

**STUDIES ON *STREPTOMYCES MOBARAENSIS*
TRANSGLUTAMINASE FOR BIOTECHNOLOGICAL
APPLICATIONS**

SYEDA WARISUL FATIMA



**DEPARTMENT OF CHEMISTRY
INDIAN INSTITUTE OF TECHNOLOGY DELHI
MARCH 2023**

© Indian Institute of Technology Delhi (IITD), New Delhi, 2023

**STUDIES ON *STREPTOMYCES MOBARAENSIS*
TRANSGLUTAMINASE FOR BIOTECHNOLOGICAL
APPLICATIONS**

by

**SYEDA WARISUL FATIMA
DEPARTMENT OF CHEMISTRY**

Submitted

in fulfillment of the requirements of the degree of Doctoral of Philosophy

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

MARCH 2023

Dedicated to my Parents

CERTIFICATE

This is to certify that the thesis entitled “**Studies on *Streptomyces mobaraensis* transglutaminase for biotechnological applications**” being submitted by **Ms. SYEDA WARISUL FATIMA** to the Indian Institute of Technology Delhi for the award of the degree of *Doctor of Philosophy* in Chemistry is a record of bonafide research work carried out by her.

Ms. Syeda Warisul Fatima has worked under my guidance and supervision and has fulfilled the requirements for the submission of the thesis, which, to my knowledge, has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

Date:
Place: New Delhi

Dr. S. K. Khare
Professor of Biochemistry
Department of Chemistry
Indian Institute of Technology Delhi

Acknowledgements

The exigent yet exhilarating odyssey of the publication of my treatise for the Ph.D. degree has been a wonderful experience. The **Almighty** has been kind and merciful in showering continued blessings. I am grateful to Him.

Embarking on the amazing roller coaster ride presented by the Doctor of Philosophy journey was a worthy one. A plethora of emotions gushed like an adrenaline rush all through upheavals. I am privileged to take this moment to unwrap my Ph.D. thesis voyage and thank all those who endured and supported me through this scientific ordeal. My Ph.D. - the tiny three abbreviated word holds the unwavering commitment and generosity of many well-wishers, friends, relatives, and teachers.

I am more than proud to convey my sincere gratitude and warm thanks to **Prof. Sunil Kumar Khare**, who served as my supervisor. He has motivated and groomed me to become an independent researcher by harnessing my scientific prowess. He has tapped the potential of critical reasoning from a blooming researcher. He always inspired me to work toward what a talented and dedicated scientist can accomplish. He always had faith in me and kept cheering for Golden hands, an energy-pack, bright smiling girl! The love and parental care of his family showered is fondly acknowledged.

I would extend my deep admiration and thanks to my thesis advisory and research committee members, especially **Prof. Amulya K Panda**, **Prof. Shashank Deep**, and **Prof. Tanmay Dutta**. They generously boosted me and gave their valuable time to offer me constructive

suggestions during my course of Ph.D. work. Moreover, they offered me helpful criticism, which assisted me in developing a broader understanding of the research work.

My special thanks go to **Prof. Siddharth Pandey**, Head of the Department of Chemistry, for moral, academic, and logistic support all through. I am indebted to **Prof. Ravi Shankar**, **Prof. Ashok K. Ganguly**, **Prof. Anil J. Elias**, and **Prof. N.D. Kurur**, Former Heads of the Department of Chemistry, IIT Delhi, for providing all the necessary facilities, constant tutelage, and valuable guidance.

I am incredibly grateful to the collaborators for sharing their knowledge and insight to tackle my technical and scientific problems: **Prof. Moshahid Alam Rizvi**, Department of Biosciences, Jamia Millia Islamia, New Delhi; **Prof. Ritu Kulshreshtha**, Department of Biochemical Engineering and Biotechnology, IIT Delhi, and other prominent scientists namely **Prof. Sagar Sengupta** of cancer studies at National Institute of Immunology, New Delhi. I owe special thanks to them for their generous permission to work in their laboratories and use instrumental facilities. There is no way to express how much it meant to me to be associated with versatile laboratories.

Special thanks to my **Enzyme and Microbial Biochemistry (EMB) Laboratory**, all the current and former EMB lab students, and the visitors I have encountered during my wonderful stay. These colleagues, friends, and coworkers/peers have greatly motivated me during my Ph.D. tenure.

I'm so grateful to my super seniors: **Dr. Anshu Gupta**, **Dr. Ruchi Gaur**, **Dr. Ram Karan**, **Dr. Arvind Sinha**, **Dr. Chetna Joshi**, **Dr. Rajeshwari Sinha**, and **Dr. Sumit Kumar**. I am thankful to my wonderful seniors and contemporaries for such a caring and supportive team: **Dr.**

R. Hemamalini, Dr. Ayesha Sadaf, Dr. Jasneet Grewal, Dr. Shubhrima Ghosh, and Dr. Neerja Yadav. My sincere gratitude to all of the current and former learned postdocs in the lab: **Dr. Shivani Chaturvedi, Dr. Arti Pal, Dr. Razi Ahmad, and Dr. Sunaina Singh,** for their scientific contributions and encouragement.

Special thanks go to **Dr. Rameshwar Tiwari** for his constant encouragement, thoughtful discussions, and continuous help throughout my research journey, **Dr. Kumar Pranaw** for his timely encouragement for sharing critical inputs on my research, **Dr. Amrik Bhattacharya** for minutely going through the draft and continued advice. Special praise goes to **Mr. Shahenvaz Alam,** who has chipped in to lift me, calm and support me as my best friend and a great companion to celebrate each accomplishment in life.

My sincere thanks to all the juniors: **Mrs. Nikky Goel, Mr. Nitin Srivastava, Ms. Pooja, Ms. Ankita Maurya, Ms. Huma Fatima, Mrs. Sukriti Srivastava, Ms. Tanya, Ms. Deeksha Gopaliya, Ms. Saniya Zaidi, Ms. Simran Kundral, Mr. Bibhuti Das, and Mr. Sachin Wagh** for establishing a vibrant work atmosphere and for all of the pleasant moments we had together in the lab.

Moreover, I am obliged to the administrative staff for their prompt support and assistance but also for kind care: Academics division, Central Library, Industrial Research and Development, and Accounts office. I have especially benefited from the truly professional support of the technical and administrative staff: **Mrs. Dimple, Mr. Ashish, Mr. H.K.V. Chopra, and Mr. J.P. Singh** are memorable for their genuine care.

I am highly obliged to the Central Instrumentation (CRF) and Nano-Scale Research Facility (NRF), IIT Delhi, and their technical team for providing me with the necessary infrastructure and assistance for carrying out crucial parts of my experimental analyses. I thank all

the staff members of the Instrumentation Lab, M.Sc Lab, M. Tech Lab, B. Tech Lab and Chemistry Office, Department of Chemistry, and IIT Delhi for their cooperation and assistance. I extend my sincere thanks to all the academics who have shaped me into the individual I am today.

I am striven to express my appreciation to the Department of Science and Technology (DST), Government of India, New Delhi, for awarding the prestigious **DST INSPIRE fellowship** for research. I would like to extend my warm credit for awarding the prestigious RETA Travel grant, sponsored by IIT Delhi, for attending an international conference.

Last, of all, I owe special thanks with love to my father, **Dr. Syed Aijazuddin**, for constantly inspiring me and enlightening me to be like him in all my pursuits. I realized my dream of becoming a Ph.D. Scholar after seeing him as a perfect soul with his extraordinary efforts. Also, thanks to my mother, **Mrs. Zarrin Aijaz**, for always supporting me unconditionally. My dearest siblings, **Abeer**, **Nayel**, and **Manaam**, for always being there for me; I owe them a lot. My heartiest appreciation extends to my new family, **Alam's family**. I thank my mother-in-law, **Mrs. Ishrat Jahan** for her love and affection throughout. My sincere thanks extend to my brother-in-law, **Mr. Sarfaraz Alam** and sister-in-law, **Mrs. Rehana Khatoon** and special thanks to my nephew, **Shehryar (Sharry)** for the joyful moments spent together.

Finally, I thank with love to my beautiful family for their unconditional trust, kindness, and endless patience which made all my efforts worth the struggle. A special reverence for my late grandparents, uncles, and aunts, who had a firm belief in me.

Cheers to my **Noo Noo Bills** family, who always stood beside me and who have enriched my life by extending their paws.

Lastly, I would like to cherish the significant role my colleagues, professors, friends, and family played knowingly or unknowingly in making this six-year-long journey a memorable piece for me to treasure forever.

Syeda Warisul Fatima

Abstract

The thesis encompasses the detailed study of transglutaminase produced by the actinomycetes *Streptomyces mobarensis* NCIM 5208, and its novel applications. Microbial transglutaminases (MTGases) are major cross-linking enzymes which modify the properties of cellular proteins by creating iso-peptide bonds among them. This enzyme has huge potential for applications in the pharmaceutical, food, textile, biomedical, and cosmetics industries. However, these applications necessitate them to be stable at high temperature, and adverse pH environment vis-à-vis their viable production and cost-effective availability, which would otherwise limit wide application in industries. An enzyme of microbial origin is now commonly exploited to overcome the shortcomings of conventionally produced transglutaminases, gaining colossal attention for diverse applications. Therefore, current research efforts have been directed to the bio-process development of MTGase production, regulatory mechanism, nano-immobilization, and their application in tissue scaffold and drug delivery.

MTGase, being nature's biological glue on account of forming ϵ -(γ -glutamyl) lysine bonds in proteins; however, low productivity and the high cost of production are the major bottlenecks for industrial MTGase production. The present work dealt with these obstacles by enhancing the MTGase production through media engineering using *S. mobaraensis* via a waste valorization approach utilizing agro-wastes. Enhancement of MTGase production vis-à-vis cost reduction was attempted using wheat bran as a carbon source. During the course of the research, bulk production of microbial MTGase by media engineering and overexpression of recombinant MTGase from *S. mobaraensis* was investigated. Initially, *S. mobaraensis* was grown under solid-state fermentation (SSF) conditions, and media components were selected by the 'one factor at a time' approach, and a 2.15-fold increase in MTGase production was achieved. The optimized conditions revealed a cost-effective process for MTGase production by utilizing a lignocellulosic residue (wheat bran) as the main carbon source for SSF. Further,

the main selected growth parameters were optimized by statistical Box-Behnken design. The resulted growth factors included Protease: 39.14 IU, MgCl₂: 0.10 M, CTAB: 0.08%, and Inoculation size: 2.1 x 10⁵ CFU (2%) led to the increase of MTGase production up to 4-fold (12.949 ± 0.061 IU/g) compared with un-optimized conditions. In addition, the effect of key regulators in augmenting transcriptional expression of MTGase was elucidated by Real-Time quantitative PCR to unravel the regulatory mechanism of MTGase synthesis in *S. mobaraensis*.

The work also intended to explore the nano-immobilization of MTGase for developing stable enzyme bioconjugates. The thesis further deals with various applications of this MTGase preparation for cross-linking food proteins and developing tissue scaffolds using casein and gelatin-carbon nanotubes as model systems. The structural and functional changes during/after cross-linking were studied by SDS-PAGE, Circular Dichroism (CD), Fluorescence Spectrometry (FS), Fourier-Transform Infrared Spectroscopy (FTIR), and Attenuated Total Reflectance (ATR). Hence, the innovative production of MTGase was shown to be useful in effective cross-linking casein protein subunits. MTGase was immobilized on carbon nanotubes with high immobilization efficiency (58%) and reusability. The nano-immobilized formulations of MTGase possessed enhanced biochemical and kinetic attributes, which were applied for bioinspired hydrogel scaffold formation.

Furthermore, cloning, overexpression, and purification were attempted as an alternate approach to overproduce MTGase. Moreover, *MTGase* and *pro-MTGase* gene sequences were successfully amplified from *S. mobaraensis*. The ORF encoded for a protein of 412 and 331 amino acids which was cloned in the pET-22b (+) vector for overexpression in *Escherichia coli* BL21 (DE3). The comprehensive study was conducted on the biochemical and structural characterization of purified and recombinant *MTGases*, that hold tremendous applications.

As an innovative part of the thesis, the novel nanoflowers of MTGase could be developed by exploiting the inherent cross-linking activity of this enzyme. Nanoflowers are nano-crystals with floral architecture formed by intra-molecular interactions between the enzymes/proteins. They are endowed with diverse functionalities for biocatalytic applications. The designing of bio-catalytically active enzymatic MTGase nanoflowers (NFs) from the microbial source at a low temperature of 4 °C has been patented. The study features a new and elegant approach in enzyme immobilization. The novelty in the present work relates to the method of synthesis, the designing of the product, and the process optimization to get the desired features in the NFs.

The multifunctional facets of NFs, including simple design and preparation, size tunability, and resistance to enzymatic degradation, allowing customized cargo loading (heavy metals/pollutants/drugs), specific recognition, and internalization into target cancer cells were worked out and formed the major highlights of the thesis.

MTGase NFs-based targeted delivery has been investigated on cancer cell models: Glioblastoma (LN-229) and Breast (MCF-7). Preliminary results showed the novel enzymatic nanoflowers have a unique morphology and enhanced enzyme stability. The shape of a hollow immobilized enzyme in the form of a cage could act as a nanocarrier for drug delivery. The framework of NFs was optimized to get the desired nanosize (< 50 nm) flowers. The MTT assay and wound healing after the treatment of these MTGase NFs gave positive results in restricting the migration of cancer cells. The cytotoxic effects of MTGase NFs were tested. There was no significant cell death in normal (non-cancerous murine fibroblasts L-929 cells), whereas, in all the cancer cell lines, visible aggregation could be observed. The results in both cases of brain and breast cancer cell lines (LN-229 and MCF-7) showed the efficacy of delivery, loss of viability, and apoptosis of the cancerous cells. These NFs are safe for usage as a nanocarrier with cargo loaded on it; hence, MTGase NFs as the magical bullet comes into

action. These findings point towards effective, low toxicity, and site-targeted, accurate anti-cancer drug delivery via these MTGase NFs.

To conclude, the cost-effective production will not only enhance the yield of this enzyme but also lead to tremendous applications in wide arrays of human welfare in food bioprocessing, pharmaceutical, tissue engineering, and therapeutics.

The following research goals were accomplished:

- **Molecular Glue of the Millennium:** The transglutaminase developed with enhanced value.
- The **transcriptional regulatory mechanism** of how the critical regulators modulate the MTGase enzyme production was elucidated.
- The **nano-immobilized** MTGase was investigated for application in the design of **biocompatible tissue hydrogel scaffolds**.
- The study highlights the **designing of MTGase nanoflowers**, which was manifested in **bioremediation** applications.
- **Developed a suitable nanocarrier** that can effectively **deliver cargo** in both *in vitro* and *in vivo* systems and have a sustained cargo release profile.
- To **inhibit aberrant expression of miRNAs** and thereby significantly impede tumor growth/cancers.
- Overall, the long-term goal was to **devise a novel, powerful MTGase-based nanotherapy** as an adjuvant to the existing chemotherapy and successfully inhibit cancer progression. Hence, the significance of cross-linking enzyme MTGase was achieved as a **multifaceted therapeutic alternative in treating cancers**.

Thus, the applicability of MTGase produced by various approaches justified the MTGase as an enigmatic enzyme in myriad realms of applications.

सारांश

थीसिस में एक्टिनोमाइसेट्स *स्ट्रेप्टोमाइसेस मोबेरेंसिस* एनसीआईएम 5208 और इसके नव अनुप्रयोगों द्वारा उत्पादित ट्रांसग्लूटामिनेज का विस्तृत अध्ययन शामिल है। माइक्रोबियल ट्रांसग्लूटामिनेस (एमटीजीएस) प्रमुख क्रॉस-लिंकिंग एंजाइम हैं जो उनके बीच आईएसओ-पेप्टाइड बॉन्ड बनाकर सेलुलर प्रोटीन के गुणों को संशोधित करते हैं। इस एंजाइम में फार्मास्यूटिकल्स, भोजन, कपड़ा, बायोमेडिकल, रोग उन्मूलन और सौंदर्य प्रसाधन अनुप्रयोगों में भारी क्षमता है। हालांकि, इन अनुप्रयोगों को उच्च तापमान और प्रतिकूल पीएच वातावरण पर स्थिर होने की आवश्यकता है, जो अन्यथा उद्योगों में व्यापक अनुप्रयोग को सीमित करेगा। माइक्रोबियल मूल का एक एंजाइम अब आमतौर पर वांछनीय गुणों की प्राप्ति के लिए कमियों को दूर करने के लिए शोषण किया जाता है और विभिन्न अनुप्रयोगों के लिए भारी ध्यान आकर्षित करता है। इसलिए, वर्तमान अनुसंधान प्रयासों को एमटीजीएस उत्पादन, नियामक तंत्र और उनके अनुप्रयोग के बायोप्रोसेस विकास के लिए निर्देशित किया गया है। एमटीजीएस, प्रोटीन में δ -(γ -ग्लूटामिल) लाइसिन बांड बनाने के कारण प्रकृति का जैविक गोंद होने के कारण, हालांकि, कम उत्पादकता और उच्च लागत औद्योगिक एमटीजीएस उत्पादन के लिए प्रमुख बाधाएं हैं। इस कार्य ने कृषि-अपशिष्टों का उपयोग करके अपशिष्ट मूल्यांकन दृष्टिकोण के माध्यम से एस मोबेरेंसिस का उपयोग करके मीडिया इंजीनियरिंग के माध्यम से एमटीजीएस के उत्पादन को बढ़ाकर इन बाधाओं से निपटा। कार्बन स्रोत के रूप में गेहूं के चोकर का उपयोग करके लागत में कमी की तुलना में एमटीजीएस के उत्पादन को बढ़ाने का प्रयास किया गया था। अनुसंधान के दौरान, माइक्रोबियल एमटीजीएस के थोक उत्पादन और एस मोबेरेंसिस से पुनः संयोजक एमटीजीएस की अति-अभिव्यक्ति की मीडिया इंजीनियरिंग द्वारा जांच की गई थी। प्रारंभ में, एस मोबेरेंसिस को ठोस अवस्था किण्वन (एसएसएफ) स्थितियों के तहत उगाया गया था और मीडिया घटकों को 'एक समय में एक कारक' दृष्टिकोण द्वारा चुना गया था और एमटीजीएस उत्पादन में 2.15 गुना वृद्धि हासिल की गई थी। अनुकूलित स्थिति ने एसएसएफ के लिए मुख्य कार्बन स्रोत के रूप में लिग्नोसेल्यूलोसिक अवशेषों (गेहूं की भूसी) का उपयोग करके एमटीजीएस उत्पादन के लिए एक लागत

प्रभावी प्रक्रिया का खुलासा किया। इसके अलावा, मुख्य चयनित विकास मापदंडों को सांख्यिकीय बॉक्स-बेहनकेन डिजाइन द्वारा अनुकूलित किया गया था। परिणामी विकास कारकों में प्रोटीज शामिल थे: 39.14 यू.एमजीसीएल 2: 0.10 एम, सीटीएबी: 0.08% और टीकाकरण आकार: 4 गुना (12.949 + 0.061 आईयू/एमएल) में 2.02 मिलीलीटर संयुक्त राष्ट्र-अनुकूलित स्थितियों की तुलना में एमटीजीएस उत्पादन में। इसके अलावा, एमटीजीएस संश्लेषण के नियामक तंत्र को उजागर करने के लिए वास्तविक समय मात्रात्मक पीसीआर द्वारा एमटीजीएस की ट्रांसक्रिप्शनल अभिव्यक्ति को बढ़ाने में प्रमुख नियामकों के प्रभाव को स्पष्ट किया गया था। थीसिस आगे खाद्य प्रोटीन के क्रॉस-लिंकिंग और मॉडल सिस्टम के रूप में कैसिइन और जिलेटिन-कार्बन नैनोट्यूब का उपयोग करके ऊतक मचान विकसित करने के लिए इस एमटीजीएस तैयारी के विभिन्न अनुप्रयोगों से संबंधित है। क्रॉसलिंकिंग के दौरान/बाद में संरचनात्मक और कार्यात्मक परिवर्तनों का अध्ययन एसडीएस-पेज, परिपत्र द्विवर्णता (सीडी), प्रतिदीप्ति स्पेक्ट्रोमेट्री (एफएस), फूरियर-ट्रांसफॉर्म इन्फ्रारेड स्पेक्ट्रोस्कोपी (एफटीआईआर) और क्षीणित कुल प्रतिबिंब (एटीआर) द्वारा किया गया था।

अतः, एमटीजीएस के अभिनव उत्पादन को प्रभावी क्रॉस-लिंकिंग कैसिइन प्रोटीन सबयूनिट्स में उपयोगी दिखाया गया था। एमटीजीएस को उच्च स्थिरीकरण दक्षता (58%) और पुनः प्रयोज्यता के साथ कार्बन नैनोट्यूब पर स्थिर किया गया था। एमटीजीएस के नैनो-स्थिर योगों ने जैव रासायनिक और गतिज विशेषताओं को बढ़ाया था जो बायोइंस्पायर्ड हाइड्रोजेल पाड़ गठन के लिए लागू किए गए थे। काम का उद्देश्य स्थिर एमटीजीएस के विकास के लिए क्लोनिंग, ओवरएक्सप्रेशन, शुद्धिकरण और स्थिरीकरण का पता लगाना भी था। इसके अलावा, पूर्ण और समर्थक एमटीजीएस जीन अनुक्रमों को सफलतापूर्वक प्रवर्धित किया गया था। ओआरएफ को 412 और 331 अमीनो एसिड के प्रोटीन के लिए एन्कोड किया गया है जिसे एस्चेरिचिया कोलाई बीएल 21 (डीई 3) में ओवरएक्सप्रेशन के लिए पीईटी -22 बी (+) वेक्टर में क्लोन किया गया था। शुद्ध और पुनः संयोजक एमटीजीएस के जैव रासायनिक और संरचनात्मक

लक्षण वर्णन पर व्यापक अध्ययन किए गए थे, जिसमें जबरदस्त अनुप्रयोग हैं। काम का अभिनव हिस्सा: एंजाइम प्रोटीन के बीच इंटर-आणविक इंटरैक्शन द्वारा गठित पुष्प वास्तुकला के साथ नैनो-क्रिस्टल हैं। वे जैव उत्प्रेरक अनुप्रयोगों के लिए विविध कार्यक्षमताओं से संपन्न हैं। 4 डिग्री सेल्सियस के कम तापमान पर माइक्रोबियल स्रोत से जैव उत्प्रेरक रूप से सक्रिय एंजाइमेटिक एमटीजीएस नैनोफ्लॉवर (एनएफ) की डिजाइनिंग का पेटेंट कराया गया है। अध्ययन में एंजाइम स्थिरीकरण में एक नया और सुरुचिपूर्ण दृष्टिकोण है। काम में नवीनता एनएफ में वांछित सुविधाओं को प्राप्त करने के लिए संश्लेषण, उत्पाद डिजाइनिंग और प्रक्रिया अनुकूलन की विधि से संबंधित है। सरल डिजाइन और तैयारी, आकार ट्यूनेबिलिटी, और एंजाइमी गिरावट और विकृतीकरण के प्रतिरोध सहित एनएफ के बहुआयामी पहलुओं, कार्गो लोडिंग (भारी धातुओं / प्रदूषक/ लक्षित कैंसर कोशिकाओं (अंजीर 100, 100 मिलीग्राम / दवाओं) के लिए कार्यक्षमताओं के अनुकूलित समावेश की अनुमति दें, जिससे लक्षित कैंसर कोशिकाओं में कार्यक्षमताओं, विशिष्ट मान्यता और आंतरिककरण के अनुकूलित समावेश की अनुमति मिलती है। एमटीजीएस-नैनोफ्लॉवर-आधारित लक्षित वितरण की जांच कैंसर सेल मॉडल पर की गई है: ग्लियोब्लास्टोमा (एलएन 22 9) और स्तन (एमसीएफ -7)। प्रारंभिक परिणामों से पता चला है कि उपन्यास एंजाइमेटिक नैनोफ्लॉवर में एक अद्वितीय आकृति विज्ञान और बड़ी हुई एंजाइम स्थिरता है। पिंजरे के रूप में एक खोखले स्थिर एंजाइम का आकार दवा वितरण के लिए नैनोकैरियर के रूप में कार्य कर सकता है।

एनएफ के ढांचे को वांछित नैनोसाइज (< 50 एनएम) प्राप्त करने के लिए अनुकूलित किया गया था। इन एमटीजीएस नैनोफ्लॉवर के उपचार के बाद एमटीटी परख और घाव भरने में कैंसर कोशिकाओं के प्रवास को प्रतिबंधित करने में सकारात्मक परिणाम दिए। एमटीजीएस एनएफ के साइटोटाॉक्सिक प्रभावों का परीक्षण किया गया था। सामान्य (गैर-कैंसर म्यूरिन फाइब्रोब्लास्ट्स एल -929 कोशिकाओं) में कोई महत्वपूर्ण कोशिका मृत्यु नहीं थी, जबकि सभी कैंसर सेल लाइनों में, दृश्यमान एकत्रीकरण देखा जा

सकता है। ये एनएफ नैनोकैरियर के रूप में उपयोग के लिए सुरक्षित हैं, जिस पर कार्गो लोड किया जाता है, इसलिए एमटीजीएस एनएफ की जादुई गोलियां कार्रवाई में आती हैं। ये निष्कर्ष इन एमटीजीएस एनएफ के माध्यम से प्रभावी, कम विषाक्तता और लक्षित (साइट-विशिष्ट), सटीक एंटी-कैंसर दवा वितरण की ओर इशारा करते हैं। निष्कर्ष निकालने के लिए, लागत प्रभावी उत्पादन न केवल इस एंजाइम की उपज को बढ़ाएगा, बल्कि खाद्य बायोप्रोसेसिंग, फार्मास्यूटिकल्स, ऊतक इंजीनियरिंग और चिकित्सीय में मानव कल्याण की विस्तृत श्रृंखलाओं में जबरदस्त अनुप्रयोगों को भी जन्म देगा। निम्नलिखित शोध लक्ष्यों को पूरा किया गया था: मिलेनियम का आणविक गोंद: ट्रांसग्लूटामिनेज को बढ़े हुए मूल्य के साथ विकसित किया गया। महत्वपूर्ण नियामकों द्वारा एमटीजेज एंजाइम उत्पादन को कैसे संशोधित किया जाता है, इसका ट्रांसक्रिप्शनल नियामक तंत्र स्पष्ट किया गया था। नैनो-स्थिर एमटीजीएस की जैव संगत ऊतक हाइड्रोजेल मचानों के डिजाइन में आवेदन के लिए जांच की गई थी। अध्ययन एमटीजीएस नैनोफ्लार के डिजाइन पर प्रकाश डालता है, जो बायोरेमेडिएशन अनुप्रयोगों में प्रकट होता है। एक उपयुक्त नैनो-वाहक विकसित किया जो इन-विट्रो और इन-विवो सिस्टम दोनों में प्रभावी ढंग से कार्गो वितरित कर सकता है और एक निरंतर कार्गो रिलीज प्रोफाइल है। एमआईआरएनए की विचलित अभिव्यक्ति एवम ट्यूमर के विकास को रोके , कुल मिलाकर इस तरह हमारा दीर्घकालिक लक्ष्य मौजूदा कीमोथेरेपी के सहायक के रूप में एक उपन्यास, शक्तिशाली एमटीजीएस आधारित नैनोथेरेपी तैयार करना था और कैंसर की प्रगति को सफलतापूर्वक रोकना था। इसलिए, कैंसर के इलाज में बहुआयामी चिकित्सीय विकल्प के रूप में क्रॉस-लिंकिंग एंजाइम एमटीजीएस का महत्व प्राप्त किया गया था। इस प्रकार, विभिन्न दृष्टिकोणों द्वारा उत्पादित एमटीजीएस की प्रयोज्यता ने असंख्य स्थानों में एक गूढ़ एंजाइम के रूप में एमटीजीएस को उचित ठहराया।

Table of Contents

Certificate	i
Acknowledgements	ii
Abstract	vii
Table of Contents	xi
List of Figures	xxii
List of Tables	xxxii

CHAPTER 1 INTRODUCTION

1.1	Rationale of the thesis	5
1.2	Objectives	9
1.3	Scope of the thesis	10
1.4	Review of literature	14
1.4.1	Microbial Transglutaminase: An enigmatic cross-linking enzyme with diverse functions	14
1.4.2	Harvesting transglutaminases from different sources	17
1.4.3	Mechanism of action of MTGase	17
1.4.4	MTGases unique importance	19
1.4.5	Structural and enzymatic properties of MTGase	19
1.4.5.1	Sequence analysis for visualization of structure and active site of MTGase family	21
1.4.6	Developing MTGase for modified functions and attributes	23

1.4.7	Production of transglutaminase: Past and recent approaches	23
1.4.7.1	Solid-state fermentation process for MTGase production	26
1.4.7.2	Cloning of MTGase	26
1.4.7.3	Purification of MTGase	28
1.4.8	Immobilization of MTGase	29
1.4.8.1	Multifunctional enzymatic nanoflowers: A tale of dynamic transformation in immobilization approach	31
1.4.9	Applications of altered nutriment in the food and nutraceutical industry	35
1.4.10	Food regulatory status of MTGase-modified products	42
1.4.11	A multifaceted biotechnological application of MTGase	43
1.4.12	Current insight and futuristic vistas of MTGase	44

CHAPTER 2 PROCESS OPTIMIZATION FOR MAXIMIZING TRANSGLUTAMINASE PRODUCTION FROM *STREPTOMYCES MOBARAENSIS*

CHAPTER 2A SOLID-STATE FERMENTATION USING AGRO-INDUSTRIAL WASTES AS A SUBSTRATE FOR COST-EFFECTIVE PRODUCTION

2A.1	Introduction	47
2A.2	Materials and Methods	49
2A.2.1	Materials	49
2A.2.2	Microorganism	49
2A.2.3	Production of MTGase	49
2A.2.3.1	Seed culture preparation	49
2A.2.4	Transglutaminase assay	50
2A.2.5	Screening of medium components under solid-state fermentation (SSF)	51

2A.2.5.1	Enzyme extraction	51
2A.2.6	Selection of parameters by using One Factor at a Time (OFAT) experimental approach	52
2A.2.7	Optimization of media constituents by using Box-Behnken design with Response Surface Methodology (RSM)	52
2A.2.8	Estimation of dry weight biomass	53
2A.2.9	Morphological and structural characterization of <i>S. mobaraensis</i>	53
2A.3	Results and Discussion	54
2A.3.1	Effect of time course on production of MTGase enzyme by <i>S. mobaraensis</i>	54
2A.3.2	Enhancement of MTGase production from <i>S. mobaraensis</i> by OFAT methodology	55
2A.3.2.1	Optimization of different media components	55
2A.3.3	Evaluation of MTGase production by using lignocellulosic biomass in SSF mode	56
2A.3.4	Effect of nitrogen sources on MTGase enzyme production	57
2A.3.5	Effect of moisture content	58
2A.3.6	Effect of inoculum size	58
2A.3.7	Effect of divalent salts	59
2A.3.8	Effect of protease	59
2A.3.9	Effect of surfactants on MTGase enzyme production	60
2A.3.10	Enhancement of MTGase enzyme by statistical optimization tool Response Surface Methodology (RSM)	62
2A.3.11	Morphological characterization of <i>S. mobaraensis</i> grown under optimized conditions under SSF	68
2A.3.12	Techno-economic analysis of MTGase production process with agro-wastes recovery	69
2A.4	Conclusions	71

CHAPTER 2B TRANSCRIPTIONAL STUDIES FOR CORRELATION WITH TRANSGLUTAMINASE PRODUCTION

2B.1	Introduction	72
2B.2	Materials and Methods	74
2B.2.1	Materials	74
2B.2.2	Microorganism and culture condition for MTGase production	74
2B.2.3	Transglutaminase assay	74
2B.2.4	Estimation of dry cell mass	75
2B.2.5	Morphological response of MTGase-producing <i>S. mobaraensis</i>	75
2B.2.6	Assessment of modulators regulating MTGase production	75
2B.2.7	Transcriptional level analysis of MTGase production	76
2B.2.7.1	RNA isolation, cDNA synthesis	76
2B.2.7.2	Real-time Quantitative PCR	77
2B.2.8	Statistical analysis	77
2B.3	Results and Discussion	77
2B.3.1	Growth performance of <i>S. mobaraensis</i> for MTGase production for dry cell mass estimation	77
2B.3.2	Morphological characterization of <i>S. mobaraensis</i> grown under optimized conditions	78
2B.3.3	Enhancement of MTGase production from <i>S. mobaraensis</i> using different modulators	79
2B.3.4	Evaluation of transcriptional pattern of MTGase produced from <i>S. mobaraensis</i>	81
2B.3.4.1	Analysis on various supplemented differentially transcripts of MTGase	81
2B.4	Conclusions	86

CHAPTER 3 PURIFICATION, CHARACTERIZATION AND APPLICATIONS OF TRANSGLUTAMINASE IN CROSS-LINKING OF FOOD PROTEINS

3.1	Introduction	87
3.2	Materials and Methods	89
3.2.1	Materials	89
3.2.2	Purification of MTGase	89
3.2.3	SDS-PAGE gel electrophoresis and zymography of purified MTGase	89
3.2.4	Enzymatic characterization of purified MTGase	90
3.2.5	Application of purified MTGase	90
3.2.5.1	Enzymatic cross-linking action of MTGase on casein	90
3.2.5.1.1	Preparation and treatment of milk samples	90
3.2.5.1.2	Silver staining of cross-linked casein by MTGase	91
3.2.6	Cross-linking assessment by spectroscopic techniques	91
3.2.6.1	Circular Dichroism spectra measurements: Far-UV CD spectroscopy	91
3.2.6.2	Intrinsic fluorescence studies by Fluorescence spectroscopy	92
3.2.6.3	Fourier Transform Infrared Spectroscopy-Attenuated Total Reflection (FTIR- ATR)	92
3.3	Results and Discussion	92
3.3.1	Purification of MTGase	92
3.3.2	SDS analysis and zymography	93
3.3.3	Enzymatic characterization of purified MTGase	93
3.3.3.1	Thermal and pH profile: Optima and stability	93
3.3.4	Cross-linking of casein by purified MTGase: Structural and chemical characterization of cross-linking	95
3.3.4.1	SDS gel electrophoresis	96
3.3.4.2	Silver staining to validate the cross-linking action mediated by MTGase	97

3.3.4.3	MTGase-mediated cross-linked casein protein secondary and tertiary structure prediction	98
3.3.4.3.1	Far-UV CD spectroscopy	98
3.3.4.3.2	Fluorescence spectroscopy	98
3.3.4.3.3	FTIR-ATR spectroscopy	99
3.4	Conclusions	104

CHAPTER 4 CLONING AND EXPRESSION OF *STREPTOMYCES MOBARAENSIS* TRANSGLUTAMINASE

4.1	Introduction	106
4.2	Materials and Methods	108
4.2.1	Strains, plasmids, reagents and culture conditions	108
4.2.2	Designing of primers and amplification of MTGase from <i>S. mobaraensis</i>	108
4.2.2.1	Gene amplification of MTGase by PCR	109
4.2.2.2	Sequence analysis of MTGase	110
4.2.3	Cloning and overexpression of MTGase gene in <i>E. coli</i>	110
4.2.4	Quantification of recombinant MTGase activity	111
4.2.4.1	MTGase assay	111
4.2.4.2	Recombinant protein analysis by SDS-PAGE and RT-qPCR	111
4.2.5	Characterization of recombinant <i>MTGase</i>	112
4.3	Results and Discussion	112
4.3.1	Cloning of <i>MTGase</i>	112
4.3.1.1	Subcloning in pGEM-T Easy vector	113
4.3.1.2	Sequence analysis	116
4.3.2	<i>MTGase</i> overexpression in <i>E. coli</i> . BL21 (DE3)	118
4.3.2.1	MTGase assay	119

4.3.2.2	Recombinant protein analysis by SDS-PAGE and RT-qPCR	120
4.3.2.3	Characterization of recombinant <i>MTGase</i>	123
4.4	Conclusions	126

CHAPTER 5 APPLICATION OF TRANSGLUTAMINASE FOR DEVELOPMENT OF TISSUE

SCAFFOLDS

5.1	Introduction	127
5.2	Materials and Methods	129
5.2.1	Materials	129
5.2.2	Enzyme production	130
5.2.3	Determination of MTGase activity	130
5.2.4	Surface modification, functionalization and bioconjugation of MWCNTs	131
5.2.4.1	Oxidation of MWCNTs	131
5.2.4.2	Modification of oxidized MWCNTs with EDC	131
5.2.4.3	Conjugation of MTGase on EDC modified MWCNTs	131
5.2.5	Structural and functional characterization of functionalized MWCNTs and immobilized MTGase	132
5.2.5.1	Determination of kinetic properties of free and immobilized MTGase	133
5.2.5.2	Determination of deactivation rate constant of free and immobilized MTGase	133
5.2.6	Reusability of immobilized MTGase	133
5.2.7	Applications of immobilized MTGase bioconjugates for designing of the hydrogel scaffold	134
5.2.7.1	Biocompatible materials screening for designing of hydrogel scaffold	134
5.2.7.2	Optimization of the amount of biomaterial as a matrix for hydrogel scaffold formation	134
5.2.8	Structural characterization of prepared hydrogel	134

5.2.9	Swelling ratio of prepared hydrogel	135
5.2.10	Statistical analysis	135
5.3	Results and Discussion	135
5.3.1	Immobilization of MTGase on functionalized MWCNTs via EDC-NHS coupling	135
5.3.2	Structural and functional characterization of immobilized MTGase	138
5.3.3	Kinetic characterization of free and immobilized MTGase	141
5.3.4	Thermal inactivation kinetics of free and immobilized MTGase	144
5.3.5	Reusability of immobilized MTGase	145
5.3.6	Application of immobilized MTGase bioconjugates for designing hydrogel scaffold	146
5.3.6.1	Screening of biocompatible material for hydrogel scaffold	146
5.3.6.2	Amount of biocompatible material optimization	148
5.3.7	Structural characterization of prepared hydrogel	148
5.3.8	Swelling ratio of prepared hydrogel	149
5.4	Conclusions	151

CHAPTER 6 DEVELOPMENT OF TRANSGLUTAMINASE NANOFLOWERS AND APPLICATION IN HEAVY METALS BIOREMEDIATION

6.1	Introduction	152
6.2	Materials and Methods	154
6.2.1	MTGase production for designing nanoflowers	154
6.2.2	Synthesis of MTGase nanoflowers	155
6.2.3	Characterization of MTGase nanoflowers	155
6.2.4	Application of MTGase nanoflowers as nanocarrier in bioremediation	156
6.3	Results and Discussion	156
6.3.1	Designing the enzymatic MTGase nanoflowers	156
6.3.1.1	Insights into MTGase nanoflowers	156
6.3.2	Optimization of MTGase nanoflower design and their characterization	157

6.3.3	Physico-chemical attributes of MTGase nanoflowers	160
6.3.4	Comparative assessment of MTGase nanoflowers with hybrid nanoflowers	162
6.3.5	Biochemical attributes of MTGase nanoflowers	165
6.3.6	Validation of MTGase nanoflowers for bioremediation applications	166
6.4	Conclusions	170

CHAPTER 7 APPLICATIONS OF ENZYMATIC TRANSGLUTAMINASE NANOFLOWERS IN CANCER THERANOSTICS

CHAPTER 7A TRANSGLUTAMINASE NANOFLOWERS AS AN ALTERNATIVE NANOMEDICINE FOR BREAST CANCER THERANOSTICS

7A.1	Introduction	171
7A.2	Materials and Methods	174
7A.2.1	Cell cultures	174
7A.2.2	Cytotoxicity assay of MTGase nanoflowers	174
7A.2.3	Clonogenic assay of MTGase nanoflowers	175
7A.2.4	Cell migration assay of MTGase nanoflowers	175
7A.2.5	DNA cell cycle analysis of MTGase nanoflowers	176
7A.2.5.1	Assessment of cell cycle phases of MTGase nanoflowers	176
7A.2.6	FACS analysis: Apoptosis assay of MTGase nanoflowers	177
7A.2.7	Statistical analysis	177
7A.3	Results and Discussion	177
7A.3.1	Characterization of MTGase nanoflowers for suitability as the drug potential	177
7A.3.2	Cytotoxicity assessment of MTGase nanoflowers on cancer cell lines	178
7A.3.3	Clonogenic assay of MTGase nanoflowers	182
7A.3.4	Inhibition of breast cancer invasion by wound healing assay	184
7A.3.5	DNA cell cycle analysis: MTGase nanoflowers stimulated G ₀ /G ₁ phase arrest of cell cycle	186
7A.3.6	MTGase NFs induced apoptosis by Annexin V Apoptosis assay	189
7A.4	Conclusions	193

CHAPTER 7B NANOFLOWER-BASED DELIVERY OF SYNTHETIC MIRNA INHIBITORS TO GLIOBLASTOMA BRAIN CANCER CELLS

7B.1	Introduction	194
7B.2	Materials and Methods	197
7B.2.1	Chemicals and materials	197
7B.2.2	Cell cultures	197
7B.2.3	Methods	197
7B.2.3.1	Rational synthesis and functionalization of MTGase NFs	197
7B.2.3.2	Physico-chemical characterization of MTGase NFs	198
7B.2.3.3	Complexation studies of MTGase NFs: anti-miR-210 formation	199
7B.2.3.4	Encapsulation efficiency of MTGase NFs as the nucleic acid nanocarrier	199
7B.2.3.5	Heparin competition assay	199
7B.2.3.6	Serum stability assay	200
7B.2.3.7	Cellular uptake	200
7B.2.3.8	Flow cytometry	200
7B.2.3.9	Fluorescence microscopy	201
7B.2.3.10	Statistical analysis	201
7B.3	Results and Discussion	202
7B.3.1	Evaluation of MTGase NFs as an anti-miR nanocarrier	202
7B.3.2	Characterization of MTGase NFs functionalized with PEI	202
7B.3.3	Complexation of MTGase NFs with anti-miRs	205
7B.3.4	Encapsulation efficiency of MTGase NFs as a nucleic acid nanocarrier	206

7B.3.5	MTGase NFs protect anti-miRs from heparin displacement	208
7B.3.6	MTGase NFs protect anti-miRs from serum-mediated degradation	208
7B.3.7	Cellular uptake studies of the MTGase NFs: anti-miR-210 complex	209
7B.3.8	MTGase NFs: anti-miR-210 delivery inhibits cellular proliferation and migration	211
7B.4	Conclusions	213
CHAPTER 8 OVERALL CONCLUSIONS		214
Future Perspectives		217
References		218
List of Publications		255
Brief Bio-Data		261

List of Figures

Figure No.	Description	Page No.
Figure 1.1	Mode of action of microbial transglutaminase (MTGase)	18
Figure 1.2	Structural visualization of MTGase	20
Figure 1.3	Phylogenetic relationship of MTGases	21
Figure 1.4	Magical shot in the form of enzymatic nanoflowers: a bloom for biotechnological applications	34
Figure 1.5	The versatile cross-linking action of MTGase as a protein stapler in food industries	36
Figure 1.6	Overview of biotechnological and industrial applications of MTGase	44
Figure 2A.1	Time course of MTGase production by <i>S. mobaraensis</i> along with the activity under SSF conditions	55
Figure 2A.2	Screening of various agro-industrial residual wastes for SSF substrate	57
Figure 2A.3	One factor at a time' experiments for optimizing the conditions for maximizing the MTGase production from <i>S. mobaraensis</i> under SSF conditions. [A] Moisture content ratio (w/v); [B-C] Nitrogen sources providing similar amount of nitrogen content (%); [D] Inoculum volume (ml); [E] Divalent salts (%); [F] Protease (IU) and [G] Surfactants (%)	61

Figure 2A.4	Response surface plots of Box-Behnken design for optimization of MTGase enzyme production by <i>S. mobaraensis</i> NCIM 5208. The contour plot shows the interaction between [A] Protease and MgCl ₂ , [B] Protease and CTAB, [C] Protease and Inoculation size, [D] MgCl ₂ and CTAB, [E] MgCl ₂ and Inoculation size, [F] CTAB and Inoculation size	66
Figure 2A.5	Growth of <i>S. mobaraensis</i> on wheat bran as a substrate under SSF condition after 10 days of incubation. [A-C]: SEM images were taken of raw wheat bran [A] and <i>S. mobaraensis</i> cells grown on wheat bran at 15000×; [B] and 30000× [C] of magnifications. [D]: TEM micrographs showing the growth of bacteria	68
Figure 2B.1	Growth performance of <i>S. mobaraensis</i> for MTGase production under shake flask conditions	78
Figure 2B.2	Morphology characterization of <i>S. mobaraensis</i> by TEM and SEM micrographs. [A] TEM image shows the mycelium growth on rod-shaped bacteria that has chains of spores upon maturity. [B] SEM analysis of mycelia differentiation of <i>S. mobaraensis</i> recorded at 5 KX magnification	79
Figure 2B.3	Effect of various modulators supplemented for maximizing the MTGase production from <i>S. mobaraensis</i> under fermentation conditions [A] CTAB (%), [B] Magnesium Chloride (M) and [C] Protease (IU). Asterisk denote statistical significance, derived from t-test: *p ≤ 0.05	80
Figure 2B.4	Transcriptional expression of MTGase production from <i>S. mobaraensis</i> under different modulators. The differential pattern in	81

the MTGase gene transcripts was observed, where, PC is a positive control. Upregulators augmented the copy number, whereas downregulators had an inhibitory effect on biosynthesis of MTGase

Figure 2B.5	Summary of bioaugmentation approach by critical factors linked with the production of MTGase	85
Figure 3.1	SDS gel analysis depicting purified enzyme and zymography of MTGase. Lane 1: Protein molecular marker. Lane 2: Purified MTGase enzyme, Lane 3: Zymogram of MTGase enzyme	93
Figure 3.2	Thermal properties of the purified MTGase enzyme from <i>S. mobaraensis</i> [A] Effect of temperature on the MTGase activity. [B] Thermostability of MTGase enzyme upto 45 min incubation. Effect of pH on the [C] activity and [D] pH stability of MTGase	94
Figure 3.3	Electrophoretogram of milk proteins cross-linked by purified MTGase	96
Figure 3.4	Silver staining and quantification of MTGase mediated cross-linked casein products by ImageJ software	97
Figure 3.5	CD spectra of cross-linked casein protein by MTGase	98
Figure 3.6	Fluorescence casein (untreated mediated cross-linked casein protein	99
Figure 3.7	FTIR spectra of casein (untreated), and enzyme-treated with purified MTGase	101
Figure 3.8	Overview of MTGase mediated casein polymerization	103
Figure 4.1	[A] Genomic DNA isolation of MTGase from <i>S. mobaraensis</i> run on 1.2% agarose. [B] PCR amplification of MTGase gene from <i>S. mobaraensis</i> genomic DNA by using primer pairs having restriction	113

	enzyme sites (NdeI and XhoI). [C] Double restriction digestion of recombinant plasmids	
Figure 4.2	[A] Blue-white screening of PCR products; transformants and non-transformants on X-gal IPTG LB-Amp plates. [B] Verification on re-streaking the transformed colonies. Transformation efficiency was high which yielded 10 clones (3 blue and 7 white transformed colonies). [C] These clones were scaled-up for plasmid isolation and subjected to restriction digestion with NdeI and XhoI for cloning and overexpression. [D] Double restriction digestion profile of pET 22b (+) vector	115-116
Figure 4.3	Construction of the pET 22b (+) overexpression vector with MTGase gene	117
Figure 4.4	Phylogenetic analysis based on the MTGase amino acid sequence of <i>S. mobaraensis</i> with the reference sequences retrieved from GenBank. The tree was constructed using the neighbor-joining method using MEGA 4.0 software	118
Figure 4.5	[A] MTGase production by the recombinant BL21 (DE3) and the wild strain	119
Figure 4.6	MTGase activity in different fractions	119
Figure 4.7	MTGase screening using a Hydroxamate plate assay (A) BL21 (DE3) transformed cell (B) Wild strain <i>S. mobaraensis</i>	120
Figure 4.8	SDS-PAGE analysis of MTGase from the recombinants- <i>E. coli</i> BL21(DE3). Lane 1-uninduced cells, Lane 2-induced cells and Lane M-protein marker	120

Figure 4.9	RT-qPCR analysis validation for MTGase expression performed on total RNA extracted. [A] Agarose gel demonstrated MTGase encoding transcripts that were specifically overexpressed in recombinant stage in the transcriptome data over wild type. [B] The relative mRNA levels of MTGase were determined by RT-qPCR studies. Each value presents the mean \pm SD of three replicates (n = 3)	121
Figure 4.10	Schematic representation of MTGase's maturation by co-expression in <i>E. coli</i> . The order/orientation of gene (A) pro-MTGase peptide + MTGase (B) The co-expression of MTGase + pro-MTGase. The active MTGase expressed in first case, highlighting the folding being assisted by the pro-region	122
Figure 4.11	Bioinformatics analysis using I-TASSER and Phyre 2 tools for structural studies of MTGase	124
Figure 5.1	Surface modification, functionalization and bioconjugation of MWCNTs for immobilization of MTGase	136
Figure 5.2	Scanning electron microscopy images of Pristine MWCNTs (A) magnification-15000 \times ; (B) magnification-30000 \times ; immobilized MTGase (iMTGase) onto MWCNTs (C) magnification-30000 \times and (D) magnification-50000 \times , bar 200 nm	139
Figure 5.3	FTIR spectra of pristine MWCNTs, functionalized MWCNTs and immobilized MTGase (iMTGase) onto MWCNTs	140
Figure 5.4	Thermal inactivation kinetics study of free and iMTGase at 60 $^{\circ}$ C	144

Figure 5.5	Reusability of iMTGase	145
Figure 5.6	Scanning electron micrograph of gelatin hydrogel scaffolds without treatment of MTGase (control) (A) magnification-15000×; scaffolds design with iMTGase as a cross-linker; (B) magnification-50000× and enlarged view of iMTGase treated scaffolds; (C) magnification-50000×, bar 3 μm	148
Figure 5.7	Effect of iMTGase content on equilibrium swelling ratio as a function of time: A plot between swelling ratio vs. time of swelling for gelatin hydrogels comprised of 0.9% NaCl	149
Figure 5.8	Overview of the development of innovative biocompatible hydrogel scaffolds with immobilized MTGase onto MWCNTs	150
Figure 6.1	(A) Schematic representation of the synthesis of enzymatic MTGase nanoflowers; (B) Characterization of the design process of MTGase NFs: SEM images of the organic nanoflowers obtained from an aqueous solution of MTGase, Cu ²⁺ , PBS at pH 7.4	158-159
Figure 6.2	Illustration of the architecture of floral nanostructure and physico-chemical attributes captured by FESEM, TEM micrographs, AFM, XRD and TGA analyses	161
Figure 6.3	[A] Control-MTGase NFs without any cargo loaded; [B] heavy metals and dye remediated from waste water effluents.	166
Figure 6.4	The application of MTGase nanoflowers in remediation with simulated as well as real waste-water effluents. FESEM-EDX spectra of metals adsorbed MTGase NFs: Inset shows the control (dye) without any treatment, EDX analysis of NFs showing the	167

property of absorbing heavy metals from effluent samples (d)
Photograph of vials containing an aqueous mixture of {MTGase
NFs + EBT dye; 3.0 mg/ml: 70.0 mg/l} after allowing to set for 24
h showing the % of adsorption of EBT dye by MTGase NFs

- Figure 6.5 Intact MTGase nanoflowers structure preserved after 168
functionalization and after subjected to harsh environment
- Figure 7A.1 Cytotoxicity induced by microbial transglutaminase nanoflowers. 179
Breast cancer (MCF-7), skin (HaCaT), and colorectal (HCT-116)
cells cell line was treated with indicated MTGase NFs
concentrations, and percent cytotoxicity was assessed at 24 h post-
treatment. Results are expressed as mean \pm S.D of three replicates
- Figure 7A.2 Cytotoxic effect of transglutaminase nanoflowers on control cells. 180
*****p*- value < 0.01**
- Figure 7A.3 Cell viability and IC₅₀. MTT assay showing the viability of MCF-7 181
cells after exposure to increasing concentrations of
transglutaminase nanoflowers, CTGase-commercial mammalian
source of transglutaminase nanoflowers; MTGase-indigenous
microbial transglutaminase nanoflowers (inset-microscopic images
after treatment for MTT)
- Figure 7A.4 Effect of MTGase NFs on colony formation (a) culture dishes with 183
stained colonies of a representative experiment; (b) statistical
results of colony-forming assays presented as colonies number in
respect of untreated cells used as control. The data in the graphs are
expressed as mean number \pm SD of three different experiments.
******p*-value < 0.001**

Figure 7A.5	Effect of MTGase NFs on MFC-7 cells invasion and migration properties. (a) MCF-7 breast cancer cells were seeded in 12-well plates. (b) Cell migration was assessed at time intervals of 0, 6, 12, 24 and 48 h post wound creation. Wound width was calculated at indicated times. Results are expressed as mean \pm S.D of three replicates. ***p-value < 0.001	185
Figure 7A.6	Effect of MTGase NFs against cell cycle progression (a–c): breast cancer MCF-7 cells were treated with IC ₅₀ microbial transglutaminase nanoflowers concentration for 24 h, and the effect on cell cycle progression was assessed through FACS using propidium iodide. (d) Cell progression was restricted in G1/G0 stage of the cell cycle by MTGase NFs treatment. **p-value < 0.01	188
Figure 7A.7	Detection of apoptosis of MCF-7 cells after treatment with MTGase NFs: breast cancer MCF-7 cells were treated with IC ₅₀ MTGase nanoflowers concentration for 24 h. Annexin V/PI staining combined with flow cytometry analysis was used to count apoptotic cells. (A) Dot plots showing apoptosis of MCF-7 cells in response to exposure to MTGase NFs. (B) Percentage of cell population of live apoptotic and necrotic was compared. Graphs represent means \pm SD of data from three independent biological replicates	190
Figure 7A.8	Overview of MTGase NFs as anti-cancer drug	192
Figure 7B.1	Physicochemical characterization of MTGase NFs: (A) Schematic representation of the synthesis process of MTGase NFs; (B) FESEM, and (C) TEM photomicrographs of MTGase NFs functionalized with PEI; (D) DLS image representing	204

hydrodynamic diameters of MTGase NFs before and after complexation with anti-miR; (E) FTIR analysis of PEI functionalized MTGase NFs

- Figure 7B.2 *In vitro* complexation of MTGase NF: anti-miR-210 and stability analyses: (A) MTGase NF: anti-miR-210 complexation at various (M/M) ratios; (B) FESEM image of MTGase NF: anti-miR-210 nanocomplex at 1:100 (M/M); (C) Anti-miR encapsulation efficiency of MTGase NFs; (D) Zeta potential of MTGase NFs at various stages of synthesis and complexation; (E) Heparin competition assay; (F) MTGase NF: anti-miR-210 Serum stability assay 207
- Figure 7B.3 Cellular uptake and transfection efficiency of MTGase NF: anti-miR-210 complex: (A) Flow cytometric analysis of FAM-labelled MTGase NF: anti-miR-210 delivery to U87-MG cells; (B) Cellular uptake of FAM-labeled MTGase NF: anti-miR-210 complex; (C) qRT-PCR analysis of miR-210 knockdown upon cellular delivery of MTGase NF: anti-miR-210 nanocomplex 210
- Figure 7B.4 MTGase nanoflowers as delivery agent: A novel, biocompatible nanocarrier (inhibitors/drugs) for GBM cancer cells 212
-

List of Tables

Table No.	Description	Page No.
Table 1.1	Commonly employed microbial strains for production of MTGase	25
Table 1.2	MTGase production by genetic engineering	28
Table 1.3	Limitations and challenges prevailing in the current drug delivery vehicles	32
Table 2A.1	Box-Behnken design matrix for the experimental design and respective response for MTGase enzyme production from <i>S. mobaraensis</i> NCIM 5208	63
Table 2A.2	Regression analysis for the production of MTGase enzyme by <i>S. mobaraensis</i> NCIM 5208 for quadratic response surface model fitting after ANOVA analysis	64
Table 2A.3	Summary of the optimized SSF-RSM conditions for enhanced production of MTGase	67
Table 2A.4	Preliminary comparison of MTGase production in SSF over SmF	69
Table 3.1	Enzymatic characterization of purified MTGase enzyme	95
Table 4.1	Prediction of critical residues for MTGase stability by I-Mutant 3.0	125
Table 5.1	Immobilization of MTGase on functionalized multi-walled carbon nanotubes: Optimization of binding of MTGase onto MWCNTs with immobilization efficiency	137

Table 5.2	Kinetic parameters of free MTGase enzyme and immobilized MTGase on functionalized MWCNTs	142
Table 5.3	Screening of matrix for hydrogels formation	147
Table 6.1	Comparative properties of MTGase nanoflowers with conventional nanoflowers	164
Table 6.2	Kinetic Parameters of Free MTGase enzyme and immobilized nanoflowers	165
