I dedicate the Ph.D thesis in memory of my beloved

Maa: Late Rumi Hazarika Neog and

Deuta: Bhabendra Nath Neog

DECLARATION

I hereby declare that the thesis entitled "Asiatic fresh water mud eel

(Monopterus cuchia) gut derived microbial enzyme, its molecular assessment and

industrial application" has been submitted to Tezpur University in the Department

of Molecular Biology and Biotechnology under the School of Sciences for partial

fulfilment for the award of the degree of Doctor of Philosophy in Molecular Biology

and Biotechnology. This is an original work carried out by me. Further, I declare that

no part of this work has been reproduced elsewhere for award of any other degree.

Date: 05-02-2024

Place: Tezpur University

(Panchi Rani Neog)

Registration No.: TZ201042, 2019

iii



TEZPUR UNIVERSITY

(A Central University established by an Act of Parliament)

DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY

Tezpur-784028, Assam, India

Dr. Bolin Kumar Konwar Senior Professor

E-mail: bkkon@tezu.ernet.in

Ph. No.: 91-3712-275402 (O)

+91-9957516231 (M)

CERTIFICATE OF THE SUPERVISOR

This is to certify that the thesis entitled "Asiatic fresh water mud eel (Monopterus cuchia) gut derived microbial enzyme, its molecular assessment and industrial application" submitted to the School of Sciences, Tezpur University in partial fulfilment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology is a record of original work carried out by Miss. Panchi Rani Neog under my personal supervision and guidance.

All helps received by her from various sources have been duly acknowledged. No part of this thesis has been reproduced elsewhere for award of any other degree.

Date: 05-02-2024

Place: Tezpur University

(Bolin Kumar Konwar)

Supervisor

Acknowledgements

I wish to express most sincere gratitude to my supervisor Sr Prof. Bolin Kumar Konwar for giving me an opportunity to undertake Ph.D. in his laboratory and for his expert guidance and constant support.

I would also like to thank the Head, Department of Molecular Biology and Biotechnology, Tezpur University and my Doctoral Committee members – Prof. Robin Doley, (Department of Molecular Biology and Biotechnology, TU), Dr. Venkata Satish Kumar Mattaparthi, (Department of Molecular Biology and Biotechnology, TU) Prof. S. C. Deka, (Dept of FET) for their valuable advice during my Ph.D. tenure.

I thank all the faculty members and their Ph.D. students, non-teaching staff and all passed out seniors of the MBBT department for helping me throughout my Ph.D. tenure.

I would like to sincerely thank my lab mates Pranab Mudoi, Kritartha, Mimi, Shreaya, Nigar, and Runumi for their support. I also like to thank all the passed-out project students of our laboratory.

I express my gratitude to my friends Muzamil, Mohit, Sushmita, Chainee, Sharmilee and Shubham who helped me in all possible ways during my tenure.

I wish to acknowledge Tezpur University Authority for allowing me to register for the Ph.D programme and providing me Research & Innovation grant vide Memo No. DoRD/RIG/10-73/1592-A dated 07/01/2021).

Last but not the least, I am greatly indebted to my father, sister and other members of the family, who have always supported and encouraged me during my good and bad times.

LIST OF FIGURES CHAPTER 1				
	CHAPTER 2			
Fig. 2.1.	Monopterus cuchia	19		
	CHAPTER 4			
Fig. 4.1.	Proteolytic activity of the isolates on skim milk agar plates	53		
Fig. 4.2.	Secondary screening of the isolates for protease production	54		
Fig. 4.3.	Morphological characterization of the protease-producing isolates	56		
Fig. 4.4.	Molecular identification of the protease-producing isolates	58		
Fig. 4.5.	Protease activity and growth optimization of B. safensis (PRN1)	60		
Fig. 4.6.	Protease activity and growth optimization of B. safensis (PRN1)	61		
Fig. 4.7.	Protease activity and growth optimization of B. safensis (PRN1)	62		
Fig. 4.8.	Industrial applications of crude protease	63		
Fig. 4.9.	Purification of the protease from Bacillus safensis PRN1	65		
Fig. 4.10.	Effect of pH and temperature on enzyme activity	66		
Fig. 4.11.	Effect of detergents on protease activity	68		
Fig. 4.12.	Gel electrophoresis	69		
Fig. 4.13.	A schematic illustration of the recombinant construct	70		
Fig. 4.14.	Fig. 4.14. Restriction digestion evaluation of a positive clone			
Fig. 4.15.	12% SDS-PAGE gel demonstrating expression profiling of 6xHis-	71		
	tagged KNBS ^{SP1} enzyme in <i>E. coli</i> BL21 (DE3).			
Fig. 4.16.	Purification of 6xHis- KNBS ^{SP1} enzyme	72		
Fig. 4.17.	Multiple sequence alignment of KNBS ^{SP1}	74		
Fig. 4.18.	Influence of pH and temperature on enzyme activity and stability of	75		
	the rKNBS ^{SP1}			
Fig. 4.19.	Effect of metal ions on the rKNBSSP1 stability and its compatibility	77		
	of with commercial detergents			
Fig. 4.20.	In silico analysis	79		
Fig. 4.21.	Docking studies	80		

Ligplot analysis of KNBS^{SP1} - caesin complex

Determination of Industrial Applications of $rKNBS^{SP1}$

mgDNA isolated from the intestinal tissue of M. cuchia

Fig. 4.22.

Fig. 4.23.

Fig. 4.24.

81

82

83

LIST OF TABLES

	CILL DEED 4				
	CHAPTER 1				
Table 1.1	Classification of <i>M. cuchia</i>	3			
Table 1.2	Proteases for detergent industry	13			
Table 1.3	Proteases for leather industry	14			
CHAPTER 2					
Table 2.1	Therapeutic uses of <i>M. cuchia</i>	24			
	CHAPTER 3				
Table 3.1	PCR amplification parameters	41			
	CHAPTER 4				
Table 4.1	Zone of hydrolysis (ZOH- mm) in skim milk and gelatin agar	55			
	plates				
Table 4.2	Morphological characterisation of protease-producing	57			
	isolates				
Table 4.3	Biochemical characterization of protease-producing isolates				
Table 4.4	% homology and Gene Bank accession no. of the isolates	59			
Table 4.5	Zone of inhibition (mm)				
Table 4.6	Purification of the protease enzyme from Bacillus safensis	65			
	PRN1				
Table 4.7	Effect of various inhibitors and surfactants on the protease	67			
	enzyme. The relative activity of the non-treated enzyme was				
	taken as a control (100%).				
Table 4.8	Purifications of the recombinant protease His6-rKNBS ^{SP1} of	72			
	B. safensis strain PRN1				
Table 4.9	Effects of different inhibitors, surfactants and oxidizing agent	76			
	on KNBS ^{SP1} stability				
Table 4.10	Kinetic values of KNBS ^{SP1} towards azocasein	78			
Table 4.11	Ramachandran plot				
Table 4.12	Interactions present between the KNBS ^{SP1} with the target	80			
	molecule				
Table 4.13	Comparative analysis of <i>M. cuchia</i> blood with human blood	84			

ABBREVIATIONS

Abbreviations	Name
3D	Three dimensional
μg	Microgram
μl	Microlitre
μM	Micromolar
%	Percentage
v/v	volume by volume
$^{\circ}\mathrm{C}$	Degree centigrade
BSA	Bovine serum albumin
BLAST	Basic local alignment search tool
bp	Base pair
CFS	Cell free supernatant
CaCl ₂	Calcium chloride
CuSO ₄	Copper Sulfate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DFP	Diisopropylfluorophosphates
EDTA	Ethylene diamine tetra acetic acid
FeSO ₄	Ferrous sulfate
g/L	Gram per litre
Н	Hour
Hb	Hemoglobin
$HgCl_2$	Mercuric chloride
H_2O_2	Hydrogen peroxide
IDT	Integrated DNA Technologies
IPTG	Isopropyl β- d-1-thiogalactopyranoside
I-TASSER	Iterative Threading ASSEmbly Refinement
kb	Kilobase
KDa	kilodalton
KH ₂ PO ₄	Potassium dihydrogen phosphate
L	Litre
LB	Luria-Bertani Broth

LOMETS Local Meta-Threading Server

mg/mL Milligrams per millilitre

M Molar

Min Minute

mL Millilitre

mM Millmolar

mm Millimeter

MCV Mean cell volume

MCH Mean cell hemoglobin

MCHC Mean cell hemoglobin concentration

MEGA Molecular evolutionary genetics analysis

MTCC Microbial Type Culture Collection

MgSO₄ Magnesium sulfate
MnSO₄ Manganese sulfate

NB Nutrient broth

NCBI National Centre for Biotechnology Information

NaOH Sodium hydroxide

NaH₂PO₄ Sodium phosphate monobasic

Na₂HPO₄.7H₂O di-Sodium hydrogen phosphate heptahydrate

NaCl Sodium chloride

Ni-NTA Nickel-Nitrilotriacetic acid

NH₄Cl Ammonium chloride

Nm Nanometer

OD Optical density

PCR Polymerase chain reaction

PAGE Polyacrylamide gel electrophoresis

PCV Packed cell volume

PDB Protein Data Bank

PMSF Phenyl methane sulfonyl fluoride

PPI Protein-protein interaction

Rpm Rotation per minute

RP-HPLC Reverse phase high performance liquid chromatography

RAPD Random amplified polymorphic DNA

RBC Red blood cells

RFLP Restriction fragment length polymorphism

RT Retention time

SDS Sodium dodecyl sulphate

SDF Structure data file

Tris-HCl Tris (hydroxymethyl) aminomethane

TSB Tryptone soya broth

UV-VIS Ultraviolet-visible spectroscopy

ZnSO₄ Zinc sulfate