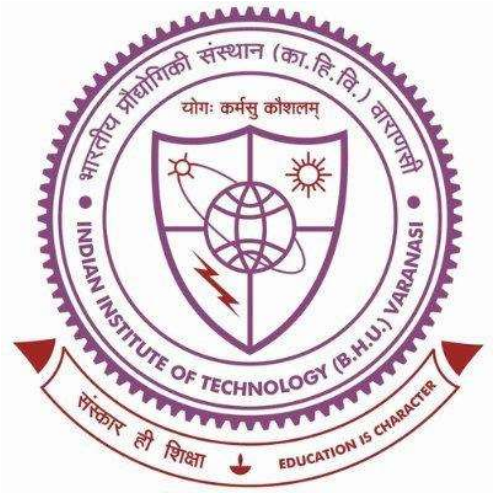
L-asparaginase production from microbial source, purification, characterization and application



Thesis submitted in partial fulfilment for the

Award of Degree

Doctor of Philosophy

By

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SCHOOL OF BIOCHEMICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY
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CERTIFICATE

It is certified that the work contained in the thesis titled " ***L-asparaginase production from microbial source, purification, characterization and application*** " by *"Deepankar Sharma"* has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.

It is further certified that the student has fulfilled all the requirements of Comprehensive Examination, Candidacy and SOTA for the award of Ph.D. Degree.

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I, **Deepankar Sharma**, certify that the work embodied in this thesis is my own bonafide work and carried out by me under the supervision of **Dr. Abha Mishra** from July 2016 to December 2022, at School of Biochemical Engineering Indian Institute of Technology (BHU), Varanasi. The matter embodied in this thesis has not been submitted for the award of any other degree/diploma. I declare that I have faithfully acknowledged and given credits to the research workers wherever their works have been cited in my work in this thesis.

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It is certified that the above statement made by the student is correct to the best of my/our knowledge.

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(DEEPANKAR SHARMA)

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
°C	Degrees Celsius
A_{280}	Absorbance at 280 nm
AHA	Aspartyl β-hydroxamate
ALL	Acute lymphoblastic leukemia
AO	Acridine orange
ANN	Artificial neural network
ANOVA	Analysis of Variance
BBD	Box-Behnken design
CCD	Central composite design
CD	Circular dichroism
cfu	Colony forming units
CSL	Corn steep liquor
DEAE Sepharose	Diethylaminoethyl Sepharose
DMSO	Dimethyl Sulfoxide
EC	Enzyme classification
EDTA	Ethylene diamine tetra acetic acid
FBS	Fetal bovine serum
FDA	Food and Drug Administration
g	grams
gds	gram of dry substrate
h	hour

HMF	Hydroxymethylfurfural
IU	International Unit
IEC	Ion exchange chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
k_{cat}	Turnover number
k_{cat}/K_m	Specificity constant
kDa	Kilo Dalton
K_m	Michaelis-Menten constant
LM	Levenberg-Marquardt algorithm
M	Molar
MSE	mean squared error
mg	milligram
mL	milliliter
min	minutes
MTCC	Microbial Type Culture Collection
MTT	4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCBI	National Center for Biotechnology Information
nm	nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBD	Plackett Burman design
PI	Propidium iodide
rpm	Revolutions per minute
RMSE	Root mean squared error
RPMI 1640	Roswell Park Memorial Institute medium

RSM	Response surface methodology
SCG	Scaled conjugate gradient
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography
SSF	Solid state fermentation
SmF	Submerged fermentation
TCA	Trichloroacetic acid
UV	Ultraviolet
V_{max}	Maximum velocity of the enzymatic reaction
w/v	Weight/volume
µg	microgram
µL	microliter
µM	micro molar

Preface

L-asparaginase is an amidohydrolase enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. It is an indispensable enzyme that plays versatile roles ranging from the therapeutic drug to the food processing agent. The L-asparaginase enzyme can be produced under both the solid-state and submerged cultivation conditions. The currently utilized L-asparaginase preparations for diverse applications suffer from several disadvantages both in terms of usage and the overall production costs. Considering the expenses in the enzyme production processes, the initially required substrates or raw materials account up to 30 percent of the total production costs. The similar nature is also seen in the production process of L-asparaginase enzyme in which expensive carbon (glucose) and nitrogen sources (yeast extract, peptone, and tryptone) are utilized for the production. So, there is certainly a need to lower down the expensive production costs of the L-asparaginase enzyme preparations.

Secondly in terms of usage, the utilization of current therapeutic formulations leads to several immunological side-effects including hepatotoxicity, nephrotoxicity, pancreatitis, coagulative abnormalities and many others. (a) All these immunological side-issues arise due to the high intrinsic L-glutaminase co-activities associated with these L-asparaginases. The same L-asparaginase enzyme in addition to L-asparagine hydrolysis also breakdown L-glutamine and up to 10% intrinsic L-glutaminase activities have been reported. (b) The treatment regimen requires multiple different types of L-asparaginases as the enzyme preparation from the single microorganism will be rapidly cleared during the subsequent doses by the host immune system. One of the solutions to this problem is to explore new microbial sources of L-asparaginase preparations that show low/negligible glutaminase activity and also shows anti-leukemic properties.

Thus, the current L-asparaginases have multiple side effects and their production processes are also expensive in nature. The present thesis work is an effort to decipher the solutions to some of the bottlenecks associated with the L-asparaginases. The entire thesis is focussed on the L-asparaginase enzyme and is divided into 7 chapters. Chapter 1 (Introduction) of the thesis starts with the general introduction on L-asparaginases, their applications, the bottlenecks associated with the L-asparaginases and the objectives of the current research work. Chapter 2 (Literature review) covers the literature survey on the L-asparaginases in which their different production sources, applications in diverse sectors and the various optimization strategies employed in the industries for enhancing the production are discussed in detail. Chapter 3 describes the utilization of a novel low cost agro-industrial substrate (niger de-oiled cake) for the L-asparaginase production to overcome the production cost challenge, and also compares the statistical and machine learning strategies employed for the enhanced production of L-asparaginase enzyme. Chapter 4 represents the bench-scale utilization of soluble industrial substrates that can be effectively employed to lower the production cost in submerged fermentation for L-asparaginase production and also reports a new bacterial source (*Bacillus indicus*) that produces the L-asparaginase enzyme with desired kinetic properties, negligible glutaminase activity and potent anti-leukemic activity. Chapter 5 describes the purification and characterization procedures employed for the L-asparaginase enzyme from *Bacillus indicus* MTCC 4374. Chapter 6 reports the evaluation of the anti-leukemic potential of the purified L-asparaginase against acute lymphoblastic leukemia (MOLT-4) cell line and lastly, Chapter 7 represents the final summary and the conclusions of the experimental work.