions. In all animal plasma membrane, it contributes to form membrane domain (lipid rafts) which may have a significant role in the maintenance of membrane rigidity and fluidity (Sevcsik and Schutz, 2016; Kumar and Chattopadhyay, 2016).

Cholesterol serve as a precursor molecule for synthesis of various steroidal hormones like testosterone, progesterone and cortisone endocrine cells in testis, ovary, and adrenal gland by series of transformation known as steroidogenesis (Cerqueira *et al.,* 2016). Intracellular nuclear receptors are activated by steroid hormones which regulate the process of transcription in cell nucleus leading to expression of thousands of genes which have significant role in regulation of various life processes. Furthermore, steroidal hormone has significant role in development of fetus brain, respiratory and cardiovascular system (Solano and Arck, 2020). However, in human blood and organs the accumulation of high concentration of cholesterol is associated with development of different diseases. Epidemiological studies showed that social and biological factors are associated with increased health risks. According to world health organization (WHO), lifestyle is linked with 60% of health risk factors. Millions of people encounter diseases, disability, and death due to adaptation of an unhealthy lifestyle. Several diseases are linked with unhealthy lifestyle such as overweight, joint, and skeletal problems, hypertension, obesity, cardiovascular and metabolic diseases. Multiple factors such as environmental, genetics and aging are associated with some of the disorders. Cholesterol a widely studied lipid, is

considered as a major risk factor in the development of mentioned diseases and involved in progression of age-related diseases or lysosomal storage diseases (Farhud, 2015).

Recently it has been revealed that high cholesterol level has direct relation with cancer cell malignancy or cancer progression may also be influenced by dysfunction of cholesterol metabolism. Cholesterol-enriched microdomains are thought to be linked with progression of several types of cancer that may activate the cancer related signaling Hedgehog pathway (Huang *et al.,* 2016; Luchetti *et al*., 2016). Beside in cell membrane cholesterol also functions in cytoplasm. Some studies reported that molecular target of rapamycin complex 1 (mTORC1) activation by lysosomal cholesterol via SLC38A9-Niemann-Pick C1 signaling complex (NPCSC) results in enhanced proliferation of cells and metastasis (Kim *et al.,* 2017). Cholesterol has a major role in pathogen host interactions, contributing to survival and virulence of pathogen into host. Some obligatory intracellular pathogen such as *Anaplasma phagocytophilum* lacks the cholesterol biosynthesis gene and acquires the host cell cholesterol for survival and infection. Regulation of cholesterol plays an important role in mitigation of several pathologies (Xiong *et al*., 2019).

Among the age-related diseases, cerebrovascular diseases are the one affecting large number of populations. Cholesterol was observed as a key component involved in progression of arterial plaques (Linton *et al*., 2019). Throughout the circulation, arterial endothelial lining regulates the hemostasis and inflammation when respond to molecular and mechanical stimuli (Gimbrone and Garcia-Cardena, 2016). Impairment of endothelial cells initiate the formation of atherosclerotic lesions mostly occur at atherosclerotic prone areas resulting in reduced expression of nitric oxide synthase (NOS), superoxide dismutase (SOD) with increased oxidative stress which affect the integrity of endothelial barrier (Malekmohammad *et al.,* 2019). Accumulative effect of all these processes boost the buildup of subendothelial atherogenic low density lipoproteins (LDL), chylomicrons and very low-density lipoproteins (VLDL). Activation of endothelial cell is increased in atherosclerotic susceptible area by nuclear factor kappa B pathway resulting the expression of proinflammatory receptors, monocytes, and cytokines (Yurdagul *et al.,* 2013; Fan *et al*., 2017). Along with endothelial cells activation, increased reactive oxygen species (ROS) production (Incalza *et al*., 2018) causes the oxidation of LDL and formation of 7-keto cholesterol (7-KC) (Figure 1.2) which is one of the oxidized derivative widely observed in atherosclerotic lesions (Negre-Salvayre *et al*., 2019). Cholesterol and its oxidized derivatives accumulate due to catabolic insufficiency of lysosome. Accumulation of these

compounds inactivate the vacuolar-ATPases leading to drop in pH which may alter membrane structure and properties of membrane bound proteins (Cox *et al*., 2007; Batch *et al*., 2008). Along with mechanical stimuli different molecular stimuli such as advanced glycosylation products, cytokines, oxidized lipoproteins, and pathogenic molecules are also reported to be involved in activation of endothelial cells (Ungvari *et al*., 2006).

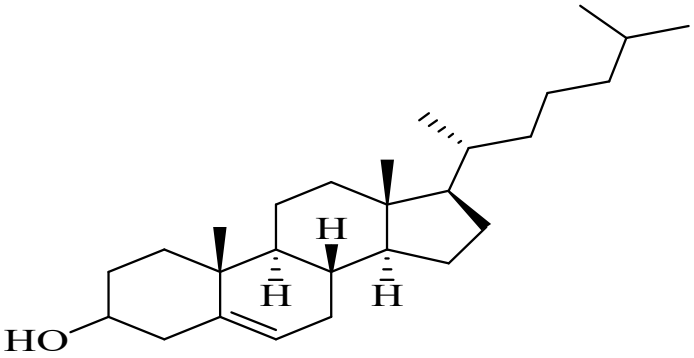


Figure 1.1 Structure of cholesterol (M/Z= 386.654).

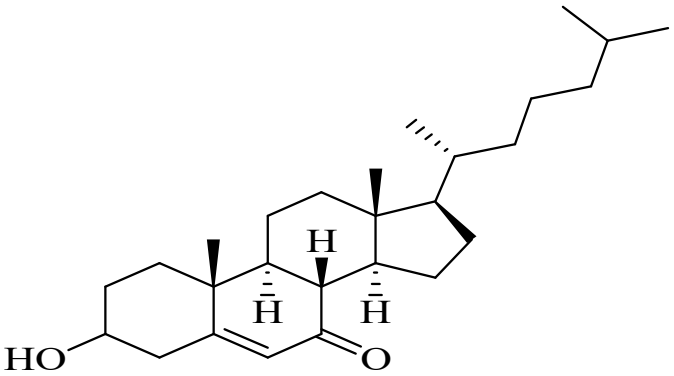


Figure 1.2 Structure of 7-ketocholesterol (7-KC) (M/Z= 400.637).

In human natural means failed to remove these recalcitrant offending compounds therefore medical bioremediation is an option to target such accumulated compounds by using exogenous enzymes. Due to degradative capability of microbes their enzymes could be used as therapeutic option for pathologically aggregated compounds like cholesterol, 7-ketocholesterol and lipofuscin involved in development of several age associated diseases (de Grey and Archer, 2001). The approach of medical bioremediation is a proposed way to treat the aging diseases by targeting the specific disease associated compounds through potential microbial enzymes. To achieve the goal of either enzyme replacement therapy (ERT) or gene therapy, it is necessary to identify, purify and engineered the therapeutic enzyme to make it compatible in mammalian cell environment (de Grey, 2002).

Natural environment, benefiting the human in several ways from long time and improved the socioeconomic status, health and age across the geographical boundaries (Hurly and Walker, 2019). An important aspect is the exploitation of different natural products from animals, plants, marine and microbial sources for the treatment of different diseases since ancient time (Mushtaq *et al*., 2018). New and emerging analytical techniques with computational software opened the new era to explore the new microbial products and process the natural compounds to derive the novel and innovative drugs (Thomford *et al*., 2018). To search the different and novel microbes from environment for their use in medical field is the major research area now a day.

Microbes are considered as one of the most potential sources of diverse and multifunctional enzymes. Properties like thermo-stability, multi functionality, high yields, enhanced product modification, economic feasibility, continuous production without seasonal fluctuation, consistency, pH stability, cheap growth media and high catalytic activity makes the microbial enzyme as potential candidates for use in various biotechnological processes in different conditions. Microbial enzymes are the major contributors in the field of biochemical investigation, diagnosis and treatment of some deadly diseases (Gurung *et al*., 2013). By using different techniques such as recombinant DNA technology, high throughput sequencing and omics several microbial therapeutic enzymes have been developed and used for therapeutic applications (Thapa *et al*., 2019).

Cholesterol oxidase is a flavoprotein belonging to flavin-dependent oxidoreductase superfamily and classified into two major classes based on binding of flavin adenine

dinucleotide (FAD). Class I enzyme have noncovalently bound FAD while the second form of enzyme have covalently liked FAD cofactor (Kumari and Kanwar, 2012). Due to the sequence difference first form of cholesterol oxidases is assigned to glucose-methanol choline oxido-reductase and second form of cholesterol oxidases are the part of vanillyl alcohol oxidase family respectively (Dijkman *et al*., 2013). Oxidation of cholesterol to cholestenone is catalyzed by cholesterol oxidase in three sequential steps. In the first reaction at third carbon of the steroid ring dehydrogenation of hydroxyl group take place with loss of 3 β-hydroxy hydrogen and 3 α-hydrogen. Oxidized FAD cofactor of the enzyme is converted to reduced form by gaining two generated electrons in the process followed by its reaction with molecular oxygen to produce H_2O_2 and to restore the original enzyme in oxidized form. The generated hydrogen peroxide in assay mixture coupled with 4-aminoantipyrine and phenol in the presence of peroxidase resulting into quinoneimine dye tracked at 500 nm. In the last step isomerization of double bond take place resulting cholest 4-ene-3one from cholest 5-ene-3-one as a final product (Figure 1.3) (Moradpour and Ghasemian, 2016; Doukyu, 2009).

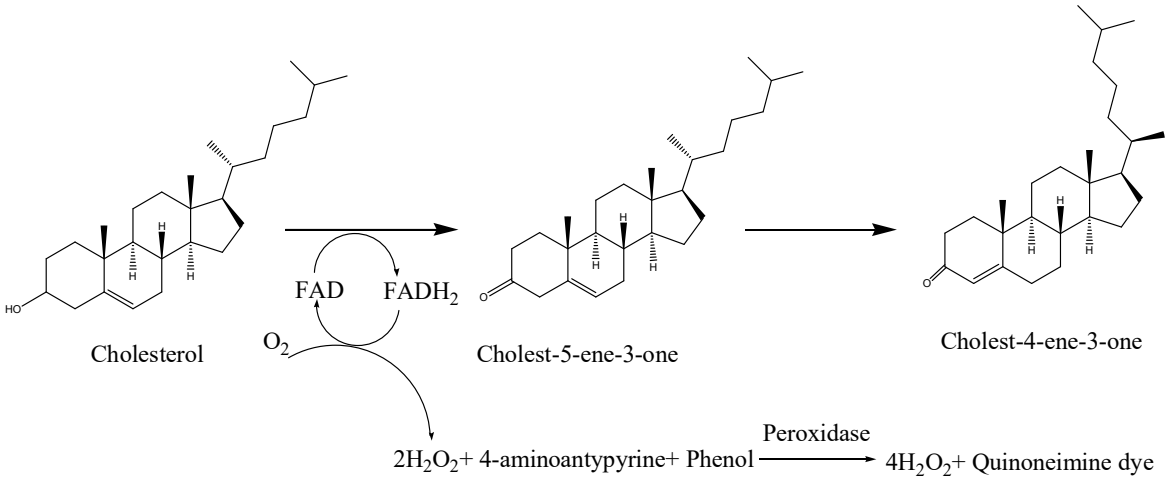


Figure 1.3 Cholesterol oxidase chemical reaction and coupled chromogenic assay. (Wu, *et al*., 2015).

Cholesterol oxidase are produced by several bacterial species including *Actinomycetes, Mycobacterium, Chormobacterium, Arthrobacter, Bacillus species, Rhodococcus* and *Pseudomonas*. Cholesterol oxidase gained significant attention due to their wide applications in different areas such as clinical, pharmaceutical, agriculture and in food industries (Devi and Kanwar, 2017). Majority of bacterial species can produce cell bound cholesterol oxidase, but some bacteria could produce the enzyme in extracellular form which is more convenient for isolation and purification. The production of cholesterol oxidase could be affected by different factors such as various carbon and nitrogen sources, pH, temperature, incubation time and the use of surfactants to increase the solubility of cholesterol for bacterial utilization (Yehia *et al*., 2015). The enzyme for therapeutic purpose is required in very low amount but must be highly pure and specific. Potential therapeutic enzymes are produced very carefully to ensure the highest purity and prevent from any kind of contamination (Taipa *et al*., 2019). Cholesterol oxidase could be purified through series a of purification steps including precipitation either by salt or solvent and various chromatographic techniques such as ion exchange chromatography, size exclusion chromatography (El-Naggar *et al*., 2017; Reiss *et al*., 2014).

Several novel isolates have been identified with potential to produce extracellular cholesterol oxidase with wide industrial and clinical applications (Niwas *et al*., 2013). In future these isolates can be further investigated by genetic studies for identification of gene and purification of cholesterol oxidase and to explore the optimal conditions for maximum production would help taking a significant step toward the industrial mass production of this enzyme (Lashgarian *et al*., 2016).

Additionally, engineered therapeutic enzymes need successful delivery to the target compound in human cell lysosomes (Vega-Vasquez *et al*., 2020). One of the major limitation with therapeutic enzyme is its inability to recognize the target and may act equally on off-target cells leading to potential toxicity (Peer *et al.,* 2007). Engineered devices are used to transport the pharmaceutical compound in a controlled manner (NIH, 2016). Successful delivery of therapeutic drug to target cell or tissue is possible either by direct conjugation of targeting moiety to protein or attachment on carrier molecules. In recent years, much attention has been paid to development of nano-delivery systems (ud Din *et al.,* 2017). Variety of materials such as synthetic polymers, proteins, inorganic metallic salts and polysaccharides can be used to prepare the nanocarriers (Mirza and Siddiqui, 2014). Now a days construction of biological nano carriers are an emerging

technology, which are formed as a part of a natural phenomenon, including viral capsids and membrane vesicles, or are derived from other living sources (Veneziano *et al.,* 2016).

Another potential barrier to use enzyme as a therapeutic drug, is the activation of immune response. Immune cells clear the therapeutic protein to undetectable level resulting reduced efficacy of treatment. Fortunately, some means are there to mitigate the antigenic response. Attachment of polyethylene glycol (PEG) alter the pharmacokinetics and bio-distribution of drug molecule. PEG conjugated proteins have low immunogenicity and longer shelf life in the circulation (Sathyamoorthy and Magharla, 2017). PEGs with methoxylated and hydroxyl groups at one or both ends are widely used in conjugation and the functional properties of conjugated molecules is adversely affected by the PEGylation chemistry and reaction conditions (Gupta *et al.,* 2018). Regulatory T-cells activation is another strategy to make the recipient antigen tolerant which is frequently used for patients with transplantation. Foxp3 is considered a crucial transcription factor for generation of natural regulatory T-cell (nTreg) such as CD4+CD25+ (Cobbold and waldmann, 2013).

Aim and Objectives

Aim of the current study is to identify, purify and clone the microbial cholesterol oxidase and to investigate their role in medical bioremediation. Objectives of the current research study are;

1) Isolation and identification of cholesterol and 7-ketocholesterol degrading bacteria.
2) Degradation studies and optimization of operational parameters affecting degradation kinetics using bacterial isolates.
3) Purification of cholesterol oxidase from selected bacterial isolates.
4) Cloning, expression and purification of synthetic cholesterol oxidase.
5) Characterization of genetically engineered cholesterol oxidase.

REVIEW OF LITERATURE

The modern lifestyle, including lack of exercise and diet high in fat leading to obesity are contributing factors of different diseases in a population. Obesity has been declared as a pandemic associated with public health problems by WHO in 2000. Some of the major diseases like heart disease, cancer and type 2 diabetes are linked to the diet containing high amount of lipids (Fenwick *et al.,* 2019). Lipids, a class of organic compounds which includes fat, oil, hormones, and some other membrane components are hydrophobic in nature and very crucial for the normal cell functions. Fat a type of lipid provides thermal insulation and serve as energy storage for organisms. Some lipids like steroid hormones work as chemical messengers between cells and tissues. The phospholipids bilayer are present in the membrane of cells and different organelles within it. These different membranes separate the cells from the environment and serves as compartment within the cell for different functions (Ahmed and Ahmed, 2018; Amin *et al*., 2019).

There are two pathways of lipoproteins based on their source. The dietary lipids incorporate via exogenous lipoprotein pathway into chylomicrons in intestine. Free fatty acids are released in the circulation by the action of lipoprotein lipase on triglycerides carried by chylomicrons which is metabolized in adipose tissue and muscles. The liver takes the generated chylomicrons remnants while VLDL form in the liver through endogenous lipoprotein pathway initially (Olivecrona, 2016; Wolska *et al*., 2017). The VLDL containing triglycerides are metabolized by lipoprotein lipase in adipose and muscle tissues and form IDL and free fatty acids. LDL is produced by the metabolism of IDL which is then taken up by different tissues including liver. The nascent HDL formed by the liver and intestine through reverse cholesterol transport. Mature HDL formation takes place by acquiring the phospholipids and cholesterol effluxes by the cells. The transportation of cholesterol by HDL to liver is carried either by direct interaction with hepatic class B, type I scavenger receptor (SR-B1) or by Cholesteryl ester transfer protein (CETP) facilitated process to LDL or VLDL. The efflux of cholesterol from macrophages to HDL is the major contributor in protection of atherosclerosis development (Ossoli *et al*., 2015; Brown *et al*., 2018).

2.1 Role of cholesterol in body

Cholesterol is a soft and waxy compound in nature with four fused alicyclic rings. It is an essential lipid for mammalian cells having tightly regulated homeostasis. Cholesterol

contributes to many roles for the proper functioning and survival of cells (Rohrl and Stangl, 2018; Huff and Jailal, 2017) Cholesterol is an important and integral part of the plasma membrane of cell and maintain its stability. Due to its chemical composition in lipid bilayer it plays an important role in membrane fluidity leading to condensing effect for lipids packing in membrane to create the special region with rich cholesterol called membrane rafts. Membrane rafts are well known for their involvement in signaling of different processes of the cell including immune regulation, fusion events, membrane trafficking and cell cycle control (Andrade and Luciana, 2016; Zumerle *et al*., 2017).

Apart from the main role of cholesterol in stability and cellular organization, it also play an important role as precursor molecule of different biologically active molecules like bile acids, vitamin D and steroid hormones which are involved in regulation of cellular metabolism, signal transduction and intra and extra cellular communication (Liu, 2009). Cholesterol has the important constituent of membranes of the brain, nervous system, spinal cord and also have an important component of insulating myelin sheath. Woolette in 2011, reported the role of cholesterol in embryonic and fetal development. Recently it has been reported that cholesterol has also an association with bone marrow and eye lenses (Zampelas and Magriplis, 2019).

2.2 Biosynthesis of cholesterol

A complex process is involved in cholesterol biosynthesis which is highly controlled and regulated. Many enzymes are required for this process. Biosynthesis of cholesterol is initiated with formation of mevalonate from acetate. Initially the thiolase enzyme catalyze the reaction to condense the two acetyle-CoA molecules to form an acetoacetyl-CoA. Another enzyme, 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase (HMGCS) form HMG-CoA by catalyzing the reaction between an acetyl-CoA and already synthesized acetoacetyl-CoA. Finally, the HMG-CoA reductase act on HMG-CoA and reduce it to mevalonate (Cerqueira *et al*., 2016).

Mevalonate is converted into two molecules of activated isoprene by subsequent steps and after a series condensation reaction, squalene is formed, which is a biochemical precursor molecule of all the steroids. After several reactions, squalene converted to lanosterol and finally after the several reactions the lanosterol is transformed into cholesterol (Figure 2.1) (Sitaula and Burris, 2016).

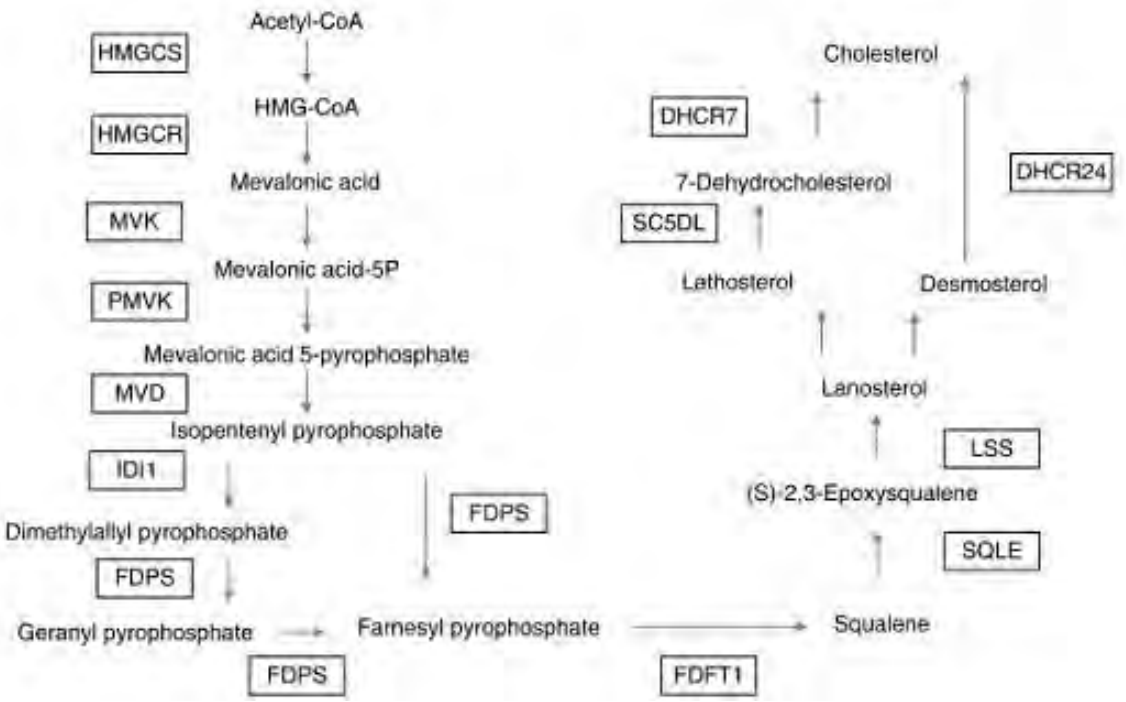


Figure 2.1 Pathway of cholesterol biosynthesis (Sitaula and Burris, 2016).

2.3 Cholesterol accumulation and associated diseases

Normally the cholesterol is metabolized by the body for several vital functions including for the synthesis of certain hormones, vitamin D and fat dissolving bile acid. But unfortunately, sometimes the unwanted cholesterol deposition may occur in different cell types which hinder the normal body functioning. There are several known factors associated with the cholesterol deposition in the body including genetics, metabolic disorders, obesity, smoking and ageing.

The causes of deposition of cholesterol and the risk factors associated with different diseases are studied by different research groups. High fat diet is a major risk factor for development of metabolic syndromes, characterized by hyperlipidemia, hypertension, obesity and hyperglycemia which contribute to arteriosclerosis, a life-threatening condition (Ford *et al*., 2002). Nonalcoholic fatty liver disease which includes the number of disorders from the common and reversible steatosis to life taking cirrhosis (Angulo, 2002). The advance fibrosis was developed in high fatty diet fed rabbits in experimental setup (Ogawa *et al*., 2010). de Munter *et al.,* (2013) reported the accumulation of cholesterol in synovial macrophages is due to the intake of high fatty diet and lack of LDL receptors which may lead to metabolic syndrome associated osteoarthritis. In 2016, de Munter *et al*., also reported that the cholesterol rich diet leads to the development of osteoarthritis in experimental mice model. High cholesterol diet fed mice and hamsters developed the hepatic inflammation which could cause the nonalcoholic steatohepatitis (NASH) (Lai *et al*., 2016). Ho *et al*., (2019) examined the surgical specimens of the liver and reported that cholesterol and its oxidized derivatives can cause the portal inflammation and fibrosis which leads to the nonalcoholic fatty liver diseases (NAFLD).

Hyperhomocystinemia, a condition of increased level of homocystine in blood plasma, occur as genetic metabolic disorder which play an important role in the enhanced production of hepatic cholesterol by activation of transcription factors. Woo *et al.*, (2005) reported the mechanism of hyperhomocystinemia enhanced hepatic cholesterol synthesis in animal model. They concluded that the increased level of cholesterol in hyperhomocystinemic rats is because of the over expression of the mRNA and increased enzyme activity of HMG-CoA reductase. Some of the studies showed that the increased level of LDL cholesterol is due to the genetic mutations in regulating genes. Mutations in the LDL receptor gene and codon 3500 of apoB gene have both shown the increase level

of LDL cholesterol, which is a major risk factor of coronary heart disease. Heterozygosity for an LDL receptor defect approximately doubles the LDL level, which is a common mutation and occurs in 1/500th person in general population (Truong *et al*., 2017).

High concentration of lipid can cause serious damage in different organs of the body. The studies conducted by Gong *et al.,* (2020) reported the membrane thickening and vascular degeneration of endothelial cells in apolipoprotein E-Knockout mouse due to the glomerular lipid deposition. The role of hyperlipidemia in glomerulosclerosis is also reported by the other research groups (Cases and Coll, 2005; Hickson *et al*., 2016).

2.3.1 Aging diseases

The major cause to drive the aging and age-related diseases are the accumulation of the waste in the various organelles of the body, due to decline in the catabolic functions as a result these aggregates hinders the cellular activities. There are several examples of the accumulated aggregates including lipofuscin, β amyloid plaques and fatty deposits, with pronounced detrimental effects and are ubiquitous markers of the aging.

2.3.2 Age related neurodegenerative diseases

The role of cholesterol and its derivatives in the development of age-related neurodegenerative diseases has been studied. The improper metabolism of cholesterol affects the production and clearance of tau phosphorylation and Aβ sheets and precede to onset of memory loss known as Alzheimer disease (Gamba *et al*., 2019; Sato and Morishita, 2015). Testa *et al*., (2016) reported the oxysterols (7-KC, 27-OHC and 7β-OHC) presence in Alzheimer's disease brains. Moreover, Chen *et al*., (2019) reported the impact of 27-OHC on the membrane permeability of lysosomes and pyroptosis. Merino-Serrais *et al*., (2019) demonstrated the impact of 27-OHC, that it induces the synaptic dysfunction resulting improper neuron morphology.

2.3.3 Age related macular degeneration

One of the ageing diseases, known as age-related macular degeneration have diverse environmental and genetic factors. Rodriguez and Larrayoz in 2010, reported 7-KC an oxidized derivative of cholesterol generated in retina is one of the most potential risk in the development of age-related macular degeneration (AMD). They speculated that the 7-KC might induced the chronic inflammation that may causes the loss in function, death and transformation of the cell.

2.3.4 Cholesterol and cancer

Beside the role in other diseases, cholesterol also plays a key in the development of cancer. The high level of cholesterol in serum is positively correlated with the development of cancers in different organs, such as rectal, colon, testis and prostate (Radisauskas *et al.,* 2016; Murai, 2015). Cholesterol, as vital component of the plasma membrane of the cell, it may be related to the receptors of the membrane by which the oncogenic signaling cold be directly activated. The increases level of blood cholesterol and high fat high cholesterol diet is found to be effective in the development of cancer. Both the external and internal cholesterol can activate the oncogenic Hedgehog pathway and mTORC1 signaling respectively (Ding *et al*., 2019). Garcia-Estevez and Moreno-Bueno, (2019) also reported the role of in development of breast cancer. In tumors for the development of cancer where the increased level of cholesterol is needed, the sterol regulatory element-binding protein 2 (SREBP2), the master transcription factor and the enzymes of the mevalonate pathway are highly upregulated (Lewis *et al*., 2015). Cai *et al* (2019) reported RAR-related orphan receptor gamma (RORγ) as another transcription factor involved in the activation of the cholesterol biosynthesis which progress the development of breast cancer.

2.3.5 Cholesterol and atherosclerosis

Cardiovascular diseases are the leading cause of death worldwide (Jiang *et al.,* 2013), causing approximately 17 million deaths per year (Mozaffarian *et al*., 2016). The leading risk factors of cardiovascular diseases (CVDs) includes hypertension, hyperlipidemia, obesity and lack of physical activities which represents more than 90% risks to CVDs collectively (Flora and Nayak, 2019). Atherosclerosis, one of the major underlying cause of CVDs, is mainly triggered by hypercholesterolemia. In these conditions the low-density lipoprotein is accumulated in walls of arteries and modified via oxidation (Glanz *et al*., 2020). In the development of atherosclerosis, the different processes like cholesterol accumulation and oxidation, endothelial cells activation, inflammatory mediators and macrophage accumulation which formed the positive feedback loop resulting the formation of atherosclerotic plaque (Poston, 2019). Some studies indicated the role of cytotoxic lymphocytes in the development of atherosclerotic lesions. Different cell types are involved in the activation of cytotoxic lymphocytes including cytokine, macrophage and dendritic cells. Once they activated, secretion of pro inflammatory cytokine takes place which play important role in the formation of atherosclerotic vulnerable lesions (Kyaw *et al.*,2017).

Normally the exogenous and endogenous biomolecules are processed in a central catabolic organelle known as lysosome. Different biomolecules including lipid are routed to this organelle for hydrolysis and redistribution by the action of different lysosomal proteins including Lysosomal acid lipase and NPC proteins (Welch *et al.,* 2007; Zhang *et al*., 2008; Du *et al*., 2002). In humans the complete absence or reduced enzymatic activity of Lysosomal acid lipase is also linked with different diseases like Wolman's disease and premature atherosclerosis vascular disease (Reynolds, 2013). Jerome (2010) demonstrated the role of lysosome in cholesterol degradation. He treated the macrophages cell lines with different modified lipids which induced the disruption of lysosomal function and observed the accumulation of cholesteryl esters inside the lysosomes. Emanuel *et al.,* (2014) also reported the effect of cholesterol and oxidized LDL on lysosomal dysfunction in macrophages due to the enlarged size of lysosome and increased pH which effect the degradation capacity of lysosomes. The increased Lipids uptake by the macrophagic cell also cause dysfunction of lysosome by the formation of crystals of cholesterol which affect the membrane integrity and activate the inflammation (Sergin *et al.,* 2015).

The oxidized derivatives of cholesterol which are produced by enzymatic or non-enzymatic reaction are also the major contributors in the progression of atherosclerosis (Gargiulo *et al.,* 2017). The initial event in the onset of atherosclerosis is the dysfunction of vascular endothelium. Oxysterols are the compound which can change the endothelium function by interacting with the membrane and alter the membrane bound enzyme's function involved in maintaining the rigidity of endothelium (Duran *et al*., 2010). Wong *et al*., (2011) reported the effect of different oxysterols including 7-KC, 7α hydroxycholesterol (7α-OHC) and 7β hydroxycholesterol (7β-OHC) on the vasodilatory response in the rabbit aortic rings which was because of the direct interaction of oxysterol with nitric oxide resulting the reduced bioavailability of nitric oxide. An apoptotic pathway in ECs is triggered by the 25-OHC by inhibiting the endothelial nitric oxide synthase (eNOS) which results in the dysfunction of endothelial functions (Ou *et al.,* 2016). The effected endothelial cells by oxysterols like 7-KC, can regulate the several other proteins involved in angiogenesis and blood coagulation (Rosa-Fernandes *et al*., 2017). Ward *et al*., (2017) also reported the confirm involvement of 7-KC in protein expression involved in foam cells formation, inflammatory responses and cell death.

Progression of atherosclerotic plaques is dependent on the dysfunction or apoptosis of the macrophages. Oxysterols can activate the process of apoptosis by induction of ROS

(Sottero *et al*., 2009). Leonarduzzi *et al.,* (2006) demonstrated the role of 7-KC in reactive oxygen species (ROS) production leading to apoptotic vascular cell death. (Larsson *et al*., 2006) also reported the effect of different oxysterols including 27 hydroxycholesterol (27-OHC), 7β hydroxycholesterol (7β-OHC), 25-OHC and 7-KC on U937 cells. They used the mixture of oxysterols, observed the apoptotic effect on cell and indicated that accumulation of oxysterols in atherosclerosis plaque are cytotoxic. It has been demonstrated that accumulation of cholesterol and its oxidized derivatives contribute to atherosclerotic plaque formation due to dysfunctional autophagy (Li *et al*., 2016).

2.4 Cholesterol lowering therapies

There are numerous problems associated with high cholesterol in humans which may be because of high cholesterol accumulation. Maintaining the level of cholesterol in the body is one of the major concerns to control/treat the diseases caused by high cholesterol level. To reduce the cholesterol levels, there are several classes of drugs available. Statin is one of the major cholesterol lowering drug approved for treating the disorders related with lipids and can also be used for lowering of LDL cholesterol level. Statin act to inhibit the activity of HMG-CoA reductase which ultimately decrease the cholesterol contents in liver and increase the regulation of LDL receptors. Although number of the patients can tolerate the statin but some side effects including headache, muscle complications, increased risk of diabetes and drowsiness are associated with statin therapy while mental confusion and memory loss are reported in serious cases (Mancini *et al*., 2016). Number of studies carried out in several patients using a variety of statins demonstrated that atherosclerotic cardiovascular disease has been reduced with statin therapy (Baigent *et al*., 2005; Feingold and Grunfeld, 2000). reported the efficacy of statin which reduce the mortality due to coronary heart disease.

The level of cholesterol is decreased by inhibiting its absorption in intestine by a drug, ezetimibe. Primarily ezetimibe is used with statin as a combination therapy mostly when statin alone is not functioning properly or in statin intolerant patients (Shapiro, 2018). It also has antitumor activity which inhibit the absorption of cholesterol by interrupting Niemann pick C1-like 1 (NPC1L1) mediated cholesterol influx studied in prostate cancer model (Solomon *et al.,* 2009). Besides its role in lowering the cholesterol level, some of the GI side effects have been observed including constipation. Florentin *et al*., (2008) demonstrated the associated side effects of ezetimibe in the patients of

hypercholesterolemia, mainly myopathy along with mild increase in the liver transaminases.

The level of LDL could be controlled in plasma by certain inhibitors, one such type of inhibitor is proprotein convertase subtilisin/kexin type 9 (PCSK9). The role of PCSK9 in LDL metabolism was first reported by Abifadel *et al*., in 2003. Normally the expression of PCSK9 takes place in liver and then released in circulation where it binds to the LDL receptor and change its metabolism. Theses PCSK9 bound LDL receptors are transported to lysosomes where its degradation takes place leading to the increased plasma cholesterol levels (Lambert *et al*., 2012). The United State Food and Drug Administration (US-FDA) approved the use of two PCSK9 inhibitor in patients with high risk of cardiovascular disease or statin intolerant. Sabatine *et al*., (2015) reported the efficacy of evolocomab, a PCSK9 inhibitor, in patients with high LDL cholesterol. They found that evolocomab efficiently reduced the LDL level by 61% in one year medication and also significantly reduced the cardiovascular events. Giuglianu *et al.,* (2017) also reported the efficacy evolocomab in reduction of cardiovascular events in patients with stable CVDs.

Cholesterol level can also be reduced by the inhibition of the activity of microsomal triglyceride transfer proteins, which is involved in the transfer of triglycerides to apolipoprotein B for the generation of chylomicrons and VLDL. Cuchel *et al.,* (2013) reported the use of lomitapide, which inhibit the activity of microsomal triglyceride transfer proteins, reduced 50% LDL cholesterol from baseline in hypercholesterolemia patients. Along with the reduction of LDL cholesterol they also observed the adverse events including gastrointestinal symptoms and increased level of aminotransaminase which was then resolved by dose reduction. Davis and Miyares., (2014) also reported more than 50% LDL cholesterol reduction when lmitapide alone or in combination was used in clinical trials.

In 2013 mipomersen was approved for the treatment of hypercholesterolemia. It is antisense oligonucleotide which bind specifically to apolipoprotein B to block the synthesis of translational product which ultimately results the reduced production of certain lipoproteins including LDL (Docherty and Padmanabhan, 2014). Raal *et al.,* (2010) reported the effectiveness of mipomersen in reduction of LDL lipoprotein in patients with high cholesterol in combination with high dose of statin. Some of the side effects are also associated with the use of mipomersen including reactions at the drug injection site and

increased liver toxicity due to the enhance production of alanine aminotransferase. Mipomersen is also reported to halt the VLDL formation which increase the risk of fatty liver. Gouni-Berthold and Berthold (2015) observed the fatty liver in 5 to 20% patients treated with mipomerson. Another study was performed by Akdim *et al*., (2010), to check the effectiveness and side effects of mipomersen in familial hypercholesterolemia patients. They found that significant reduction of LDL cholesterol approximately from 21 to 34% from baseline by using the highest doses of mipomersen. They also reported certain side effects associated with high dose mipomersen which cause local injection site reaction, transaminase elevation, stetotic liver and hepatomegaly.

2.5 Microbial degradation of cholesterol

As the endogenous enzymes fail to degrade the accumulated cholesterol and its oxidized derivatives which leads to the development of certain diseases in humans. Some of the known therapies including statin and some inhibitor which lowers the cholesterol level by various means are already in practice. But the associated risks and side effects of those drugs are not negligible. Microorganism are known for their capability to degrade the number of recalcitrant compounds. Different microbes are reported which can degrade the compounds associated with different pathologies. One of the complex groups of compound is steroids, which is ubiquitous in environment and also the cause of different diseases in human.

Due to the availability of steroidal compounds in environment, different kind of microorganisms can degrade and use them for their energy requirements. In nature several microorganisms exist with unlimited biotechnological potential due to their high catabolic diversity and ubiquitous nature (Madigan *et al*., 2010). Several new approaches have been developed for the discovery of microorganisms and their catabolic enzymes (Baldrian and Lopez-Mondejar, 2014). Different studies were carried out to isolate the cholesterol or their derivatives degrading microorganisms and in 1944 microbial cholesterol degradation was first reported by Turfitt (Jangala and Pulipati, 2020). In production of steroidal precursors, the microbial transformation of cholesterol is considered as an effective tool as compared to synthetic methods (Giogi *et al.*, 2019). Cholesterol has been catabolized to several ketonic derivatives by the action of many enzymes. Cholesterol oxidase is one of the initial enzyme involved in cholesterol degradation and reported in a large number of bacterial species including *Streptomyces, Rhodococcus, Nocardia, Mycobacterium* and *Ballicus*

(Kreit., 2017). Yazdi *et al.,* (2000) reported the isolation of *Agrobacterium sp*. M4, from soil which was found to degrade cholesterol efficiently. They also detected and identified 4-cholestene-3-one, which is the first oxidized product in cholesterol degradation. Kim *et al.,* in 2002 isolated *Bacillus subtilis* SFF34 from Korean fish and reported the production and purification of cholesterol oxidase and cholesterol degradation by the enzyme. *Gordonia cholesterolivorans*, was isolated from sewage sludge which was capable to degrade different steroidal compounds including cholesterol, cholestenone, stigmasterol and ergosterol. Unique cholesterol oxidase genes were also identified from *G. cholesterolivorans* with intracellular enzymatic activity (Drzyzga *et al*., 2011). This study showed that *G. cholesterolivorans* can only use long chain steroidal compounds for degradation while in other studies De Las Heras *et al*., (2009) reported *Rhodococcus rubber* with a potential to degrade thirteen various steroidal compounds with short, long or no carbon side chains. Liu *et al*., (2011) isolated one hundred twenty-six strains from multiple samples with their capability to use the cholesterol as the only carbon source for growth in mineral salt medium (MSM). On basis of thin layer chromatography (TLC) analysis 13 strains were found with capability to utilize cholesterol. One of the strain with highest capability to degrade cholesterol was identified as *Gordonia neofelifaecis* from faeces of wild cat (*Neofelis nebulosa*). Different intermediates were identified in the cholesterol biotransformation and in presence of 2,2'-dipyridyl major metabolite was androsta-1,4-diene-3,17-dione. The complete degradation of cholesterol was achieved. Another study conducted in 2014 by Saranya *et al.,* reported the isolation of *Pseudomonas, Bacillus* and *Streptomyces* species from the waste of soap and vegetable oil industries. They used these isolates for the degradation of cholesterol and purified the cholesterol oxidases.

The abilities of probiotic bacteria were checked for the cholesterol degradation. Kulkarni *et al.*, (2013) tried to isolate the probiotic based cholesterol transforming bacteria. Different lactobacillus species with the ability to grow on cholesterol medium were isolated from cow milk samples. In another study *Lactobacillus sakei* GMK01, *Lactobacillus rhamnosus* GMK02 and *Leuconostoc mesenteroides* GMK03 were isolated from lamb meat and checked for their ability to produce cholesterol oxidase. The cell free supernatants of all three isolates were able to reduce the cholesterol by more than 80% (Khirala, 2015). *B. pumilus* W8 and *B. safensis* W1 isolated from oil contaminated soil as a potential cholesterol degrading microorganism were reported from the current studies. Different factors affecting the cholesterol degradation were studied and around 80% cholesterol

reduction was achieved under optimized conditions. Furthermore, both strains were reported as potential source of cholesterol oxidase (Wali *et al*., 2019).

In aerobic cholesterol degradation, the first step is oxidation of cholesterol by which cholest-5en-3-one is formed and then isomerized to cholestenone (Ghosh *et al.,* 2018). This reaction is carried by the enzyme cholesterol oxidase or 3β-hydroxysteroid dehydrogenase/isomerases. After the oxidation reaction the aliphatic chain of cholesterol is removed which, leads to the formation of a C22-oic acid and then release an acetyl-CoA and two molecule of propionyl-CoA (Wilbrink *et al*., 2011).

Bacterial catabolism of cholesterol by any bacterial strain has not been fully elucidated yet, the metabolic pathway could be postulated by combining biochemical and molecular studies already carried out in various microorganisms (Garcia *et al*., 2012). After the first step of cholesterol oxidation, cholestenone is formed, and then the removal of C17-alkyle chain takes place resulting in the 17- ketosteroids androsta-4-ene-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD) formation (Kreit, 2017). This process of side chain degradation in cholesterol is similar to the fatty acids β-oxidation (Garcia *et al*., 2012). 9a-Hydroxylation is catalyzes by the action of 3-ketosteroide dehydrogenase in *M. smegmatis* (Andor *et al*., 2006), or 3-ketosteroide-9-hydroxylase yielding 9α-hydroxyandrosta-1,4-diene-3,17-dione (van der Geize *et al*., 2008; Capyk *et al*., 2009b) and then by some non-enzymatic transformation 3-hydroxy-9,10-secoandrosta-1,3,5 (10)-trien-9,17-dione (3-HSA) is formed. The 3-HAS is subsequently transformed by hydroxylation leading to 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-DHSA) catalyzed by oxygenase from *C. testosteroni* (Horinouchi *et al*., 2018). An enzyme meta extradiol diooxygenase (*hsaC*) identified in from *R. jostii* RHA1 or *M. tuderculosis* act on catechol derivative and produce 4,9-DHSA (Yam *et al*., 2009). The hydrolysis of 4,9-DHSA takes pace by the action of HsaD in *M. tuderculosis* leading to the formation of 2-hydroxyhexa-2,4-dienoic and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic (DOHNAA) acids (Lack *et al.*, 2010). 4,9-DHS could also be transformed to 2-hydroxyhexa-2,4-dienoic acid which is then further metabolized by the action of hydratase into 4-hydroxy-2-oxo-hexanoic acid. The enzyme aldolase catalyze the formation of pyruvic acid and propionaldehyde which is subsequently metabolized to the end product propionic acid by the action of aldehyde dehydrogenase (Horinouchi *et al*., 2005). DOHNAA could also be metabolized to succinic acid through sequential degradation steps

catalyzed by coenzyme A and nicotinamide adenine dinucleotide phosphate, NADPH-dependent dehydrogenase (Figure 2.2) (Van der Guiz *et al.,* 2011).

Information about anaerobic steroid degradation and mechanism is limited comparatively. From anaerobic sludge an isolated betaproteobacterium identified as *Sterolibacterium denitrificans* was able to mineralize cholesterol by using nitrates as terminal electrons acceptors (Tarlera and Denner, 2003). Chiang *et al*., (2007) represented the first study of complete anaerobic metabolism of steroidal compounds. Some common but also some different intermediate of aerobic pathway were used to propose the initial reaction outlines. They used *Sterolibacterium denitrificans* for anoxic metabolism of cholesterol and reported that the degradation take place by oxidation of ring A, followed by hydroxylation of side chain via oxygen independent pathway. Warnke *et al.,* (2018) also reported that the *Sterolibacterium denitrificans*, a denitrifying bacterium for the degradation of cholesterol anaerobically. They reported that *S. denitrificans* can degrade the cholesterol anaerobically by number of the enzymes in which some these are reported for aerobic bacterial degradation pathway. *S. denitrificans* was used as model organism to establish an anaerobic, 2,3-*seco* cholesterol catabolic pathway (Figure 2.3) (Wang *et al*., 2013; Wang *et al*., 2014). This process includes the uptake of substrate, modification of sterane, ring and side chain degradation. Across the outer membrane, cholesterol uptake is mediated by long-chain fatty acid like transporters (Lin *et al*., 2015; Warnke *at al*., 2017). In periplasm oxidation of cholesterol ring and isomerization of carbon-carbon double bond take place by the action of cholesterol dehydrogenase/isomerase, generating cholest-4-en-3-one (Lin *et al.,* 2015; Chiang *et al.,* 2008). C25 dehydrogenase (C25DH) catalyses the hydroxylation of cholest-4-en-3-one at C-25, yielding 25-hydroxycholest-4-en-3-one (Dermer and Fuches, 2012) and then 26-hydroxycholest-4-en-3-one is generated by the process of isomerization which undergo through oxidation producing cholest-4-en-3-one-26-oic acid. Through β-oxidation reactions the subsequent side chain degradation take place to produce AD (Wang *et al*., 2013). The coenzyme A-ester metabolites and genes involved inside chain degradation has been identified (Warnke *et al*., 2017) and for side chain activation, substrate specificity of acyl-CoA synthetase prior to each β-oxidation is now fully elucidated (Warnke *et al*., 2018).

By two sequential redox reactions AD is transformed into (1-AD) and androstan-1,3,17-trione is generated when a bifunctional enzyme hydratase/dehydrogenase (Yang *et al*., 2016) introduced a water molecule between C-1 and C-2 of 1-AD. 1,17-dioxo-2,3-seco-

androstan-3-oic acid (DSAO) is a characteristic intermediate which is produced by hydrolytic cleavage of trione structure (Wang *et al.*, 2013; Yang *et al.*, 2016). The transformation of all the androgen metabolites take place by 17-hydroxysteroid dehydrogenase (Yang *et al.*, 2016). DSAO activation by acyl-CoA synthase lead to generation of DASO-CoA, which is further degraded into 2,5-seco-3,4-dinorandrost-2,5,17-trione (2,5-SDAT) by an aldolic reaction (Wang *et al.*, 2014). Acyl-CoA hydrolase/reductase are involved in the remaining rings degradation which is highly similar in both aerobic and anaerobic cholesterol catabolism (Crowe e*t al*., 2017).

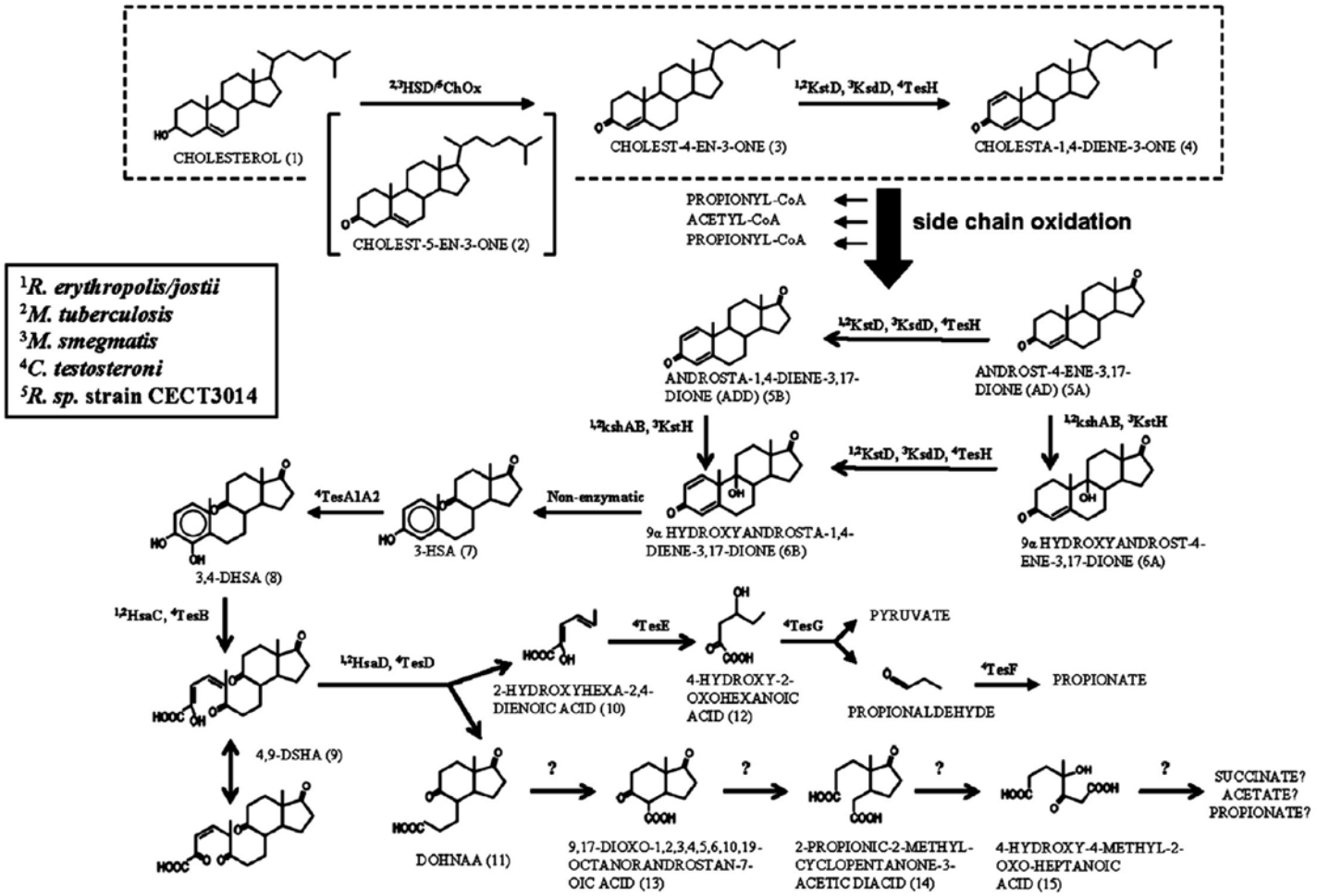


Figure 2.2 Proposed pathway of microbial cholesterol degradation (Garcia *et al*., 2012)

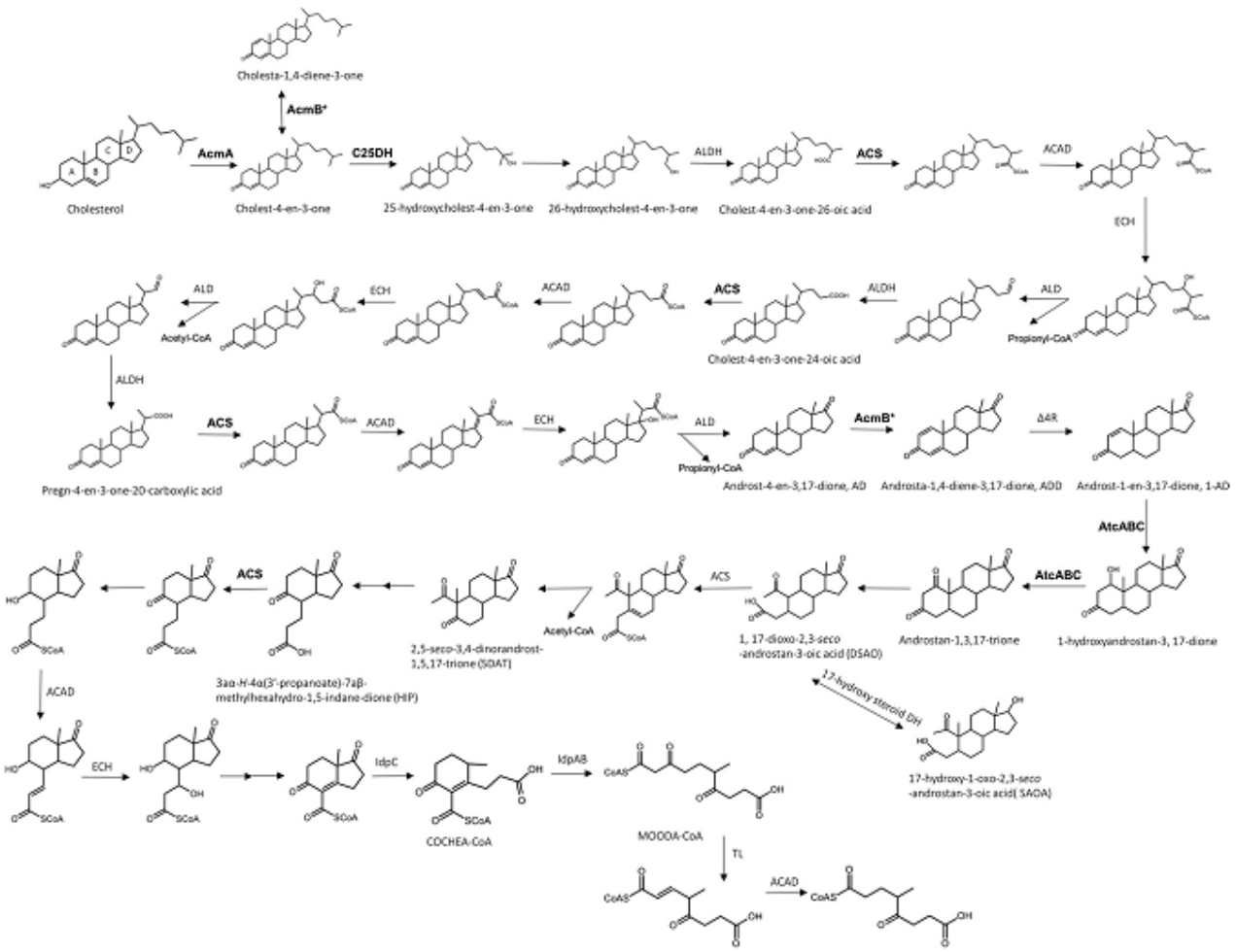


Figure 2.3 Anaerobic cholesterol catabolism by 2,3-*seco* pathway in *Sterolibacterium denitrificans* (Wei *et al*., 2018).

2.6 Degradation of 7-ketocholesterol

Beside cholesterol some of its oxidized derivatives are studied to be involved in the pathogenesis of different diseases. 7-KC is one of the major cytotoxic oxidized derivatives of cholesterol which interferes with cellular homeostasis. Along with cholesterol degradation different microorganisms are also reported which are capable to catalyze the 7KC. Methieu *et al.,* (2008) isolated number of microorganisms from activated sludge or soil, including *Rhodococcus sp*. RHA1, *Proteobacterium* Y-134, *Pseduomonas aeruginosa, Nocardia nova* and *Sphingomonas sp*. JEM-1 using 7-KC as the only energy and carbon source. They reported that biosurfactant producing *Nocardia nova* was the fastest degrader. Mathieu *et al*., (2010) compared the gene expression in *Rhodococcus jostii* RHA1, grown separately on 7KC, pyruvate and cholesterol. They reported the *Rhodococcus jostii* RHA1 mineralization of 7-KC and by using transcriptomic analysis it was concluded that cholesterol and 7-KC utilizes same key catabolic genes, indicating common degradation rout. Recently few studies showed that microbial enzymes could mitigate the cytotoxicity of 7-KC resulting the increased cell viability. Mathieu *et al.,* (2012) reported the increased cell viability with short term treatment of *Chromobacterium* cholesterol oxidase. A potential degrader strain *Pseudomonas aeruginosa* PseA identified by Ghosh and Khare, (2016) degraded 88% 7-KC under optimized conditions and some metabolites were detected with electrospray ionization mass spectrometry ESI-MS. *In-vitro* degradation studies with cell free supernatant indicated that cholesterol oxidase is one of the potential degrading enzyme. Same group used the previously isolated *Rhodococcus erythropolis* MTCC 3951 strain for 7-KC degradation, which has already been reported to degrade the xenobiotic compounds including benzene and biphenyls. Under optimized conditions this strain was able to degrade 93% of 7-KC along with production of certain metabolites. To assess the enzyme involvement, 7-KC was treated with intra and extracellular extracts and the hydrolysis of compound was observed. They identified several enzymes produced by *Rhodococcus erythropolis* in presence of 7-KC including dehydrogenase, lipase, cholesterol oxidase and reductase (Ghosh and Khare, 2017). Perveen and Ahmed, (2016) isolated *Serratia marcescens* IP3, *Streptomyces auratus* IP2 from soil and *Thermobifidafusca* IP1 from manure piles. HPLC analysis showed that all the environmental isolates were potential degrader of 7-KC resulting its mineralization.

2.7 Therapeutic biological enzymes

The role and application of enzymes in medical and industrial use is a growing field. The utilization of different enzymes for diagnostic and therapeutic purposes is increasing with the advantages because of their specific targets and conversion of multiple substrates. Because of these characteristics of the enzymes, different enzymes are used in medical problems alone or in combination with other therapies for the treatment of diseases. Using of enzymes for treatments of different medical problems as a drug forms the basis for it use for therapeutic purposes.

Therapeutic enzymes were first described by de Duve in 1959 for the diseases in genetic deficiencies as replacement therapy (Vellard, 2003). The first enzyme, Actavase1, as a therapeutic agent was approved in 1987 by Food and Drug Administration (FDA) for heart attack patients caused by clot in coronary artery (Aiuti, 2002). Another therapeutic enzyme Adengen1 bovine adenosine deaminase treated with polyethylene glycol (PEG), was approved in 1990 by the FDA for the treatment of chronic deficiency of adenosine deaminase (ADA) which leads to severe combined immunodeficiency disease (SCID) (Mechler *et al*., 2015).

The production of medically important enzymes of microbial origin have several advantages as consistent, economically feasible, high yield and easy for modification of products and its optimization. Different microbial enzymes have received significant attention for their therapeutic role in diseases.

In diagnostic and pharmaceutical industries, various enzymes have significant role. These enzymes are used for the diagnosis of different metabolic diseases. Several enzymes are used for the quantitative determination such as Glucose oxidase for diabetes; sarcosine oxidase and creatinase for creatinine; lipase and glycerol kinase of triglycerides; urease for urea and urate oxidase for uric acid in body (Kumar *et al*., 2019; Le Roes-Hill and Prins, 2016). Enzymes are extensively used in analytical procedures like ELISA (Mane and Tale, 2015) and used in the field of genetic engineering. As the DNA polymerase is used in polymerase chain reaction for DNA amplification and different restriction endonucleases are used for site specific cleavage of nucleic acid for cloning purposes (Di Felice *et al*., 2019; Vimal and Kumar, (2019). The use of radioactive compounds in various immunoassays have been replaced with the advancement in enzyme technology for the determination of various hormones and proteins (Kazeto *et al*., 2019). Enzymes and

enzyme rich food for consumption can prevent different diseases and aging processes. Enzymes have vital role in every cell biochemical function and the lack of such enzymes increased the aging process (Kaur and Sekhon, 2012). Some health disorders caused by genetic problems which are associated with deficiency of certain enzymes could be treated by the enzymes of microbial origin (Anbu *et al.,* 2017). The enzyme sacrosidase is given orally to facilitate the sucrose digestion in patients with inherited deficiency of sucrase-isomaltase (Puntis and Zamvar, 2015). Phenylketonuria is caused by accumulation of phenylalanine in blood, due to mutation in phenylalanine-4-hydroxylase gene. Increased accumulation of phenylalanine is neurotoxic and can cause the mental retardation. Recombinant phenylalanine ammonia lyase is used subcutaneously to revert the accumulated phenylalanine (Babaoglu Aydas *et al.,* 2016). Some of the other enzymes including rhodanese, acid protease and dextranase may be used for cyanide poisoning, alimentary dyspepsia and tooth decay, respectively (Swami and Shah, 2017; Ren *et al*., 2018; Chaudhary and Gupta, 2012).

L-Asparaginases have an important role in therapy of acute lymphoblastic leukemia and also it inhibits the formation of acrylamide during food processing. It hydrolyzes the l-asparagine into l-aspartate and ammonia. The depletion of L-asparagine results in cytotoxicity of leukemia cells (Zuo *et al.,* 2015; Lopes *et al.,* 2017). However, the normal cells are able to synthesize the asparagine and it affected very less when its depletion occurs by treatment with L-asparaginase. Streptokinase is used to dissolve the fibrinogen of blood clots in lungs and heart arteries. Deep vein thrombosis is treated by the administration of streptokinase, urokinase and tissue plasminogen activator at the site of clots directly or into vein (Watson *et al*., 2016).

Some microbial enzymes have bactericidal activity against several microorganisms. This information gives the idea to search and screen the biological and other chemical drugs for microbial infections. Some microbial enzymes with therapeutic and diagnostic potentials have been reported. Lipases could be used to support the digestion and been used in treatment of digestive allergies and gastrointestinal disturbances. Lipases also have a role in therapy of malignant tumors as they can activate the necrotic factors in tumors. Lovastatin synthesized by a lipase produced by *Candida rugosa* can decrease the cholesterol level in serum. Some proteolytic enzymes from microbial sources also have therapeutic potential against burnt skin cells. Vibrilase TM produced by *Vibrio proteolyticus* is found very effective against the burned skin cells (Gurung *et al*. 2013). The action of certain

therapeutic enzymes on damaged nerves has been studied. Chondroitinase and hyaluronidase both can regenerate the damaged nerve cells and tissues by acting on chondroitin sulphate proteoglycans which inhibit the signaling pathway (Rosenzsweig *et al*., 2019; Raghavan *et al*., 2016). Applications of therapeutic enzymes to cure the different diseases are summarized in Figure 2.4.

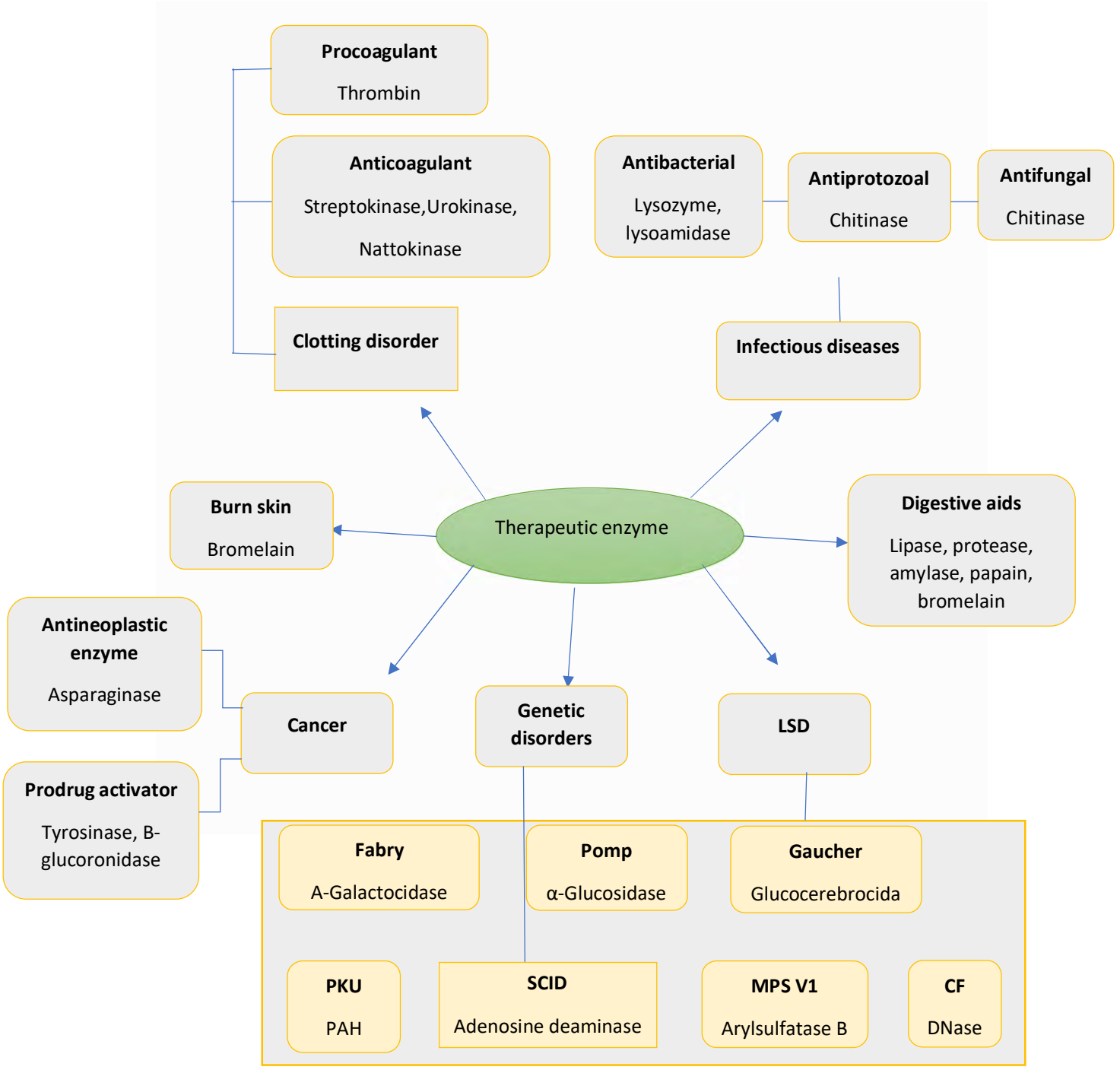


Figure 2.4 Human disorders treatment by therapeutic enzymes.

2.8 Cholesterol oxidase

Bacterial cholesterol oxidase is an enzyme widely studied in degradation of sterols. It is a flavoprotein that catalyzes the oxidation reduction reactions. Beside the role of cholesterol oxidase in degradation of steroidal compounds it has been used in determination of cholesterol levels in serum and food, in the process of biocatalysis and as an insecticidal protein**.** In different samples including blood or serum, human bile, gall stones, cell membrane and in food samples (Isobe *et al*., 2003; Pollegioni *et al*., 2009) cholesterol oxidase is used as an analytical tool to detect LDL, HDL, total and esterified cholesterol. In human blood the normal level of cholesterol is about less than 200 mg/dL-1 with ~70% in esterified form. An imbalance in cholesterol level is associated with several pathologies that increases the public concern about the importance of cholesterol concentration determination.

Enzymatic assay is used to check the serum cholesterol level, in first reaction free cholesterol is released by cholesterol esterase, which is then oxidized by cholesterol oxidase resulting into cholestenone and hydrogen peroxide followed by enzymatic oxidative coupling of H_2O_2 with colored compound which is then measured by spectrophotometer (Toyama *et al*.,2002). Biosensors have the capability to measure the specific substance concentration in a desired sample, to monitor the blood cholesterol level, impelled the need to develop the cholesterol biosensors. Recently immobilized cholesterol oxidase is used to design the electrochemical biosensors for serum and food cholesterol determination. However, in market only few cholesterol biosensors have been launched due to critical parameters optimization including enzyme immobilization, stabilization and design of instrumentation. The efforts towards cholesterol biosensors development led to commercialization of some biosensors such as Cholestrak by AccuTech, CardioChek by Polymer Technology Systems Inc. and Biosafe's cholesterol test kit etc (Arya *et al*., 2008).

Microbial proteins with insecticidal properties have great importance in pest controlling strategies. To control the pests, genetically modified plants has been developed with ability to produce the insecticidal proteins. Highly competent protein in *Streptomyces* culture filtrates was first discovered by Purcell *et al*., (1993) which can kill boll weevil larvae and later identified as cholesterol oxidase. It has been revealed that cholesterol oxidase disrupts the membrane of midgut epithelial cells by oxidizing the membrane sterol and cholesterol leading to larval death (Doukyu, 2009). Protein is also active against cotton insect pests,

such as pink bollworm, tobacco budworm and corn earworm. Corbin *et al* (2001) transformed the cholesterol oxidase gene from Streptomyces with tobacco plant. They observed strong insecticidal activity in the transgenic leaf extracts when treated with boll weevil larvae.

Certain microbial enzymes are involved in the degradation of cholesterol and cholest-4-ene-3-one is produced by the action of cholesterol oxidase which may undergo through oxidation and resulting 1, 4-androstadiene3,17-dione (ADD) and 4-androstene-3, 17-dione (AD), important precursor molecules for hormone synthesis (Giorgi *et al.,* 2019). Some studies showed the antifungal drug synthesis by cholesterol oxidase. Mendes *et al*., (2007) observed the involvement of *Streptomyces natalensis* cholesterol oxidase in synthesis of 26-membered macrolide known as antifungal antibiotic, used to prevent the mold contamination in food industries. It has been demonstrated that polyene macrolide is also used for corneal fungal infections by interaction with fungal membrane, disrupting the membrane functions Pollegioni *et al*., (2009). Another group Wang *et al.,* (2016) demonstrated that *pimE* gene regulate the cholesterol oxidase production, which act as a signaling protein for natamycin biosynthesis. They also reported the improved production of natamycin with increased production of cholesterol oxidase by insertion of an extra copy of *pimE* gene in well-known macrolide producing strain *Streptomyces gilvosporeus.* Jang *et al*., (2011) reported 2.4-fold increase in natamycin production by overexpressing the regulatory *pimM* gene in *Streptomyces natalensis*. Recently cholesterol oxidase is studied to use in antitumor therapies. Both *in vivo* and *in vitro* irreversible apoptosis of lungs cancer cells was seen by Liu *et al.,* (2014) when treated with *Bordetella spp* cholesterol oxidase. Extracellular signal-regulated kinase 1/2 and protein kinase B phosphorylation inhibition was detected upon treatment with cholesterol oxidase. Furthermore, reactive oxygen species (ROS) generation, down regulation of B-cell lymphoma and production of hydrogen peroxide leading to release of cytochrome C and activated caspase-3 was studied with cholesterol oxidase treatment. They concluded that cholesterol oxidase causes the irreversible apoptosis by generating the ROS or reducing the cholesterol concentration.

All microbial cholesterol oxidases are not same as they are slightly different in their structures, as a result they follow slightly different scheme for production of product. In most of the flavo enzymes repeating glycine residues are present as a consensus sequence followed by presence of glutamic acid or aspartic acid indicating the presence of a nucleotide-binding fold (Leferink *et al*, 2009). Class I cholesterol oxidase have nucleotide-

binding fold due to the presence of glutamate along with almost identical consensus sequence of glycine residues while in the case of class II, they lack the nucleotide binding fold due to the absence of consensus sequence. Both the classes of cholesterol oxidase have one substrate binding domain along with one FAD-binding domain and hydrophobic pockets considered as a steroid ring backbone binding site.

The process of genetic engineering is employed to better understand the catalytic activity of cholesterol oxidase from several microorganisms. The industrial applications of cholesterol oxidase have been hindered due to poor understanding about substrate selectivity, enzyme activity and thermal instability. Using molecular stimulations combined with mutagenesis and structural comparison of wild type and mutants can be used to achieve *Pimelobacter simplex* cholesterol oxidase (PsChO) activity and substrate specificity (Qin *et al.,* 2017).

To develop thermostable cholesterol oxidases, several attempts have been made by using methods of site directed and random mutagenesis (Ghasemian *et al.* 2008; Sun *et al.,* 2011). Multiple target properties of cholesterol oxidase have been improved by using a variety of directed evolution approaches. Therefore, these engineered cholesterol oxidases may be attractive for several biotechnological applications.

High cost is one of the problems in production of enzyme-based drugs, to overcome this issue the best method is to increase the yield of enzymes. Enzyme production could be enhanced by constructing an efficient vector to overexpress the desired segment of DNA Cholesterol oxidase gene in several bacterial isolates has been identified, cloned, purified and characterized (Pollegioni *et al*., 2009). Cloning and expression method were used by Fujishiro *et al*., (2002) to isolate the cholesterol oxidase encoding gene from *Brevibacterium sterolicum. Bam* HI-digested expression plasmid pUC19 was used for cloning of cholesterol oxidase gene and transformed into *E. coli* MM294. Recombinant protein was purified by using DEAE-Cellufine column with a molecular mass of 47.5 kilodalton (kDa). While the optimum temperature and pH was 55°C and 6.5 respectively. Hejazi *et al.,* (2007) cloned the *Streptomyces luridus* cholesterol oxidase encoding gene into pTZ57R/T vector. They sequenced and reported the oxidoreductase domain of cholesterol oxidase with 272 amino acids encoded by 818 nucleotides. From *Chryseobacterium gleum* DSM cholesterol oxidase producing gene *choA* was cloned into pQE-30 vector and expressed in *Escherichia coli* JM109. Due to 8% rare codon in *choA*

gene, an additional transformation of pRARE2 plasmid was done with expression *E. coli* JM109. Recombinant His-tagged cholesterol oxidase was purified by size exclusion chromatography, exhibiting an optimum pH at 6.75 and optimum temperature of 35°C (Reiss *et al.,* 2014). Three different *Escherichia coli* host strains *BL21(DE3)*, *Rosetta-gami2(DE3)* and *BL21(DE3) pLysS* were used by Fazaeli *et al.,* (2018) for transformation of pET24b(+) expression plasmid containing *Streptomyces sp*. SA-COO *ChoA*. To prevent the misfolded protein accumulation in inclusion bodies due to high transcription rate, pLyS plasmid was used to continuously produce phage T7 lysozyme. They also studied the effect of culture media, concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG), incubation temperature, pre- and post-induction time on production of recombinant cholesterol oxidase. Under optimized conditions maximum recombinant cholesterol oxidase with N-terminal His tag was produced and purified by using nickel affinity chromatography. Doukyu and Aono, (2001) used the genomic DNA of previously isolated *Burkholderia cepacia* ST-200 as a cholesterol oxidizing and cyclohexane tolerant strain. They demonstrated that cholesterol oxidase from ST-200 is highly stable or resistant to organic solvents. Cholesterol oxidase gene from ST-200 strain was cloned in pHSG398 vector plasmid, sequenced, transformed and expressed in *E.coli*, while same group Doukyu *et al.,* (2008) isolated the cholesterol oxidase producing *Chromobacterium sp*. DS1 from soil. Furthermore, they purify and characterize the cholesterol oxidase and reported the stability of enzyme at high temperature and in presence of organic solvents and detergents. Fazaeli *et al.,* (2019) cloned the cholesterol oxidase gene from *Chromobacterium sp.* DS1 and enhanced production of recombinant enzyme was achieved under optimized conditions. Study on Arthrobacter simplex cholesterol oxidase encoding gene *(choAA)* was carried out by Chen *et al.,* (2006). Gene of 1653 base pairs was cloned in pQE31 and pPICZA vector, transformed in *E. coli* and *Pichia pastoris* respectively in order to express 502 amino acid products. They observed that signal sequence deletion enhances the cholesterol oxidase expression in *P. pastoris*. Cholesterol oxidase gene *(choR)* from *Rhodococcus sp.* PTCC 1633 previously isolated by Yazdi *et al.,* (2001) was cloned into pET23a and expressed in *Escherichia coli BL21(DE3) pLysS* (Ghasemian *et al.,* 2009). One-step purification method was used for recombinant protein purification. They found that recombinant protein has isoelectric point of about 9.0 and absorption spectrum maxima at 280, 380, and 460 nm, indicating that cholesterol oxidase is a flavoprotein.

Cholesterol oxidase gene *choD* from *Streptomyces lavendulae* YAKB-15 was identified by Yamada *et al.,* (2019) using a draft genome sequence and showed 82% identity with *Streptomyces sp.* SA-COO cholesterol oxidase gene. In order to find out the basic kinetic parameters they cloned the synthetic gene codon in a modified pBAD vector then expressed in *E. coli* and purify the protein by affinity chromatography. Furthermore, enhanced production was achieved in *Streptomyces albus* J1074 by using pSET-152-based expression system compare to wild YAKB-15 strain. Beside the increased efficiency of expression, *Streptomyces albus* allowed the production of cholesterol oxidase in a medium without containing yeast cells which is essentially required by *Streptomyces lavendulae* YAKB-15.

The activity of purified cholesterol oxidase from *Streptomyces aegyptia* was evaluated against five different human cancer cell lines including breast cancer, cervical epithelioid carcinoma, rhabdomyosarcoma, hepatocellular carcinoma, and colon carcinoma by El-Naggar *et al.,* (2018). Selective index of the cancer cell lines treated with cholesterol oxidase range from 1.262 to 3.26 which is significantly higher than doxorubicin. They also checked the anticancer activity of cholesterol oxidase in mice model having solid tumor. Mice were injected with cholesterol oxidase and doxorubicin alone and in combination which resulted the inhibition of tumor growth by 60.9%, 72.9% and 97% compared to the control model. 4-cholesten-3-one, an oxidized product of cholesterol catalyzed by cholesterol oxidase, reported to reduce the viability of breast cancer cell lines such as MDA-MB-231 and MCF-7 cells by increasing the expression of ATP-binding cassette sub-family A member 1 (ABCA1) and ATP-binding cassette sub-family G member 1 (ABCG1) and lowering the regulation of fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), acetyl-CoA carboxylase 1 (ACC1) and 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) (Elia *et al*., 2019). Another study on cytotoxicity of cholesterol oxidase purified from *Streptomyces sp.* against nasopharyngeal, breasts and ovarian cancer cell lines was carried out by Kavitha and Savithri, (2020) using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. They observed that cholesterol oxidase has significant anticancer activity against all the three cell lines at very low concentration.

MATERIAL AND METHODS

3.1 MEDIA AND SUBSTRATE

Cholesterol was purchased from Amresco and 7-ketocholesterol was acquired from Sigma-Aldrich. M9 medium was used in all degradation experiments**.** Luria Bertani (LB) was used in all the expression experiments. Media and chemical compounds were purchased from DIFCO laboratories (Michigan, USA). Agarose based sulfolink coupling resin from thermo fisher and DEAE Sepharose Fast Flow from sigma was used for protein purification.

3.2 ISOLATION AND IDENTIFICATION OF CHOLESTEROL DEGRADING BACTERIA

3.2.1 Environmental sampling

Several soil samples were collected with the help of sterile spatula in sterile falcons from Sadiqabad, Kuri road graveyard, Rawalpindi, Pakistan; Fimkassar oil field, Chakwal, Pakistan and from model town ghee mills Islamabad, Pakistan. Water sample from the effluent of the ghee industry was also collected. All the samples were stored overnight in refrigerator at 4℃.

3.2.2 Isolation of cholesterol degrading bacteria

Each soil (1g) and water sample (1ml) was added in 10 ml of sterilized distilled water and the suspension was used as an inoculum. M9 media (5 ml) supplemented with cholesterol (1mg/ml) as a sole carbon and energy source was prepared and inoculated separately by the stock suspension of the soil and water. All the inoculated tubes were incubated at 30℃ and 250 rpm for 24 hours.

All positive M9 broth samples (100 µl) were inoculated and spread on M9 agar plates containing cholesterol as the only carbon source and incubated at 37℃ for 4-7 days. Every distinct colony was sub-cultured on nutrient agar plates. Sub culturing was repeated multiple times till to obtain the pure colonies. To further confirm the cholesterol utilization each purified colony was inoculated in M9 media with cholesterol as a source of energy and incubated under shaking conditions at 250 rpm for 30 hours. All the samples were run in triplicate. Samples were collected at interval of 3 hours and analyzed for bacterial growth using spectrophotometer. Two pure colonies with maximum potential to grow in the presence of cholesterol were further selected for optimization studies.

3.2.3 Taxonomic characterization of bacterial isolates

3.2.3.1 Biochemical characterization

3.2.3.1.1 Gram staining

Gram staining was performed according to standard protocol to identify the gram positive and gram-negative organisms.

3.2.3.1.2 IMVIC Tests

Indole test

Tryptophan broth was prepared according to standard procedure and distributed in the test tubes (5 ml in each). All test tubes were inoculated with overnight bacterial isolates and incubated for 24 hours at 37°C. After incubation 0.5 ml of Kovac's reagent was added in all the inoculated tubes to observe the ring formation.

Table 3.1 Composition of tryptophan broth.

Content	Quantity (g/L)
Casein enzymic hydrolysate	10.0
Sodium chloride	5.0
DL-tryptophan	1.0
pH	7.5

Methyl red test

Methyl Red Vogus Proscur (MRVP) broth was equally distributed in test tubes and all test tubes were inoculated with bacterial strains and incubated for 24 hours. After incubation five drops of methyl red was added in all test tubes and observed for immediate development of red color.

Table 3.2 Composition of MRVP broth.

Content	Quantity (g/L)
Buffered peptone	7.0
Glucose	5.0
Dipotassium phosphate	5.0

Voges Proskauer test

MRVP broth was prepared. Broth was distributed in the test tubes and incubated at 37°C for 24 hours. After incubation 5% (six drops) of alpha-naphthol (Barritt′s reagent A) in absolute ethanol was added and mixed well. Then two drops of 40% potassium hydroxide (Barritt′s reagent B) was added in all the tubes and observed for the development of pink-red color on surface within 30 minutes.

Simon citrate test

Simon citrate media was inoculated in test tubes and solidified in slanted position. All the slants were streaked with bacterial isolates and incubated for 24 hours. The color change was observed after incubation**.**

3.2.3.1.3 Triple Sugar Iron (TSI)

The slants TSI media were inoculated by first stabbing through center of the medium to the bottom of tubes and then streaked on the slant surface and incubated at 37°C for 24 hours. The color change was observed after incubation.

3.2.3.1.4 Catalase test

To confirm the production of catalase production a drop of 3% hydrogen peroxide was placed on slide then with the help of sterilize loop the overnight bacterial colony was picked and mixed in hydrogen peroxide to observe the bubble formation.

3.2.3.2 Molecular characterization

For identification of bacterial isolates 16S rRNA sequencing analyses were performed. Fresh culture on LB plates of six bacterial isolates (GS2, GS3, GS6, GS7, GSS, GS9) from graveyard soil, one isolate (W1) from oil contaminated soil, three isolates (UP, LSB, DS)

from ghee contaminated soil and one isolate (WS) from water contaminated with ghee industry effluent were subjected to DNA isolation using standard protocols and sent to GENVIZ- solid science superior service, for 16S rRNA gene sequencing.

3.2.3.3 Phylogenetic analyses

To study the closely related specie for identification of bacterial isolates, the results of sequencing were subjected to Blast search at nucleotide collection database, National Centre for Biotechnology Information (NCBI). Fast alignment sequence tool (FASTA) files were generated from sequence files, interpreted in sequin software and sequence data was submitted to NCBI gene bank to obtain the accession numbers.

3.3 BIODEGRADATION STUDIES

All the bacterial strains were tested for their abilities to degrade cholesterol in shake flask experiments and then degradation conditions were optimized for maximum degradation for selected bacterial isolates.

3.3.1 Degradation parameters optimization

Certain degradation parameters were optimized for two selected strain W1 and W8. All the optimization experiments were carried out in M9 media (10 ml), inoculated with 1% (v/v) overnight culture and incubated under shaking conditions at 30°C and 250 rpm. Several parameters affecting the cholesterol degradation were studied such as incubation temperature (°C), incubation time (days), effect of metal ions, pH and substrate concentration.

3.3.2 Analysis of cholesterol

To calculate the cholesterol utilization in all the optimization experiments, enzymatic colorimetric cholesterol oxidase peroxidase method was used by utilizing cholesterol estimation kit (Merck) (Kulkarni *et al.*, 2013). Reaction mixture was prepared according to the manufacturer's instructions and 10 µL of cell free supernatant (CFS) was added, mixed well by inversion, and then incubated for 10 minutes at 30°C. The absorbance of standard and test was checked against the blank at ƛ 505 nm, and concentration of cholesterol was determined by using the following formula.

$$cholesterol\ [mg/dl] = Absorbance\ of\ test/Absorbance\ \ of\ \ standard\ \ X\ \ Conc.\ \ of\ \ standard\ [mg/dl].$$

3.3.3 Degradation of cholesterol and 7-ketocholesterol by bacterial isolates

Isolated pure strains were grown in M9 broth (10 ml) containing cholesterol and 7-ketocholesterol (1mg/ml) as a single carbon and energy source and incubated under optimized conditions. Samples were collected at the end of incubation and analyzed for degradation.

3.3.3.1 Degradation analysis

From transformation experiments, culture broth was transferred to sterile Eppendorf and centrifuged at 10,000 rpm for 10 minutes to separate the cells. Cell free supernatant was extracted with a mixture of hexane/isopropanol (3:2). Solvent was mixed with the sample (equal ratio) by vigorous vertexing, the two phases were separated and the organic phase containing the degradation products were collected and dried, samples were dissolved in hexane and analysed by HPLC.

3.3.3.2 HPLC detection method

To evaluate the cholesterol degradation, extracted samples were dissolved in hexane and filtered, and 20 µl was analyzed by reverse phase HPLC using Waters HPLC system with UV detector and C18 column, at ƛ 210 nm and hexane/acetonitrile (60:30) was used as mobile phase with a flow rate of 0.72 ml/min, while 10 µl of 7-ketocholesterol samples were analyzed on same HPLC system at ƛ 233 nm with a flow rate of 0.75 ml/min.

3.3.3.3 GCMS detection method

To further analyze the degradation products of cholesterol, samples were analyzed on Leco TruTOF GC-MS with an EI source and an Agilent 7890 GC with a 7693 autosampler and a split/splitless injector. The GC was equipped with a 30 m RTX-5 capillary column with 10 m guard column, 0.25 mm ID, and 0.5 µm film thickness (Restek). Helium was used as a carrier gas with an inlet pressure of 79 PSI and a flow rate of 1 ml/min. Samples were analyzed by injection of 1 µl of sample in split mode with a split ratio of 10:1, a septum purge of 3 ml/min, and an inlet temperature of 300°C. The initial column temperature of 100°C was held for 2 minutes then raised to 330°C at a rate of 20°C/min and held for 5 minutes. The transfer line temperature was held at 280°C and the source temperature was 250°C. The electron energy was -70 eV and the mass spectra were recorded 35-600 m/Z at a scan rate of 20 spectra/s. The structure of metabolites was determined by using Chromatof version 4.72 and NIST library.

3.4 SCREENING OF ISOLATES FOR CHOLESTEROL OXIDASE

All the purified isolates were screened for qualitative and quantitative assays of cholesterol oxidase according to Allain *et al*., (1974).

3.4.1 Qualitative assay

Colony staining method was done on the grown colonies of all identified strains along with *E. coli* as a control, to confirm the Cholesterol oxidase (ChO) production. The filter papers were dipped into the solution containing 1.7% 4-aminoantipyrin, 6% phenol, 0.5% cholesterol and 3000 U/L horseradish peroxidase (HRP) in 100 mM potassium phosphate buffer (pH 7.0). After that, soaked discs were placed on fresh bacterial colonies and incubated at 37°C overnight. ChO activity of tested colonies was confirmed by development of red color on the plate where the discs were placed (Bholay *et al*., 2013).

3.4.2 Quantitative assay

Quantitative cholesterol oxidase assay for all the identified organism was run in triplicate in 96 well microtiter plate. Activity was checked in different media including M9, LB and 2xLB supplemented with cholesterol (1 g/L) after 24 and 48 hours of incubation at 37°C. The working concentration of the assay was 87 mM Potassium phosphate buffer pH (7.0) 0.89 mM cholesterol, 1.4 mM 4-aminoantipyrine, 0.34% tween 80, 21 mM phenol, and 5U/ml horse radish peroxidase (Fazaeli *et al*., 2019). Assay mixture was shaken for 30 seconds, incubated at 37°C and developed red color was assessed by plate reader at 500 nm. The activity was calculated by using the following formula

$$U/ml = \frac{\Delta OD/min \ \ (\Delta ODtest - \Delta ODblank) \times Vt \times df}{13.78 \times ½ \times Vs}$$

Vt = Total volume

Vs = Sample volume

df = Dilution factor

½ = Half mole of quinoneimine dye produces one mole of H_2O_2

13.78 = Millimolar extinction coefficient of quinoneimine dye

3.4.3 Purification of cholesterol oxidase

On basis of maximum production of cholesterol oxidase, ammonium sulphate precipitation was carried out for *Psychrobacter sp.* GS3, *B. subtilis* LSB (40, 60 and 80%) and *B. safensis* W1 (70, 80 and 90%). 250 ml conical flask containing 100 ml of 2xLB supplemented with cholesterol (1 g/L) and inoculated with 1 ml of 24 hours fresh culture and incubated for four days at 35℃, 250 rpm. The culture was centrifuged at 10000 x g for 30 minutes to remove the cells. The cell free supernatant was passed through filter of 0.2µm. Calculated amount of ammonium sulphate salt was added separately to the respective flask containing the cell free supernatant under stirring condition placed on magnetic plate. In order to separate the precipitates, the broth was centrifuged at 10000 x g for 30 minutes at 4℃. Supernatant was discarded and the pallet was dissolved in 25 mM Tris (pH 8.0) buffer. The dissolved precipitates were injected in dialysis cassette (0.5-3 ml) with pore size (10K), placed each in a separate beaker containing 25 mM Tri (pH 8.0) buffer and left in that buffer for four hours followed by buffer change and left overnight for dialysis. All the precipitation experiments were carried out at 4℃.

3.4.3.1 Enzyme activity

Cholesterol oxidase activity of all the precipitated proteins was checked according to the procedure described previously.

3.4.3.2 Protein estimation

Total protein content was calculated by the method described by Bradford (1974) using the bovine serum albumin as a standard. Each standard (150 µl) and unknown sample (150 µl) was pipetted in a clean, clear microplate wells. 300 µl of MQ water was used as a blank and 1x Bradford dye (150 µl) was added in all the wells containing samples and BSA standard dilutions. All the samples were run in triplicate and the plate was incubated at room temperature for 5 minutes and absorbance was recorded at 595 nm/450 nm, 25℃ for five minutes by using plate reader.

3.4.3.3 Sample preparation for gel electrophoresis

The samples were prepared before loading on the gel. 20 µl of each sample was mixed with 20 µl of 1x SDS loading dye, mixed well and boiled the sample on heat block for 5 minutes at 100℃.

3.4.3.4 SDS-PAGE analysis

The precipitated dialyzed proteins collected at different ammonium sulphate percentage were run on 12% SDS-PAGE. 4 µl of standard (unstained protein Marker, thermo scientific) and 10 µl of sample was loaded in the respective wells. Gel was run in 1x SDS running buffer at 120 V for 15 minutes, then increased the voltage to 180 and run till to all the dye remove through gel followed by staining with 0.1% (w/v) coomassie blue (sigma) solution for 20 minutes. To carry out the destaining, gel was kept overnight in DI water then scanned by using scanner.

3.4.4 Large scale production and purification of cholesterol oxidase from *B. safensis* W1and *B. subtilis* LSB

3.4.4.1 Production of cholesterol oxidase

24-hour fresh culture of the *B. safensis* W1 and *B. subtilis* LSB were inoculated in 2xLB (1L) each 500 ml in 1L flask with 1% overnight inoculum, supplemented with cholesterol (1 g/L) and incubated at 35℃, 250 rpm for 48 hours. After incubation, the cells were removed centrifugation at 10000 x g for 30 minutes.

3.4.4.2 Precipitation of protein

The cell free supernatant was passed through 0.2µm filter and precipitated with the optimized ammonium sulphate concentration that was 40% for LSB and 80% for W1. The precipitates were left overnight at 4℃ and centrifuged at 10000 x g for 30 minutes. Precipitates were dissolved in 3ml of 25mM Tri (pH 8.0) buffer and dialyzed overnight at 4℃ against the same buffer.

3.4.4.3 Chromatographic purification

For purification of precipitated protein diethylaminoethyl (DEAE) sepharose anion exchange chromatography (AKTA Pure 25) was used. 10 ml precipitates of W1 and LSB was syringe filtered (0.2µ) and loaded on 5 ml DEAE Sepharose column. Three range of UV was set UV1 at 280 nm UV2 at 254 nm and UV3 at 410 nm. Two buffers A1: 25 mM Tris HCl, pH8.0 and buffer B1: 25 mM Tris HCl + 500 mM NaCl, pH 8.0 were used in gradient mode. Initially column was equilibrated with 10 cV (column Volume) A1 buffer (25 mM Tris HCl, pH 8.0) followed by injection of 10 ml sample. Column was washed with 5 cV of A1 buffer then elusion was carried out by using 20 cV of 0 % B1 to 100% B1

in gradient mode. Total of 41 fractions of each loaded sample was collected. The fractions with peaks were analyzed for cholesterol oxidase activity and total protein content and further verified on 12% protein gel.

3.5 CLONING AND EXPRESSION OF SYNTHETIC CHOLESTEROL OXIDASE GENE IN *ESCHERICHIA COLI*

Cholesterol oxidase gene *ChO* (accession no. MH892608) from *Chromobacterium sp*. was synthesized by Integrated DNA Technology (IDT) and cloned into pET21 vector with 54% GC content.

3.5.1 Cloning of *ChO* in pET21 vector

Synthetic construct of cholesterol oxidase was PCR amplified by using the primers pET-NpuC-*ChO*.F.GA

(5' ATGGCTTCATAGCTCATAATACCTGCAGTCAGCCGAACAAC 3') and pETNpuC-*ChO*.R.GA

(5' TTGTTAGCAGCCGGATCTCAGAGACCGAGAGAATCAAGCAGC 3'). PCR reaction mixture total volume (50 µl) contained 22.5 µl of distilled water, 10 µl of 5 x Q5 reaction buffer, 10 µl of 5 x Q5 high GC, 2.5 µl of forward primer (10 µM), 2.5 µl of reverse primer (10 µM), 1 µl dNTP's (10 mM), 1 µl of template synthetic DNA and 0.5 µl of Q5 polymerase [New England Biolabs (NEB)].

In PCR conditions total of 25 cycles were used with initial denaturation at 98°C for 30 seconds followed by primer annealing at 58°C for 30 seconds and ended with polymerization at 72°C for 30 seconds. On 1% agarose gel PCR amplified product was run along with 1kb plus DNA ladder at 100 V for 30 minutes. The band (2381 bp) was excised from gel under UV illuminator with sterile sharp cutter. For digestion of band 1ml of QC buffer was added in sterile Eppendorf containing gel band and incubated for 10 minutes at 50°C. After incubation mixture was vortexed for proper mixing and purified by miniprep silica spin column (Takara Bio USA, Inc.).

3.5.1.1 PCR amplification of pET vector

pET21 vector was PCR amplified by using the primers pET-NpuC.F (5' TGAGATCCGGCTGCTAACAAAG 3') and pET-NpuC.R (5' ATTATGAGCTATGAAGCCATTTTTGAG 3'). PCR reaction mixture total volume (50

µl) contained 22.5 µl of distilled water (DI), 10 µl of 5 x Q5 reaction buffer, 10 µl of 5 x Q5 high GC, 2.5 µl of forward primer (10 µM), 2.5 µl of reverse primer (10 µM), 1 µl dNTP's (10mM), 1 µl of plasmid DNA and 0.5 µl of Q5 polymerase.

In PCR conditions total of 25 cycles were used with initial denaturation at 98℃ for 30 seconds followed by primer annealing at 58℃ for 20 seconds and ended with polymerization at 72℃ for 4 minutes and 30 seconds. On 1% agarose gel PCR amplified product was run along with 1kb plus DNA ladder at 100 V for 30 minutes. PCR amplified product was purified by miniprep silica spin column (Takara Bio USA, Inc.).

PCR amplified and purified products of insert and vector each with a volume of 17µl was mixed separately with 0.5µl of sterile water, 0.5 µl of Dpn1 and 2 µl of cut smart buffer followed by incubation at 37℃ for 45 minutes to allow the digestion of parent vector. After Dpn1 digestion 3.4 µl of insert and 1.6 µl of vector was mixed gently with 5 µl of DNA assembly master mix to clone the *ChO* construct into pET backbone by a single thermal reaction known as Gibson assembly reaction (10 µl). The reaction mixture has T5 exonuclease, which generate the sticky ends and expose the complementary sequence for annealing followed by 3' extension and gaps filling on the annealed region by the action of fusion polymerase. Finally, ligase seals the remaining gaps and links the DNA fragments together. To confirm the ligation 5 µl from Gibson assembly rection was mixed with engineered DH5α competent cells (50 µl) and spread on the ampicillin (100 µg/ml) resistant LB plate under sterile conditions than plate was incubated overnight at 37℃. Six colonies were selected from ampicillin plate and inoculated into 4 ml of LB ampicillin resistant broth (100 µg/ml) and incubated overnight at 37℃. plasmid DNA was purified from all the six samples by miniprep silica spin column. pET-NpuC-*ChO* construct was verified by analytical digestion with Sca1 and Nhe1 followed by 1% agarose gel run. Further pET-NpuC-*ChO* was verified by sequencing (Genwiz).

3.5.2 *ChO* expression and purification

pET-NpuC-*ChO* construct was transformed into different expression cells including *SHT7(E. coli* K12 cells suitable for T7 protein expression), *BLR, BL21* and *Origami* (Novagen). All the expression cells were grown in Luria-Bertani with double concentration (2xLB) supplemented with ampicillin (100 µg/ml) and incubated at 37℃ except of *SHT7* incubated at 30℃, 250 rpm till to growth OD600 reached to 0.6-0.8. At that point 100 µl of 1M isopropyl-β-D-thiogalactopyranoside (IPTG) was added and incubated overnight at

20℃, 250 rpm. After incubation cells were harvested by centrifugation (6000 x g) for 20 minutes. Harvested cells were suspended in 1x phosphate buffer saline (PBS) at a ratio of 1 ml of buffer for 10 ml pallet (1:10) and lysed using ultrasonic homogenizer (Q500-sonicator). Cell pallet collected from 1L expression medium was sonicated in four rounds with 15 cycles each, and whole sonication process was carried out on ice. Centrifuge (16000 x g) was used to separate the cell debris and supernatant was collected. The expression of *ChO* was checked in whole and clear lysate by running the samples on 12% SDS-PAGE.

3.5.2.1 Optimization, expression and solubility of recombinant protein

Different parameters including post induction temperature (20, 30, 37℃), expression media such as Super Broth (SB) and Terrific Broth (TB) were used for cells expression. Additionally, post incubation temperature (20, 30, 37℃), post induction incubation time (4,8,16 hrs), and different concentration of IPTG (0.1 mM, 0.25 mM, 0.5 mM, 1 mM) were checked for expression and solubility of *BLR* and *Origami* expressed protein. All the optimization experiments were performed in 100 ml expression medium supplemented with 1% overnight inoculum.

3.5.2.1.1 Gel electrophoresis

In all expression experiments the protein expression and solubility were monitored using 12% SDS-PAGE and run at 120 V for 15 minutes followed by 180 v for next 1 hour and 30 minutes. Protein gel was stained with Coomassie stain (sigma) solution (0.1% w/v) and destained in distilled water under shaking conditions. For determination of molecular masses, the prestained protein ladder (10-170 kDa) (Thermo Scientific) was used as a protein standard.

3.5.2.2 Purification of recombinant protein

The *BLR* and *Origami* expressed recombinant cholesterol oxidase with an N-terminal self-cleaving split intein tag was purified at room temperature (25℃) and at 37℃ by passing through Npu trapped gravity column (10 ml), that provides the purification of tagged protein from homogenized cell lysate. Washing was done with column buffer (AMPD/PIPES, 20 mM AMPD+20 mM PIPES+200 mM Nacl) (pH 8.5) (3 ml), then with cleavage buffer (AMPD/PIPES 20 mM AMPD+20 mM PIPES+200 mM Nacl (pH 6.2) (3ml). After washing multiple samples of resin mixed with cleavage buffer was collected

at interval of 0, 1, 3 hours and overnight. Finally, elusion of tag less protein was carried out with a simple shift in buffer pH from 8.5 to 6.2 (200 µl).

All the samples collected during purification was analyzed on SDS-PAGE according to the method described previously. Protein concentration was determined by method of Bradford assay and cholesterol oxidase activity was checked according to previously used method.

3.5.3 Modification of protein for fast cleavage

To get the fast cleavage of protein, first amino acid of cholesterol oxidase was changed from threonine to phenylalanine. Plasmid DNA was PCR amplified by using the primers *ChO*. F (5' TTCTGCAGTCAGCCGAACAACTT 3') to amplify the N-terminal of insert and pET-NpuC-*ChO*. R (5' ATTATGAGCTATGAAGCCATTTTTGAG 3').

PCR reaction mixture total volume (50 µl) contained 22.5 µl of deionized (DI) water, 10 µl of 5 x Q5 reaction buffer, 10 µl of 5 x Q5 high GC enhancer, 2.5 µl of forward primer (10 µM), 2.5 µl of reverse primer (10 µM), 1 µl dNTP's (10 mM), 1 µl of plasmid DNA and 0.5 µl of Q5 polymerase.

In PCR conditions total of 25 cycles were used with initial denaturation at 98℃ for 10 seconds followed by primer annealing at 58℃ for 20 seconds and ended with polymerization at 72℃ for 6 minutes. DNA agarose gel was run followed by miniprep purification and Dpn1 digestion according to methods described previously.

In order to phosphorylate the DNA before ligation, 20 µl reaction mixture was prepared containing 2 µl of template DNA, 15 µl of DI water, 2 µl of T4 DNA ligase buffer (10x) and 1 µl of T4 polynucleotide kinase, incubated at 37℃ for 45 minutes followed by ligation by addition of 0.5 µl of T4 DNA ligase and incubated at room temperature for one hour. Transformation of pET-NpuC-*ChO* (5 µl) was carried out in DH5α cells (50 µl) and grown on ampicillin resistant LB plate, incubated at 37℃ for 18 hours. Two colonies from LB plate was further inoculated separately into ampicillin (100 µg/ml) LB broth (4 ml) incubated overnight at 37℃. Plasmid DNA was miniprepped, run on agarose gel (1%) and further verified by Genwiz sequencing.

3.5.4 Expression and purification of modified recombinant cholesterol oxidase

Synthetic construct of *ChO* was expressed in *E. coli* BLR cells (Novagen) transformed with pET-NpuC-*ChO* (+F) and expressed under optimized conditions in terrific broth (TB),

induced by 0.1mM (100mM) IPTG and incubated overnight at 20℃. The cells were harvested, lysed and protein was purified according to protocol described previously. Enzyme activity of fast cleaved cholesterol oxidase was checked, and total protein content was determined by Bradford method using BSA as a standard.

3.6 CHARACTERIZATION OF RECOMBINANT CHOLESTEROL OXIDASE

The activity of Purified cholesterol oxidase was measured by using 0.1 mM potassium phosphate buffer under various pH (3-9) and incubated at 30°C for 30 minutes. To determine the optimum thermal activity of ChO the enzyme activity was assayed at various range of temperatures (20°C-100°C). The recombinant cholesterol oxidase activity was also checked in 0.1% of different detergents (Triton-x100, sodium dodecyl sulphate, Tween 80, sodium cholate) and in various solvents (methanol, ethanol, isopropanol, dimethyl sulfoxide (DMSO), ethyl acetate, acetone).

3.6.1 Prediction of recombinant cholesterol oxidase epitopes

To predict the immunogenicity of recombinant protein, B cells and T cells epitopes were analyzed by using Immune Epitope Database (IEDB) (http://tools.iedb.org/main/). Amino acid sequence of recombinant cholesterol oxidase was added to IEDB and graph was generated for prediction of immunogenic amino acids.

3.6.2 Cell toxicity assay of cholesterol oxidase

The purified cholesterol oxidase of *Bacillus safensis* W1*and Bacillus subtilis* LSB strains and recombinant cholesterol oxidase was assessed for its anticancer activity (*in vitro*) using sulforhodamine B(SRB) assay (Vichai and Kirtikara, 2006) against three human cancer cell lines namely, prostate cancer (DU-145), breast cancer (MCF-7) and colon carcinoma (HT-29). Lyophilized purified cholesterol oxidase was dissolved in sterile deionized water (26 U/ml) in order to prepare the stock solution. Using sterile DI water different dilutions were made from the stock with concentration of (13 U/ml, 6.5 U/ml, 3.25 U/ml, and 1.65 U/ml). Taxol was used as a positive control along with DMSO as a negative control. All the samples were run in triplicate.

Prior performing the assay the cells with 80% confluency grown in 75 ml tissue culture flasks containing minimal essential medium MEM (eagle) was removed, and all the cells were washed three times with sterilized phosphate buffer saline (PBS). To dissociate the cells 1ml of trypsin (0.08%) was added and incubated at 37℃ for 10 minutes followed by

cell dilution (1 ml) with 3ml of media and cell concentration was determined by counting in hemocytometer chamber under a microscope. To obtain an appropriate cell seeding density, $5x10^4$ cells/ml were used. Cell suspension (190 µl) was added into sterile 96-well clear flat bottom plate and incubated overnight followed by addition of each dilution of the sample (10 µl) in the respective well then incubated for 72 hours. Cell fixation was carried by addition of 100 µl of 20% (wt/vol) cold trichloro acetic acid (TCA) in each well and incubated at 4℃ for 30 minutes. Plates were washed gently under slow running tape water and let to air dry followed by addition of 100 µl of 0.4% (wt/vol) sulforhodamine B (SRB) solution and left at room temperature for 30 minutes. After incubation, the unbound dye was removed by washing the plates four times with 1% (vol/vol) acetic acid and dried at room temperature. After staining and drying 200 µl of 10 mM Tris base solution (pH 10.5) was added in each well to solubilize the bound dye in 30 minutes and OD at 510 nm was recorded in a microplate reader.

3.6.3 Determination of cholesterol degradation with purified enzyme by enzymatic assay

To check the catalytic activity of cholesterol oxidase purified from bacterial isolates and synthetic construct, assay mixture was prepared according to method described in a section 3.4.2. The generation of chromogenic compound was tracked at 500 nm on plate reader.

RESULTS

4.1 ISOLATION AND IDENTIFICATION OF CHOLESTEROL DEGRADING BACTERIA

Environmental samples (soil and water) collected from graveyard soil of Rawalpindi, ghee contaminated soil of Islamabad and one from oil contaminated soil of Chakwal, Pakistan, showed growth (turbidity) when inoculated in M9 media supplemented with cholesterol as single carbon and energy source (Figure 4.1). From M9 broth 100 µl of each sample was inoculated on M9 agar plate showed appearance of different colonies (Figure 4.2) and each distinct colony was further purified by enrichment culture technique (Figure 4.3). In total of twelve isolates were purified from different samples, were named as GS2, GS3, GS6, GS7, GSS, GS9 (isolated from Kuri road graveyard), LSB, UP, DS (isolated from model town ghee contaminated soil), WS (isolated from ghee contaminated water) and W1 (isolated from Fimkassar oil field). W8 was already isolated from oil contaminated soil and identified as *Bacillus pumilus*.

Purified isolates were further assessed for their growth in M9 broth with cholesterol as the only source of carbon, to check the utilization of the tested compound, broth turbidity was considered as positive results for the growth and was further confirmed by growth OD_{600} by using spectrophotometer (Figure 4.4). All the isolates showed growth but with variable degree. W8 and W1 showed maximum growth OD of approximately 1.25-1.2 after 30 hours of incubation followed by LSB, GS9 and GSS with OD of 0.866, 0.811, 0.8, respectively. Lowest growth values of 0.59 and 0.61 was recorded with WS and GS2.

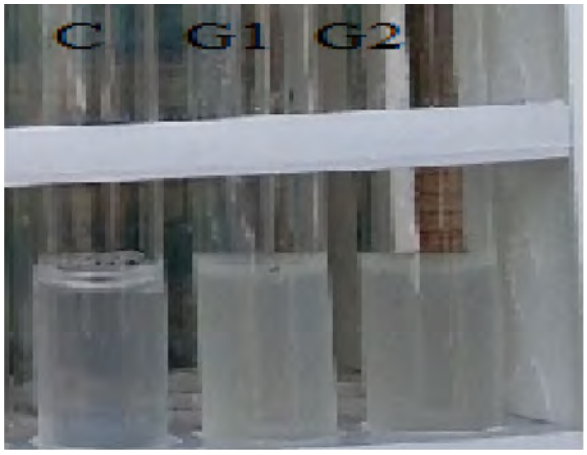


Figure 4.1 M9 media with cholesterol as single carbon and energy source showing growth.

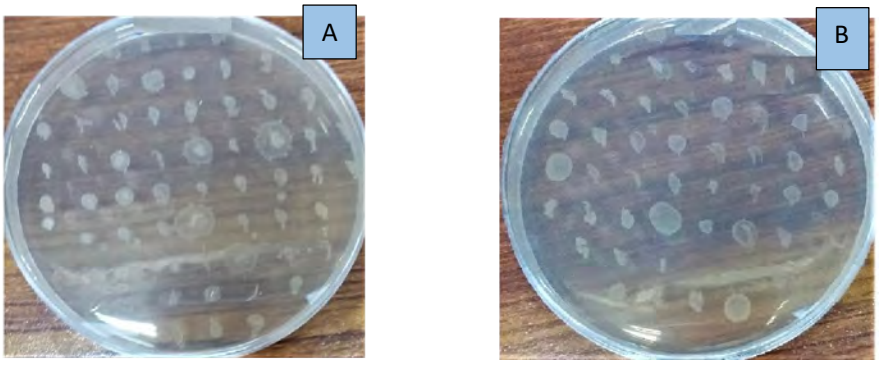


Figure 4.2 Bacterial growth on M9 agar plate supplemented with cholesterol as only carbon source, inoculated from M9 broth of Graveyard soil (A) and ghee contaminated soil (B).

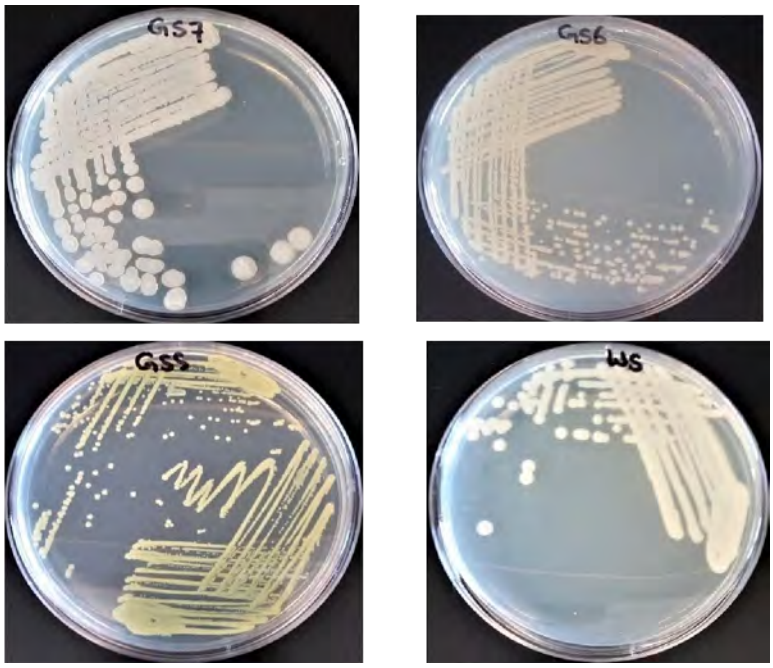


Figure 4.3 Morphological characteristics of some isolates on LB media purified by sub culturing technique.

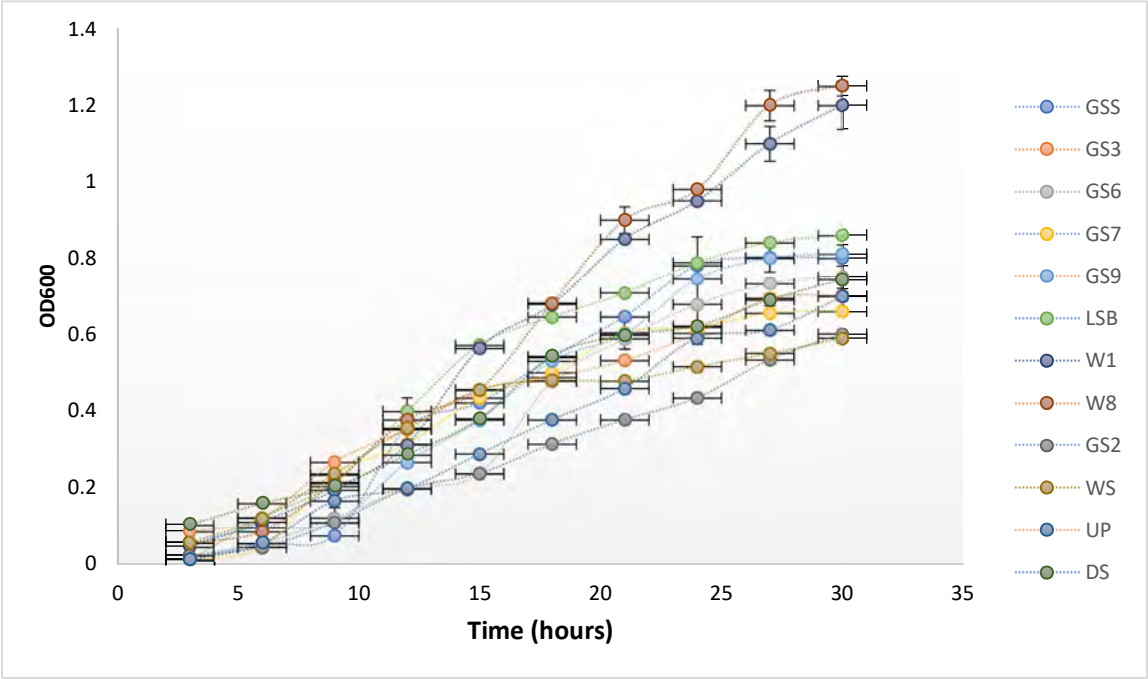


Figure 4.4 Growth of bacterial isolates in M9 media having cholesterol as sole source of carbon and energy.

4.1.1 Taxonomic characterization of cholesterol degrading bacteria

Taxonomic analysis of all the strains were carried out. Colony morphology of GS2, GS9, DS were off-white large colonies and creamy in nature while W8 and GS7 showed the same morphology but sticky in nature. The morphology of W1, GS3, GS6, LSB, appeared small to medium size colony with light to off-white color while GSS and UP appeared with different morphological characteristics of colored (green) and bright colonies. Bright, round, and sticky colonies of WS were observed (Table 4.1). All the isolates were found gram negative except of LSB, W1 and W8 which were gram positive. Furthermore, different biochemical characteristics were analyzed All the isolates were indole negative and only GS2, GS9 were methyl red positive and rest of the isolates were negative. Majority of the isolates were VP negative but with four positive isolates including W1, W8, LSB and WS. GS2 and GS9 failed to utilize the Simon citrate while others showed the citrate utilization. Furthermore, GS6 failed to ferment any of the sugar in TSI test while other isolates were positive for variable sugar fermentation. Most of the isolates showed positive results for catalase production while, UP and GS3 were negative for catalase (Table 4.2).

Further molecular identification was done on the basis of 16S rRNA and NCBI BLAST was used for analysis of sequencing and phylogenetic trees were constructed. Based on 16S rRNA sequences and NCBI blast percent similarity, the bacterial isolate W1 was identified as *Bacillus safensis*, GS2 as *Stenotrophomonas pavanii*, GS3 as *Psychrobacter sp*., GS6 as *Moritella sp.*, GS7 as *Vibrio sp*., GSS as *Shewanella peaeleana* and GS9 as *Moritella sp*., while LSB was identified as *Bacillus Subtilis*, UP as *Psychrobacter sp*., WS as *Klebsiella sp*. and DS was identified as *Vibrio sp*. (Table 4.3; Figures 4.5-4.15).

Table 4.1 Morphological characteristics of isolates.

Isolates	**Colony morphology**
W1	Off-white small size colonies with irregular margin
W8	off-white, sticky, medium to large size colony with irregular margin
GS2	Off-white, creamy, large colonies with small white center
GS3	Off-white, bright, small size round colonies
GS6	Small, round, light color colonies
GS7	Light color, large sticky colonies with wide center
GS9	Off-white, large irregular colonies
GSS	Green, small size, bright, round colonies
LSB	Off-white, round shape, small to medium size colonies
UP	Light green, bright colonies with opaque center
DS	Off-white, large, creamy, round colonies
WS	Bright, round, sticky colonies

Table 4.2 Biochemical characterization of isolates.

Isolates	Gram Staining	Indole test	Methyl red test	Vogues Proskauer test	Simon citrate test	TSI test	Catalase test
W1	+	-	-	+	+	++	+
W8	+	-	-	+	+	+	+
GS2	-	-	+	-	-	+++	+
GS3	-	-	-	-	+	+	-
GS6	-	-	-	-	+	---	+
GS7	-	-	-	-	+	+++	+
GS9	-	-	+	-	-	+++	+
GSS	-	-	-	-	+	+++	+
LSB	+	-	-	+	+	+	+
UP	-	-	-	-	+	+	-
WS	-	-	-	+	+	+++	+
DS	-	-	-	-	+	+++	+

Table 4.3 Molecular identification of the isolates.

Code	Accession no	Strains name
W1	MW785042	*Bacillus safensis*
GS2	MN443620	*Stenotrophomonas pavanii*
GS3	MN421797	*Psychrobacter* sp.
GS6	MN421796	*Moretella* sp.
GS7	MN421799	*Vibrio* sp.
GSS	MN443612	*Shewanella pealeana*
GS9	MN421800	*Moretella* sp.
LSB	MN032374	*Bacillus subtilis*
UP	MN420817	*Psychrobacter* sp.
WS	MN420814	*Klebsiella* sp.
DS	MN396664	*Vibrio* sp.

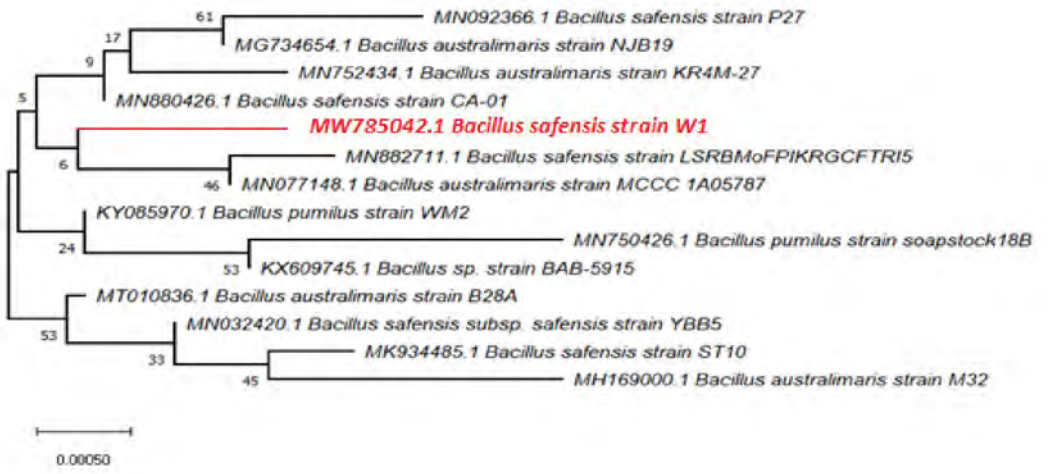


Figure 4.5 Phylogenetic tree of *Bacillus safensis* W1, isolated from oil contaminated soil.

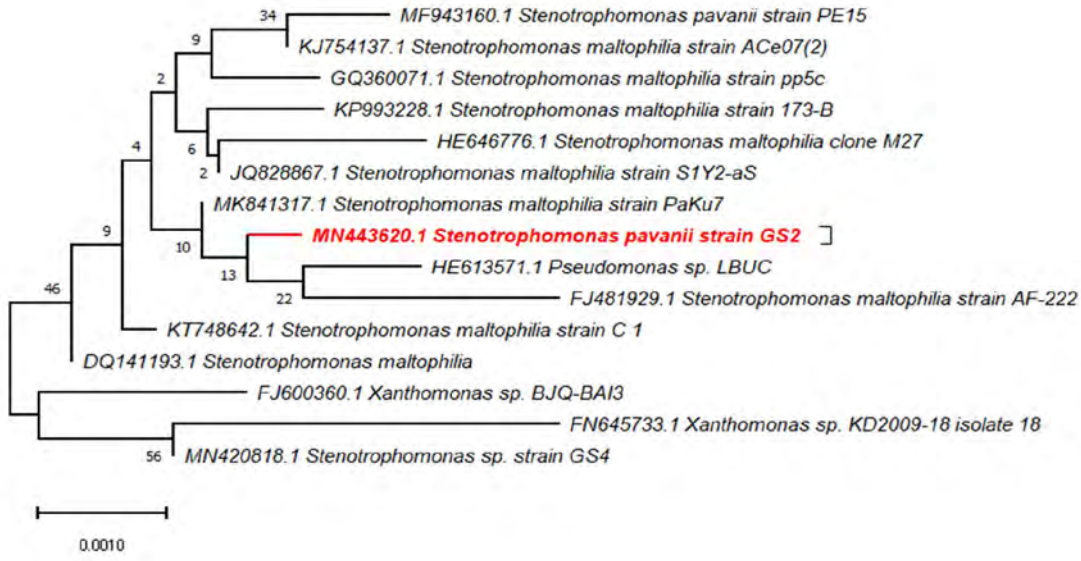


Figure 4.6 Phylogenetic tree of *Stenotrophomonas pavanii GS2* (accession number MN443620), isolated from graveyard soil.

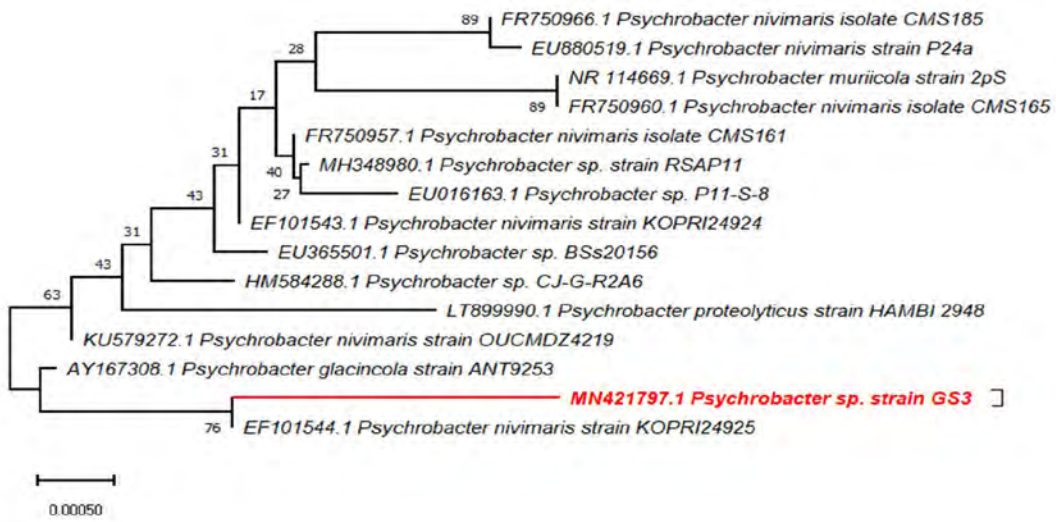


Figure 4.7 Phylogenetic tree of *Psychrobacter sp.*GS3 (accession number MN421797), isolated from oil graveyard soil.

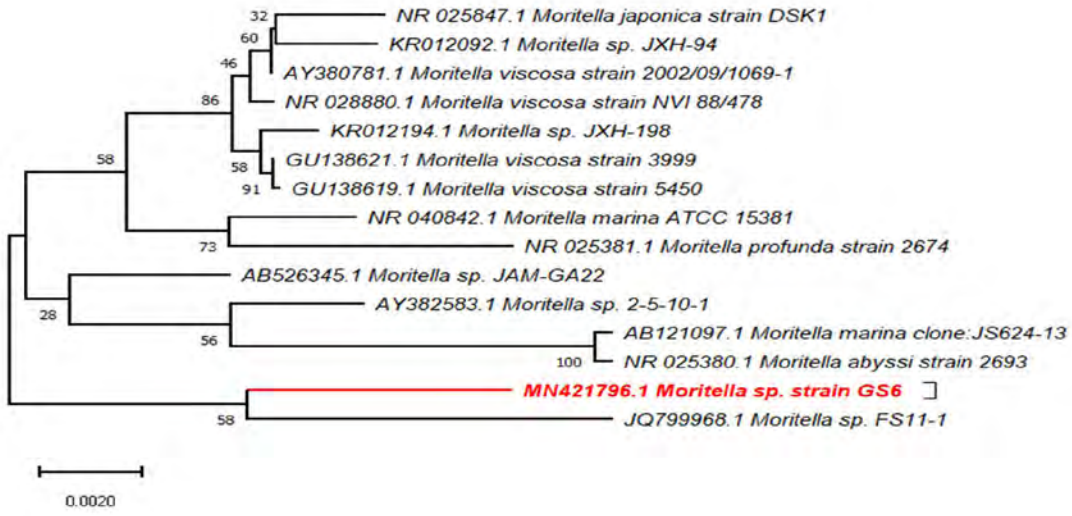


Figure 4.8 Phylogenetic tree of *Moretella sp.* GS6 (accession number MN421796), isolated from graveyard soil.

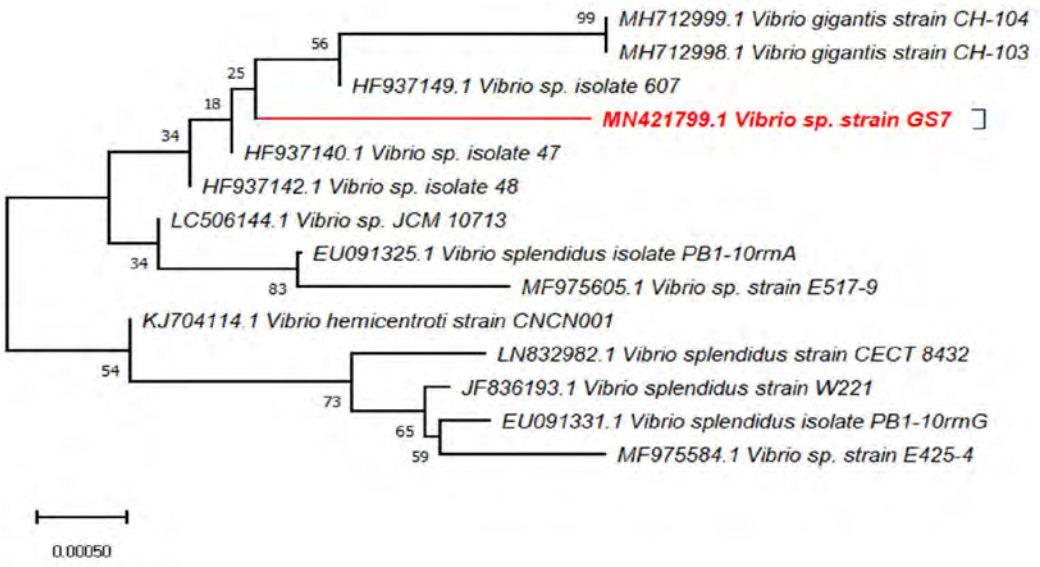


Figure 4.9 Phylogenetic tree of *Vibrio sp.* GS7 (accession number MN421799), isolated from graveyard soil.

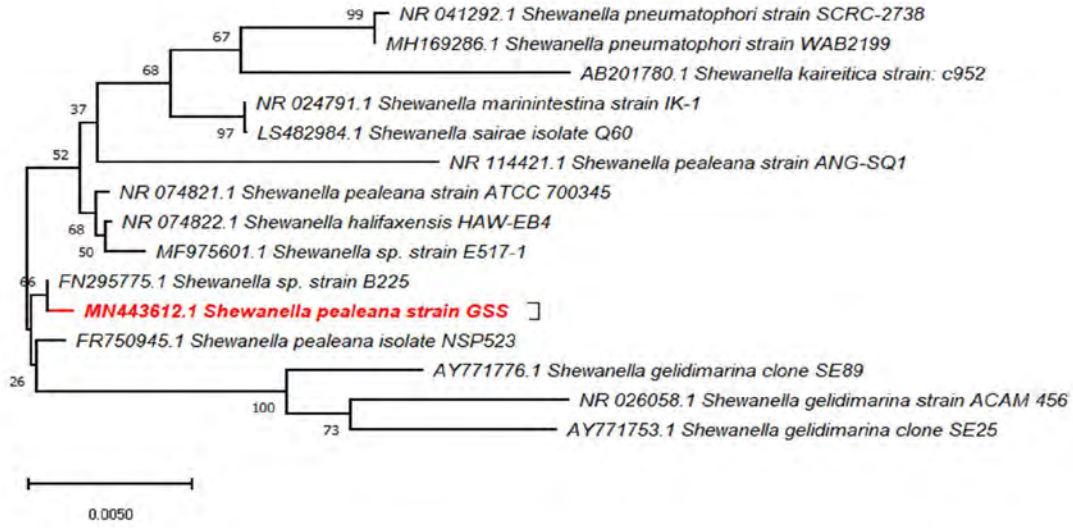


Figure 4.10 Phylogenetic tree of *Shewanella pealeana* GSS (accession number MN443612), isolated from graveyard soil.

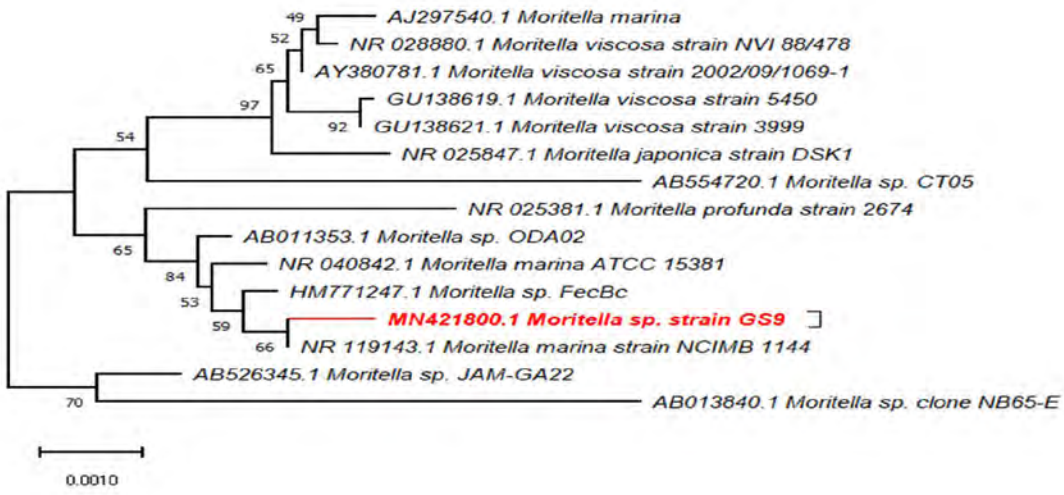


Figure 4.11 Phylogenetic tree of *Moretella sp.* GS9 (accession number MN421800), isolated from graveyard soil.

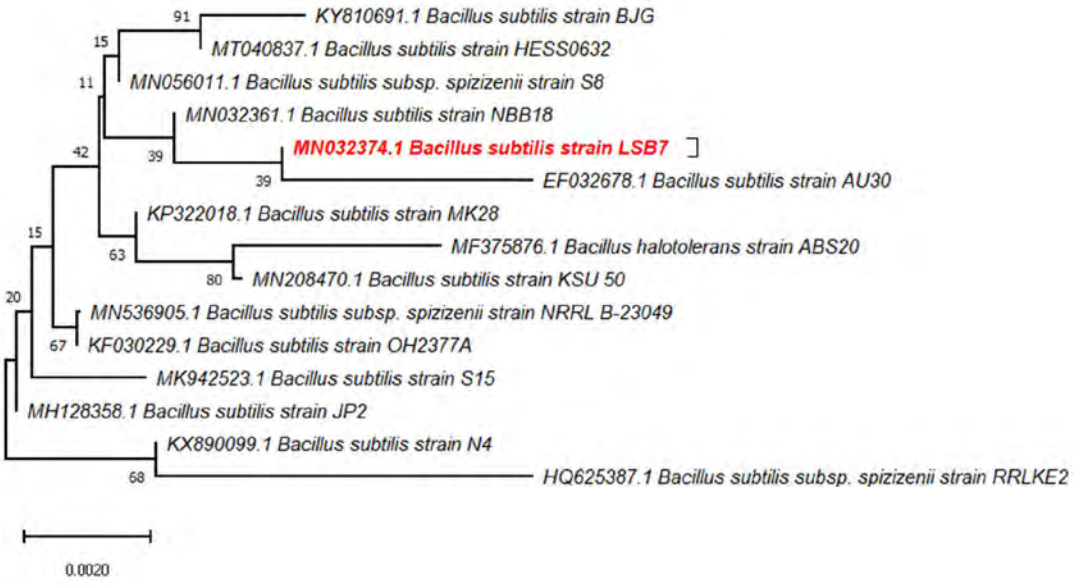


Figure 4.12 Phylogenetic tree of *Bacillus subtilis* LSB (accession number MN032374), isolated from ghee contaminated soil.

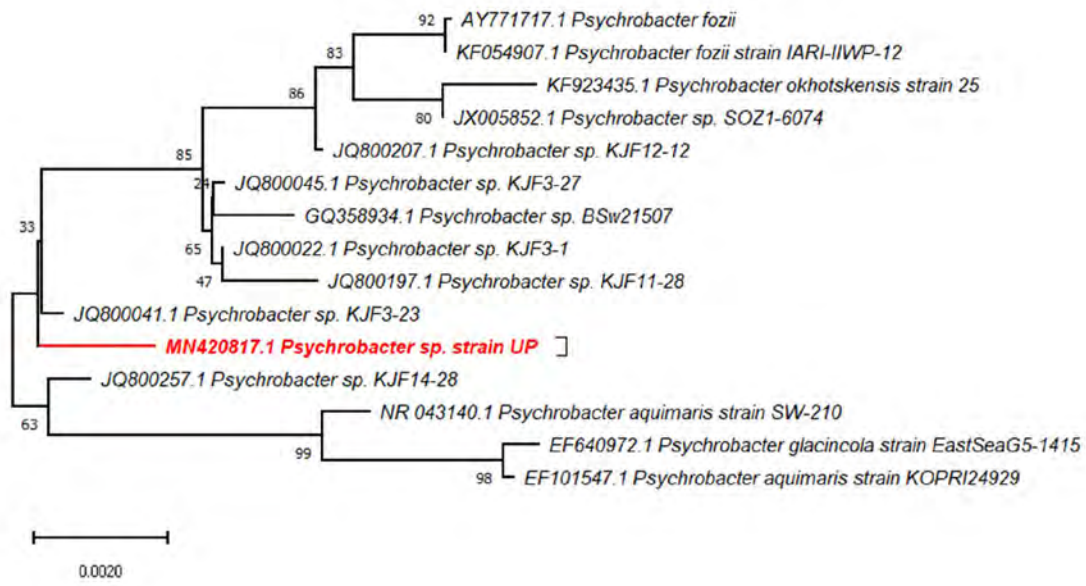


Figure 4.13 Phylogenetic tree of *Psychrobacter sp.* UP (accession number MN420817), isolated from ghee contaminated soil.

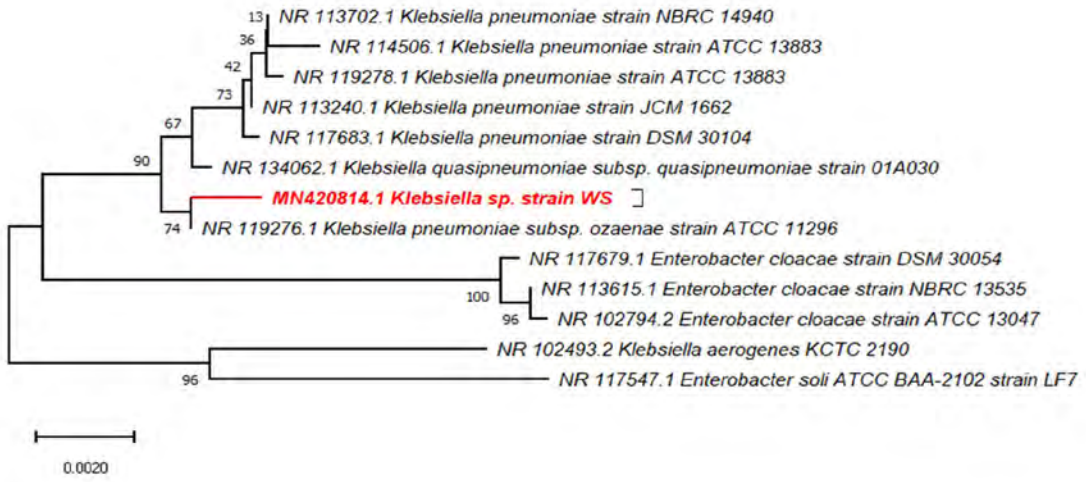


Figure 4.14 Phylogenetic tree of *Klebsiella sp.* WS (accession number MN420814), isolated from ghee contaminated water.

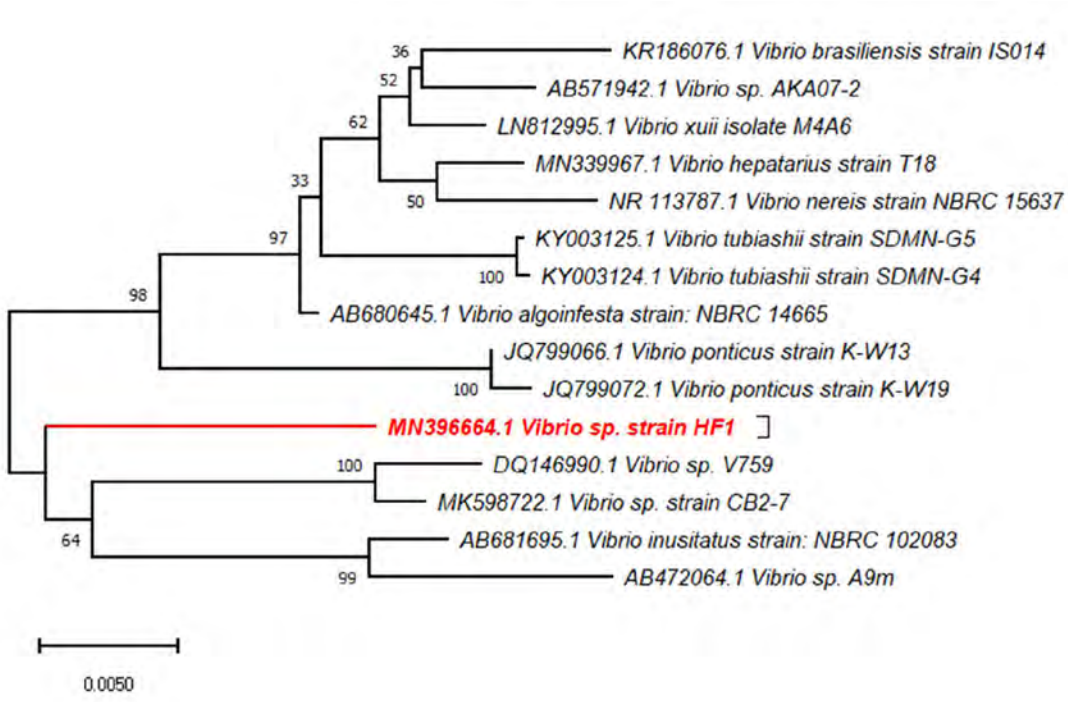


Figure 4.15 Phylogenetic tree of *Vibrio sp.* DS (accession number MN396664) isolated from ghee contaminated soil.

4.2 BIODEGRADATION STUDIES OF CHOLESTEROL

4.2.1 Optimization studies of cholesterol degradation

Two purified isolates including *B. pumilus* W8 and *B. safensis* W1 (isolated from oil contaminated soil) were selected for optimization studies on basis of their best growth. Several factors including incubation time, temperature, pH, effect of metal ions, different concentration of cholesterol was checked for optimum degradation of cholesterol.

Progressive increase in degradation was observed with increasing incubation time and maximum reduction of cholesterol (69% by W1, 70% by W8) was achieved after four days of incubation. On further incubation constant reduction graph was achieved and no more degradation was found (Figure 4.16a).

For cholesterol biotransformation optimum temperature was 35ºC and 57% and 60% reduction were recorded with *B. safensis* W1 and *B. pumilus* W8. Increased cholesterol degradation was observed with increase in temperature while on further increase (45ºC) the percent degradation was decreased (Figure 4.16b).

Biodegradation of cholesterol by *Bacillus safensis* W1 and *Bacillus pumilus* W8 at several pH revealed that highest degradation by both isolates was observed at pH 7. After four days of incubation at pH 7, 62% cholesterol utilization by *B. safensis* W1 and 59% by *B. pumilus* W8 was recorded (Figure 4.16c). Least degradation was observed at more acidic pH as compare to less acidic and basic pH.

Bacterial isolates were also checked for their capability to utilize the different concentration of cholesterol. Gradual increased cholesterol degradation was there with increased concentration of cholesterol and optimum concentration was found 1 g/L, while on further increase the degradation was decreased. 55% degradation by *B. safensis* and 58% by *B. pumilus* W8 was recorded at optimum concentration of cholesterol (Figure 4.16d).

Furthermore, effect of various metals on degradation was studied and found that presence of some metal ions such as $MgSO_4$ and $CaCl_2$ favors the cholesterol degradation with both of isolates. 60% degradation by *B. safensis* W1 in presence of $CaCl_2$ while 62% degradation by *B. pumilus* W8 in presence of $MgSO_4$ was recorded. Less degradation was there in presence of $BaCl_2$and $CoCl_2$ (Figure 4.16e).

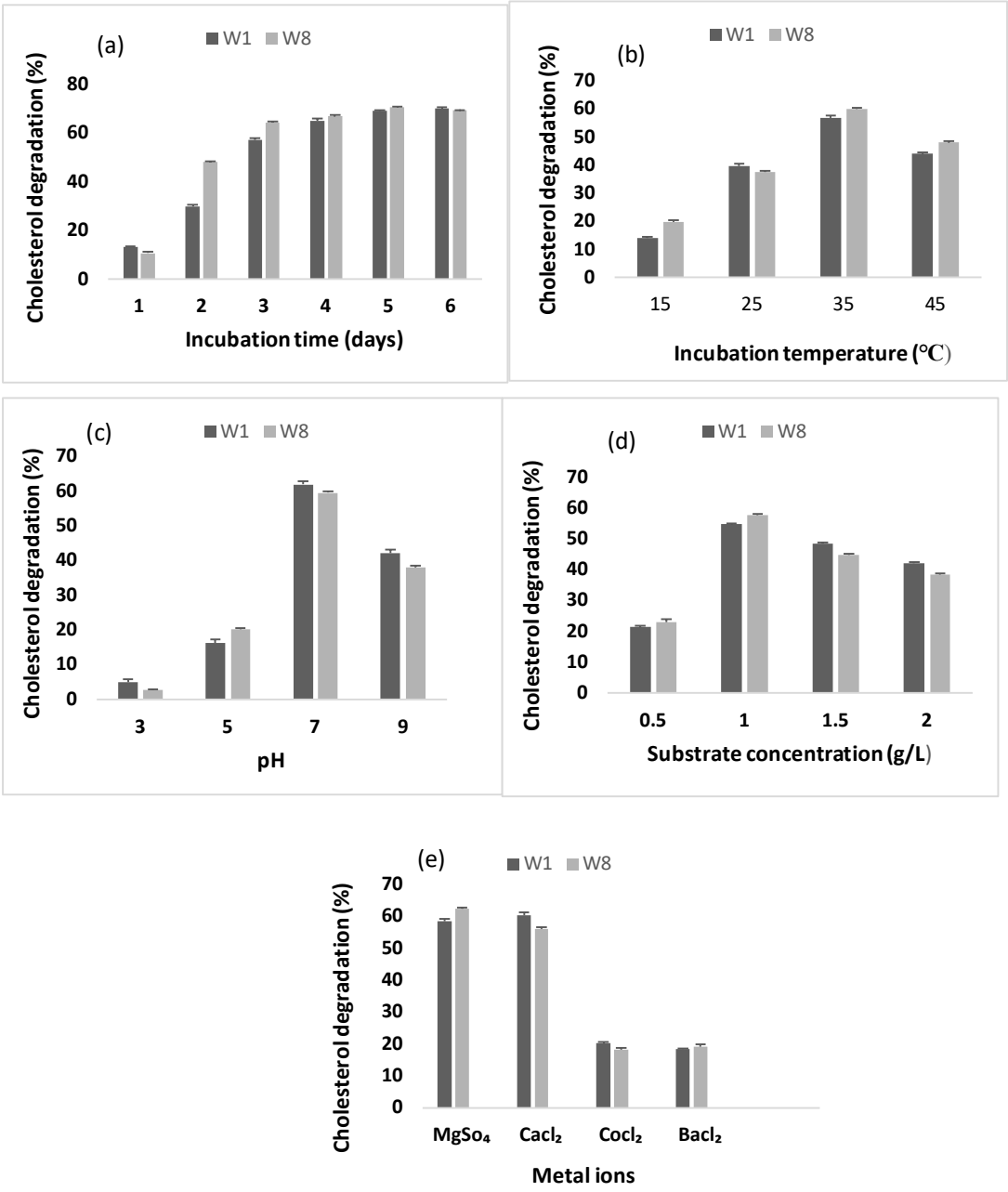


Figure 4.16 Effect of incubation time (1-6 days) at 30°C, pH 7, and 1% cholesterol as a substrate (a), incubation temperature (15-45°C) at pH 7 and 1% cholesterol concentration for four days of incubation (b), pH (3-9) at 35°C, pH 7, and 1% cholesterol for four days incubation (c), substrate concentration (0.5-2 g/L) at 35°C, pH 7, and 1% cholesterol for four days incubation (d), metal ions ($MgSo_4$, $Cacl_2$, $Cocl_2$, $Bacl_2$) at 35°C, pH 7, and 1% cholesterol for four days incubation (e) on degradation of cholesterol by *B. safensis* W1 and *B. pumilus* W8.

4.2.1.1 HPLC analysis of cholesterol and 7-ketocholesterol degradation

Broth cultures of some isolates were extracted and run on HPLC to confirm the cholesterol biodegradation. In all cases, cholesterol peak appeared with reduced area at retention time of 2 minutes and no other peaks were detected in *B. safensis* W1 (Figure 4.17a), *B. pumilus* W8 (Figure 4.17b), *Stenotrophomonas pavanii* GS2 (Figure 4.17c), *Psychrobacter sp*. GS3 (Figure 4.17d), *Moretella sp*. GS6 (Figure 4.17e) treated samples, while appearance of new peak at retention time of 6.8, 7.6 minutes were detected with *Vibrio sp*. GS7 (Figure 4.17f) and *Shewanella pealeana* GSS (Figure 4.17g) treated samples. Two new peaks at retention time 8.0 and 8.8 minutes were observed with *Moretella sp*. GS9 (Figure 4.17h) treated cholesterol sample. The whole cells treated extracts of W1, W8, GS2, GS3, GS7 GSS and GS9 exhibited 58, 86, 16, 44, 57, 51 and 61% as shown in Figure (4.18).

Based on optimum cholesterol degradation *B. safensis* W1, *B. pumilus* W8, *Moretella sp*. GS6 and *Shewanella pealeana* GSS were selected for biodegradation of 7-ketocholesterol and exhibited 70%, 90%, 86% and 92% reduction (Figure 4.19) when analyzed by HPLC.

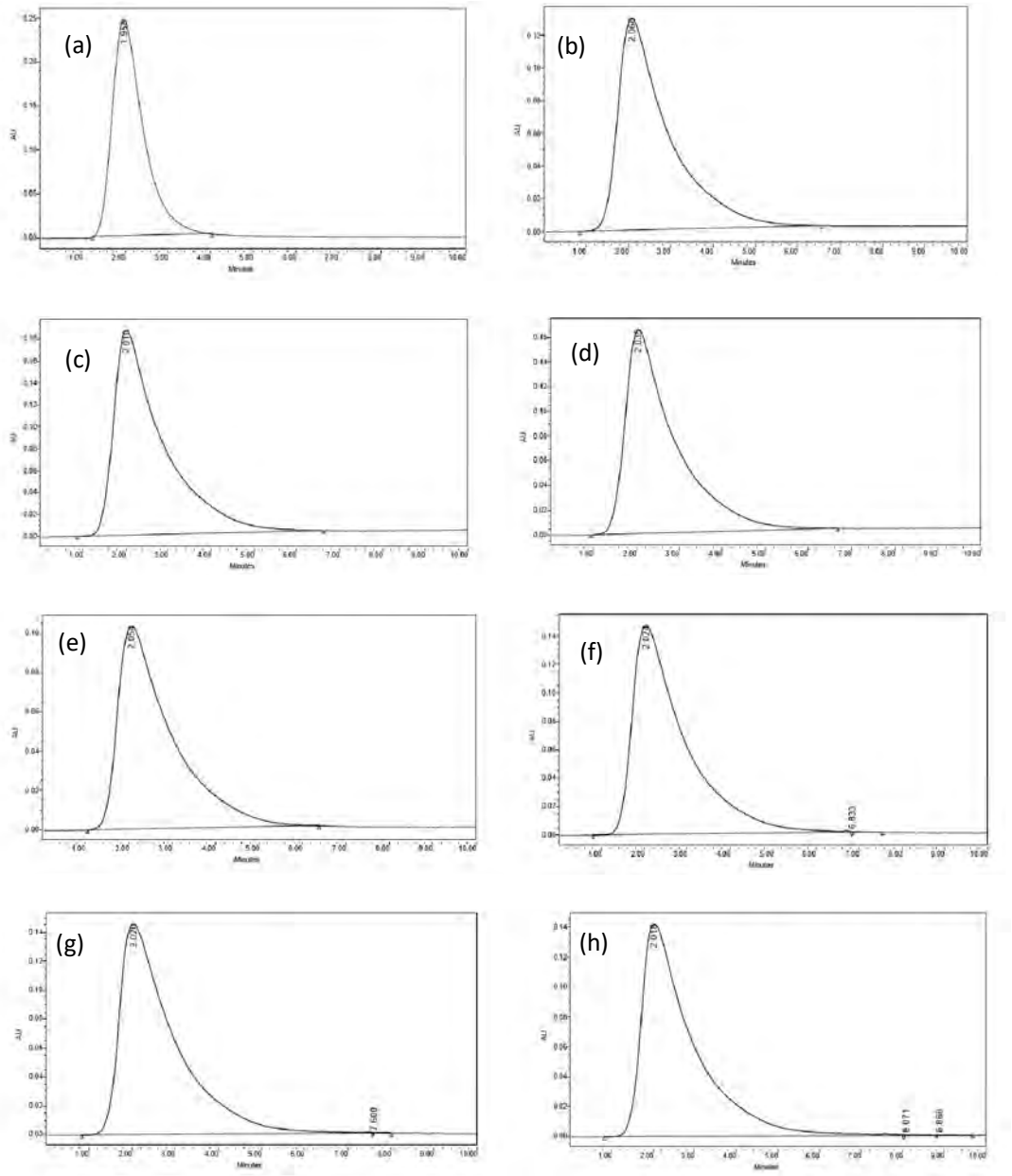


Figure 4.17 HPLC chromatogram of cholesterol extracted from culture medium of *B. safensis* W1 (a), *B. pumilus* W8 (b), *Stenotrophomonas pavanii* GS2 (c) , *Psychrobacter sp.* GS3 (d), *Moretella sp*. GS6 (e), *Vibrio sp*. GS7 (f), *Shewanella pealeana* GSS (g*), Moretella sp*. GS9 (h).

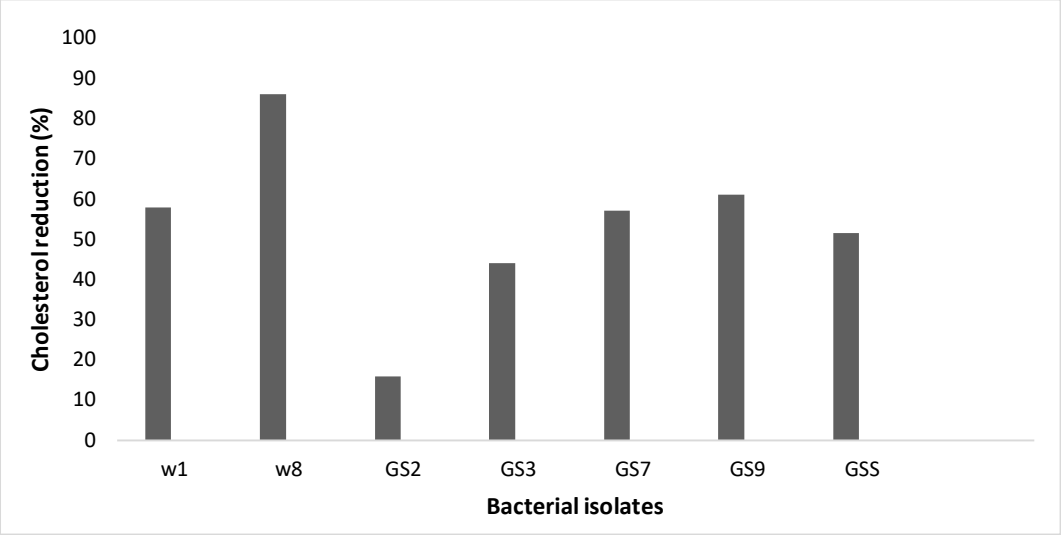


Figure 4.18 Cholesterol reduction by environmental isolates in M9 media supplemented with only carbon source of cholesterol.

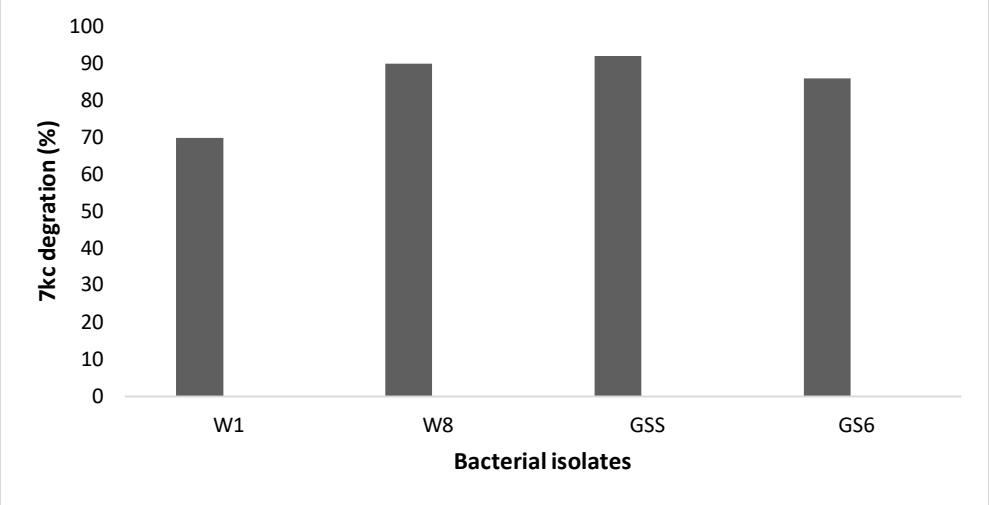


Figure 4.19 Reduction of 7-ketocholesterol by environmental isolates in M9 media supplemented with only carbon source of cholesterol.

4.2.1.2 GCMS analysis of Cholesterol metabolites

The broth extracts of some isolates including *B. subtilis* LSB, *Psychrobacter sp*. UP, *Vibrio sp.*DS (isolated from ghee contaminated soil), *Moretella sp* GS6, *Psychrobacter sp*. GS3 (isolated from graveyard soil) and *B. safensis* W1 (isolated from oil contaminated soil) were run on GCMS to identify the degradation metabolites. Cholesterol peak was detected at retention time of 16.8 minutes in control chromatogram (Figure 4.20). In all treated samples number of peaks appeared with some common metabolites along with different metabolites with significant reduction of cholesterol (Table 4.4). Hexadecenoic acid at retention time 10.7 minutes was detected as a common metabolite in all the treated samples. One significant new peak was observed in *B. subtilis* LSB treated sample and identified as a 2-dodecene-1-yl-succinic anhydride along with a minor peak of hexadecenoic acid (Figure 4.21). whereas two minor peaks identified as fatty acids such as octadecanoic acid and hexadecenoic acid were detected in *Vibrio sp.* DS treated cholesterol sample with no other significant peaks when analyzed on GCMS (Figure 4.22). One of prominent peak of 26,27-Dinorergosta-5,23-dien-3-ol was appeared with *Psychrobacter sp*. UP treated sample along with presence of hexadecenoic acid as a minor peak (Figure 4.23). Some minor peaks were appeared in sample treated with *Moretella sp*. GS6 including octadecanoic acid along with common metabolite hexadecanoic acid (Figure 4.24), while appearance of significant peak corresponding to 7-oxo-cholesterol in *Psychrobacter sp*. GS3 treated sample with minor peak of hexadecenoic acid (Figure 4.25). *B. safensis* W1 treated cholesterol sample analysis on GCMS showed the minor peaks of common fatty acids identified as octadecanoic acid and hexadecenoic acid (Figure 4.26). GCMS spectra of some metabolites detected in our samples including hexadecenoic acid, octadecanoic acid and 7-oxo cholesterol are shown in Figure 4.27.

Table 4.4 Cholesterol metabolites detected by GCMS analysis.

Isolates	**Metabolites**	**Retention time (RT)**
LSB	Hexadecanoic acid	10.7
	2-dodecene-1-yl-Succinic anhydride	14.2
DS	Hexadecanoic acid	10.7
	Octadecanoic acid	11.7
UP	Hexadecanoic acid	10.7
	26,27-Dinorergosta-5,23-dien-3-ol	17.1
GS6	Hexadecanoic acid	10.7
	Octadecanoic acid	11.7
GS3	Hexadecanoic acid	10.7
	7-oxo-cholesterol	12.8
W1	Hexadecanoic acid	10.7
	Octadecanoic acid	11.7

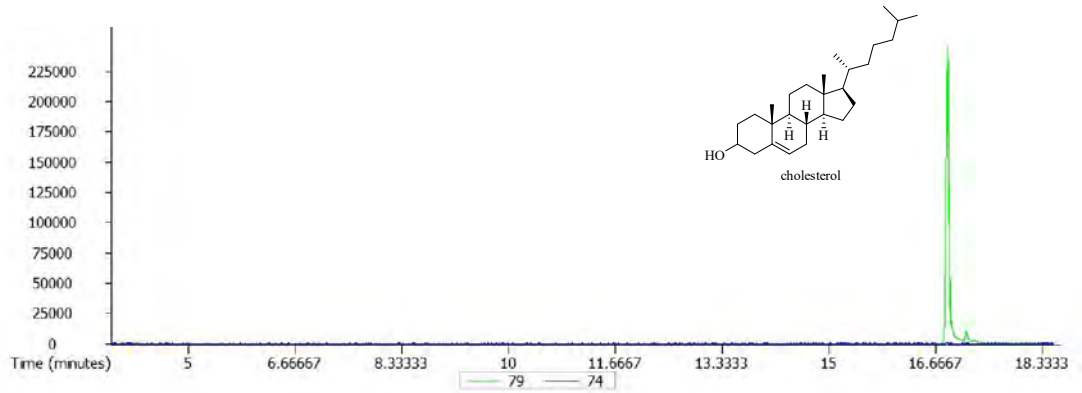


Figure 4.20 GCMS chromatogram of cholesterol (control)

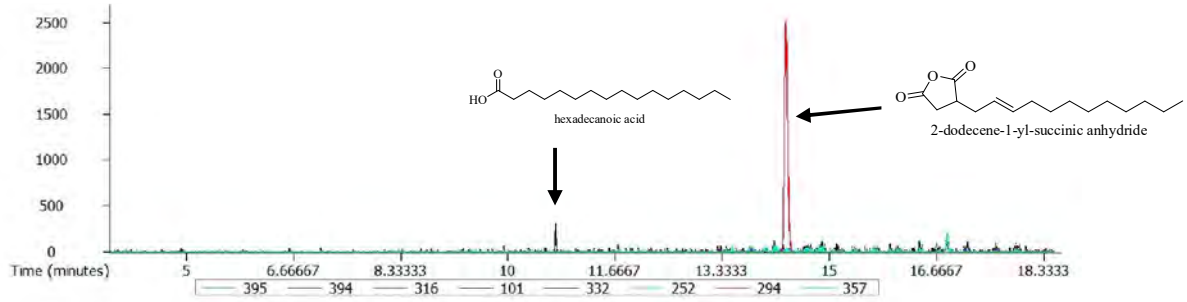


Figure 4.21 GCMS chromatogram of *B. subtilis* LSB treated cholesterol

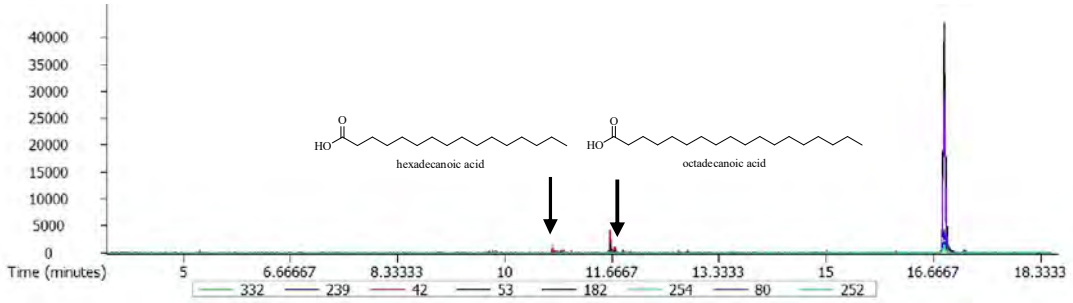


Figure 4.22 GCMS chromatogram of *Vibrio sp.* DS treated cholesterol.

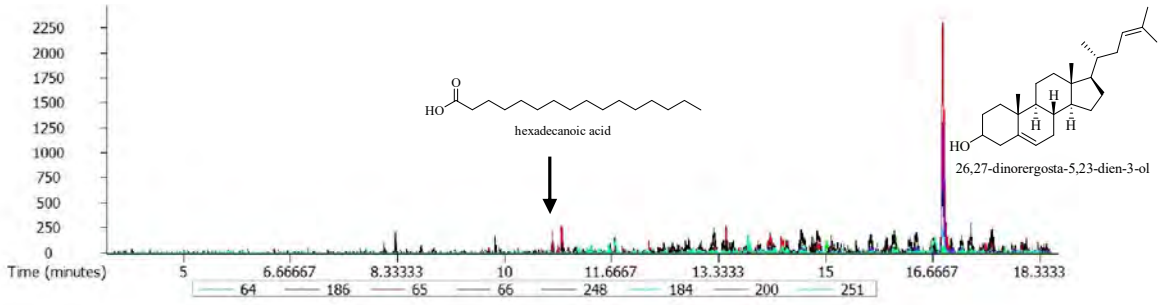


Figure 4.23 GCMS chromatogram of *Psychrobacter sp* UP treated cholesterol.

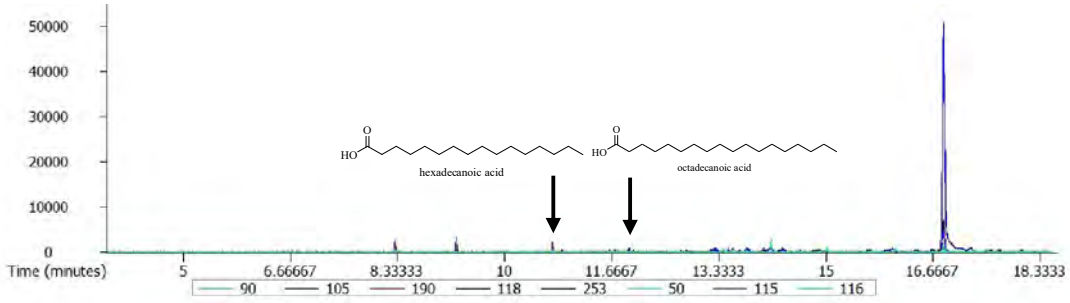


Figure 4.24: GCMS chromatogram of *Moretella sp.* GS6 treated cholesterol.

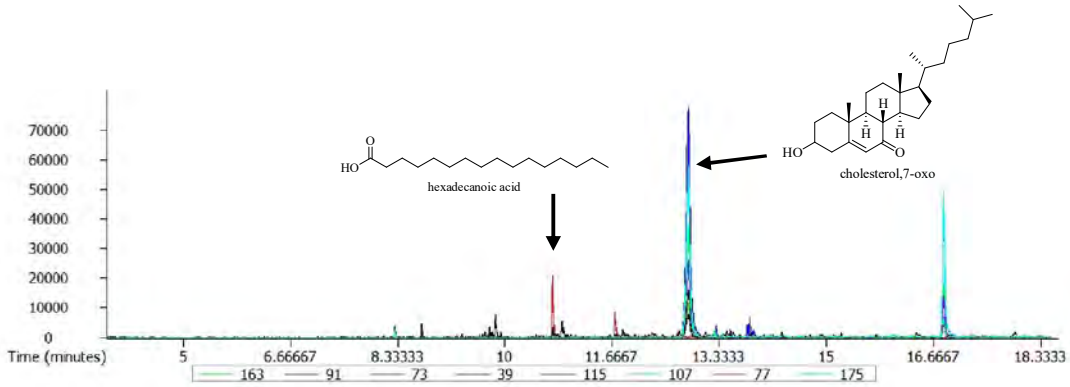


Figure 4.25 GCMS chromatogram of *Psychrobacter sp.* GS3 treated cholesterol.

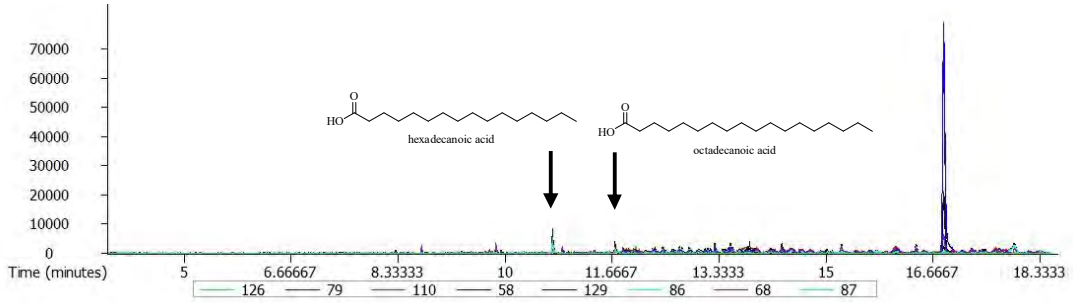


Figure 4.26 GCMS chromatogram of *B. sefensis* W1 treated cholesterol.

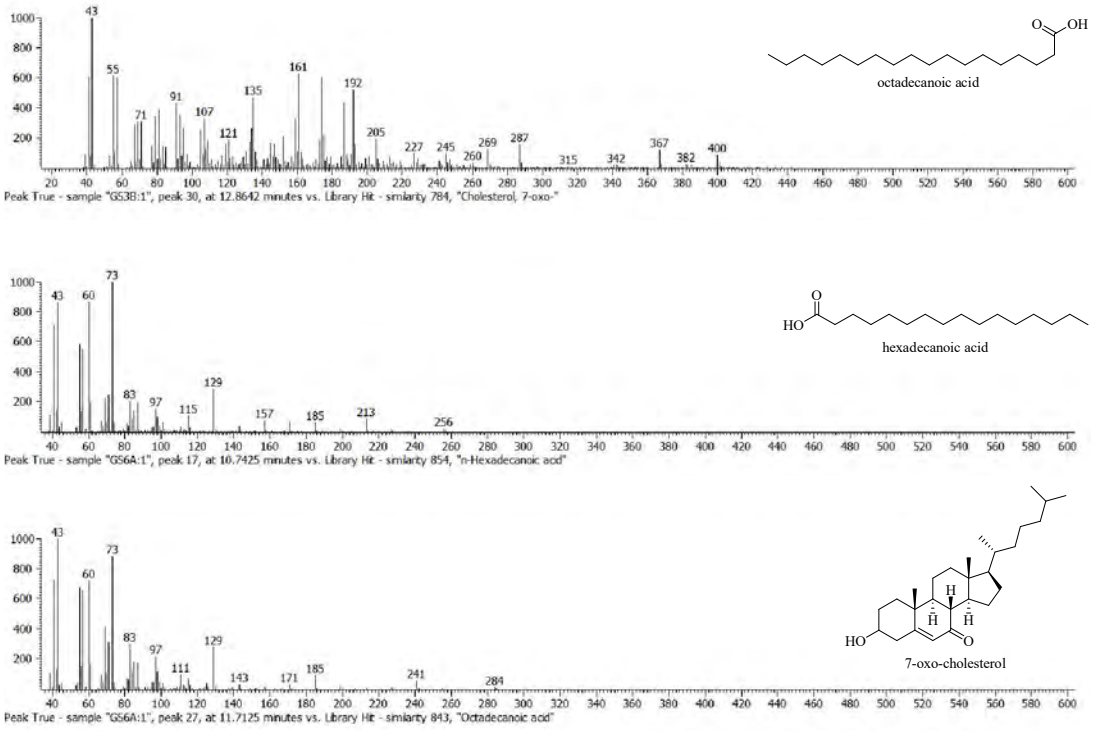


Figure 4.27 GCMS spectra of major cholesterol degradation products.

4.3 SCREENING OF ISOLATES FOR CHOLESTEROL OXIDASE

4.3.1 Qualitative analysis

All the isolated strains showed the cholesterol oxidase production by development of red color in cholesterol oxidase assay plates compared to control plate (*E. coli*) *Bacillus safensis* W1 showed intense red color indicating it as an excellent strain for production of cholesterol oxidase (Figure 4.28 a-d).

4.3.2 Quantitative analysis

Cholesterol oxidase production of all the isolated bacteria was quantified by using different production media. Maximum production was achieved in 2xLB (Figure 4.29a) after 48 hours of incubation followed by1xLB (Figure 4.29b) and least production was recorded in M9 media (Figure 4.29c). Among all isolates LSB, W1 and GS3 was found efficient cholesterol oxidase producers. 0.336 U/ml in 2xLB, 0.127U/ml in 1xLB was produced by LSB, 0.140 U/ml in 2xLB and 0.130 U/ml in 1xLB was produced in 1xLB while GS3 produced 0.186 U/ml, 0.054 U/ml in 2xLB and 1xLB respectively.

4.3.3 Precipitation and purification of cholesterol oxidase

Based on maximum enzyme activity in cell free supernatant *Bacillus subtilis* LSB, *Bacillus safensis* W1 and *Psychrobacter sp.* GS3 were selected for purification. The selected strains showed activity at different percent of ammonium sulphate saturation and optimum activity of, 1.277 U, 0.695 U and 0.906 U were achieved at 80, 60, and 40% by *Bacillus safensis* W1 (Figure 4.30a), *Psychrobacter sp.* (Figure 4.30b) GS3 and *Bacillus subtilis* LSB (Figure 4.30c), respectively. Furthermore, number of bands appeared on 12% SDS-PAGE in all the precipitated proteins (Figure 4.31).

Further purification of cholesterol oxidase from *B. safensis* W1 and *B. subtilis* LSB was carried out by using DEAE-Sepharose an anion exchange chromatography. Number of fractions were collected from both *B. safensis* W1 (Figure 4.32a) and *B. subtilis* LSB (Figure 4.32b) and fractions with peaks were used for determination of enzyme activity (Figure 4.32c) and protein content but maximum specific activity 75 U/mg was achieved in fraction 33 of W1. In other case pooled fraction of 4 and 5 of LSB showed maximum activity of 56 U/mg. Furthermore, purity of ChO in fractions of maximum activity of both

strains were analyzed on 12% SDS-PAGE and single band with size of 58 kDa from *B. safensis* W1 (Figure 4.33a) and 22 kDa (Figure 4.33b) from *B. subtilis* LSB was appeared. Activity of cholesterol oxidase with purification steps are shown in Figure 4.33c.

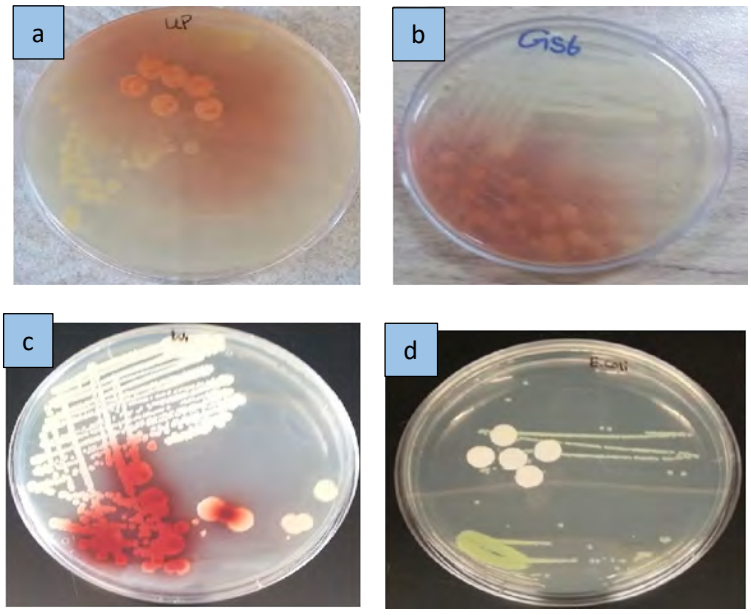


Figure 4.28 Qualitative detection of cholesterol oxidase from *Psychrobacter sp.* UP (a), *Moretella sp*. GS6 (b), *Bacillus safensis* W1 (c) by development of red color due to production of chromogenic compound compared to *E.coli* (d) plate as a control.

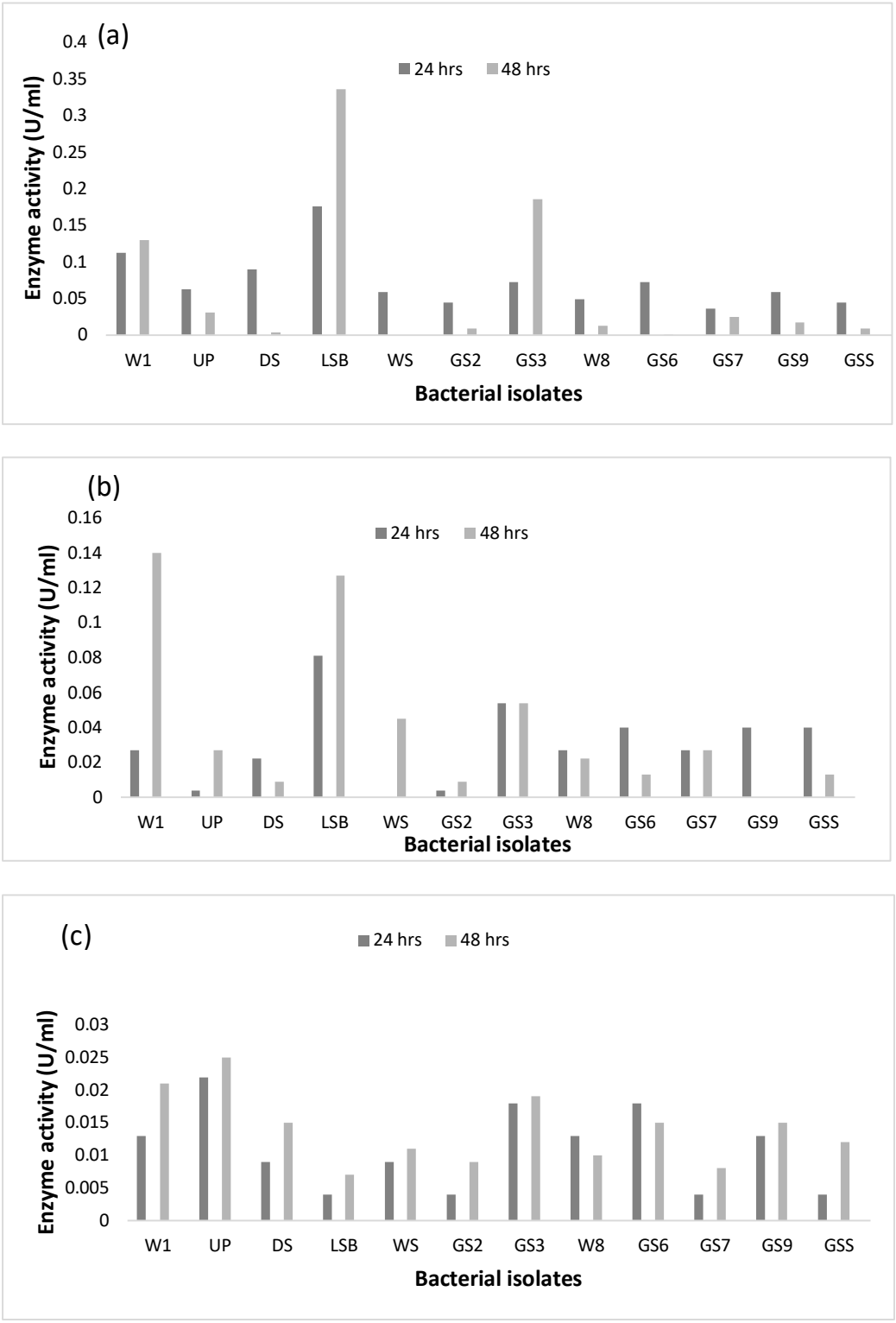
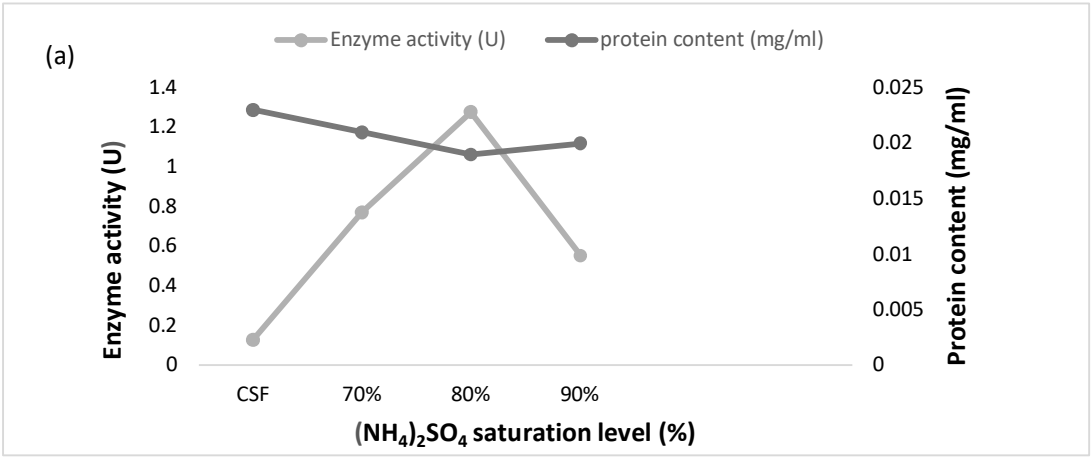
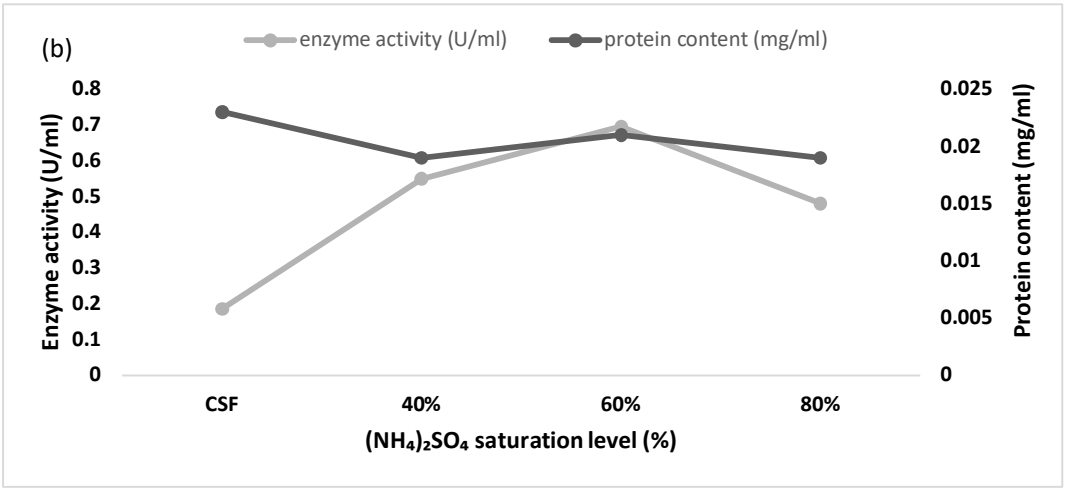


Figure 4.29 Cholesterol oxidase production after 24hrs and 48 hrs interval in 2xLB (a), 1x LB (b) and M9 (c) media.





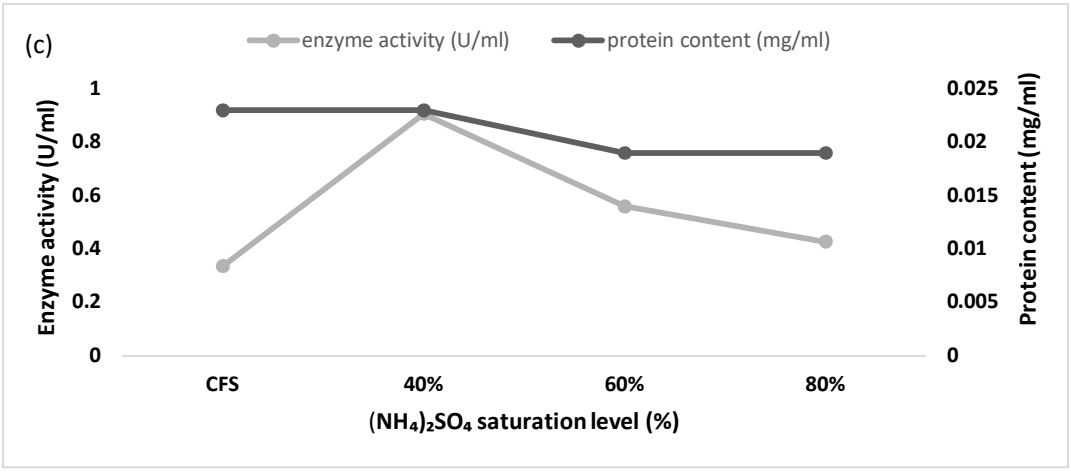


Figure 4.30 Protein content and enzyme activity of *Bacillus safensis* W1 (a), *Psychrobacter sp*. GS3(b) and *Bacillus Subtilis* LSB, (c) at different percent of NH_4SO_4 saturation.

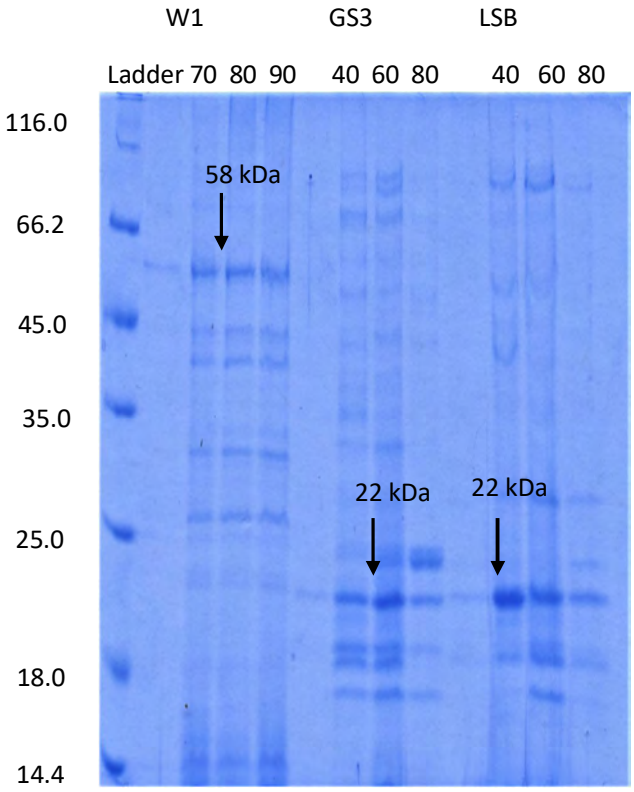
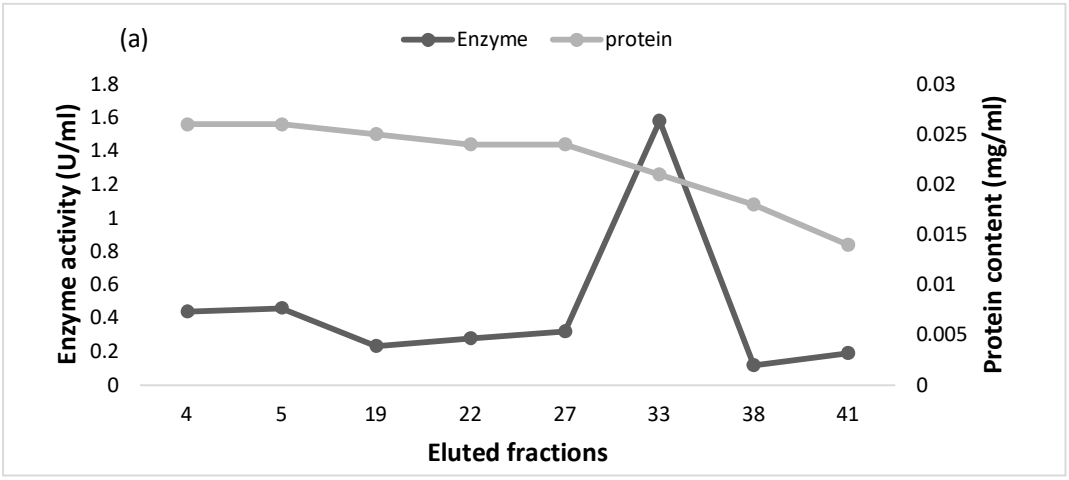
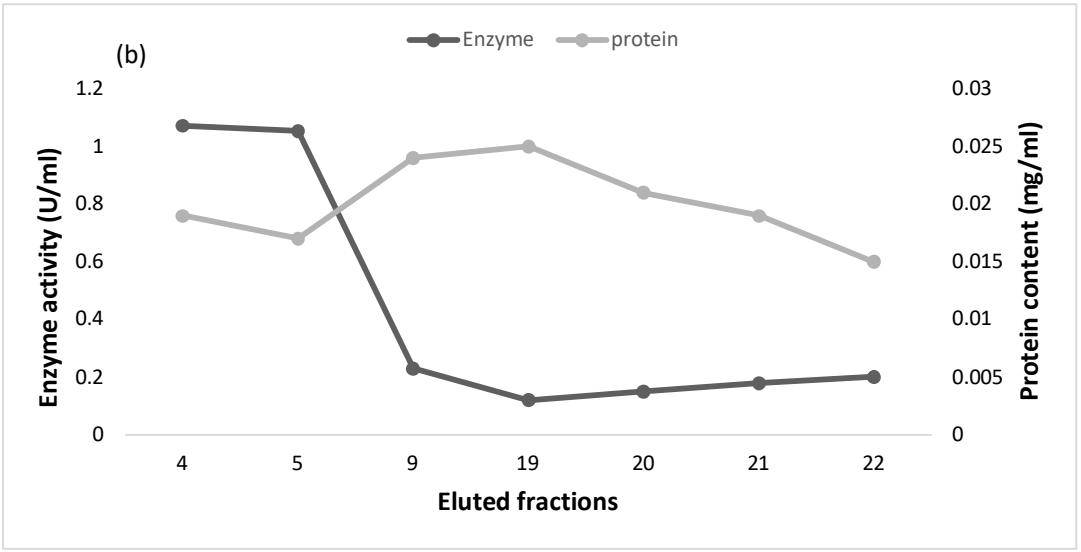


Figure 4.31 SDS PAGE analysis of precipitated protein of *B. safensis* W1 (70, 80, 90%), *Psychrobacter sp.* GS3 (40, 60, 80%) and *B. subtilis* LSB (40,60, 80%).





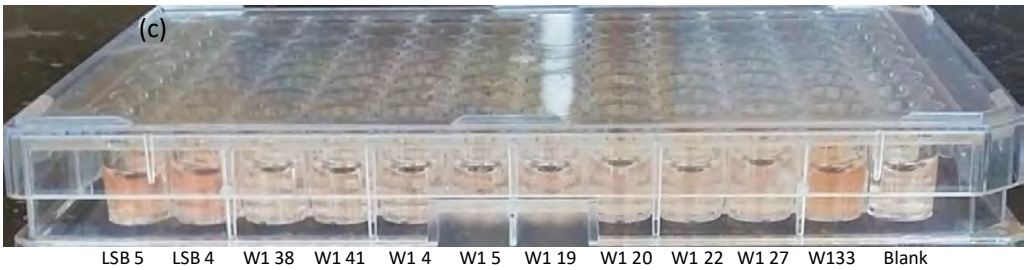


Figure 4.32 Elusion profile of column chromatography for cholesterol oxidase produced by *Bacillus safensis* W1(a) *and Bacillus subtilis* LSB (b) and quantification of cholesterol oxidase in plate assay (c).

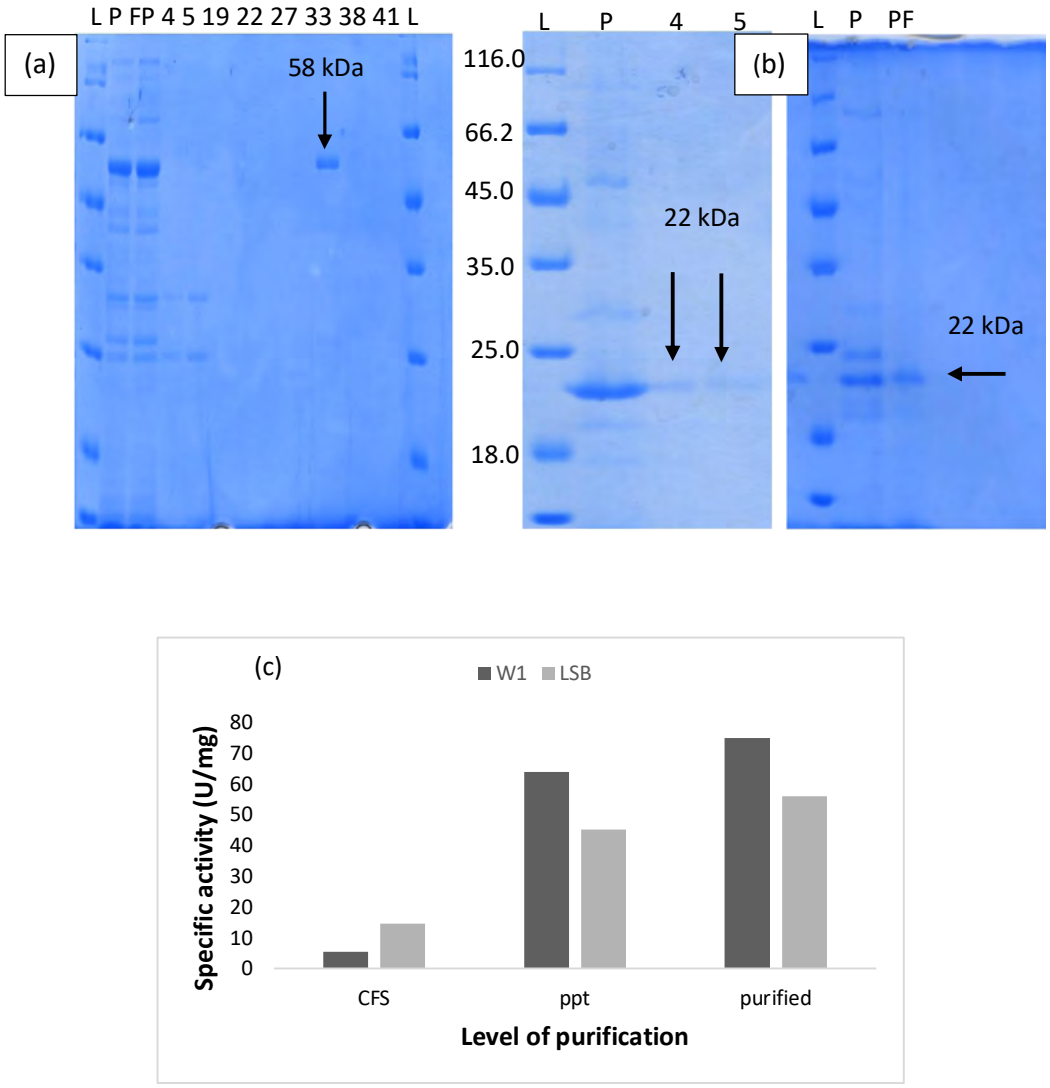


Figure 4.33 Purified CHO by using DEAE sepharose anion exchange chromatography (AKTA Pure 25) of *Bacillus safensis W*, (L represents ladder and P (precipitated protein) FP (filtered precipitates) number of fractions (lane 4-41) [a] *and Bacillus Subtilis LSB,* (L represents ladder, P (precipitated protein) number of fractions (4, 5), PL (pooled fractions) [b] and enzyme activity with purification [c].

4.4 CLONING AND EXPRESSION OF SYNTHETIC CHOLESTEROL OXIDASE GENE IN *E. COLI*

4.4.1 Cloning of *ChO* in pET21 vector

Based on several studies number of cholesterol oxidase gene (*ChO*) has been identified and cloned from different bacteria. Cholesterol oxidase (ChO) of *chromobacterium sp.* was used as a model and planned to be cloned in pET vector with novel split intein tag. Further the tag less protein was expressed, purified, and characterized.

Initially cholesterol oxidase gene of the *chromobacterium sp*. DS1, was synthesized and PCR amplified by making the use of primers. Band of *ChO* gene was excised from agarose gel and purified by miniprep silica column. pET21 vector was also PCR amplified followed by miniprepped purification. Later cholesterol oxidase gene was cloned into pET21 vector by a single thermal Gibson assembly reaction. pET-NpuC-*ChO* construct was transformed in competent *E. coli* DH5α cells. Transformed cells were then plated with ampicillin resistant LB plate. Large number of colonies were appeared on plate. Six colonies were selected and inoculated into ampicillin resistant broth. From all the six samples plasmid DNA was miniprepped and the band of 7075bp was appeared when analyzed on DNA gel. To confirm the *ChO* gene in minipreped plasmid DNA double digestion with *NHe1* and *Sca1* was performed and run on DNA gel. Agarose gel results showed a band of 2381bp corresponds to *ChO* gene and 4695 bp correspond to pET21 vector. Among the six colonies, five showed the successful insertion of *ChO* gene while one colony failed on basis of agarose gel analysis.Plasmid DNA with two bands and with highest concentration (117 µg/ml) was further verified by sequencing (Figure 4.34).

4.4.2 Transformation of pET-NpuC-*ChO* construct in different *E. coli*

For expression pET-NpuC-*ChO* construct was transformed into *E. coli* competent strains including *BLR, BL21, Origami* and *SHT7*. Initially all the transformed cells were grown for expression in double concentration of Luria-Bertani (2xLB) broth with ampicillin (100 µg/ml) at 37℃ except of *SHT7* grown at 30℃ and then all were induced with 100 mM IPTG when OD_{600} reached to 0.6-0.8. After induction, the temperature of all the cultures were shift to 20℃ and further incubated overnight. Cells were harvested by centrifugation at 6000 x g for 20 minutes then lysed by ultrasonic homogenizer. Whole lysate and clear lysate of all the expression experiments were run on 12% SDS-PAGE and the expression band in a range of 58 kDa was only appeared with *BLR* (Figure 4.35).

4.4.3 Optimization of *ChO* gene expression

For expression and solubility of cholesterol oxidase different parameters were optimized including, expression media, post induction temperature, post induction time and different concentration of IPTG. Expression cells were grown in Super broth (SB) (Figure 4.36) and Terrific broth (TB) (Figure 4.37) at different post induction temperature (20℃, 30℃, 37℃). Expression of *BLR* and *Origami* was achieved in TB media at low post induction temperature (20℃).

Furthermore, cells of *BLR* and *origami* were harvested at different time interval (4, 8 and 16 hours) after induction. Expression was seen at all time interval with low solubility, but significant expression was there after 16 hours of incubation when the samples were run on protein gel (Figure 4.38). To enhance the solubility of cholesterol oxidase different concentrations of IPTG (0.1-1 mM) were used and optimum solubility was achieved at lowest concentration (0.1 mM) (Figure 4.39).

4.4.4 Purification of recombinant cholesterol oxidase

NpuC tagged recombinant cholesterol oxidase expressed by *BLR* and *origami* was purified at room temperature (25℃) by passing through gravity column. All samples collected during purification was run on SDS-PAGE which include whole and clear lysate (WL, CL), sample flow through column (FT), wash 1,2 (W1, W2), samples collected at different times (0, 1, 3, 16 hours), eluted samples (E1, E2, E3) and sample from resin to check the bind protein (RBP). single protein band of about 58 kDa was seen in the eluted fractions corresponding to *BLR* (Figure 4.40a) and *Origami* (Figure 4.40b) expressed ChO while significant amount of protein was seen still bind to resin.

To elute the bind protein all the above purification steps were repeated and carried out at 37℃. There was no effect of increased temperature on elusion of resin bind protein and its activity (Figure 4.41). Specific activity of the purified cholesterol oxidase was 33 U/mg and 26 U/mg expressed by *Origami* and *BLR*, respectively.

4.4.5 Modification of enzyme by protein engineering

Fast elusion of recombinant ChO from resin was achieved by changing the first amino acid from threonine to phenylalanine. Plasmid DNA was amplified by making the use of forward primer for cholesterol oxidase gene. Further DNA was digested, phosphorylated, and ligated by sequential treatment with DPn1, T4-polynucleotide kinase and T4 DNA

ligase. Ligated DNA was transformed in DH5α cells and miniprepped plasmid DNA showed a band of 7075 bp when analyzed on agarose gel and further confirmed by sequencing. pET-NpuC-*ChO* construct was expressed in *BLR* and purified under optimized conditions. Significant elusion of protein from resin was seen with one amino acid change when sample was run on SDS-PAGE (Figure 4.42). With fast elusion the activity of recombinant protein was significantly increased and recorded as 41 U/mg (Figure 4.43).

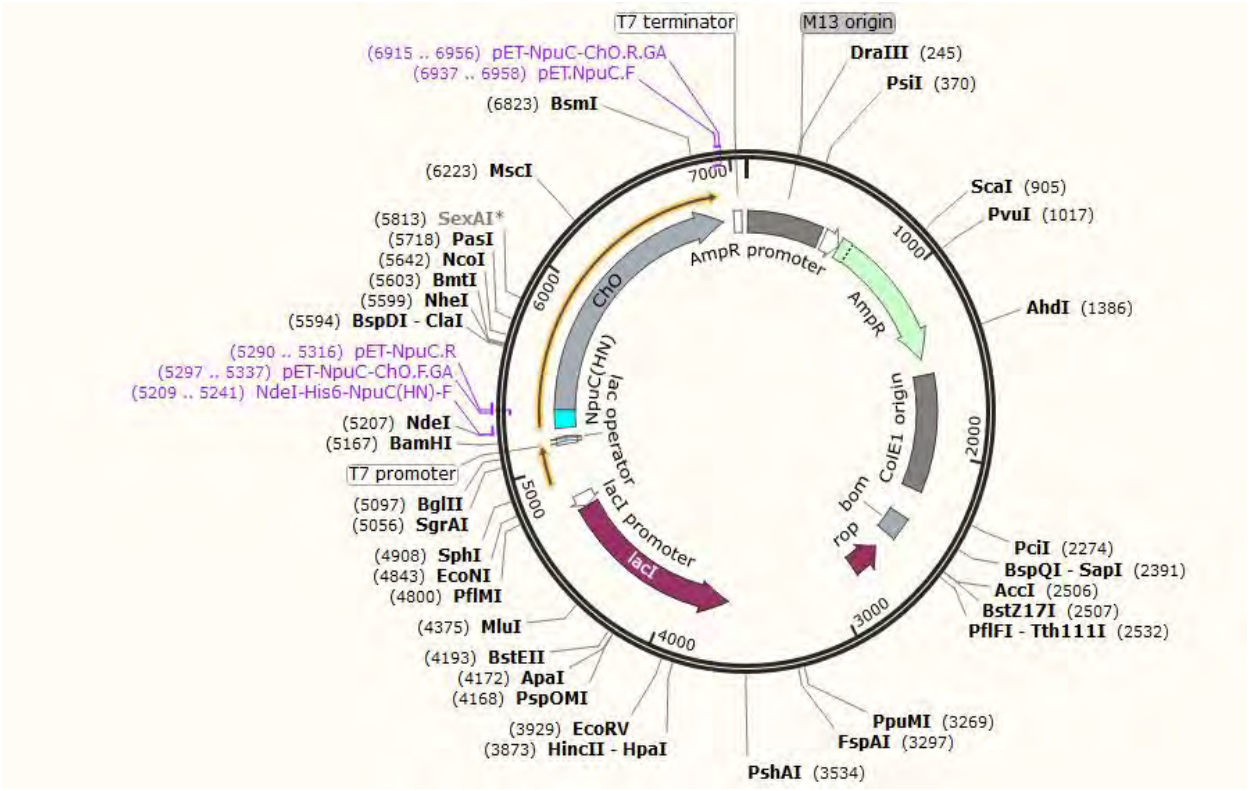


Figure 4.34 Plasmid pET-NpuC-*ChO* construct.

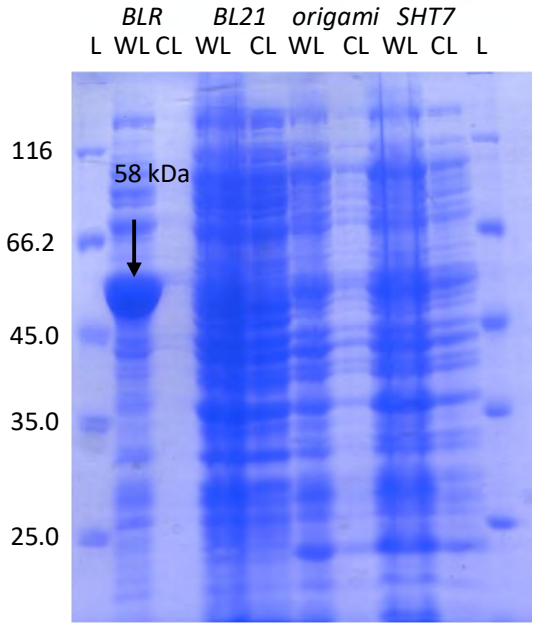


Figure 4.35 Expression of PET-NpuC-*ChO* in *BLR, BL21*, *Origami* and *SHT7* (in whole and clear lysate) in LB media, L represent protein ladder, WC represents whole lysate and CL represents clear lysate.

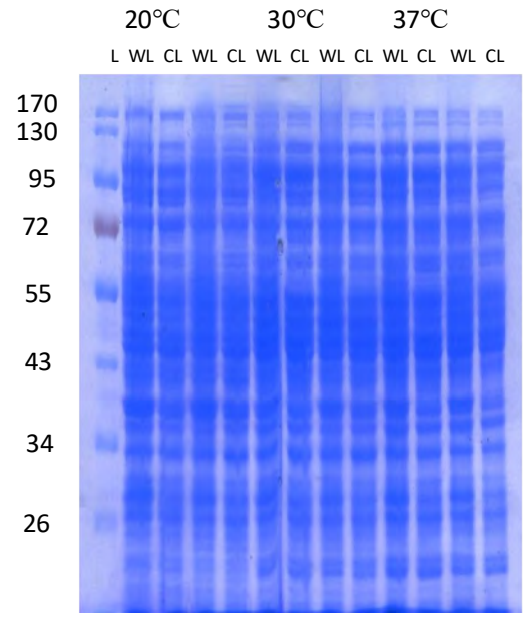


Figure 4.36 Expression of pET-NpuC-*ChO* in *BLR*, and *origami* in SB media at different post induction temperature. L represent the prestaind ladder and first lane under each temperature represent the whole lysate (WL) and clear lysate (CL) of *BLR* expression followed by *Origami* expression.

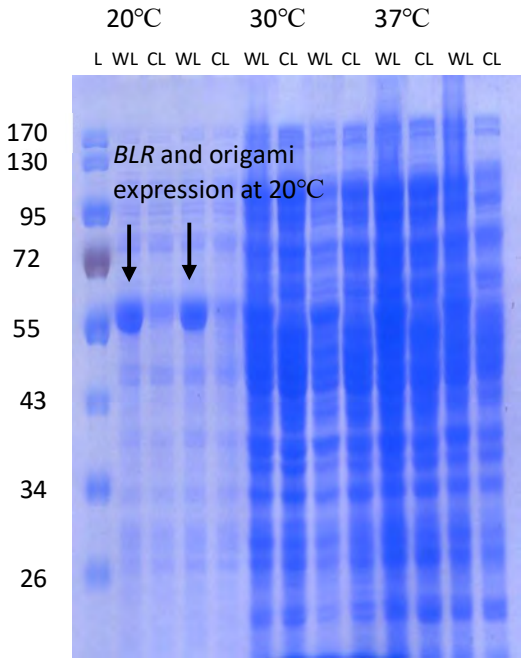


Figure 4.37 Expression of pET-NpuC-*ChO* in *BLR*, and *Origami* in TB media at different post induction temperature. L represent the prestaind ladder and first lane under each temperature represent the whole lysate (WL) and clear lysate (CL) of *BLR* expression followed by *Origami* expression.

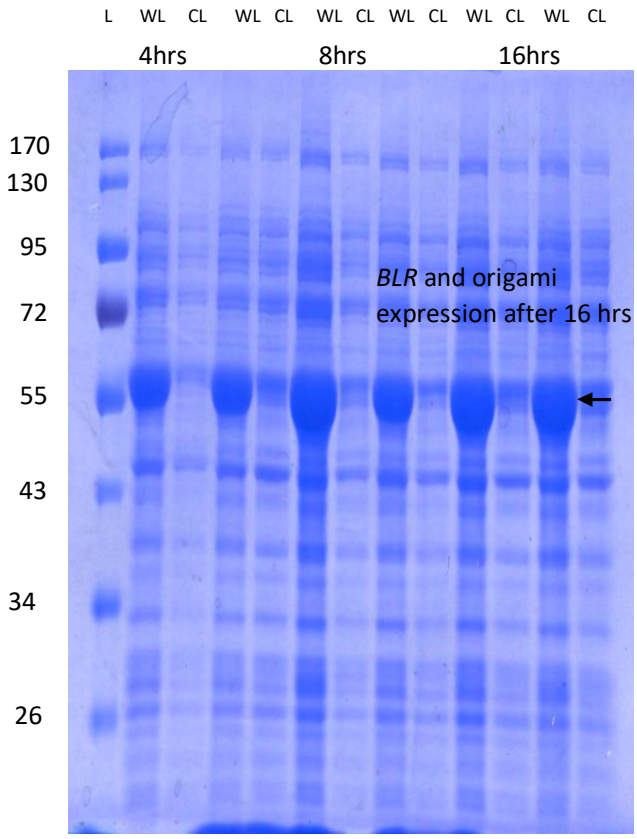


Figure 4.38 Expression of *BLR* and *Origami* at different post induction time in optimized TB media. L represent the prestained ladder and first lane under each incubation time represent the whole lysate (WL) and clear lysate (CL) of *BLR* expression followed by *Origami* expression.

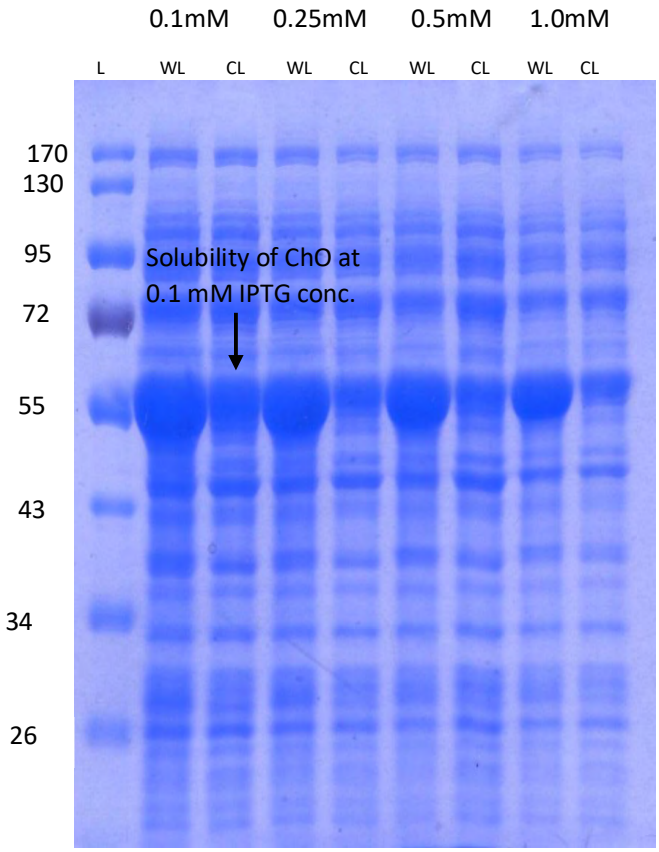


Figure 4.39 Expression of *BLR* at different concentration of IPTG (0.1 mM-1.0 mM), L represents ladder, WL and CL represent whole lysate and clear lysate of each concentration of inducer.

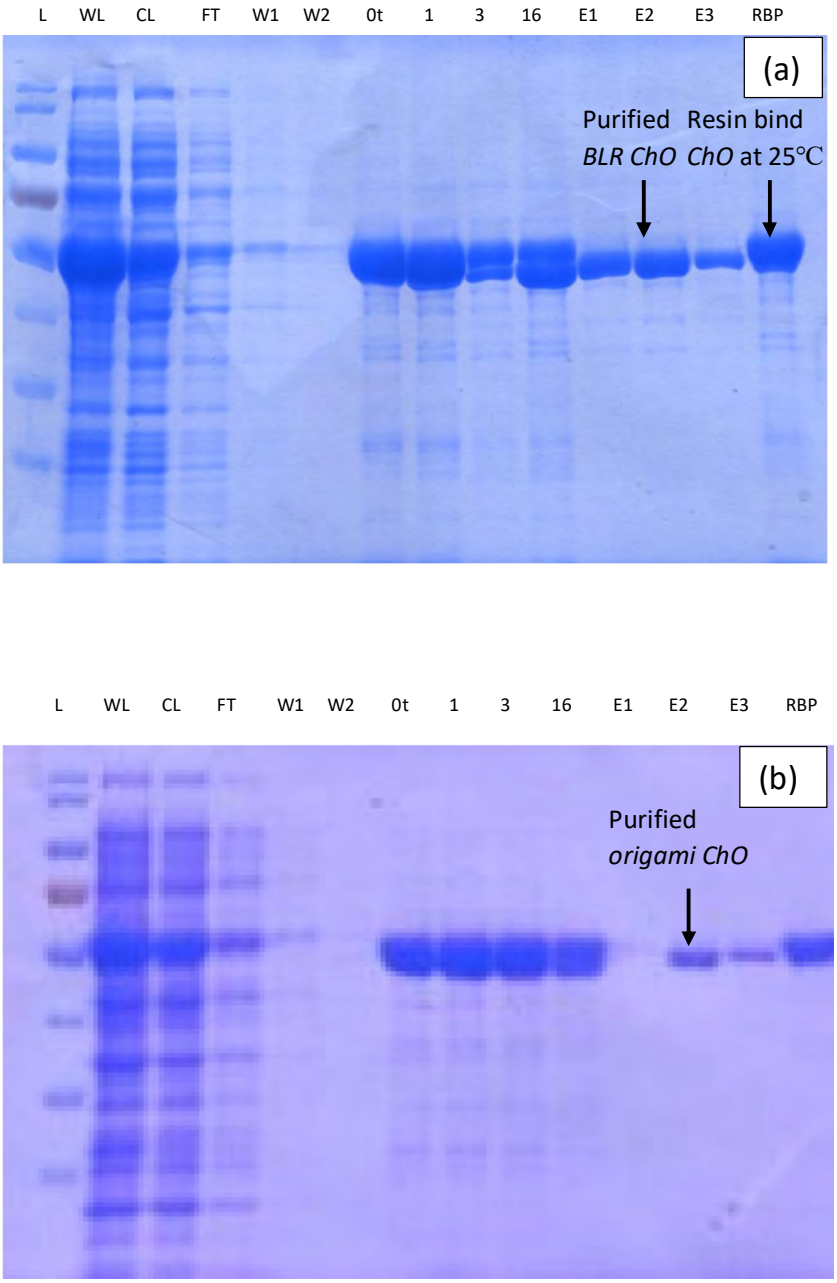


Figure 4.40 Purification of *BLR* (a) and *Origami* (b) expressed protein at room temperature (25°C), WL and CL represent whole and clear lysate, FT (flow through), W1, W2 (wash 1and 2), time of sample collection in hours (0, 1, 3,16), E represents Elusion (1-3) and RBP represents the resin bind protein after elusion.

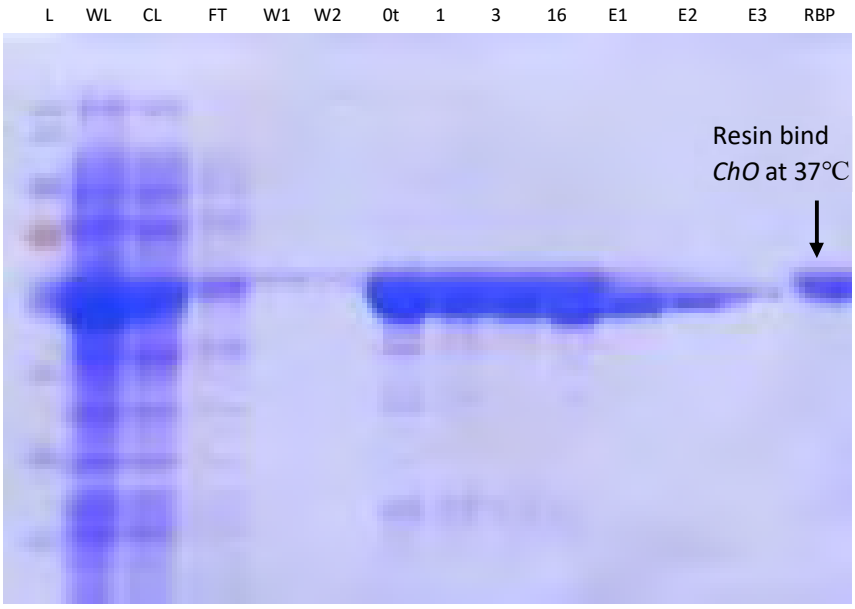


Figure 4.41 Purification of cholesterol oxidase at 37°C. WL and CL represent whole and clear lysate, FT (flow through), W1, W2 (wash 1and 2), time of sample collection in hours (0, 1, 3,16), E represents Elusion (1-3) and RBP represents the resin bind protein after elusion.

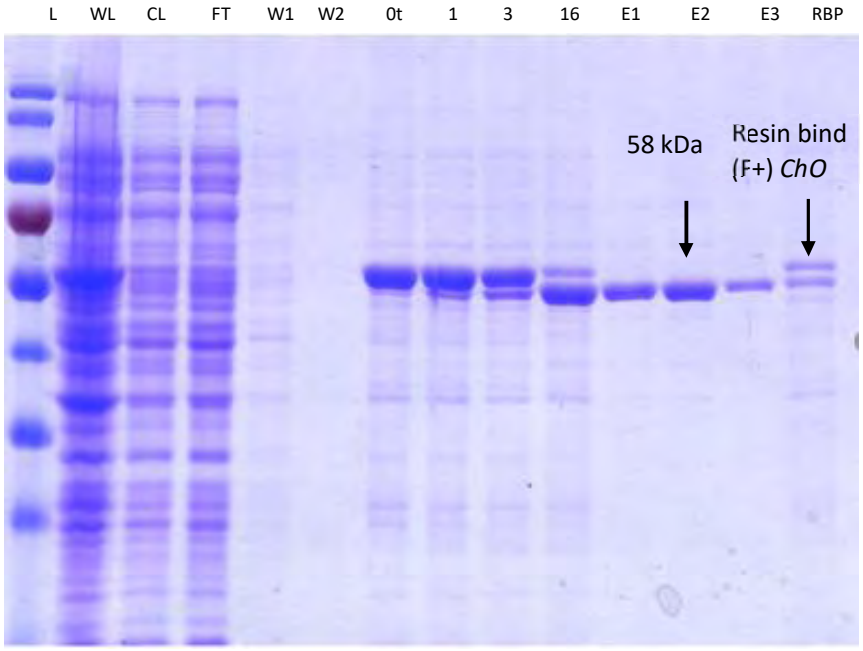


Figure 4.42 Engineered cholesterol oxidase purification. WL and CL represent whole and clear lysate, FT (flow through), W1, W2 (wash 1and 2), time of sample collection in hours (0, 1, 3, 16), E represents Elusion (1-3) and RBP represents the resin bind protein after elusion.

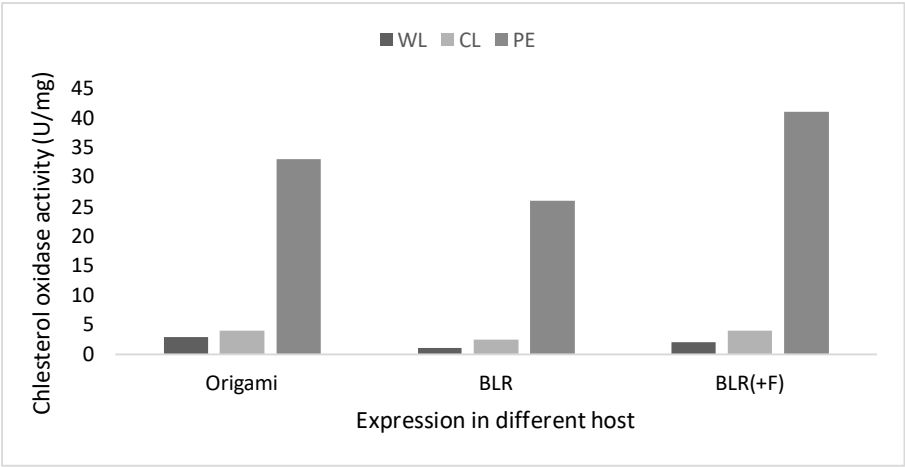


Figure 4.43 Activity of native and engineered recombinant cholesterol oxidase.

4.5 CHARACTERIZATION OF CHOLESTEROL OXIDASE

4.5.1 Kinetic parameters

For evaluation of cholesterol oxidase kinetic parameters, a range of pH (3-9), temperature (20-100℃), different solvents and detergents were employed with constant enzyme concentration (0.05 mg/ml). The activity of all reactions was monitored at 500 nm along with controls having PBS in place of enzymes and used as a blank. Enzyme activity was seen at all pH and temperatures with optimum activity at pH 6.0 and 60℃ while in case of different solvents and detergents maximum activity was recorded with methanol and in presence of tween 80 (Figure 4.44).

4.5.2 Immunogenicity prediction

Furthermore, immunogenicity of recombinant enzyme was determined by subjecting the amino acid sequence to IEDB. B cells epitopes were detected with an average threshold line with a value of 0.505 and number of amino acids were detected above the average line with maximum value of 0.585 corresponding to immunogenic response by activation of B cells while rest of amino acids fall below average line with minimum value of 0.211 and considered as non-immunogenic (Figure 4.45). Peptides predicted with B-cell immunogenic epitopes are represented in table 4.5. Along with B cell epitopes high value of 4.772 was predicted for T cells epitopes.

4.5.3 Cytotoxicity assay of cholesterol oxidase

To evaluate the cancer chemo preventive potential of bacterial cholesterol oxidase, number of cell lines were utilized. Total of three samples of lyophilized cholesterol oxidase purified from *Bacillus safensis* W1, *Bacillus subtilis* LSB and recombinant cholesterol oxidase of *Chromobacterium sp*. (*BLR* expressed) was used for evaluation of anticancer activity using SRB assay against three cancer cell lines. The obtained results revealed that recombinant and purified cholesterol oxidase showed variable degree of inhibitory activity against the tested cell lines. Recombinant cholesterol oxidase and cholesterol oxidase of *Bacillus safensis* and *Bacillus subtilis* at 13 U/ml inhibited the cell viability of colon cancer (HT-29) cells by 95.4, 92.2, 68.3% (Figure 4.46a) and prostate cancer (DU-145) cells by 96.6, 86.8, 81% (Figure 4.46b) respectively while in case of breast cancer cells (MCF-7), 80 % inhibition was seen with recombinant cholesterol oxidase, 63.8% with *B. safensis* W1, and 55.8% (Figure 4.46c) with *B. Subtilis* LSB was recorded.

4.5.4 Enzymatic assay of purified cholesterol oxidase for determination of cholesterol degradation

Treatment of cholesterol with purified cholesterol oxidase of Bacillus safensis W1 and Bacillus subtilis LSB and recombinant engineered ChO showed the development of intense red color in enzymatic assay reaction mixture (Figure 4.47). These results indicated the production of cholest-4-ene-3-one along with H_2O_2 as the initial reaction products by the action of cholesterol oxidase and further coupling reaction of H_2O_2 with assay mixture resulted the development of red Quinoneimine dye. Purified cholesterol oxidase of *B. safensis* W1*, B. subtilis* LSB and recombinant protein (*BLR* expressed) showed the maximum activity of 75 U/mg, 56 U/mg, and 41 U/mg, respectively.

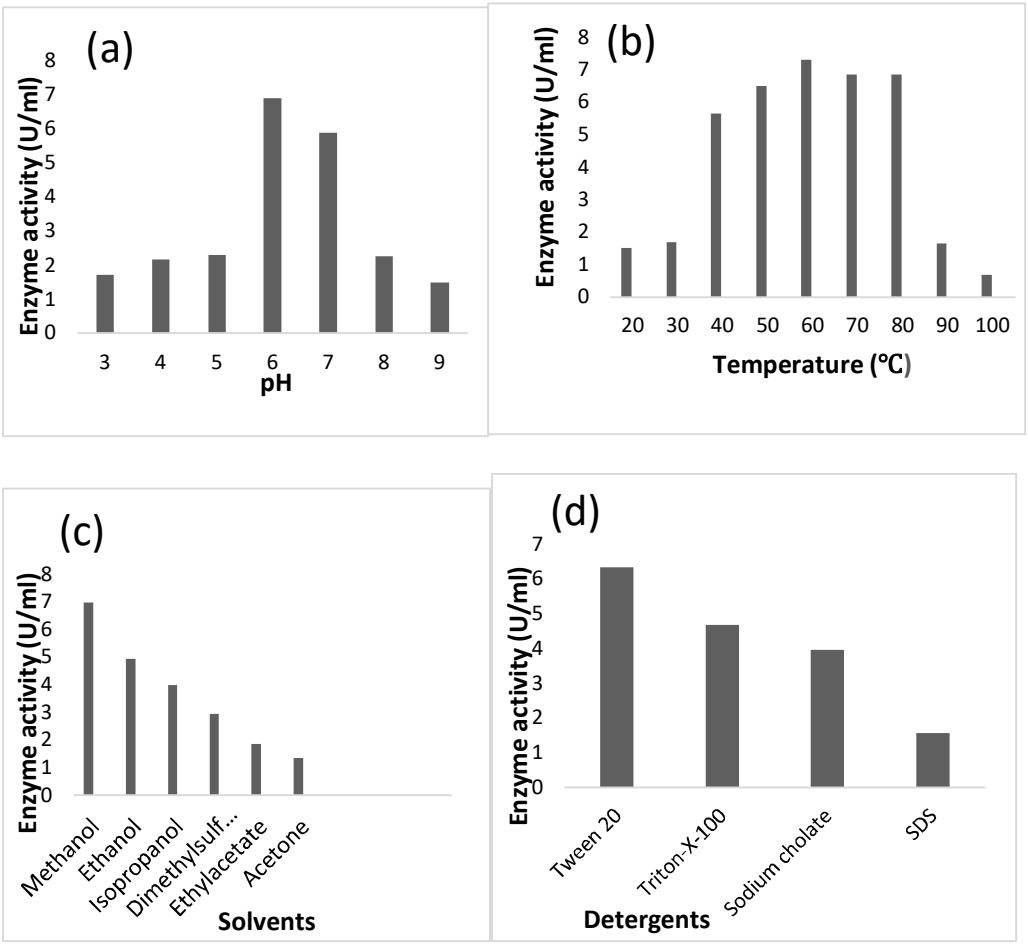


Figure 4.44 Activity of cholesterol oxidase at different pH (a), temperature (b) in the present of solvents (c) and detergents (d).

Table 4.5 Predicted peptides of B cells immunogenic epitopes.

Predicted peptides:

No.	Start	End	Peptide	Length
1	16	29	KQSFKNWAGDIKVD	14
2	33	45	TCAPRSADEVVKV	13
3	47	50	NWAK	4
4	59	68	RGMMHNWSPL	10
5	75	79	SCPAV	5
6	81	180	LLDTTRYLTAMSIDASGPVAKVTAQAGITMEALLTGLEKAGLGVTAAPAPGDLTLGGVLAINGHGTAIPAKGERRLAGASYGSISNLVLSLTAVVYDKAS	100
7	202	205	VGRS	4
8	207	225	IVEATLQAAPNQRLRCQSW	19
9	234	253	FAAAGSGGRTFASYLDSAGR	20
10	264	264	N	1
11	276	283	KPLFSRQT	8
12	296	314	PDEVTDLANKILSLGDGKL	19
13	319	340	GKAQFAAASAGLVATASWDLWG	22
14	354	359	LRVTAN	6
15	371	373	QRV	3
16	389	394	QQQGRY	6
17	400	401	VE	2
18	404	421	VTGLDDPSEAALSGGVAP	18
19	467	473	FNGSYAA	7
20	475	502	RPEWSKGWGYTDQAAWADSAMLQTTIPN	28
21	509	519	PAAANWDAAKA	11
22	531	537	SSPLLDS	7

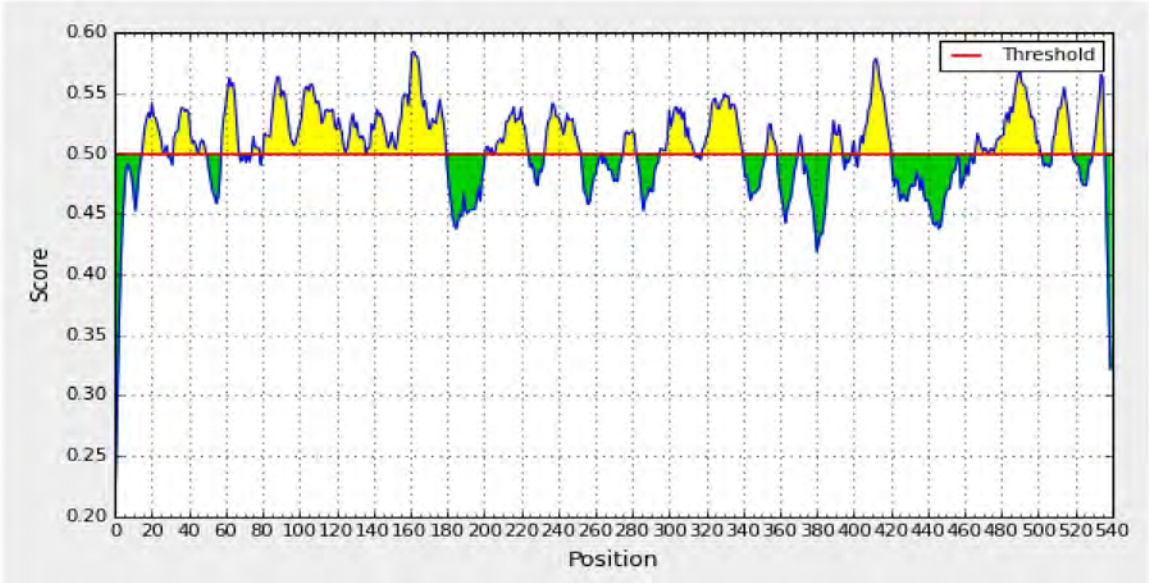
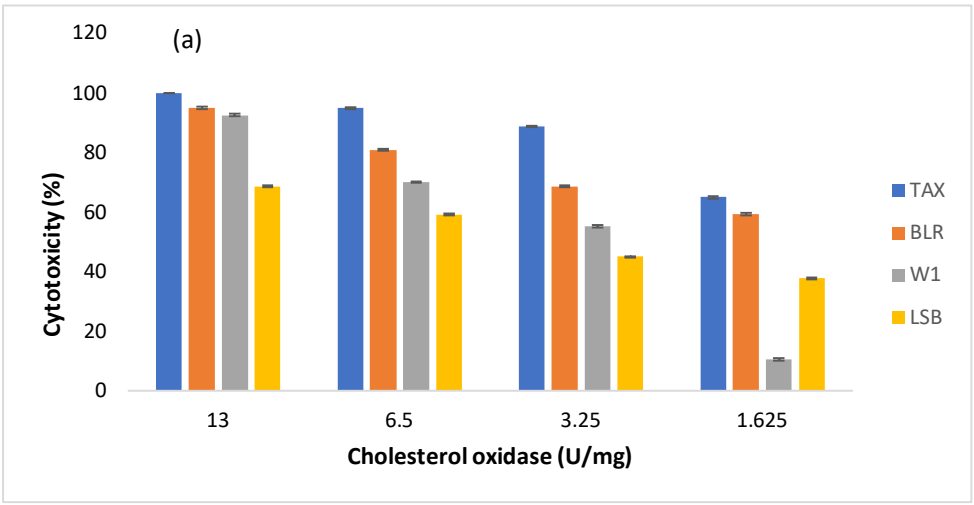
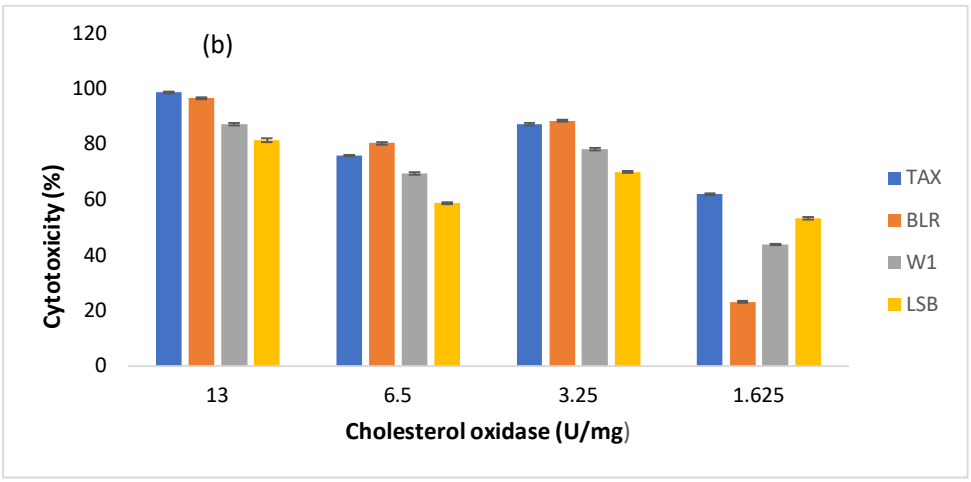


Figure 4.45 Cholesterol oxidase amino acids with B cells epitopes represented by yellow color and green color correspond to non-immunogenic epitope.





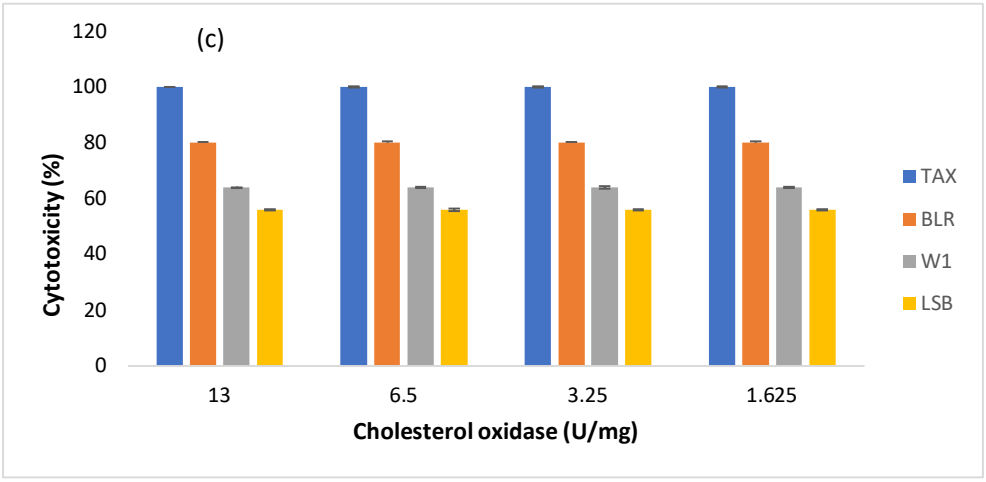


Figure 4.46 Cell toxicity assay of cholesterol oxidase against colon cancer cell lines (HT 29) (a), prostate cancer cell lines (DU-145) (b) and breast cancer cell lines (MCF-7) (c).

Figure 4.47 Reaction of purified cholesterol oxidase in enzymatic assay mixture.

DISCUSSION

Environmental pollution is one of the major problem that adversely affect the humans and other living organisms. The chemical and physical remediation processes are expensive and in majority of the cases do not result in complete removal of pollutants. Bioremediation is an attractive and promising approach to transform the toxic chemicals into less or nontoxic substances or complete removal of pollutants (Azubuike *et al.*, 2020). When bioremediation is employed to human body cells to degrade the aging cells garbage, it is termed as medical bioremediation. With increasing age, some pathogenic compounds build up at a rate that shows they are impervious or difficult to degrade by the existing enzymes resulting to improper cellular activity and functioning leading to development of certain metabolic disorders related to aging, including atherosclerosis, age related macular degeneration and neurodegenerative diseases.

Naturally, lysosome and proteasomes are responsible for biological macromolecules degradation. Different pathways, including endocytic, autophagic and phagocytic, facilitate the degradation of unwanted byproducts and macromolecules to maintain the normal cell functions (Trivedi *et al*., 2020). The intrinsic deficiency of the degradative process may lead to the accumulation of waste materials such as oxidized proteins, lipofuscin and undegradable material (Barbosa *et al*., 2019).

By identifying the exogenous enzymes with capability to degrade the disease-causing accumulated waste compounds, came up as a revolutionary therapeutic option to treat the aging diseases. Engineering of the specific enzyme for targeted cell type delivery in human cells either by enzyme replacement therapy or gene therapy is used as a mean of treatment for aging diseases. Some common aging diseases associated with compound accumulation include alzheimer disease, AMD, atherosclerosis, and cancer. Alzheimer disease is linked with accumulation of abnormal neurofibrillary tangles (NFT) and Aβ deposits. Accumulation of drusen and lipofuscin are the prominent features of the AMD while accumulation of cholesterol and its oxidized derivative specially 7-ketocholesterol are associated with development of atherosclerosis (Mathieu *et al*., 2009) and recently (Huang *et al*., 2020) it has been reported that cholesterol also play an important role in development of certain types of cancer. Without disturbing the normal homeostasis, the mentioned disease associated compounds can be targeted through microbial enzymes. Therefore,

removal of these build up pathogenic compounds by any treatment strategy would serve as a promising candidate in treatment of metabolic disorders.

Soil contaminated with fatty compound including oil, animals and humans remains is an ideal source for the isolation of bacterial isolates which could be used for the degradation of cholesterol and their derivative compounds (Banerjee *et al*., 2018; Khiralla, 2015). In this context, in current study the degradation of cholesterol and 7-ketocholesterol, implicated in pathogenesis of atherosclerosis, was tried through a number of environmental isolates. Soil from graveyard and contaminated with oil and ghee were investigated for the isolation of cholesterol degrading bacteria. The isolated strains having the cholesterol degrading abilities were identified on the basis of 16S rRNA gene sequencing as *Bacillus pumilus* W8, *Bacillus safensis* W1, *Bacillus subtilis* LSB, *Klebsiella* sp WS, *Psychrobacter* sp. GS3 and UP, *Stenotrophomonas pavanii* GS2, *Moretella sp.* GS6 and GS9, *Vibrio sp.* GS7 and DS, *Shewanella pealeana* GSS (Table 4.3).

Genus *Bacillus* is distributed widely in the environment and able to grow and colonize in different niches. The ability of *Bacillus* to adapt different environmental conditions is due to their modification of fatty acids (Diomonde *et al*., 2015). Previously different strains of *Bacillus* have been isolated from contaminated soil and other samples for cholesterol degradation. Kumari and Kanwar (2013) reported the *Bacillus sp*. for cholesterol degradation and similarly Kim *et al*., in 2020 isolated *Bacillus subtilis* with the cholesterol degradation capabilities. *B. subtilis* isolated from Ghee is also reported by Kumar and Kupsummay (2016) for the degradation of cholesterol. Banerjee *et al*., in 2018, isolated cholesterol degrading bacteria from oil contaminated soil. In present study, The use of contaminated soil for the isolation of bacterial strains suggest that the pre exposure and acclimatization of the isolates to the cholesterol and fatty acid compounds may lead to well adaptation to the contaminants and can be used effectively in remediation process. Beside the degradation of cholesterol, *Bacillus* genus is also reported for the biodegradation capabilities of other pollutants like pesticides including organophosphate, deltamethrin and prochloraz-manganese (Ali *et al*., 2012; Zhang *et al*., 2016; Jiang *et al*., 2019). *B. subtilis, B. amyloliquefaciens and B. cereus* were reported to degrade crude oil and petroleum products (Zhang *et al*., 2016; Wang *et al*., 2019; Christova *et al*., 2019).

Members of the Genus *Stenotrophomonas* have been reported in the biodegradation of wide range compounds including cholesterol (Banerjee *et al*., 2018), *p*-nitrophenol and 4-

chlorophenol (Liu *et al*., 2007), benzene and toluene (Lee *et al*., 2002) and EDTA (Kaparullina *et al*., 2009). Other bacteria isolated in the present study like *vibrio* is also reported previously for their degradation capacity of different organic compounds. Imron and Titah (2018) reported the *Vibrio alginolyticus* for the degradation of diesel. Peng *et al*., (2019) isolated a testosterone degrading *vibrio sp*. and reported that this specie could degrade other steroidal compound like 17β-estradiol (E2), 17β-ethinyl estradiol (EE2), estriol (E3) and cholesterol and use them as a sole source of carbon. The other bacterial strains isolated in the present study for cholesterol degradation are also reported for the degradation capabilities of different compounds like *Shewanella sp*. is reported for naphthalene degradation (Tarhriz *et al*., 2014), *Psychrobacter* for PCB catabolism (Luigi *et al*., 2007) and *Enterobacteriaceae sp*. for cholesterol degradation (Ye *et al*., 2008).

In the biodegradation studies of cholesterol by *B. pumilus* and *B. safensis*, different parameters were assessed. Based on the data the pH 7.0, 35℃ temperature, 1g/L concentration and 4 days of incubation with tween 80 were found optimum conditions for cholesterol degradation. Similar results were reported by Kumar and Kupsummay in 2016 that the degradation of cholesterol was highest at pH 7 and 37℃ temperature and when applied the cholesterol in medium with the concentration of 1 g/L by *B. subtilis* KAVK3. The incubation time for degradation of cholesterol was also analyzed previously by different research groups. Shobharani and Halimi (2015) reported that *B. licheniformis* MCC2514 showed maximum degradation of cholesterol after 4 days of incubation. *Mycobacterium* strain was also reported for the cholesterol degradation by Smith *et al*., (1993) and they reported that after four days of incubation in presence of tween 80 the maximum reduction of cholesterol was observed. Tween 80 and tween 20 are reported to enhance the degradation of cholesterol (Elbaz *et al*., 2017; Banerjee *et al*., 2018). Surfactants like tween 80 are widely used to reduce the surface tension and increase the solubility and bioavailability of insoluble compounds to increase the biodegradation efficiency (Suhail *et al*., 2019). The stimulatory and inhibitory effect of various metal ions on cholesterol degradation was also checked in order to find that either the metals have a role in binding of enzyme and substrate or may metals bind to the prosthetic group of the enzyme. $Mgso_4$ and $Cacl_2$ showed the stimulatory effect on cholesterol degradation while $Bacl_2$ and $Cocl_2$ have inhibitory effect and these findings are in agreement with Yehia *et al*., (2015).

The cholesterol degrading capability of environmental isolates were analyzed on HPLC. Various isolates showed different potential of cholesterol utilization and 58, 86, 16, 44, 57, 61 and 51% of cholesterol reduction was recorded when *B. safensis* W1, *B. pumilus* W8, *Stenotrophomonas pavanii* GS2*, Psychrobacter sp*. GS3, *Vibrio sp*. GS7, *Moretella sp*. GS9 and *Shewanella pealeana* GSS treated samples were analyzed on HPLC (Figure 4.18). Some of isolates including *Moretella sp*. GS6, *Shewanella pealeana* GSS, *B. safensis* W1 and B. *subtilis* were also checked for 7-KC degradation and significant reduction was seen when their extracts were analyzed on HPLC (Figure 4.19). Our findings are in agreement with Saranya *et al* (2013) they reported the appearance of standard cholesterol chromatogram at a retention time of 2.2 against tested (*Bacillus, Pseudomonas* and *Streptomyces* treated) samples but with less prominent peak when compared with standard indicating the cholesterol transformation. Furthermore, Andhale and Sambrani, (2006) also reported the biotransformation of cholesterol with reduced area of HPLC chromatogram of *Bacillus subtilis* treated sample. To further characterize the cholesterol metabolites of some treated cholesterol samples were analyzed on GCMS.

Several metabolites are produced when cholesterol is degraded and in the presence of oxygen various oxidized derivatives of cholesterol are generated such as α-epoxide, β-epoxide, 7-ketocholesterol, 7α-hydroxycholesterol and 7β-hydroxycholesterol. In addition, cholesterol can be degraded into other products including aldehydes, short-chain hydrocarbons, alcohols, fatty acids, and ketones (Dentas *et al*., 2015). During fermentation process of cholesterol, no significant increase of oxides was observed in the presence of bacterial cells suggesting that bacteria have also the ability to further degrade the oxidized cholesterol products into various metabolites (Kim *et al*., 2003). Oxidation of cholesterol is the initial step of cholesterol degradation then followed by dehydrogenation to form 4-androstendione (AD) which is further transformed into 1,4-androstendione and by action of reductase complex, 9-hydroxy-androsta-1,4diene-3,17-dione (ADD) is formed (Horinouchi *et al*., 2003). Further the ring degradation produces series of intermediates and propionic acid and succinic acid as final degradation products.

In our study many catabolic routes are predicted instead of one-directional pathway and several metabolites were detected on GCMS such as 7-oxocholesterol, E-9- tetradecenoic

acid, hexadecenoic acid, cis-vaccenic acid, 2-dodecene-1-yl-Succinic anhydride, octadecanoic acid, 26,27-Dinorergosta-5,23-dien-3-ol and 9,12-octadecadienoic acid in *Psychrobacter sp*. GS3, *Moretella* sp. GS6, *B. subtilis* LSB, *B. safensis* W1, *Vibrio sp*. DS and *Psychrobacter sp*. UP treated cholesterol samples (Figures 4.21-4.26). Initially, in degradation of cholesterol the oxidation takes place and number of oxidized derivatives could be generated. We find 7-ketocholesterol as an oxidation product of cholesterol in beginning keeping the rings intact. Formation of 26,27-Dinorergosta-5,23-dien-3-ol was detected as a new intermediate which may be by side chain degradation without any androsta compounds. It is concluded that cholesterol degradation by our isolates is carried out by an unknown degradation pathway without the formation of AD and ADD reported by other microbial species. Similarly, Drzyzga *et al*., (2011) reported the cholesterol degradation by *Gordonia cholesterolivorans* via unknown pathway without formation of AD and ADD compounds. According to Academia USSR, (1981) Ergosta compounds undergo through partly reduction leading to formation of androsta-1,4-diene-3,17-dione and its presence slow down the reaction that's why ergosta-3-4-diene were detected without androsta-1,4diene-3,17-dione among the transformation products. Another study on degradation of β-sitosterol by the mutant of *Rhodococcus equi* produced new compounds such as ergost-4-en-3-on-26-oic acid and ergosta-1,4-dien-3-on-26-oic acid which was not reported before (Murohisa and Iida, 1993). Furthermore, in the present study several straight chain fatty acids were detected including hexadecenoic acid and octadecanoic acid. Ghosh and Khare (2017) also reported octadecanoic acid as straight chain fatty acid in later steps of 7-ketocholesterol degradation by *Rhodococcus erythropolis*.

According to studies, cholesterol can be removed from media during bacterial growth either by directly uptake of cholesterol or co-precipitation/binding of cholesterol to living cells and by action of several extracellular enzymes secreted in media. The import of any molecule into bacterial cell is difficult due to presence of hydrophobic membrane (Brennan 2003). To facilitate the entry of cholesterol and related sterols across the problematic cell envelope, multiprotein complex known as Mce4 is reported in *Mycobacterium tuberculosis*, *Rhodococcus equi* (van der Geize *et al*., 2008b) and in *R. jostii* RHA1 (Mohn et al., 2008). Furthermore, mutants of Mce4 locus of *M. tuberculosis* and *R. jostii* failed to grow in a medium containing cholesterol as the only carbon source confirmed the requirement of Mce4 system for cholesterol import (Nazarova *et al*., 2017; Perkowaski *et al*., 2016). It has been reported that cholesterol reduction mechanism in probiotic bacteria

takes place by several bioactivities including integration of cholesterol into cell membrane, inhibition of coenzyme A reductase and production of cholesterol reductase and aldolase (Lye *et al*., 2010; Kimoto *et al*., 2002; Tsai *et al*., 2014). Recently much attention has been given to cholesterol oxidase found to be involved in catalyzation of initial steps of cholesterol. Although complete pathway of cholesterol degradation is not elucidated in any specie and it is concluded that assimilation and utilization of cholesterol in bacterial isolates varies across the specie and strains.

Microbes from contaminated soil can degrade the cholesterol and 7-ketocholesterol efficiently by their enzymes. Flavin adenosine diphosphate (FAD) containing cholesterol oxidase was expected to be involved in degradation of cholesterol and 7-ketocholesterol as this enzyme is already reported in biodegradation of cholesterol and related sterols (Kreit, 2017). The production of red color (quinoneimine dye) on assay plate proved the presence of cholesterol oxidase in all the strains under study. During cholesterol degradation by cholesterol oxidase, the molecules of hydrogen peroxide is produced which react with chromogenic indicators such as phenol and 4 aminoantipyrine in presence of peroxidase and produced red color (Lashkarian *et al*., 2010; El-Naggar *et al*., 2017; Barbosa *et al*., 2019). The production of cholesterol oxidase during cholesterol degradation is detected by color plate assay and reported by different research groups in a variety of bacterial isolates including *Burkholderia* and *Chromobacterium* (Doukyu, 2009), *Bacillus sp.* (Kumari and kanwar, 2013), *B. subtilis* (Kim *et al*., 2002).

Several factors including the composition of production medium and different sources of carbon affect the yield of cholesterol oxidase. In our study, the cholesterol oxidase production was highest by different bacterial isolates when LB was used in double concentration. Kuppusamy and Kumar (2016) reported the maximum production of cholesterol oxidase by *B. cereus* when the production medium is enriched with fructose. Sahu *et al* in 2019 studied the *Streptomyces* for cholesterol oxidase production ability and found the optimal production of ChO, in media containing yeast extract, starch, peptone and glucose in combination. These finding suggest that along with cholesterol other complex sources of carbon and nitrogen favors the ChO production indicating the direct influence of substrate on production.

In our studies number of strains showed the cholesterol oxidase activity and the promising isolates *Bacillus safensis* W1 and *Bacillus subtilis* LSB cholesterol oxidase was purified by

using DEAE anion sepharose column and characterized with a purified band of 58 kDa and 22 kDa when analyzed on 12% SDS-PAGE (Figure 4.33a, b) . Cholesterol oxidases vary in number of amino acids most of ChO have approximately 550 amino acids. Several investigators studied the different bacterial ChO and reported the different ranges of the enzyme in terms of their size which ranges from 20-61 kDa. Gopal *et al*., (2014) reported the Cholesterol oxidase from *Stenotrophomonas sp*. which has molecular weight of 20.5 kDa. Kumara and Kanwar (2015) reported a 21.5 kDa size ChO from *B. subtilis*. In some bacterial species more than one ChO have been reported. Rhee *et al*., in 2002, purified two different ChO enzyme from *Bacillus sp*. SFF34 with 36 and 37 kDa molecular size. *B. cereus* isolated from agriculture waste is reported to produce 55 kDa molecular size ChO (Jayachitra *et al*., 2012). However, the molecular weights of ChO enzyme purified from *Burkholderia cepacia* (Aunpad *et al*., 2002), *Streptomyces sp*. SA-COO (Lario and, Vrielink, 2003) *Gamma-proteobacterium* Y-134 (Isobe *et al*., 2003) and *Enterococcus hirae* (Yehia *et al*., 2015) were 58.7, 55, 58 and 60 kDa, respectively.

Genetic engineering improved the catabolic activities of microorganisms and advancement in recombinant DNA technology opened the possibilities towards genetically engineered microbes for specific functions by combining the different genes from various organisms in a single host. Several studies have been conducted to isolate the cholesterol oxidase gene from *Arthrobacter simplex, Rhodococcus Erythropolis, Brevibacterium Sterolicum, Burkholderia Cepacia* and *Streptomyces sp*. SA-COO and expressed in heterologous system (Parekh, 2012; Chandrasekaran, 2010). In present study cholesterol oxidase has been purified from *B. safensis* and *B. subtilis* successfully but due to unavailability of literature on ChO genes of these isolates we were not able to carry further studies on expression. As a model we planned to get the synthetic cholesterol oxidase gene of *Chromobacterium* DS1 cloned in pET vecrtor by Gibson assembly reaction for heterologous expression in *E. coli* with NpuC (split intein) as novel purification tag. Furthermore, the successful transformation and expression optimization of cholesterol oxidase gene was carried out.

Gibson Assembly from NEB (Ipswich, MA, USA) is one of effective cloning method based on principle of Ligation Independent Cloning LIC (Gibson *et al*., 2009). Gibson assembly is considered as one of the preferred method due to its ability of restriction independent insertion of one or more fragments of DNA in linearized vector at any position. In high-

throughput cloning Gibson assembly is convenient due to its efficiency, versatility, speed and scar less assembly with vector (Thomas *et al*., 2015).

For expression of recombinant proteins *E.coli* is considered one of the most ideal organisms and being used for production of most FDA approved therapeutic proteins. *E.coli* is a perfect choice due to its well established cell factory with characterized genome, availability of several host, cost effectiveness, accessibility of adoptable vectors and high level expression of desired protein (Joseph *et al*., 2015). According to Sezonov *et al*., (2007) *E.coli* is an advantageous expression system because of its fast growth kinetics in simple and cheap glucose salt media with least doubling time of about 20 minutes. This means high cell density cultures can easily achieved when medium is inoculated with a starter culture of 1/100 dilution. Furthermore, Shiloach and Fass, (2005) added that due to fast transformation with exogenous DNA and by using inexpensive readily available components in media for expression optimization make the *E.coli* as a preferable expression cells.

The pET plasmid system was used for gene cloning. pET vector series based on T7 phage RNA polymerase promoter are considered most powerful system for recombinant protein expression. pET vector has the capability to tightly regulate the expression and upon induction produces maximum levels of transcripts leading to desired proteins. In successful cloning 50% of the total protein expression represents the target protein (Graumann and Premstaller, 2006).

Further several strains of *E. coli* such as *BLR, BL21, SHT7* and O*rigami* were used for protein expression studies. These strains of *E. coli* are widely used for expression as *BLR* strains are Lon proteases deficient and *Origami* strains with glutathione reductase and thioredoxin reductase mutations, with these characteristics the cells are used for expression of proteins with disulfide bonds. The factors which limit the expression of gene could be disulfide bond formation and proper protein folding and this is confirmed by Xu *et al*., 2008 when they get the improved protein expression with O*rigami*.

We failed to get the expression of cholesterol oxidase in *SHT7* and *BL21* cells and expression was seen with *BLR* and *Origami* but in insoluble form. Over expressed ChO may accumulate into inclusion bodies. In recombinant bacteria metabolic burden usually occurs (Bentley *et al*., 1990). Some of the factors such as expression medium, strong

promoter, high temperature and inducer concentration enhance the transcription of recombinant protein that favors the inclusion body formation (Ahmad *et al*., 2018; Ma *et al*., 2013). Moreover, proper folding of recombinant protein that facilitates to produce the protein in soluble form could be enhanced by reducing the concentration of protein which is ultimately achieved by at low incubation temperature (Vera *et al*., 2007; Peti and Page, 2007). Considering these we employed the various strategies to express the cholesterol oxidase in soluble form and soluble protein was obtained in TB media after 16 hours of incubation at low temperature (20℃) and low inducer (0.1mM) concentration. In previous study cholesterol oxidase of same strain *Chromobacterium sp*. was expressed in BL21 strain of *E. coli* and found with maximum expression and solubility in TB media at low incubation temperature (15℃) and low inducer concentration (0.25 mM) (Fazaeli *et al*., 2018). In another study high biomass and ChO production was achieved with use of glycerol and high concentration of yeast extract in expression medium (Collins *et al*., 2013). It is concluded that presence of glycerol and high buffering capacity of TB media favors the expression of several recombinant proteins.

To enhance the production and purification of recombinant proteins, different types of tags are used to fuse with target proteins. These tags increased the solubility and stability along with decreased host cell toxicity (Arnau *et al*., 2006; Young *et al*., 2012; Karav *et al*., 2017). In present study recombinant cholesterol oxidase (under optimized conditions) is first time purified with engineered intein tag. The target protein fused either N or C-terminal to the tag release at once in response to pH shift. The critical feature of these tags is due to specific cleavage reaction that prevents the unwanted cleavage and cleaves the target protein without addition of proteases (Banki and Wood, 2005). Recently protein purification based on intervening protein (intein) enabled the high throughput applications due to its consistency, reliability, and cost effectiveness (Gierech and Wood, 2019).

Furthermore, engineered ChO by one amino acid change efficiently enhanced the cleavage of protein from resin (Figure 4.44) which ultimately significantly increased activity from 26 U/mg to 41 U/mg. For production of therapeutic proteins this new technology possibly provides a disruptive method for highly purified protein at research and manufacturing scale.

Purified recombinant cholesterol oxidase was characterized and found to be most active at pH 6 followed by neutral pH 7, at 60℃ and active in various organic solvents and detergents

with optimum activity in presence of Tween 80 and methanol (Figure 4.46). This is suitable pH required for the cholesterol oxidase activity in acidic environment of the lysosomes but with accumulation of cholesterol and its derivatives may increase the pH to more acidic to neutral (Jerome *et al*., 2008) and these results are in agreement with previous reports on recombinant cholesterol oxidase expressed and purified from different isolates (Doukyu *et al*., 2008; Fazaeli *et al*., 2018; Kavita and Savithri, 2020). Furthermore, B and T cells epitopes of recombinant cholesterol oxidase is predicted, best to my knowledge it is the first report on immunogenicity prediction of recombinant ChO. The increased utility of Immune Epitope Database (IEDB) is due to find the critical amino acid by analysis of identified epitope sequence. Furthermore, immunogenic epitopes determination by IEDB is easier, efficient, cost-effective and mimic the steps for further analysis by in vitro assay (Bryson *et al*., 2010).

Beside the role of cholesterol and its derivatives in development of atherosclerosis, cholesterol and their metabolites are considered as a contributing factor in development of cancer (McDonnell *et al*., 2014). Up-regulation of cholesterol synthesis is associated with decreased survival of the patients of acute myeloid leukemia and melanoma (Hasin *et al*., 2015; Gabitova *et al*., 2015). Furthermore, in mouse model mammary tumors was detected with increased plasma cholesterol level indicating the cholesterol as oncogenic stimuli (Llaverias *et al*., 2011). In maintenance of cancer stem cells cholesterol biosynthesis has a crucial role by activating several cancers related cellular signaling pathways (Kim, 2019) and this is confirmed by Ehmsen *et al*., (2019) when they observed the elevated cholesterol in patients derived cancer stem cells. Thus, all these evidences ensure the significant role of cholesterol in cancer progression.

The cytotoxic effect of cholesterol oxidase was studied directly against HT-29, MCF-7 and DU-145 human cancer cell lines. ChO treated cells remained colorized visibly, suggesting significant lysis of the cells and further the cell death is confirmed by tracking OD at 510 and above 90% cell toxicity was observed with 13 U/ml cholesterol oxidase concentration (Figure 4.48). In agreement of our findings there are several reports on anticancer activity of cholesterol oxidase. *Streptomyces* ChO showed activity against number of cell lines including breast cancer, colon carcinoma, rhabdomyosarcoma, (MCF-7), cervical epithelioid carcinoma and hepatocellular carcinoma and also act as an apoptosis inducer when tested in vivo (El-Niggar *et al*., 2018). Another study on cytotoxicity of marine *Streptomyces sp.* cholesterol oxidase was conducted by Kavitha and Savithri, (2019)

against Breast, Ovarian and nasopharyngeal cancer cell lines and concluded that ChO have a significant cytotoxicity potential.

It has been reported that in progression of several types of cancer cholesterol plays critical roles as cholesterol is an important part of membrane raft involved in regulation of signaling associated to cell proliferation (Kuzu *et al*., 2018). This is supported by Coogan *et al*., 2002; Li *et al*., 2006 when they found cholesterol rich membrane rafts in prostate and breast cancer cells as compare to normal counterparts. The role of membrane raft cholesterol in cancer progression is recently proved when another research group used methyl-β-cyclodextrin as a cholesterol lowering agent on breast cancer cells resulted in apoptosis induction due to disruption of lipid rafts (Badana *et al*., 2018). The strong association between cholesterol level and cancer development showed that cholesterol lowering agents such in our findings bacterial cholesterol oxidase could be used as a natural potential anticancer drug.

In this study, different bacterial isolates from different geographical locales and genera were isolated and identified having the degradation potential. It is summarized from the study that isolated strains have the capability to degrade and transform the cholesterol. Some of isolates including *B. safensis* W1*, B. pumilus* W8*, Moretella sp.* GS6 and *Shewanella pealeana* GSS were found with potential to degrade 7-ketocholesterol. Cholesterol oxidase is the initial enzyme involved in transformation of cholesterol and its oxidized derivatives. All the tested isolates were positive for ChO production and *B. safensis* W1 and *B. subtilis* LSB were found with maximum production of ChO. The expression of *ChO* of our strains was not carried out due their gene sequence is not reported yet. Therefore, the *ChO* gene was synthesized from the known sequence of *Chromobacterium sp.* then cloned, expressed, purified, and characterized. Purified cholesterol oxidase showed the maximum activity in cholesterol assay reaction by catalyzing cholesterol and releasing the degradation products. Components of assay mixture react with generated H_2O_2 leading to development of chromogenic compound. These results indicated that active cholesterol oxidase was purified from both strains and cloned expression cells. Cholesterol oxidase offers new therapeutic option for medical bioremediation with potential to degrade the disease related compounds such as cholesterol and 7-KC and have anticancer activity against several cancer cells.

CONCLUSIONS

1. Soil contaminated with fatty compounds from various sources including oil, ghee, human and animal contaminants could be a rich source for cholesterol and related compounds degrading microbes.

2. From HPLC data, the isolated strains showed the ability to degrade and utilize cholesterol and 7-ketocholesterol.

3. GCMS analysis showed the transformation of cholesterol into different metabolites by the action of bacterial catabolic enzymes with an unknown degradative pathway.

4. There is an effect of pH, temperature, incubation time, substrate concentration on degradation and transformation of cholesterol by selective bacterial isolates and optimum conditions are 7.0, 35°C, four days and 1 g/L respectively.

5. Quantitative and qualitative analysis showed the production of cholesterol oxidase by all the bacterial isolates with maximum enzyme ability of two isolates *B. safensis* W1 and *B. subtilis* LSB.

6. Cholesterol oxidase was purified from *B. safensis* W1 and *B. subtilis* LSB showing molecular weight of 58 kDa and 22 kDa respectively.

7. Cholesterol oxidase gene was synthesized from known gene sequence of *Chromobacterium* and successfully cloned in pET21, expressed, and purified.

8. There was an effect of inducer concentration (IPTG) on solubility of recombinant protein. Maximum solubility of recombinant cholesterol oxidase was achieved at lowest concentration of IPTG (0.1 mM) used as an inducer.

9. Recombinant ChO revealed the high stability at pH 6, temperature 60°C, and in the presence of methanol and tween 80.

10. Cholesterol oxidase from bacterial isolates has potent anticancer activity against different cell lines which is comparable with anticancer drug Taxol as this indicates their great potential as an anticancer drug.

11. B and T cells immunogenic epitopes of cholesterol oxidase was predicted by analyzing the amino acid sequence of protein in immune epitope database.

FUTURE PROSPECTS

- Steroid contaminated soil and water can be exploited for isolation of other sterol degrading bacterial strains.
- These strains can also be checked for other diseases associated recalcitrant compounds biodegradation and transformation.
- Cholesterol oxidase gene of *Bacillus safensis* could be identified and cloned for enhanced production of cholesterol oxidase.
- Degradation of cholesterol and 7-ketocholesterol could be checked with purified cholesterol oxidase.
- To reduce the cholesterol oxidase immunogenicity several PEG (polyethylene glycol) molecules could be used.
- There is a need to search an efficient drug delivery system to target the specific pathogenic accumulated cholesterol and 7-ketocholesterol.
- Animal models could be used to check the catalytic potential of cholesterol oxidase.
- Further characterization of cholesterol oxidase is needed to be used in cancer treatment.
- Medical bioremediation potential of cholesterol oxidase could be exploited for several aging diseases.

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