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I, **Shraddha Sahu**, certify that the work embodied in this Ph. D. thesis is my own bonafide work carried out by me under the supervision of **Prof. Rathindra Mohan Banik** for a period of **7 years and 9 months from September 2011 to June 2019** at School of Biochemical Engineering, Indian Institute of Technology (Banaras Hindu University) Varanasi. The matter embodied in this Ph. D. thesis has not been submitted for the award of any other degree/diploma.

I declare that I have faithfully acknowledged, given credit to and referred to the research workers wherever their works have been cited in the text and the body of the thesis. I further certify that I have not willfully lifted up some other's work, para, text, data, results, etc. reported in the journals, books, magazines, reports, dissertations, thesis, etc., or available at websites and included them in this Ph. D. thesis and cited as my own work.

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Place: Varanasi

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(Shraddha Sahu)

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28/06/19
Prof. Rathindra Mohan Banik
(Supervisor)

(Signature of the HOD/Coordinator with seal)

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I asked for Strength and
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I asked for Wisdom and
God gave me Problems to solve,
I asked for Prosperity and
God gave me Brain and Brawn to war,
I asked for Courage and
God gave me Danger to overcome,
I asked for Love and
God gave me Troubled people to help,
I asked for Favour and
God gave me Opportunities,
I Received Nothing I wanted,
I Received Everything I Needed!

PREFACE

Cholesterol is the most abundant lipid found in the human body including the brain, spinal cord, serum, and other tissues. Cholesterol plays important physiological functions as well as structural roles in a cell; as an essential component of cell plasma membranes, neurotransmitters, and hormones. The U.K. government, as per the 1992 Health of The Nation Report (The Health of The Nation, 1992) and the United States as per the recommendations of the National Research Council (National Research Council, 1989) have recognized the need to limit dietary cholesterol intake. The elevated level of serum cholesterol poses certain life-threatening risks to human life and also plays a major role in the onset of serious diseases like atherosclerotic plaque formation, hypertension and coronary heart disease, Alzheimer's disease, etc. With the increasing risk of these diseases, monitoring the total serum cholesterol on a routine basis is the only way to stay away from these lifestyle disorders.

The serum cholesterol analysis is usually performed using a three enzyme assay (Cholesterol esterase, Cholesterol oxidase, and Peroxidase) and an indicator dye. Cholesterol oxidase is one of the key enzymes used in the estimation of cholesterol concentration in human serum. In recent times, the medical application of Cholesterol oxidase is mainly focused on the field of clinical diagnostics for the detection of cholesterol concentrations in human serum and the development of Cholesterol oxidase based cholesterol biosensors. The present thesis focuses on different aspects of the fermentative production of cholesterol oxidase, purification and some basic physicochemical properties of cholesterol oxidase and its application in medical field.

This thesis contains nine chapters and each chapter discusses different facets of the research related to the Cholesterol oxidase enzyme. *Chapter 1 – Introduction*: introduces the reader first to the important physiological functions of cholesterol in the human body and the problems related to elevated serum cholesterol; second, the method of serum cholesterol analysis based on Cholesterol oxidase; and third the possible ways to reduce the cost of downstream processing in the commercial production of Cholesterol oxidase. An overview of Cholesterol oxidase and the rationale of the present study has been conversed. *Chapter 2: Literature Review* gives an idea about the historical background of Cholesterol oxidase viz. different microbial sources and their mechanism of action; heading towards the advancements in the methods of production, purification, and applications of Cholesterol in diverse fields. *Chapter 3: Selection of Microorganism for Cholesterol oxidase Production and Optimization of Enzyme Assay Parameters* describes about the Cholesterol oxidase produced by six different *Streptomyces sp.*; the selection of most potent cholesterol oxidase producer strain and selection of the most suitable assay method for cholesterol oxidase estimation among the various methods described and optimization of cholesterol oxidase enzyme assay parameters. *Chapter 4: Optimization of Process Parameters and Medium Components for Cholesterol oxidase Production* describes about the optimization of medium components (carbon and nitrogen sources, effect of surfactants and inducer concentration) and statistical optimization of the fermentation process parameters through Response Surface Methodology and Artificial Neural Network modeling for the over-production of Cholesterol oxidase. *Chapter 5: Partitioning of Cholesterol oxidase in Different PEG-Salt-Water Aqueous Two-Phase Systems* illustrates downstream processing aspect of the enzyme cholesterol oxidase.

Among the eight different types of PEG-Salt-Water aqueous two-phase systems designed, PEG-Ammonium sulfate-Water system was chosen for the cholesterol oxidase purification for maximizing the final yield and purity. *Chapter 6: Physicochemical properties of Partitioned Cholesterol oxidase* discusses the biochemical properties (pH and temperature optima and effect of substrate concentration) and stability of cholesterol oxidase under various conditions including pH and temperature stability and stability in the presence of various organic solvents, detergents, chelating agents and metal ions. *Chapter 7: Immobilization of Horseradish Peroxidase on Graphene oxide coated Magnetic Chitosan Beads for Applications in Cholesterol oxidase Assay* describes the details about the development of a cost-effective Cholesterol oxidase assay method by the immobilization of horseradish peroxidase onto graphene oxide coated magnetic chitosan beads, which is one of the enzyme used in the estimation of cholesterol oxidase. The preparation of graphene oxide coated magnetic chitosan beads, characterization, and biochemical properties of the soluble and immobilized horseradish peroxidase were studied. *Chapter 8: Application of Cholesterol oxidase Immobilized Chitosan Beads for Estimation of Serum Cholesterol* describes how the partitioned cholesterol oxidase was recovered from the top-phase of PEG-Ammonium Sulfate-Water two-phase system and the study of surface morphology of the bare and enzyme loaded chitosan beads. The medical application of chitosan loaded cholesterol oxidase in the serum cholesterol assay has been covered in this chapter. *Chapter 9: Conclusion* discusses the summary and conclusive outcomes of the different experiments (described in Chapter 3-8).

The thesis is the original work done and written by the author. The purification and biochemical properties of cholesterol oxidase were performed under the guidance of Prof.

Medicherla Venkata Jagannadham in the Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University. The blood collection for serum cholesterol assay was done in the Students' Health Center with the permission of Chief Medical Officer and the required ethical clearance for performing the serum assay was obtained by the Ethical Committee, Institute of Medical Sciences, Banaras Hindu University. The chapters described in this thesis have been published in part or as a whole in various peer-reviewed journals.

DEDICATED TO

SHRI KASHI VISHWANATHJI,

MY GRAND PARENTS, PARENTS

&

MY LOVING DAUGHTER

ABBREVIATIONS

AAD	Absolute Average Deviation
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
ATPE	Aqueous Two Phase Extraction
ATPS	Aqueous Two Phase System
BSA	Bovine Serum Albumin
CCD	Central Composite Design
Chox	Cholesterol oxidase
˚C	Degree centigrade
DEAE	Diethyl Aminoethyl
DMF	Dimethyl Formamide
DMSO	Dimethyl Sulfoxide
EC	Enzyme Classification
FTIR	Fourier Transform Infrared Spectroscopy
GA	Glutaraldehyde
GO	Graphene oxide
GOMC	Graphene oxide coated magnetic chitosan
h	Hour
HRP	Horseradish Peroxidase
LM	Levenberg Marquardt
MAPE	Mean Absolute Percentage Error
MC	Magnetic chitosan

ABBREVIATIONS

MLP	Multi Layer Perceptron
MSE	Mean Squared Error
MTCC	Microbial Type Culture Collection
MW	Molecular Weight
µl	Micro liter
mM	Millimolar
PAGE	Poly Acrylamide Gel Electrophoresis
PEG	Polyethylene Glycol
PGOMC	Peroxidase immobilized magnetic chitosan beads
rpm	Revolution per minute
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulphate
S. olivaceus	*Streptomyces olivaceus*
VSM	Vibrating Sample Magnetometer
XRD	X-ray diffraction
ml	Milliliter
h	Hour
s	Second
min	Minute
U/ml	Units per milliliter
R^2	Regression coefficient
M	Molar

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SYMBOLS USED

°	Degree
%	Percentage
α	Alpha
β	Beta
±	Plus minus
<	Less than
>	Greater than
θ	Theta
/	Divide
x	Multiplication
=	Equal to